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Cover picture: Potato leaves infected with *P. infestans*. Photo taken at Ultuna autumn 2010 by Anna Åsman.
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Abstract

The oomycete pathogen *Phytophthora infestans* is the causal agent of the devastating plant disease late blight on potato. Diverse type of transposons and many gene families are present in the genome which encodes the effector proteins involved in causing the pathogenicity. This plant pathogen is predicted to secret hundreds of effector proteins inside the host plant cells to promote infection. These proteins are sensed by the plant immune system in order to prevent pathogen growth. The effector proteins are divided into two main types, cytoplasmic effectors and apoplastic effectors based on their translocated status in the plant cell. In this study, the effector-encoding genes *Avr3a, Epi1, Epi10, Inf1* and *CRN8* were selected to monitor the potential *in planta* function of the effectors and to develop a stable transformation procedure for reporter gene constructs with effector gene promoters. The putative promoter sequences were derived from the 5´ regions of the oomycete genes. Primers were designed to amplify the promoter regions and the amplification was confirmed by gel electrophoresis. The reporter gene *GFP* (encoding green fluorescent protein) was chosen for analysis of their promoter activities and to facilitate studies on spatial and dynamic alteration of gene expression. Cloning was performed using the vector pTOR-eGFP containing a *ham34* promoter and a *GFP* gene. The *ham34* promoter was removed and the effector promoters were inserted in its place. A stable transformation procedure was examined using three vectors for the GFP-constructs and the five effector gene promoters. Transformants were obtained at similar frequencies with each combination of effector promoter and *GFP*; which were confirmed by gel electrophoresis. Subsequently *Agrobacterium tumefaciens* (C58) mediated transformation was tried for an *Avr3a* promoter construct. The construct was ligated into the binary vector, but the transformation of *Agrobacterium* was not successful.

Table of Contents

Introduction

Phytophthora infestans is a hemibiotrophic pathogen that exhibits two distinct phases in its life cycle: initial asymptomatic biotrophic phase and a later necrotrophic phase (Lee and Rose, 2010). In the biotrophic phase, the pathogen is likely to secrete effector proteins that suppress programmed cell death (PCD) and spoil the host defense responses. In contrast, during the necrotrophic stage secreted proteins invade the plant tissue and destroy them followed by characteristic symptoms. Leaves and tuber are the susceptible parts of the potato plant that are primarily infected by the pathogen. The disease cycle starts when the sporangium comes in contact with the susceptible parts of potato plants (Pieterse *et al.,* 1991). The pathogen thrives in warm, moist conditions and causes rapid death of plants. Symptoms first initiate as spots like water soaked lesions, typically at the edges of lower leaves. In favorable condition, the spots enlarge rapidly and appear as brown blighted areas. High humidity influences the growth of white aerial mycelium on the lower side of the leaves. Then with time the pathogen spreads to other parts of the plant for instance the stem and tuber. It is more than 150 years since the pathogen caused the Irish potato famine. On that time, one million people starved to death and two million people emigrated to America. Present worldwide loss for the late blight disease is estimated at 6.7 billion dollars per year (Haas *et al.,* 2009). In Sweden 2.5-3.0% of the agricultural land is used for potato cultivation. Remarkably 50-60% of all fungicides applied are used against *P. infestans.* The problem with *P. infestans* is substantial and new efforts are needed to secure the potato crop.

Potato

Potato (*Solanum tuberosum* L.) is the fourth largest food crop in the world and is currently an important alternative to major cereal crops (Haas *et al.,* 2009, Vleeshouwers *et al.,* 2008). The majority of the potato cultivars are; autotetraploid ($2n=4x=48$), highly heterozygous, suffer from severe inbreeding depression and are susceptible to many pests and pathogens. The Potato Genome Sequencing Consortium (PGSC, 2011) used homozygous doubled and monoploid potato clones for sequencing, where 86 % of the 844 Mb genome were assembled and assumed to have 39,031 protein coding genes.

Phytophthora infestans

Oomycetes is a specific group of eukaryotic microorganisms that includes different destructive pathogens affecting many plant species (Kamoun, 2003). Among them, members within the genus *Phytophthora* cause extensive economic losses of plants especially on potato and tomato (Erwin *et al.,* 1996). Oomycetes are fungal-like heterotrophs also referred to as water molds, and they have a distinct lineage to organisms like brown algae and diatoms (Haas *et al.,* 2009; Lamour *et al.* 2007). The oomycetes organism group is remote from the kingdom fungi and belongs to the biflagellate Stramenopiles under the kingdom Chromista (Cavalier-Smith and Chao, 2006). *P. infestans* is the most notorious and best-studied oomycete pathogen species till now. The pathogen also causes disease on other plant species such as several wild species of *Solanum* in central Mexico (Fry, 1998). Moreover, in Canada and USA, *P. infestans* has been documented to infect hairy nightshade (*Solanum sarachioides*), bitter sweet (*S. dulcamara*), petunia (*Petunia hybrida*) and pear melon (*S. muticatum*) (Fry, 1998). *P. infestans* has two ways of reproduction: asexual and sexual. In the asexual reproduction pathway sporangium germinates by releasing zoospores and this process is mostly responsible for spreading the disease late blight (Fry and Goodwin, 1997). The sexual cycle takes place when two mating types A1 and A2 are present in a population. A1 (2n) and A2 (2n) are differentiated for producing antheridium (n) and oogonium (n), respectively. When antheridium and oogonium fuse, the oospore (2n) develops that has a thick cell wall and that can survive for a long time in soil without the host plant. When winter ends, the oospores germinate by germ tube and produce sporangia.

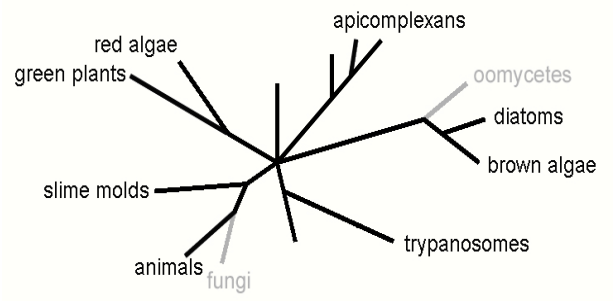

Fig. 1. Phylogenic tree of the eukaryotes. Adapted from Baldauf *et al.* (2000). The tree is made on the basis of six highly conserved proteins and shows the grouping of oomycetes with the brown algae and diatoms (heterokonts) in the Stramenopiles rather than with fungi.

The organism *P. infestans* is diploid at all stages of its life cycle except during sexual reproduction where it forms haploid nuclei within gametangia. It is heterothallic in nature which

indicates that its sexual reproduction occurs when A1 and A2 are present in the same plant tissue, fertilization can take place and oospores may be formed starting new rounds of infections.

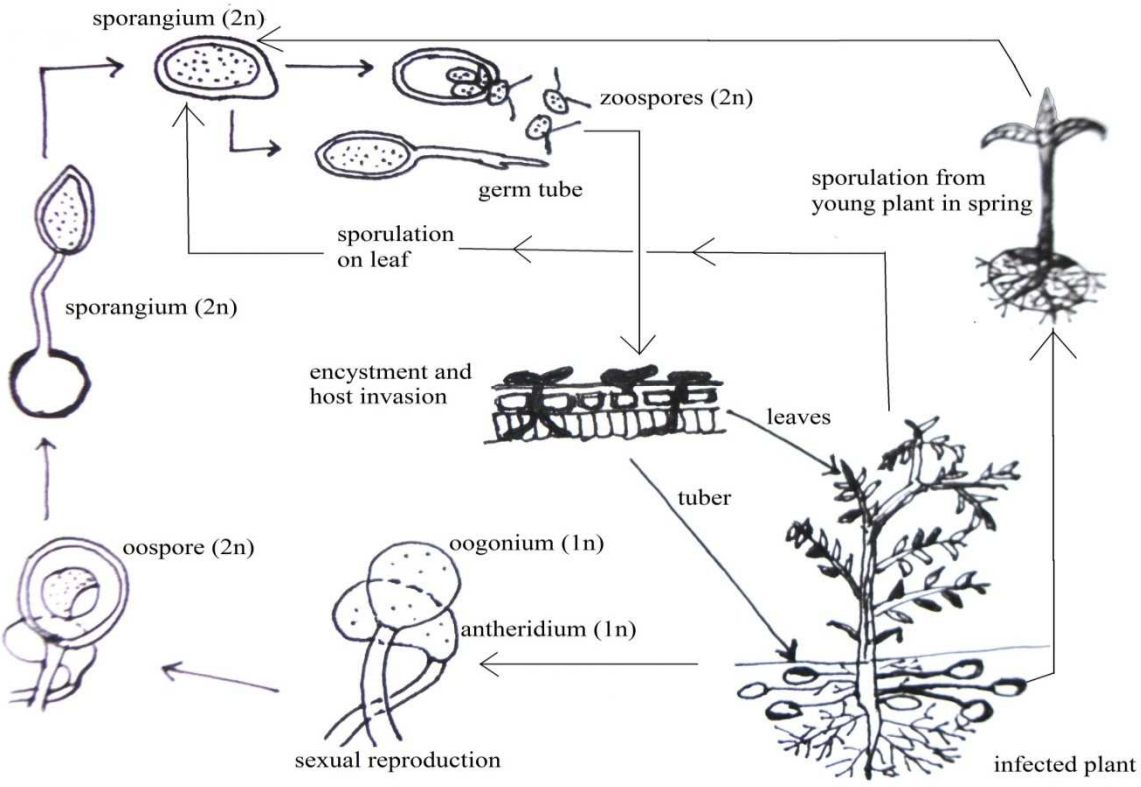

Fig. 2. Life cycle of *Phytophthora infestans,* which is the causal agent of potato late blight. It is adapted from Schumann, 1991.

The complete genome of *P. infestans* has been sequenced (Haas *et al.,* 2009). The genome is large compared to those of other sequenced oomycetes, 240 Mbp comprising about 18,100 genes. The genome sizes of other related *Phytophthora* species are 95 Mb (*P. sojae*) and 65 Mb (*P. ramorum*). The large genome size of *P. infestans* reflects its high content of transposable elements (50%) and repetitive DNA sequences, which together constitute about 74% of the genome (Haas *et al.,* 2009). Transposons are active in *P. infestans* (Judelson *et al*., 2008) and are associated with effector family expansion and genome reorganizations (Haas *et al*., 2009). Uncontrolled activities of transposable elements are however not advantageous. Proper control mechanisms have most likely evolved in *P. infestans* as in other eukaryotes (reviewed in Malone and Hannon, 2009).

Effectors

The genome of *P. infestans* codes for large numbers (>700) of effector proteins; effectors in this instance are defined as secreted pathogen proteins and other molecules that modulate plant defense mechanisms and enable parasitic colonization of plant tissue. The effector proteins are divided into two types: cytoplasmic effectors and apoplastic effectors (Kamoun, 2006). *P. infestans* secretes both types of effectors to facilitate colonization and to repress host defenses. The effectors are identified based on the occurrence of conserved domains and motifs within the N-termini of RXLR and CRN classes (Jiang *et al*., 2008). The N-terminal domain is encompassing the signal peptide and RXLR leader functions that facilitate secretion and translocation of the protein inside the host cell, whereas the C-terminal domain carries the effector activity and operates inside plant cells (Kamoun, 2006; Morgan and Kamoun, 2007).

The domain organization of apoplastic and cytoplasmic effectors is depicted in Fig. 3.

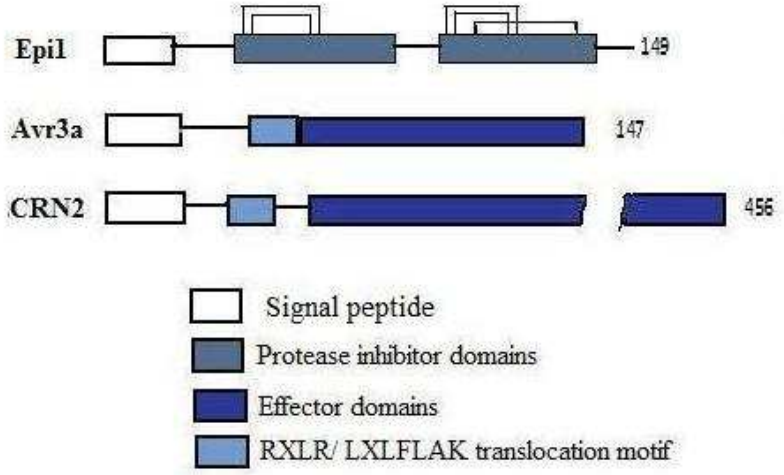

Fig. 3. Schematic representation of the domain organization of apoplastic and cytoplasmic effectors. Oomycete effectors are modular. All known effectors carry the N-terminal signal peptides for secretion that here are in white markings. The two-disulphide bridges, the Kazal domains of Epi1 are protease inhibitor domains that are shown in gray. The cytoplasmic effectors (eg. Avr3a and CRN2) have conserved motifs in their N-termini (RXLR or LXLFLAK) that are necessary for host translocation. The C-terminal domain carries the module with biochemical effector activities that modulate host defenses inside the plant cells. The dark blue color region of the effector proteins indicates the involvement in secretion and targeting the molecules. The figure is adapted from Schornack *et al.*, 2009.

Avr3a is a polymorphic member of the RXLR family that encodes at least two polymorphic secreted proteins of 147 amino acids varying in only three residues; two out of the three residues are found in the mature protein (Armstrong *et al.,* 2005). *Epi1, Epi10* and *Inf1* encode apoplastic effectors secreted into the plant extracellular space. *Epi1* and *Epi10* encode multidomain secreted serine protease inhibitors of the Kazal family (Rawlings *et al.,* 2004). In addition, the Inf1elicitin is a highly conserved 10-KD extracellular protein secreted by *P. infestans* (Kamoun *et al.,* 1993).

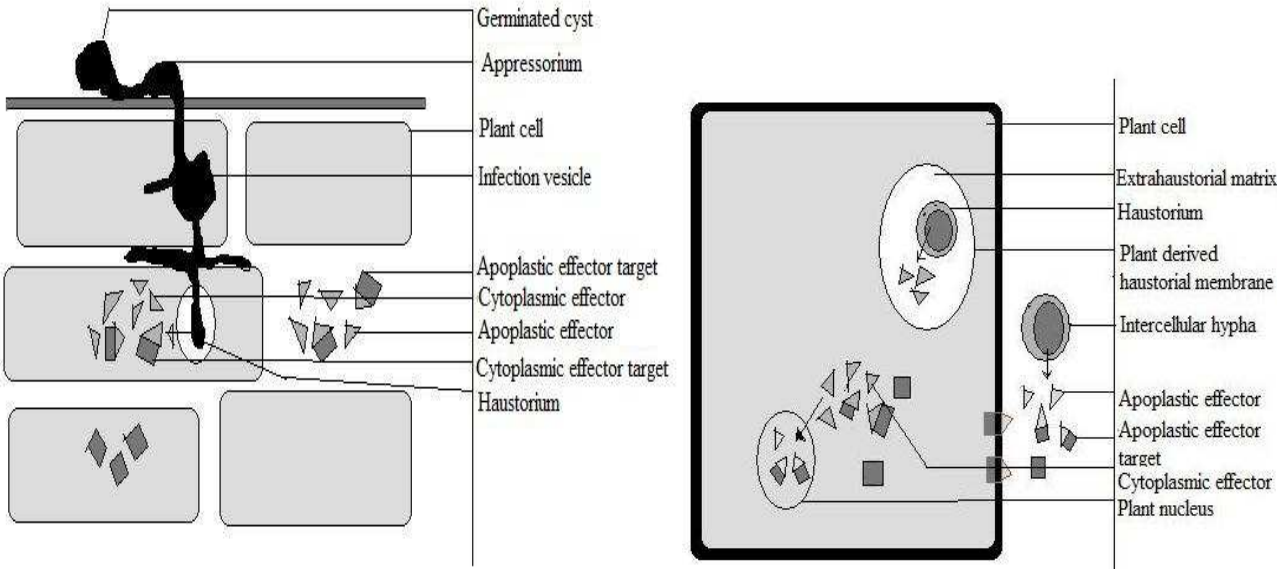

Fig. 4. To the left: Schematic representation of early stages of infection into plant cells. Plant pathogenic oomycetes secrete apoplastic effectors into the plant intercellular space and cytoplasmic effectors translocate inside the plant cell. To the right: Cross section showing the apoplastic and cytoplasmic effectors and their activity to their targets. Indeed cytoplasmic effectors can target the plant nucleus most probably using infection vesicle and haustoria that invaginate inside living host cells. In contrast, apoplastic effectors interact with extracellular targets and surface receptors. The figure is modified from Kamoun, 2006.

It has been suggested that the ubiquitin proteosome system (UPS) is contributing to the regulation of plant defense mechanisms and acts as a target for pathogen effectors. *Avr3a* has the potential to control host plant UPS functions. There are two allelic variants of *Avr3a*: Avr3a KI (containing amino acids K^{80} and I^{103}) and Avr3a EM (containing amino acids E^{80} and M^{103}) both having significantly different characteristics and activities in the plant. Avr3a KI but not Avr3a EM, triggers hypersensitive cell death in presence of the potato resistance protein R3a, although

it suppresses the hypersensitive cell death induced by another *P. infestans* effector protein Inf1 elicitin (Schornack *et al.,* 2009; Bos *et al*., 2010).

Specific plant resistant (R) proteins can be identified using effectors and classified according to distinct recognition specificities. RXLR effector genes were collected from *P. infestans* and employed in high-throughput *in planta* expression assays on wild potato germplasm to test their avirulence activities and accelerate the cloning of related *R* genes (Vleeshouwers *et al.*, 2006). Vleeshouwers and co-workers reported rapid identification and cloning of closely related orthologues of the *Solanum bulbocastanum R* gene *Rpiblb1* from *S. stoloniferum* and *S. papita*. Recently, four *R* genes (*Rpi-blb3, Rpi-abpt, R2* and *R2-like*) were identified from potato germplasms that show late blight resistance and the genes were mapped to potato chromosome IV. All four gene products recognized the RXLR effector PiAvr2 (Lokossou *et al.,* 2009).

Gene silencing

The gene knockout approach is comparatively difficult for *P. infestans* due to its diploid nature. Therefore, a number of genes have been well studied via the gene silencing strategy (Whisson *et al.,* 2005). Post-transcriptional gene silencing (PTGS) is initiated by double-stranded RNA (dsRNA). dsRNA can be produced from transposons, which are stretches of DNA that can self-replicate from inverted repeats or through the action of RNA-dependent RNA polymerase (Rdr). Gene silencing plays an important role in the gene regulation system, for example in targeting mRNA for degradation, translational repression or by altering the transcriptional activity of chromatin (Whisson *et al.,* 2005). dsRNA is processed into 18-20 bp small non-coding RNAs (Jinek and Doudna, 2009). Small interfering RNA (siRNA) and microRNA (miRNA), are two major classes of small RNA documented to date which differ in their biogenesis and mode of action (Moazed, 2009).

Most eukaryotes possess endogenous pathways to degrade viral, transposon or aberrant RNAs. In the endogenous RNAi pathway, longer dsRNAs are cleaved by RNase type III endonuclease (Dicer) generating 21-23 nucleotide siRNAs. The siRNA duplex undergoes strand separation and the antisense strand loads onto Argonaute (Ago) which is characterized by PAZ/PIWI domains (Höck and Meister, 2008). Ago is an essential part of a multi-protein RNA-induced silencing

complex (RISC). The complementary targeted mRNA is cleaved through the slicer characteristics of the Ago PIWI domain. In the transitive RNAi process, siRNAs are produced in an Rdr-dependent mechanism, forming secondary siRNAs (Sijen *et al.,* 2001).

Aim of the study

The aim of the project was to design effector vectors harboring 5 different *P. infestans* promoters to be tested in the *P. infestans*-potato interaction.

MATERIAL AND METHODS

Culturing of *P. infestans* and isolation of genomic DNA

Phytophthora infestans strain 88069 was maintained on rye medium supplemented with 2% sucrose (Caten and Jinks, 1968) at 20^{o}C. Liquid cultures in 25 Henniger synthetic medium were intiated from zoospores obtained from 2-week old cultures on rye sucrose agar at a concentration of 2 x 10^4 zoospores/ml. Cultures were incubated for 14 days at 20^{o}C to obtain mycelium for DNA extraction. Total genomic DNA was isolated from mycelia grown in liquid culture. Mycelium (10 g) was ground in liquid nitrogen to a fine powder and mixed in 5 ml of extraction buffer (200 mm Tris-HCl, pH 9.0, 100 mM NaCl, 10 mM Na_2-EDTA, 0.5% SDS, 14 mM 2-mercaptoethanol, and 200 ug/mL Proteinase K) per gramme of mycelium and incubated at 55^{o}C for 5 min. Then the mixture was extracted with 0.6 volume of water saturated phenol. The water phase was extracted once with an equal volume of phenol/chloroform/isomyl alcohol at the ratio of 25:24:1 and once with equal volume of chloroform/isomyl alcohol at the ratio of 24:1. The DNA was precipitated using 0.6 volume of 2-propanol that was dissolved in T10E1 buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA) and further purified by CsCl gradient centrifugation (Pieterse *et al.,* 1991b).

PCR primer design and PCR conditions

Five *P. infestans* effector genes (*Epi1, Epi10, Infl, CRN8* and *Avr3a*) were selected for promoter studies and gene sequence data was retrieved from www.broad.mit.edu. The following PCR primers were designed to amplify the promoter sequences (Table 1).

Table 1. Primers used to amplify the selected promoter sequences of *P. infestans*.

Gene	Size (bp)	Forward primer	Reverse primer
Epi1	639	ATGGGTCGAGTCTTCTGTGC	AAGCGCGGATTTCATGAGTG
Epi10	915	GGACAGAGCTACCACCTTCG	AGCAGACTTCATGGTAATTG
Infl	575	CCTGTTTGTATGGCATCGTG	ACGAAAGTTCATCGTGGACG
CRN8	633	CGGTAAACCGAGCCTAAGTG	GAACAAAGTAACCATATTTC
Avr3a	634	AAAGAATTCGCAATGACGACAGACGC ATTATCTG	AAAATCGATTGCCAGACGCATGG TGTGGA

The PCR mixtures (50 µl/reaction) contained: 5× Phusion HF Buffer 10 µl (Finnzymes), 1 µl of dNTPs (200 µM each), 3 µl of each primer, 2 µl of template genomic DNA, 1 µl of Phusion polymerase enzyme (Finnzymes) and 30 µl of water. In the PCR programs (Table 2), 35 cycles were run after a hot start at 98^{o}C for 30 sec.

Table 2. PCR programs used in the analysis.

Gene	**Denaturation**	**Annealing**	**Elongation**	**Extension**
Epi1	10 s, 98^{o}C	30 s, 61^{o}C	30 s, 72^{o}C	7 min, 72^{o}C
Epi10	10 s, 98^{o}C	30 s, 61^{o}C	30 s, 72^{o}C	7 min, 72^{o}C
Infl	10 s, 98^{o}C	30 s, 61^{o}C	30 s, 72^{o}C	7 min, 72^{o}C
Crn8	10 s, 98^{o}C	30 s, 58^{o}C	30 s, 72^{o}C	7 min, 72^{o}C
Avr3a	10 s, 98^{o}C	30 s, 72^{o}C	30 s, 72^{o}C	7 min, 72^{o}C

In order to examine the amplified products, they were run on a 1% agarose gel, followed by EtBr staining. 1kb ladder (Fermentas) was used for size determination of linear double-stranded DNA fragments from 500 bp to 12 kb.

Constructing plasmid vectors

The supplied pTOR-eGFP (6531 bp) DNA contained: *ham34* promoter (934 bp), *GFP* (720 bp) and terminator (549 bp) (Blanco and Judelson, 2005). Restriction sites for *Kpn*I and *Cla*I were selected to delete the *ham34* sequence. The restriction digestion mixtures contained: 2.5 µl of 10× Fast Digest buffer (Fermentas), 1 µl of each restriction enzyme (Fermentas), 1 µg of plasmid DNA and water to a final volume of 25 µl. The digestion was incubated at 37^{o}C for 1.5 hr. The linearization was monitored by running the samples in a 1% agarose gel, followed by EtBr staining. The four amplified promoter sequences of *Epi1, Epi10, Infl* and *CRN8* were digested with *Cla*I and *Kpn*I and ligated into the corresponding restricted sites of pTOR-eGFP (lacking $ham34_{\text{pro}}$). This step created 4 new plasmids: pTS1 harboring $Epi1_{pro}$, pTS2 with $Epi10_{pro}$, pTS3 with $Infl_{pro}$, pTS4 with $CRN8_{pro}$ and pTS5 with $Avr3a_{pro}$. Plasmid DNA (promoter-GFP-3') from pTS1, pTS2, pTS3 and pTS4 was isolated (Fermentas GeneJet^{TM} plasmid miniprep kit) and digested by *Kpn*I/*Nde*I and the promoter constructs (promoter-GFP-3') were cloned into pSpiro-PAC-3XHA-C (Jerlström-Hultqvist, unpublished) harboring the corresponding restriction sites. The resulting new plasmids were pLD1, pLD2, pLD3 and pLD4. The vector pSpiro-PAC-3XHA-C was used in the experiment since the required restriction site *Hind*III was not present in pTOR-eGFP. However, each gene cassette (promoter-GFP-3') from pLD1, pLD2, pLD3 and pLD4 was digested by *Kpn*I and *Hin*dIII and inserted into the corresponding sites of the binary vector pPZP200 (Hajdukiewicz *et al.,* 1994). This step produced four new plasmid constructs named pJP1, pJP2, pJP3 and pJP4.

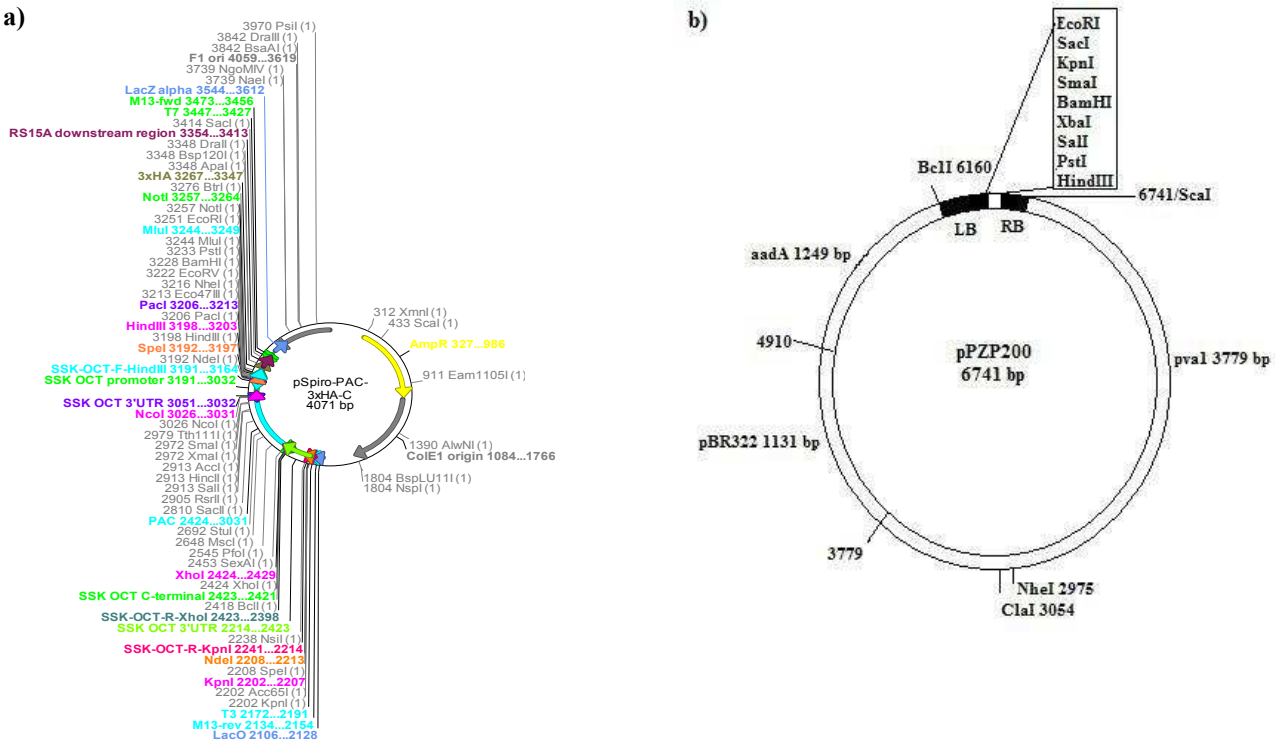

Fig. 5. a) The pSpiro-PAC-3XHA-C vector (Jerlström-Hultqvist, unpublished) and **b)** pPZP200 *Agrobacterium* binary vector; which is adapted from Hajdukiewicz *et al.,* 1994.

In the case of *Avr3a*, a modified pTOR-eGFP plasmid was used in order to clone the *Avr3a* promoter, since its promoter sequence contained a *Kpn*I site. A Multiple Cloning Site (MCS) was produced using the phagemid pBluescript II SK+ as a template for PCR amplification and the primers were pBlue F (5´- AAAGGTACCGCGCGTAATACGACTCACTA-3´) and pBlue R (5´- AAAGGTACCCGCGCAATTAACCCTCACTA-3´). *Kpn*I sites (GGTACC) were generated by adding the corresponding sequences to the primers. The PCR product was digested and ligated into the *Kpn*I site of pTOR-eGFP to create pTOR-eGFP -MCS. In parallel, *Eco*RI and *Cla*I sites were added to the *Avr3a* promoter by adding their sequences at the ends of the *Avr3a* PCR primers. After PCR amplification and restriction by *Eco*RI (GAATTC) and *Cla*I (ATCGAT), the $Avr3a_{pro}$ fragment was ligated into the vector pTOR-eGFP –MCS producing pTS5 with an $Avr3_{pro}$ -GFP -3' sequence.

The 20 µl restriction mixtures of the PCR-amplified promoters contained 2 µl Fast digest buffer (Fermentas), 0.5 µl of each restriction enzyme, 200 ng of amplification product and 15 µl water.

After incubation at 37^{o}C for 1 hr, the samples were purified following the QIAquick PCR purification kit protocol. Vector pSpiro-PAC-3XHA-C was not used in the case of *Avr3a*. Therefore the plasmid DNA of pTS5 was digested with *Eco*RI and *Pst*I and inserted in the pPZP200 binary vector directly, creating pJP5.

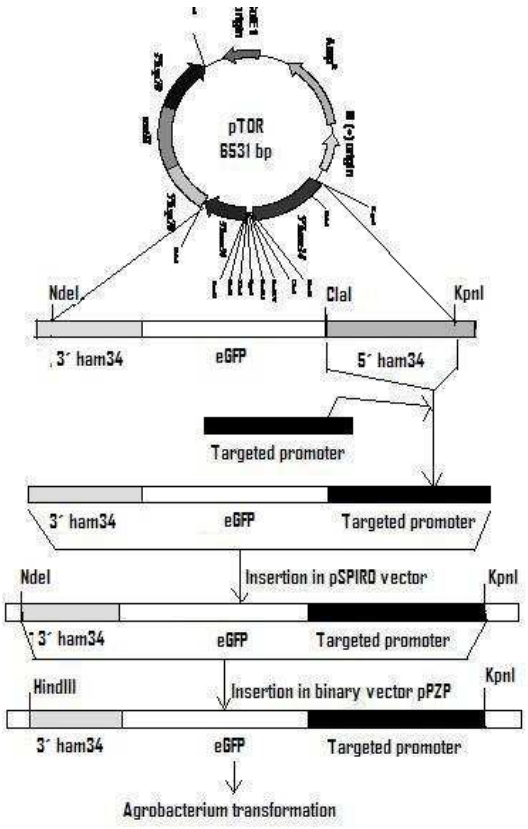

Fig. 6. Schematic representation of cloning procedure for the effector promoters (*Epi1, Epi10, Inf1* and *CRN8*).

Fig. 6 shows the whole cloning procedure step by step of the four effector promoters using three vectors. In the case of *Avr3a,* there were two exceptions: the modified pTOR-eGFP was used and the pSpiro-PAC-3XHA-C step was not followed, but the remaining steps were similar.

Sequence analysis of transformants

Three vectors (pTOR-eGFP, pSpiro-PAC-3XHA-C and pPZP-200) were used in this experiment in order to examine the effector promoter activity by GFP tagging. Sequencing was performed after two steps of cloning (pSpiro-PAC-3XHA-C and pPZP-200) and the sequencing were accomplished at Macrogen, Seol. The required sample concentration at Macrogen was 100 ng/µl

for plasmid DNA. Five clones for each promoter *Epi1, Epi10, Infl1, CRN8* and *Avr3a* were sequenced at the pSpiro-PAC-3XHA-C vector step and in the case of pPZP-200 vector only *CRN8* and *Avr3a* clones were sequenced. The sequencing results were analyzed using BLAST search of GenBank, NCBI.

Ligation conditions

The ligation formula used to calculate the amount of insert vs. vector DNA:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}}$$

A molar ratio of 1:3 for vector and insert was used in these experiments corresponding to *Epi1* 30.7 ng, *Epi10* 43.97 ng, *Infl* 27.63 ng, *CRN8* 30.42 ng of insert and 100 ng of vector. The pTOR-eGFP vector concentration was 54.2 ng/µl.

Two types of reaction mixtures were prepared: ligating sample and re-ligation control without insert DNA. The reaction mixture was in a total volume of 20 µl containing 2 µl of 10X T4 DNA ligase buffer, 1.85 µl vector DNA, 1 µl of T4 DNA ligase, calculated amount of insert (*Epi1* 3.8 µl, *Epi10* 12.93 µl, *Infl* 16.85 µl and *CRN8* 6.91 µl) and the rest of nuclease free water. The ligation reactions were incubated at room temperature for 30 min.

Transformation

To 100 µl of competent *Escherichia coli* DH5α cells, 10 µl of the ligation mix was added and to another tube, 10 µl of re-ligated vector DNA was added as a control. The samples were kept on ice for 30 min, heat shock treated at 42^{o}C for 45 sec in a water bath and followed by addition of 900 µl LB media. The samples were shaken (150-200 rpm) for 1 hour at 37^{o}C. 250 µl of each transformation mix was spread on LB agar plates with ampicillin (100 µg/ml LB) and grown overnight at 37^{o}C. Colonies were not observed on control plates indicating absence of self-ligation. Five colonies were picked for each sample reaction and transferred to glass tubes containing 4 ml LB with 4 µl ampicillin (final concentration 100 µg/ml). The tubes were incubated at 37^{o}C overnight with shaking. Plasmid DNA was isolated using the alkaline lysis method (Fermentas GeneJet^{TM} plasmid miniprep kit) and analyzed by using 1% agarose gel electrophoresis.

Agrobacterium transformation

After constructing the new binary pPZP plasmids in *E. coli*, the freeze-thaw method was applied to transfer the plasmids into *Agrobacterium*. One microgram (µg) of plasmid DNA was added to cultured *Agrobacterium tumefaciens* (C58) and kept on ice for 5 min followed by 5 min at 37^{o}C in a water-bath. One milliliter (ml) LB was added to the tube, which was incubated at 28^{o}C for 3 hrs with shaking. The tubes were centrifuged for 30 s and after discarding the supernatant the pelleted cells were resuspended in 0.1 ml LB medium. The mixture was spread on LB agar plates containing 50 µl each of the antibiotics streptomycin, rifampicin and carbenicillin, and incubated at 28^{o}C for 4 days.

RESULTS

Full-length sequences of the selected effector genes with upstream regions were retrieved from the *Phytophthora infestans* database. A promoter scan was used to identify the promoter regions. PCR primers were designed for the 5 selected promoters and the promoter regions were amplified (Fig. 8).

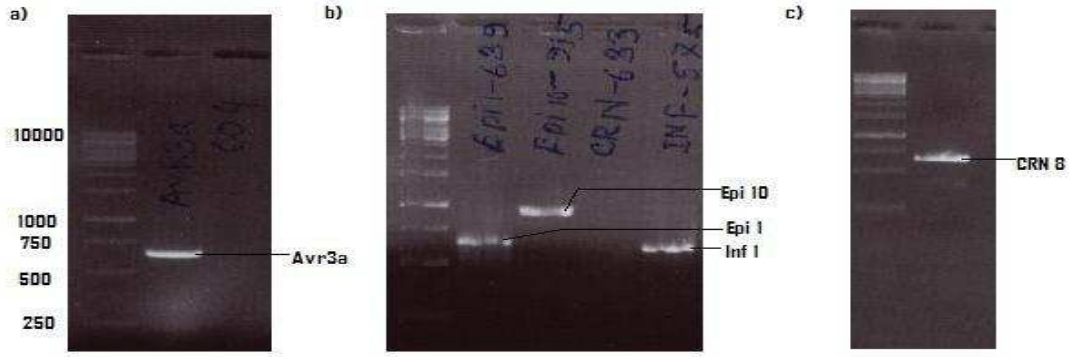

Fig. 8. Agarose gels showing the products from polymerase chain reaction amplification of *Avr3a, Epi1, Epi10, Inf1* and *CRN8* promoters. The size marker is GeneRuler™ 1kb DNA ladder, 250-10000 bp (Fermentas). A single band was detected for each of the five promoters and the estimated fragment sizes are 604 (*Avr3a*), 639 (*Epi1*), 915 (*Epi10*), 575 (*Inf1*) and 633 bp (*CRN8*).

The *ham34* promoter in the pTOR-eGFP vector was selected to be replaced with the 5 chosen promoters. The *ham34* promoter sequence was deleted using the *Kpn*I and *Cla*I restriction sites.

The designed vector pTOR-eGFP-MCS for harboring the *Avr3a* promoter was digested with *Eco*RI and *Cla*I enzymes. In the restriction digests, the *ham34* (954 bp) fragment was separated from vector DNA (Fig. 9).

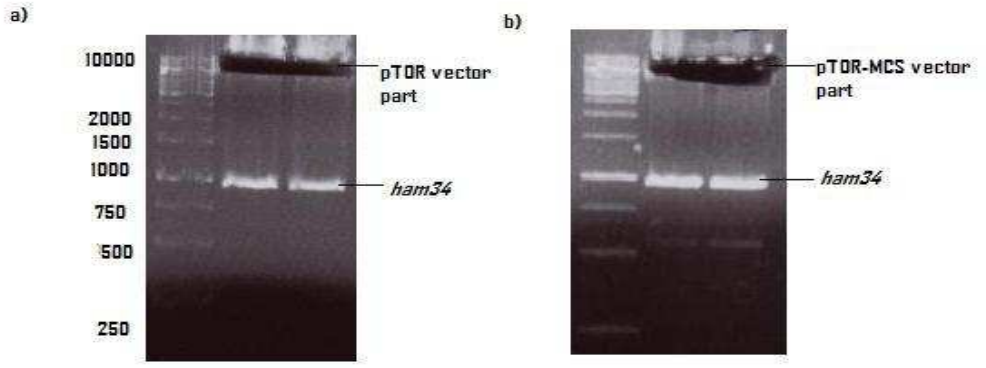

Fig. 9. Agarose gels with restriction enzyme digests to delete the *ham34* promoter a) pTOR-eGFP and b) pTOR-eGFP-MCS vector DNA. The size marker is GeneRuler™ 1kb DNA ladder (Fermentas).

When the test digestions of pTS1, pTS2, pTS3 and pTS4 (five samples of each plasmid DNA) were analyzed on 1% agarose gels, expected fragment sizes were observed for the clones *Infl* (clone 1), *CRN8* (2), *CRN8* (3), *CRN8* (4), *CRN8* (5), *Epi1* (1), *Epi1* (2), *Epi1* (3), *Epi1* (4) and for *Epi10* (1). Two representative gel pictures are shown in Fig. 10.

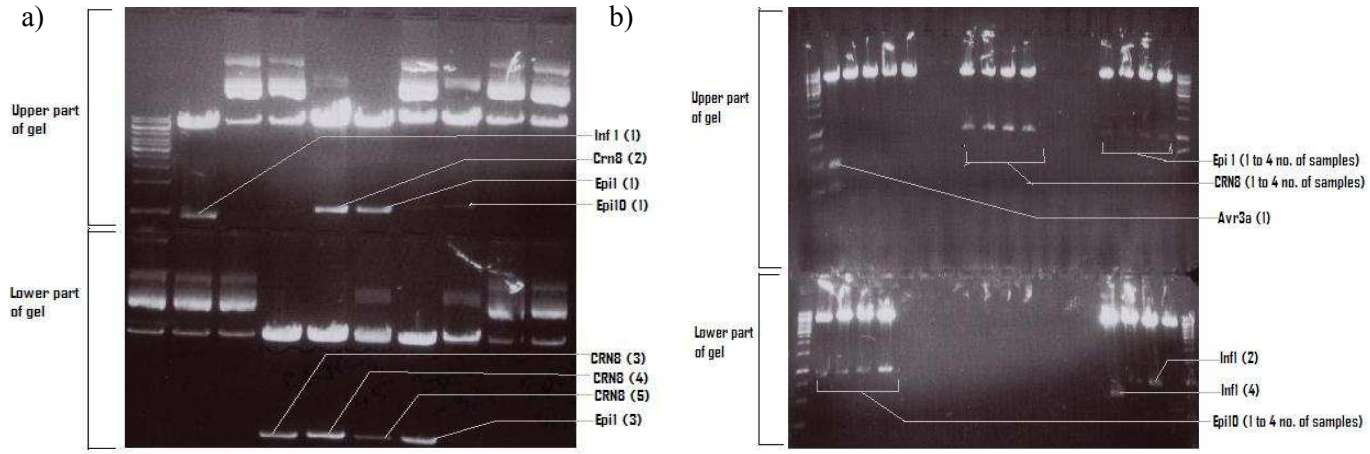

Fig. 10. Agarose gels with test digestions of pTS1, pTS2, pTS3 and pTS4 ligated plasmids. a) and b) show repeated experiment. The size marker is GeneRuler™ 1kb DNA ladder (Fermentas).

First, five clones of each construct (pTS1, pTS2, pTS3 and pTS4) were analyzed (Fig. 10a). Among them, 1 clone for *Infl*, 2 for *Epi1*, 1 for *Epi10* and 4 for *CRN8* were digested properly

and generated expected insert sizes. To improve the result, the ligation steps were repeated (Fig. 10b). It was observed that 1 clone for *Avr3a*, 4 for *CRN8* and 1 for *Epi10* were clearly digested using the enzymes *Kpn*I and *Cla*I and found to contain inserts of the expected sizes. However, on the basis of the observed fragments on gels pTS1, pTS2, PTS3 and pTS4 were digested by *KpnI/Nde*I and the gene cassettes were cloned into pSpiro-PAC-3XHA-C (Jerlström-Hultqvist, unpublished) generating the constructs pLD1, pLD2, pLD3 and pLD4.

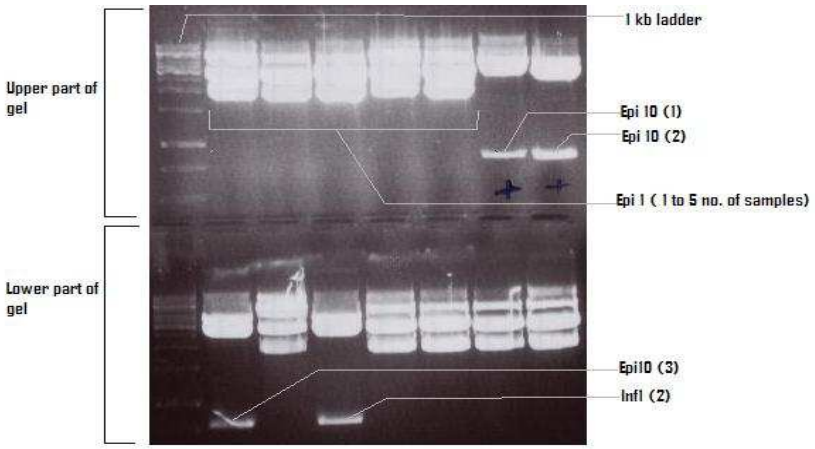

Fig. 11. Gel picture after digestion of pLD1, pLD2, pLD3 and pLD4 plasmid DNA with *Kpn*I *and Nde*I. Size marker is GeneRuler™ 1kb DNA ladder, Fermentas). The clone numbers (1 to 5) are marked in the figure.

After restriction digests of the new constructs, expected fragment sizes were obtained for the clones *Epi10* (1)*, Epi10* (2)*, Epi10* (3)*, Infl* (2) and *CRN8* (3) (Fig. 11). One clone for each promoter was selected for sequencing. The sequencing result for *CRN8* and *Epi1* sequences was not good enough and it was difficult to analyze the cloned sequences. Possibly there was some impurity so that the sequencing reaction did not work properly. However, the result was much better for *Epi10* (Epi10-GFP-3´). In total 959 nt (nucleotides) were sequenced. The length of the constructed vector was 4067 bp whereas the included *Epi10* promoter was 915 bp. However, BLAST searches at NCBI revealed high sequence identities to various vector sequences, for example the *Giardia* integration vector pc-CycB-3HA-BSR was found at nt 125-755. This sequence was matched with the pSPIROBSR-3xHA of its nt 3251-3884. Maximum identity was 99% and the gene in the *Giardia* vector was *Giardia* cyclin B. However, no insert was present from *Phytophthora* DNA. The electropherogram had sharp and clear peaks without any background noise. For *CRN8* and *Epi1*, the peaks of the electropherogram were overlapping and

sometimes unevenly spaced because of secondary structures which is common in the case of GC-rich areas. Moreover, a low signal and with a high background suggest that the sequencing reaction did not work and it may be because of poor template quality or low DNA concentration. Nevertheless, it was decided to proceed with one clone each for promoter constructs of *Epi10, CRN8* and *Avr3a* (promoter-GFP-3') and ligation into the binary vector pPZP-200. Test digestion of the resulting constructs pJP2 and pJP4 using the enzymes *Kpn*I and *Hind*III, did not give the expected bands (Fig. 12a). However, in the case of *Avr3a*, an *Eco*RI/*Pst*I double digestion of the constructs pJP5 generated the expected fragments after increasing the digestion time to 2.2 hrs (Fig. 12b). The expected Avr3a construct (promoter-GFP-3') was 1873 bp in length.

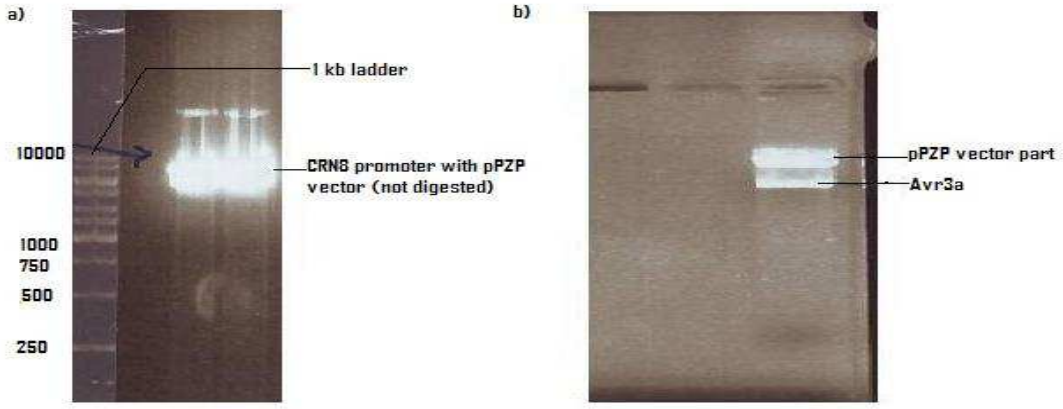

Fig. 12. Test digestion of promoter constructs in the vector pPZP (pJP2 and pJP5) a) *CRN8* promoter construct (incomplete digestion of pJP2) b) *Avr3a* promoter construct (pJP5) from the plasmid DNA of pPZP (complete digestion of *Avr3a* promoter construct). The size marker is GeneRuler™ 1kb DNA ladder, Fermentas.

The pJP5 plasmid DNA was transformed into *Agrobacterium.* The plates were checked regularly but colonies did not appear although they should grow within 2-3 days after transformation.

Discussion

The aim of this study was to develop a transformation procedure for GFP tagging of *P. infestans* effector promoters *in planta* in order to monitor their activity under laboratory conditions, something that would facilitate future host-pathogen interaction and gene disruption experiments. The required promoter sequences were amplified using the designed primers. At the first stage of cloning, deletion of the *ham34* promoter from pTOR-eGFP was confirmed by visualizing its fragment size by agarose gel electrophoresis. Test digestion of the five effector promoter constructs pTS1, pTS2, pTS3, pTS4 and pTS5 yielded the expected fragments for a number of clones as analyzed. However in the second step, when the promoter constructs had been ligated into the vector pSpiro-PAC-3XHA-C, sequencing did not show the expected result. The electropherogram for pTS1 and pTS4 represented noisy signals, which indicate the presence of contaminating DNA (the presence of two or more DNA templates in the reaction mixture). Insufficient sample denaturation before electrophoresis or enzyme slippage occurrence in homopolymer regions (Tamas *et al.,* 2008) can also be considerable factors. Most likely the construct (promoter-GFP -3' sequence) was not ligated properly with the pSpiro-PAC-3XHA-C vector DNA. In the case of pLD2, the electropherogram signal was good but the 634 nt sequence was matching the vector sequence and no insert sequence was observed. There may have been several reasons for not getting predicted sequencing result. For example, proper DNA quantification of the samples is important before submitting them for sequencing since failing to quantify DNA may lead to poor sequencing results. Low amounts of template result in poor signals that in turn direct poor base calling or lead to short sequence reads (Kircher *et al.,* 2011).

In order to increase the possibility of obtaining the intended promoter sequences, more *E. coli* colonies could have been screened, followed by test digestions of the new plasmid DNA to make sure that the ligation has worked. In this study, when the selected gene cassettes had been transferred into the binary vector pPZP-200, the test digestion showed positive results for Avr3a but sequencing indicated the presence of contaminations.

In the first step of the cloning procedure, the *ham34* promoter was to be digested out of the pTOR-eGFP vector. Incubating the plasmid restriction digest for at sufficient amount of time for

the *ham34* fragment to be removed from the vector is of highest importance (Sambrook *et al.* 1989).

A. tumefaciens mediated transformation was attempted for the Avr3a construct. The transformation is potentially efficient and a few hundred transformants can easily be obtained in a single transformation analysis (Vijn and Govers, 2003). When the Avr3a construct was transferred into *A. tumefaciens* under the selection of three antibiotics, colonies were not observed within 3 days. It is important to make sure that *E. coli* and *A. tumefaciens* plates are incubated at the appropriate temperatures.

There are several possible explanations for un-optimal ligation reaction conditions. In theory many factors are important to consider when designing gene constructs: confirmation of genomic DNA purity, making sure that the restriction enzymes are active under optimal conditions and checking the DNA concentrations before ligation (Sambrook *et al.,* 2001). Checking the ligation reactions on a gel before transformation into *E. coli* is also a good practice. Moreover, long wave UV should be used to visualize PCR products when excising them from agarose gels and the DNA should be exposed for UV for as short time as possible. Using short wave UV is very damaging to DNA (Hollosy, 2002) and may significantly reduce ligation and cloning efficiencies, particularly of DNA fragments with sticky ends. In the case of the promoter sequence restriction digests in this study, however, mid range UV was used to visualize the gel and long range UV was used for gel fragment excision. An additional problem would be subjecting the T4 DNA ligase buffer through repeated freeze-thaw cycles since this would decrease its efficiency (Michelsen, 1995). Proper pipetting of the T4 DNA ligase is also an important factor.

A negative control (ligase reaction without insert) of double digested vector should not generate colonies and would indicate whether the vector was truly cut with both restriction enzymes. In this experiment, no colonies were observed on the control plates after transformation into competent *E. coli* DH5α cells.

The observation from this study is that the primer design for cloning the targeted promoters was

successful. Furthermore, a stable transformation procedure has been examined. The experiment allows working on further research; if the cloning had worked properly then the promoter cassette would have been transferred into *Agrobacterium tumefaciens* (C58) followed by *Agrobacterium*-mediated potato transformation. Florescence microscopy would then have been used to test the transformants for *GFP* florescence (Samils *et al.,* 2006).

Conclusions

In this study, five effector promoters were selected to monitor their activity via expression of *GFP*. The reporter gene was selected due to its fluorescence capacity in living plant cells. Successful PCR amplification of the promoter sequences using the selected primers was confirmed by gel separation. The cloning procedure contained a number of steps where each step was monitored via restriction and gel electrophoresis, followed by sequencing analysis. The latter revealed poor alignments to expected gene sequences. Hypothetical possibilities influenced on sequencing results are incomplete restriction, contamination of vector DNA in fragment DNA cut out from the gels, un-optimal ligation or enzyme conditions.

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