Functional characterization of the *Phytophthora infestans* effector Scr74 in potato

MSc Major Thesis

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Abbreviation and Acronyms

ATTA - *Agrobacterium tumefaciens* transient assay

AVR - Avirulence

ETI - Effector Triggered Immunity

ETS - Effector Triggered Susceptibility

HR - Hypersensitive Response

NB - LRR- Nucleotide Binding Leucine Rich Repeats

PAMP - Pathogen Associated Molecular Patterns

PRRs - Pattern Recognition Receptors

PTI - PAMP Triggered Immunity

PVX - Potato Virus X

R gene - Resistance Gene

RLK - Receptor Like-Kinase

RLP - Receptor Like-Proteins

Scr74 - 74-amino acid secreted cysteine-rich protein with similarity to the *Phytophthora cactorum* phytotoxin PcF

Abstract

Potato is one of the most important food sources for the world population. However, potato production is hampered due to biotic and abiotic stresses. *Phythophthora infestans,* an *oomycete* causing the late blight disease, is most destructive pathogen in potato production. To colonize the host plant, the pathogen secretes effector proteins into the extracellular and cytoplasmic sites of the host cell. One of the *P. infestans* effectors secreted into the apoplast is the small cysteine-rich (Scr74) but the function of this effector during the infection process is not well studied yet. Therefore, in this study, we aimed to elucidate the role of Scr74 on the virulence level of *P. infestans* when infecting known susceptible potato cultivars. Moreover, we have examined the response of wild potato species against Scr74 effector protein. Scr74 variants, Scr74-B3b and Scr74-G1, were assayed with protein infiltration to investigate the response of wild potato species to the effectors. In addition, disease assay was conducted on detached leaves of potato cultivars Désirée and Bintje, and *N. benthamiana* to examine the extent of virulence of *P. infestans* due to Scr74 effector protein infiltration. We found that wild potato species *Solanum microdontum* subsp. *gigantophyllum* (GIG362-6), *Solanum verrucosum* (VER910-5) and *Solunum hougassi* (HCB353-8) showed a cell death response against the Scr74 effector variants indicating that the effector can trigger immunity of the host plant. Moreover, the disease assay on cv. Désirée result revealed a larger lesion area of Scr74-G1 treated spots of the leaf compared to the positive control $AVR3a^{KI}$ and negative control empty vector. Therefore, Scr74-G1 contributes to the virulence of *P. infestans*. Further virulence assay should be conducted on large number potato cultivars to infer on virulence role of Scr74 during the infection process.

Key words: *Phytophthora infestans*, apoplastic effector, Scr74, Protein infiltration, disease assay

Introduction

Biology, genetics, and economic importance of potato

Potato (*Solanum tuberosum L.*) is believed to be domesticated and widely cultivated 1800 years back in South America by the Incas (Britannica, 2016). The cultivated potato is extremely heterozygous autotetraploids (2n = 4x = 48), however, landraces of the crop are also still available in South America as diploid (2n = 2x = 24), triploid (3x = 36), or pentaploid (5x = 60) (Andersson and de Vicente, 2010). Potato is grouped under family Solanaceae that includes more than a thousand species. This family contains several known cultivated crops including eggplant (*Solanum melogena*), tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*) and pepper (*Capsicum annuum*) (Kimura & Sinha, 2008). Potato is a perennial but grown as an annual crop in several parts of the world for their tubers. Potato is mainly reproduced by vegetative organs, called tubers (Canadian Food Inspection Agency, 2012).

Potato is produced all over the world in varied seasons across different climatic zones. It was rated as the fourth most important economic crop after maize, rice, and wheat (FAO, 2015). Presently, several improved cultivars are available in the marketplace because the demand and awareness of potato nutrition and health benefits is improving. Nutrition wise, potato is a nutrient dense food which induces a potential in feeding the most food shortage prone areas and the increasing world population. Literature showed potatoes are a major source of energy and proteins comparing to other cereals. For example, the total amount of energy obtained per unit area from potato exceeds 75 % than wheat and 58 % than rice (Stephen Belyea et al., 2010).

Leading potato producing countries are China, India, Russia, Ukraine, and USA with total production of 95.9, 45.3, 30.2, 22.3 and 19.7 million tonnes respectively. From world, total production, nearly to 50% is produced in Asia. In the year 2013, approximately 375 million tonnes of potato tubers were harvested from around 19.2 million ha area (FAOSTAT, 2015). The trend of potato production and demand in developing countries, specifically in Africa, showed a gradual increase since 1992 (FAOSTAT, 2015). Moreover, production of potatoes is stretching economy of countries by utilizing the potato starch and its derivatives as a raw material in textile, paper, and food processing industries.

Challenges

There are a lot of problems arisen together with potato cultivation. Mainly different diseases threaten potato production. Potato is a host of several diseases caused by fungi (e.g fusarium wilt), viruses (e.g potato virus X), bacteria (e.g brown rot), and nematodes (e.g potato cyst nematode). Consequently, disease outbreak has been making significant economic losses on potato production. The Average loss due to disease is estimated to be 25 %, per annum. Actual total losses are estimated to vary from 24 % in Northwest Europe to more than 50 % in some parts of the Central Africa (Oerke, 2005). Besides, potatoes are vulnerable to disease due to their vegetative propagation nature that leads to several infection cycles within and across successive seasons and pathogens can potentially spread out to other areas.

Among potato diseases, the most devastating is late blight disease caused by a fungus like microorganism (oomycete), *Phytophthora infestans* (Nowicki et al., 2012). Late blight contributes to severe crop damage and losses every year estimated to be 5 billion US dollars (Haverkort et al, 2009). In Europe, it was estimated that the annual cost incurred to control and due to damage of the disease reach one billion dollars (Haverkort et al., 2008).

Once *P. infestans* entered inside the leaf, it can damage almost all aboveground parts of the plant, and leads to a complete death within few days. This disease can destroy an entire potato field within a period of 7 to 10 days (Nowicki et al., 2012). World potato production is under great threat due to the occurrence of new and aggressive isolates of *P. infestans*. Infection success of the pathogen is mainly due to its ability to reproduce both sexually and asexually. However, asexually reproduced clones mainly mediate the late blight widespread infection; the spores that helps for areal dispersion (Nowicki et al., 2012).

On the other hand, current breeding strategies for late blight disease resistant varieties of potato rely on identifying of new resistant genes from wild relatives and introgression of resistance genes into susceptible cultivars (Zhu et al., 2012). However, the breeding process is complicated due to the ploidy level (tetraploid) of potato and the ability *P. infestans* to rapidly circumvent resistance. The fact that, *P. infestans* has several effector genes and virulence promoting factors in the repeat rich and gene sparse regions of the genome that enhance its genetic variation (Raffaele et al., 2010).

Host defence mechanisms

A wide range of pathogens relies on host cells as a major source of nutrients. The host cytoplasm and organelles are a place where main molecular interactions are undertaken (Dodds & Rathjen, 2010). Unlike animals, plants lack an adaptive immune system, but they developed means of protection against harmful organisms through a wide array of structural, chemical, and protein-based defences (Freeman & Beattie, 2008). Structurally, the cell wall plays a significant role as a physical barrier that can also incorporate different chemicals activated as soon as non-self-molecules of a potential pathogen are detected by the plant. Specialized structures of a plant such as the epidermis, trichomes, and thorns can be mentioned as the mechanisms of structural defence. Chemically, plants counterpart pathogens through secondary metabolites that belong to one of the three chemical classes; namely terpenoids, phenolics, and alkaloids (Freeman & Beattie, 2008). Furthermore, proteins such as defensins, protease inhibitors and hydrolytic enzymes have a remarkable antagonistic effect to pathogens.

Plants have recognition sites to compensate their inability of the adaptive immune system in both the cell surface and in the cell cytoplasm (Dodds & Rathjen, 2010). Plant innate immunity to infecting pathogen can be summarized in a zig-zag model (Figure 2). The extracellular conserved pathogen elicitors known as pathogen associated molecular patterns (PAMPs) are recognised by the cell surface receptors called pattern recognition receptors (PRRs) (Figure 1; Dodds & Rathjen 2010). Activation of PRRs initiates PAMP triggered immunity (PTI) (Chisholm et al., 2006; Dodds & Rathjen, 2010). It is also termed as a first line of defence or basal immunity of host plants against pathogen molecules. To counteract the plant PTI, the invading pathogen release small molecules called effectors leading to the effector triggered susceptibility (ETS). The second line defence, effector triggered immunity (ETI), is triggered by the recognition of pathogen effectors through intracellular receptors. ETI is mainly mediated by the nucleotide binding leucine rich repeats (NB-LRR) proteins that are encoded by resistance (*R*-) genes (Dangl & Jones, 2001).

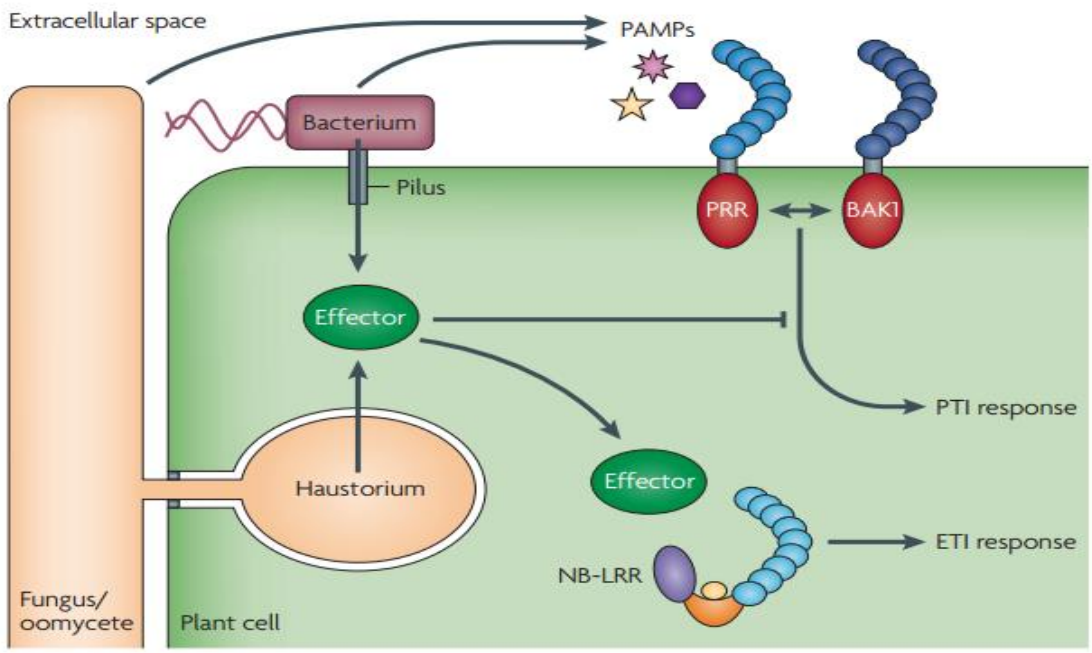

Figure 1. Schematic illustration of host plant defence mechanisms and effector-receptor interactions. Taken from Dodds & Rathjen (2010).

There are often co-evolutionary dynamics in both the pathogen effector and the intracellular receptors that makes ETI unique from PTI. Relatively, the hypersensitive response (HR) or sometimes called localized cell death is much faster and stronger in ETI comparing to PTI. Generally, there are four important phases in the plant immune system which termed as the 'zigzag' model. As per the illustration of figure 2, Phase 1 represents the interaction of PAMPs with the extracellular receptors. It indicates that recognition of PAMPs leads to PTI. Phase 2, shows the suppression of PTI by deploying pathogen effectors to exploit the host plants and finally causes an effector triggered susceptibility (ETS). Consequently, in phase 3, the host plant receptors evolve the recognition apparatus to identify pathogen effectors, which results in effector triggered immunity (ETI). In phase 4, pathogens start to change the form of effectors to overcome the immunity response (HR) of host plants (Jones & Dangl, 2006).

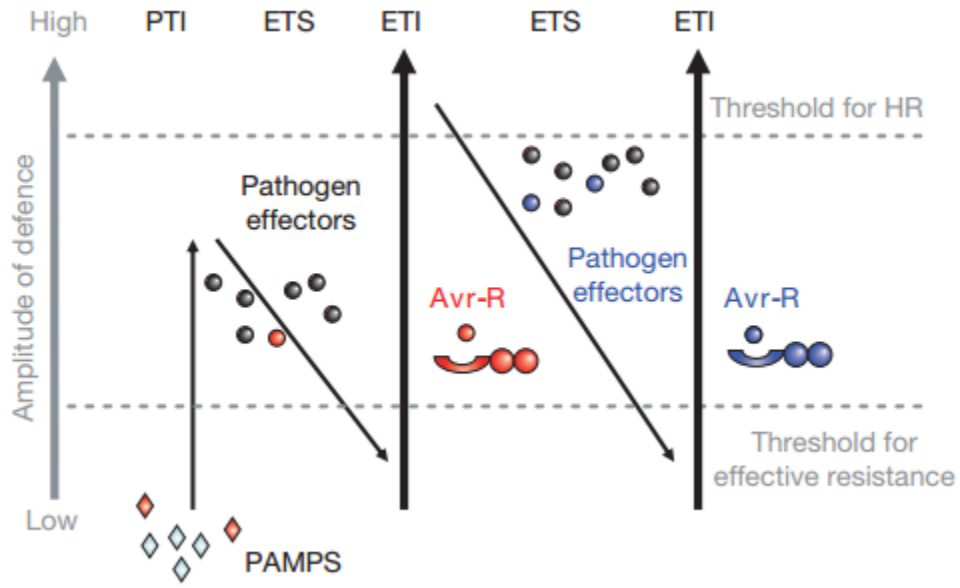

Figure 2. Zigzag model that illustrates the response of host plants to several to apoplastic and cytoplasmic effectors. Pattern triggered immunity (PTI) stands for the immune response of plants towards the pathogen associated molecular patterns (PAMPs) with the help of pattern recognition receptors (PRRs). Effector triggered susceptibility (ETS) explains the malfunctioning of the PRRs which helps pathogens to deliver their effectors at extracellular and cytoplasmic sites. Taken from (Jones & Dangl, 2006).

Pattern recognition receptors (PRRs) and resistance genes against *P. infestans*

Likewise, circumvention of other pathogen attack, plants have evolved complex multiple layers of defence responses against oomycetes (Raaymakers & Van den Ackerveken, 2016). This continuous co-evolutionary fight of plants to avoid colonization of pathogens was clearly described in the "zigzag model" of (Jones & Dangl, 2006). Plants make full use of cell surface receptors to detect extracellular signals of the apoplastic molecules of oomycetes (Raaymakers & Van den Ackerveken, 2016). Commonly, these receptors possess extracellular leucine-rich repeats that play a key role in the recognition of PAMPs. Thus, various sets of constitutive and induced defence responses are initiated. The perception of nonself patterns is mediated by the receptor like kinase (RLK) and receptor like proteins (RLP). RLKs and RLPs are membrane bound signalling molecules that contain an extracellular domain. Unlike to RLK, RLP lacks the intracellular kinase domain which is responsible for the downstream signalling (Wang et al., 2008). RLP works together with known co-receptors such as SOBIR1 and SERK3 RLKs. To date, some oomycete receptors are known in triggering innate immunity system (Hein et al., 2009). For example, the secreted proteins of *Phytophthora infestans* elicitin INF1 and necrosis and ethylene-inducing peptide 1 (Nep1)-Like proteins (NLPs) are recognised by the elicitin response (ELR) (Vleeshouwers et al. 2014, Du et al. 2015) and receptor like protein 23 (RLP23) (Albert et al.,

2015), respectively. Both ELR and RLP23 commonly require the co-receptors SERK3 and SOBIR1 (suppressor of BIRI 1) for signalling.

Extracellular effectors of oomycetes

There are some broadly known oomycete apoplastic effectors. These include enzyme inhibitors, small cysteine rich proteins, Nep1-like (NLP) family, GP42 (PEP13) Transglutaminase and cellulose Binding elicitor and Lectin-like (CBEL) (Kamoun, 2006, Summuna B. and Shahnaz E., 2014). The endoglucanase inhibitors GIP1 and GIP2 of *Phytophthora sojae* (Rose et al., 2002), serine protease inhibitors of kazal family EPI1 and EPI10 of *P. infestans* (Rawlings et al., 2007), and cysteine protease inhibitors EPIC1 and EPIC2 of *P. infestans* (Rawlings et al., 2007) are the ones which act as enzyme inhibitors (Kamoun, 2006). Small cysteine rich proteins are secreted proteins which encode less than 150 amino acids with an even number of cysteine residues (Kamoun, 2006). It includes INF1 elicitin (Kamoun et al., 1997), INF2A and INF2B elicitins (Kamoun et al., 1997) which serve as sterol scavengers (nutrient uptake), PcF (*Phytophthora cactorum-Fragaria*) (Orsomando et al., 2001), and PcF like Scr74 and Scr91 (Bos et al., 2003). Moreover, Wawra et al. (2012) categorised oomycete apoplastic effectors as, effectors acting protection against host defences and effectors acting invasion of hosts. The extracellular effectors that mediate protection involve protease inhibitors and glucanase inhibitors. For instance, EPI1 and EPI10 are among the extracellular effectors of *P. infestans* that inhibit tomato subtilisin-like protease P69B (Tian & Kamoun, 2005). In contrast, effectors mediating invasion involve several hydrolytic proteins on the extracellular space. For example, *P. infestans* secrete glycosyl hydrolases to degrade the cell wall components for an easier entrance into the host tissue (McLeod et al., 2003). Besides, small cysteine rich proteins play an important role in enhancing invasion of host cells by deploying toxins. The toxins released by pathogens to degrade host cell wall trigger innate immune system of plants. PcF are among the toxins that triggers necrosis and induces production of the defence enzyme phenylalanine ammonia lyase in the host (Orsomando et al., 2001).

P. infestans small cysteine-rich (Scr74) effectors

PcF-like Scr74 protein is a member of highly polymorphic 21 gene family of *P. infestans* (Figure 3). It was first identified from *P. cactorum*. It has a little variance in the signal peptide, much of the variance is found in the mature Scr74 protein of different isolates of *P. infestans*.

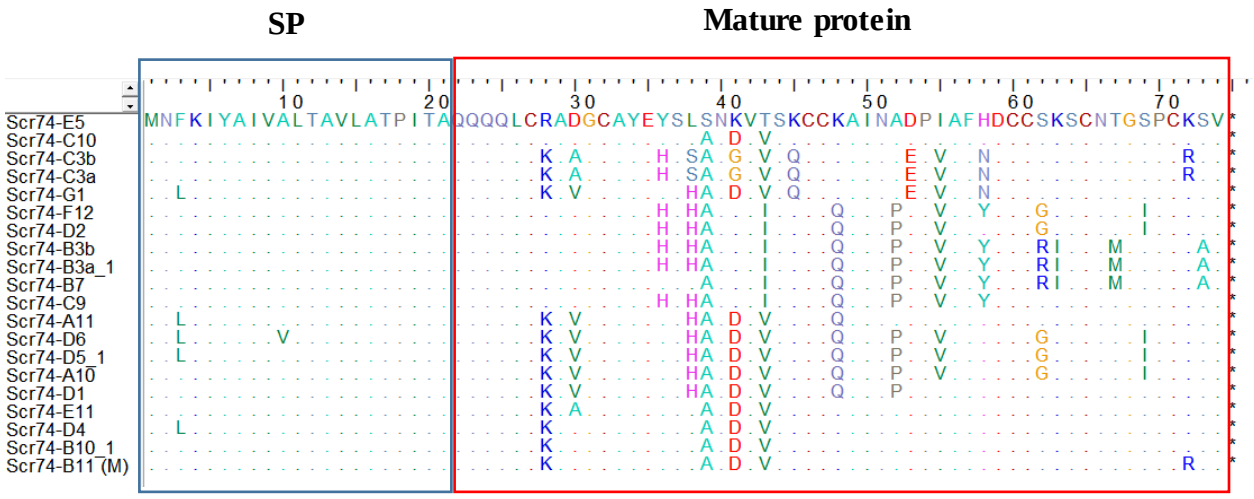

Figure 3. Conserved and polymorphic regions from twenty-one Scr74 homologs which determine the specific amino acids identified in previous study (Kassa Bittew 2014, Master Thesis).

The Scr74 allele variants are found in different copy numbers between different isolates (Figure 4). The Scr74, which is under highly diversified selection, has 74 secreted cysteine rich amino acids and 8 conserved cysteine residues in the mature protein. The expression of Scr74, which, is remarkably upregulated during the early colonization of potato (Liu et al., 2005) able to induce necrosis in tomato and strawberry (Orsomando et al., 2001). Nevertheless, the function of Scr74 effector is not clearly known.

Scattering of Scr74 variants along *P.infestans* isolates

P. infestans isolates	Scr74 variants																					
	C3[b]	B11*	C4_1	C10	B11_1*	E6_a1[c]	B3a_1a[b]	E6_1	C4[c]	B10_1[b]	B3a_2[b]	B3a_1[b]	C4_2[c]	C4_3[c]	B3a_1b[b]	D5_1a[d]	E5	C4_4[c]	H1	C3a	D5_2a[d]	D5_3a[d]
90128										×												
89148-09									×													
EC1	×																					
H3OPO4		×																				
IPO-0			×	×	×																	
IPO-C											×	×	×	×								
Katshaar																						
PIC99177							×		×													
PIC99183															×	×						
PIC99189																	×	×				
UK3928-A																			×	×	×	×
UK7824						×																

Figure 4. Variants of Scr74 effectors found at distinctive *P. infestans* isolates for which some of the isolates contain more than one duplicate of the allele. (Kassa Bitew 2014-MSc thesis and Liu et al. 2005).

The general aim of this thesis was to understand the role of Scr74 effector on host colonization of *P. infestans*. In this work, we studied the role of Scr74 variants on the virulence of *P. infestans* in different potato cultivars. We also investigated the recognition of Scr74 on different wild *Solanum* species to spot the defence reactions.

Research questions

Does the effector Scr74 influence *Phytophthora infestans* virulence in potato cultivars?

Do wild *Solanum* species show defence responses upon protein infiltration of Scr74?

Material and Methods

Plant Material and propagation

Wild *Solanum* species and potato cultivars were used in this study (Table 1). Stock explants from previous experiment was used as a source of *in vitro* plant material. Murashige and Skoog (MS20) media containing 20 g/L of sucrose, 4.4 g/L of MS salts composed of vitamins and 8 g/L of micro agar adjusted to pH 5.8 was utilised to supplement *in vitro* grown plants with the required nutrients (Murashige and Skoog 1962). Plants were kept in a climate room set to 24ºC temperature and a 16h/8h light/dark light regime. Sub culturing took place from the stock plants maintained in the climate room. Two-week old *in vitro* grown plants, with well-developed shoots and roots, were transferred to the greenhouse and planted in standard soil. The greenhouse conditions were kept under conditions 24 ºC/18 ºC, 16h/8h light/dark regime and 75 % relative humidity until the plants get ready for the entire experiment.

Table 1. List of wild *Solanum* genotypes and cultivars used to detect the recognition of Scr74 effectors also degree for virulence of *P. infestans*together with Scr74 effectors.

Solanum species	CBSG clone number	Effector family	Recognized apoplastic effector members (based on previous PVX-agroinfection screenings)
S. microdontum	MCD 360-1	INF1	INF1
S. verrucosum	VER 922-1	Scr74	Scr74-B3b/G1
	VER 989-4	Scr74	Scr74-B3b/G1/A10
	VER 910-5	Scr74	Scr74-G1
S. microdontum subsp. *Gigantophyllum*	GIG 362-6	Scr74	Scr74-B3b
S. hougassi	HOU 654-1	Scr74	Scr74-B3b
	HOU 271-1	Scr74	Scr74-B3b/G1
	HOU 272-1	Scr74	Scr74-G1
	HOU 655-1	Scr74	Scr74-B3b
	HCB 353-8	EPI	EPI1/EPIC2B
Désirée	-	-	-
Bintje	-	-	-

Pathogen growth and maintenance

Ten to fourteen days in advance of the disease assay, *P. infestans* isolates IPO-C and NL07434 were grown in a rye sucrose agar (RSA) medium containing 20 g sucrose, 60 g of rye and 15 g of agar in 1 L distilled water. Spores of the isolates were harvested by rubbing the plates with a sporulating mycelium using 10 mL cold water and transferred into a fresh 10 mL tube. The sporangia were accustomed for incubation on an ice box for 2 hours to induce zoospore formation. The discharge of zoospores was checked through a microscope, three random full squares of the haemocytometer were considered to estimate the final concentration. Consequently, the last volume was set to the concentration of 5×10^4 spores/mL and this spore suspension was used for virulence assay.

A. tumefaciens-mediated transient transformation

To perform agroinfiltration LB and YEB mediums are essential. The LB medium was prepared from 20 g of LB extract (10 g peptone, 10 g NaCl and 5 g yeast) in one litre of MQ water. While, YEB medium was prepared from 5 g beef extract, 5 g bacteriological peptone, 5 g sucrose 1 g yeast extract and 2 mL of 1M $MgSO_4$ in a litre of MQ water. Prior to use, the mediums were autoclaved in 121°C for 20 minutes. For agroinfiltration, appropriate antibiotics and *A. tumefaciens* from glycerol stock were added into 10 mL tubes that contain 3 mL of LB medium. Afterwards, Incubation took place overnight at 28°C shaking at 200 rpm. The day after, 100 µL of LB medium overnight grown inoculum were pipetted and transferred to 50 mL falcon tube that contain 15 mL of YEB medium. The YEB medium was supplemented with 1.5 µL acetosyringone, 150 µL MES buffer (2-(N-morpholino)-ethane sulfonic acid, 1 M) and 15µL of appropriate antibiotics of the plasmids as well as agrobacterium strains used. Subsequently, the inoculum was kept overnight for further growth at 28°C shaking at 200 rpm.

One day later the inoculum was centrifuged at 4000rpm for 10 minutes after its optimal growth is checked. The supernatant was then discarded and the pellet was resuspended gently with 5ml MMA medium. The MMA medium was prepared at the day of infiltration from 20 g sucrose, 5 g MS salts without vitamins, 1.95 g MES, adjusted to pH 5.6 and 1 mL of 200 mM aceteosyringone in one litre of MQ water. Afterwards, incubation took place for one to six hours at room temperature in the dark. Optical density of the inoculum was then measured using BioPhotometer (eppendorf) to adjust the final concentration of the bacteria carrying the effector gene to an OD_{600}

of 0.3. MMA medium was added to fill in the final required volume based on the measured OD_{600}. Finally, the agrobacterium suspensions were infiltrated on the abaxial side of potato as well as *N. benthamiana* leaves using 2.5 mL needleless syringe.

Virulence assays

Infection assays with different *P. infestans* isolates were performed in two susceptible potato cultivars, Bintje and Désirée with two biological repeats each. These assays were conducted on detached leaves one and two days after agroinfiltration of Scr74 effectors (pGWB14-Scr74-G1, pGWB14-Scr74-B3b) and controls (pGWB20 empty vector and pK7WG2-$Avr3a^{KI}$). The lesion size was quantified using a digital calliper as described in Vleeshouwers et al. (1999). In more detail, four fully expanded and well-developed compound leaves for every plant were transiently transformed through *Agrobacterium tumefaciens* mediated transient assay (ATTA). One and two days later, the leaves treated with ATTA were detached from their parent plant. The detached leaf petiole was then plugged into a water soaked florists foam (Oasis) and placed on top of pre-wetted filter paper which spread in a tray. Six detached leaves which contains effectors, positive and negative controls were plugged in both sides of the foam were placed in every tray. A droplet of 10 µL of the sporangia suspension was inoculated on the abaxial side of a comparative spot where the Scr74 effectors are infiltrated and on the leaflets, that are not treated with the effectors. Those trays were then packed by a transparent plastic bag for further incubation in a climate chamber adjusted at 15°C, 16 hours light and 8 hours dark photoperiod. Infection level of the *P. infestans* isolates and effectors were assessed at different time points, four, five and six days after inoculation. The lesion size was measured in two dimensions using a digital calliper. The percentage of infection efficiency was computed by dividing successful infection over the total treated detached leaves. As per the description of Vleeshouwers et al. (1999), the ellipse lesion area was calculated using the formula ($A = 1/4 \times \pi \times \text{length} \times \text{width}$), where the length is the biggest piece of the lesion and the width is the lesion size perpendicular to the length. The data was finally subjected to one-way analysis of variance (ANOVA) using SPSS 23^{th} edition software (2015). The pair wise comparison between means were analysed using fisher LSD ($P \leq 0.05$).

Western Blotting

Protein detection and analysis was executed through the comprehensively referred procedure called western blotting, also known as immunoblotting. To do this technique, initially effector

genes INF1, Scr74 variants B3B and G1 fused to HA tag were transiently expressed in *N. benthamiana* leaves using ATTA. Two days later, the leaves were harvested in liquid nitrogen and stored at -80 °C. For extraction and detection of the target protein, the leaves were homogenized and grinded with pre-chilled mortar and pestle on liquid nitrogen. The powder was then transferred into 50 mL falcon tube and mixed with radio-immunoprecipitation protein extraction buffer (RIPA). It is a modified extraction buffer that contains IGEPAL CA-630 as a detergent and complemented with a protease inhibitors cocktail EDTA-free (Roche) (Liebrand et al., 2012; Liebrand et al., 2013). Two millilitres of RIPA buffer was added to one gram of ground plant material. Afterwards, 1ml of the sample was taken into 2 mL Eppendorf and centrifuged for 20 minutes under 4 °C at 13,000 rpm. To denature, 50 µL of 4×Laemmli loading buffer (BioRad) was added to 150 µL samples at a final concentration of 1× (2.4 mL 1 M Tris, pH 6.8, 0.8 g SDS stock, 4 mL 100 % glycerine, 0.01 % bromophenol blue, 1 mL ß-mercaptoethanol and 2.8 mL water). The samples were boiled for 10 minutes on thermo shaker, which was adjusted at 95 °C. Consequently, 35 µL from each sample were loaded in a TGX 4-20 % precast gradient protein gel (BioRad) and ran for ±25 minutes in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) apparatus which was set at 250V. For SDS-PAGE, the apparatus was filled up with 1× diluted SDS-PAGE buffer (from 10× concentrate; 144 g/L glycine, 30 g/L Tris, and 10 g/L SDS). Western blotting, transfer of proteins from the gel to Polyvinylidene Difluoride (PVDF) membrane, was then performed after SDS-PAGE. Briefly, PVDF membrane was sliced with a same size of the protein gel. The membrane was then pre-wetted in MeOH for activation and helping proteins bind to the blot. Consequently, the pre-wetted PVDF membrane was transferred and soaked together with filter paper, filter pads and gel holder cassette in a tray filled up by 1× diluted transfer buffer supplemented with 100 mL/L EtOH (10×concentrated; 30 g/L Tris and 140 g/L glycine). The gel and blotting membrane were then assembled into a sandwich along with several sheets of filter paper which protect the gel and blotting membrane. The gel sandwich was assembled in the following order; the gel and transfer membrane were stacked by filter papers, filter pads and gel cassette holders on top and bottom side. The blotting was then run up to an hour at 100V. Finally, the membrane was soaked for 30 minutes in a square petri dish that hold 25 mL of TBS-T (12.1 g/L TRIS pH 7.5, 146 g/L NaCl, which was diluted 10x and 0.5 mL Tween-20 included for the final 0.05 % Tween-20 concentration), a blocking solution supplemented with 5 % skimmed milk. Then, the membrane was placed in a 50 mL falcon tube that contain a mix of 5

mL of TBS-T with 5 % skimmed milk plus αHA-HRP antibody diluted 1.5:5000 (Miltenyi Biotech). Later, the membrane was incubated overnight at 4 °C in rotating cylinder for incubation to take place. Afterwards, the membrane was removed to a square petri dish that hold TBS-T buffer and washed 3 times for 5 minutes. Finally, SuperSignal west-Fempto substrate (Thermo-Pierce) was used for chemo-luminescent signal development and placed inside G-BOX (Syngene, USA) to capture the image of western blot.

Effector protein infiltration

Purified effector proteins, Scr74-B3b and Scr74-G1 with HisHA tag, were produced in *Pichia pastoris* yeast strains previously. These effector proteins were used to test their recognition in distinctive wild potato genotypes. The leaves of wild potato species were infiltrated with 1 µM, 5 µM and 10 µM protein concentrations. Briefly, the proteins were diluted into final concentration of 1 µM, 5 µM and 10 µM for every 10 ml of MQ water depending upon their initial concentration. Thereafter, the effector proteins including MQ water as a negative control were infiltrated into the wild potato species using a needleless syringe. Totally, three plants per genotype were used. Two leaves that are not too old or too young of each plant were selected for the protein infiltration. Each effector protein was infiltrated twice in a parallel spot of the same leaf. For observing the reaction of wild potato genotypes, hypersensitive response was scored five days after infiltration. Pictures of the responses were taken on an illuminating white board with florescent light.

Detection of cell death and reactive oxygen species accumulation

Detection of *in situ* hydrogen peroxide, part of reactive oxygen species (ROS), was performed using the 3,3'-diaminobenzidine (DAB) staining method (Daudi & O'Brien, 2012). The presence and level of ROS were exploited based on the intensity of the dark brown colour staining in the leaves. For DAB staining, two weeks old *N. benthamiana* plants were used for agroinfiltration of effector proteins. A total of six plants were used to perform this study, where two middle leaves of every plant were selected for ATTA. The following day, some of the infiltrated leaves were detached and soaked overnight in 1mg DAB per millilitre of PBS solution (NaCL 8 g/L, KCL 0.2 g/L, Na_2HPO_4 1.44 g/L and KH_2PO_4 0.24 g/L pH adjusted to 7.8). Afterwards, bleaching solution (Ethanol: glycerol: acetic acid = 3:1:1) was applied repeatedly to remove the chlorophyll and destain the DAB treated leaves. Finally, pictures of the of DAB stained leaves were captured on a sheer white background under uniform lighting. In addition, cell death response due to the

recognition of the transiently expressed effectors on *N. benthamiana* leaves were scored five days after infiltration (ATTA). Normal images of the leaves were captured under background white light and under UV light.

Results

Recognition of Scr74 effector

In wild potato genotypes

To assess the Scr74 recognition in five wild potato species, purified effector proteins of Scr74 variants, Scr74-G1 and Scr74-B3B, and MQ water as a negative control were tested. Wild potato species (A) *S. microdontum* subsp. *gigantophyllum*, (GIG362-6), (B) *S. microdontum* (MCD360-1) (C) *S. verrucosum* (VER910-5) (D) *S. hougassi* (HOU655-1) and (E) *S.hougassi* (HCB353-8) leaves were infiltrated with the purified effector proteins of Scr74 variants (Scr74-B3B and Scr74-G1) and MQ water twice at a parallel spots of the same leaf. Protein infiltration screening method was employed to investigate the consistency of the results obtained from previous PVX screening on similar wild potato genotypes. In this experiment, we have observed a cell death response against Scr74-B3b in *S. microdontum* subsp. *gigantophyllum* (GIG362-6), while the *S. verrucosum* (VER910-5) and *S. hougassi* (HCB353-8) respond to Scr74-G1 infiltration (Figure 5). Besides, a cell death response against MQ infiltration was shown in *S. microdontum* subsp. *gigantophyllum* (Figure 5).

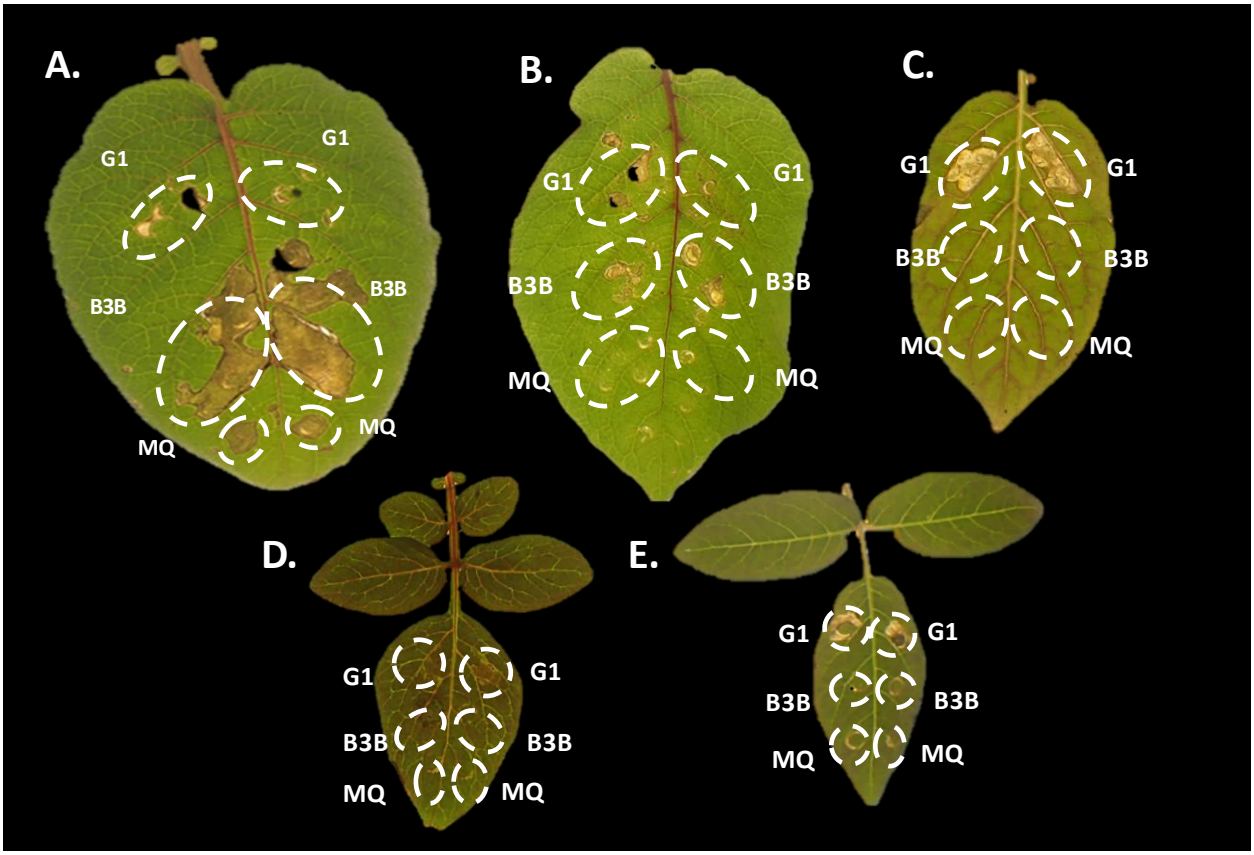

Figure 5. Scr74-G1 and Scr74-B3b protein infiltration in wild Solanum sp. Leaves of 6-week-old Solanum *S. microdontum* subsp. *gigantophyllum*, *S. microdontum*, *S. verrucosum*, *S. hougassi* and *S. hougassi* species were infiltrated with a 10µM solution of purified Scr74-G1, Scr74-B3b proteins of MQ water. Photos were taken at 5 days post infiltration. Where, (A) *S. microdontum* subsp. *gigantophyllum* (GIG362-6), (B) *S. microdontum* (MCD360-1), (C) *S. verrucosum* (VER910-5), (D) *S. hougassi* (HOU655-1) and (E) *S.hougassi* (HCB353-8)

Furthermore, the results from the protein infiltration assays were summarised and compared with previously obtained PVX-agroinfection results using the same effector proteins and the same genotypes (Table 2). Consistent with the result from protein infiltration screening, in PVX screening *S. verrucosum* showed a cell death response against Scr74-G1 whereas *S. microdontum* subsp. *gigantophyllum* respond to Scr74-B3b infiltration.

Table 2. Responses of wild potato species to *P. infestans* effector proteins through protein infiltration and PVX (potato virus X) screening methods

Genotype	Protein infiltration screening			PVX screening	
	Scr74-G1	Scr74-B3b	MQ	Scr74-G1	Scr74-B3b
GIG362-6	-	+	+	-	+
MCD360-1	-	-	-	-	-
VER910-5	+	-	-	+	-
HOU655-1	-	-	-	-	+
HCB353-8	+	-	-	-	-

N. benthamiana response to Scr74 variants

N. benthamiana plants, suitable for agroinfiltration, were used to study the response against Scr74 variants, Scr74-G1 and Scr74-B3B. Constructs carrying C'-HA-tagged Scr74-G1 and Scr74-B3b (pGWB14-Scr74-G1-3HA, pGWB14-Scr74-B3b-3HA) were previously transformed into *A. tumefaciens* strain AGL1. The extent of cell death due to the Scr74 variants was compared with the controls N' or C'-HA-tagged INF1 (pK7WG2-PR1Sp2HA-INF1 or pGWB14-INF1-3HA) or pGWB14 empty vector (EV). The INF1 was considered as a positive control because of its cell death inducing activity when infiltrated in *N. benthamiana*. Compared to the INF1-3HA (unable to induce cell death; Emmanouil Domazakis, personal communication) or EV treatments. At five days after agroinfiltration, the PR1Sp2HA-INF1 infiltration resulted in strong cell death response in *N. benthamiana* plants (Figure 6). The normal and UV light images support the visual level of cell death due to the tested effectors. Similarly, the DAB staining method also confirmed that the PR1Sp-2HA-INF1 clone has a strong cell death response and followed by INF1-3HA clone. However, *N. benthamiana* plant did not show any response to Scr74-G1 and Scr74-B3B as well as the empty vector. The lack of response to Scr74-G1 and Scr74-B3B infiltration demonstrates the absence of a receptor in *N. benthamiana* plants that can detect the Scr74 effector proteins of *P. infestans*.

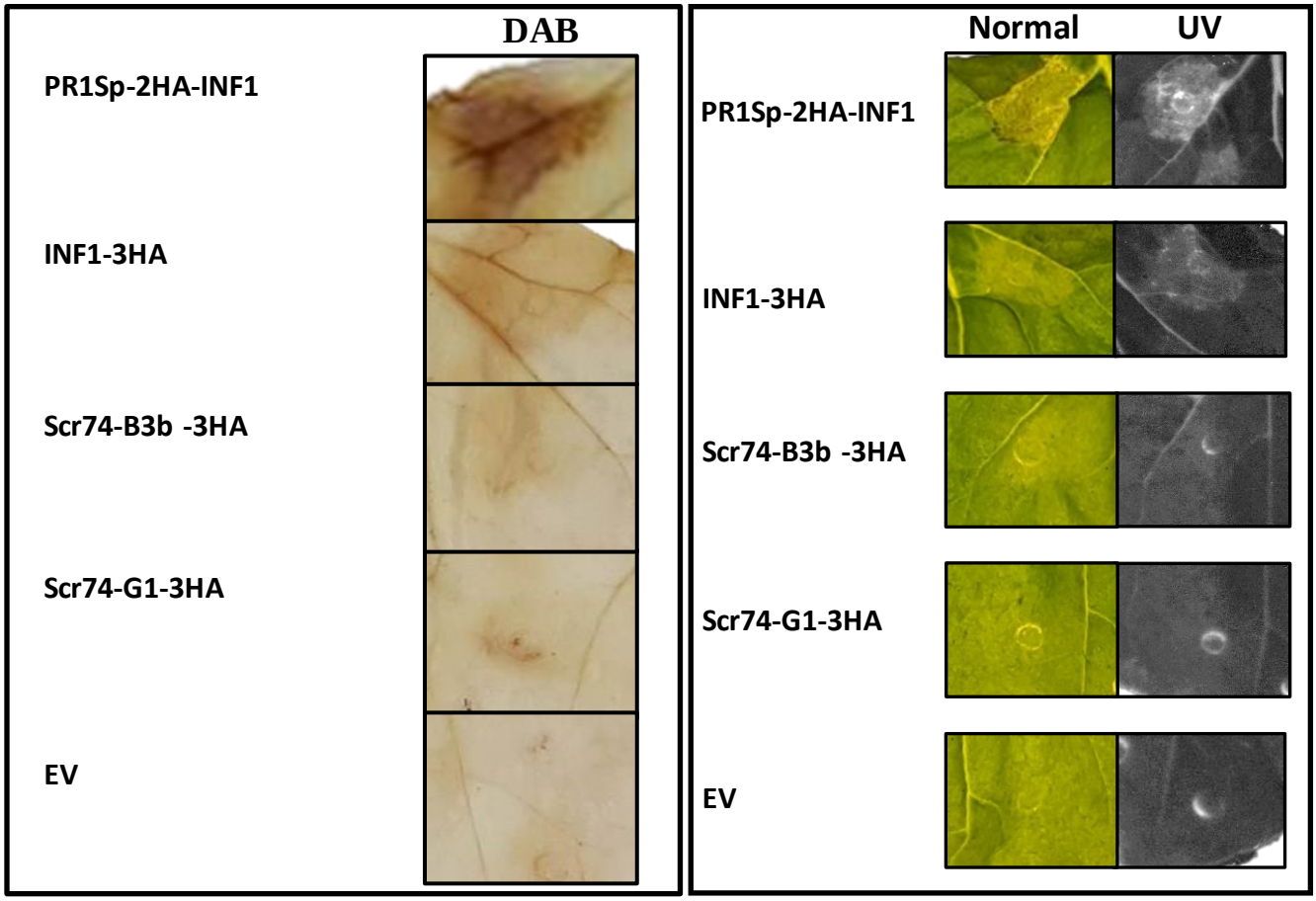

Figure 6. Scr74-G1, Scr74-B3b, PR1Sp-2HA-INF1, INF1-3HA and EV agroinfiltration in two weeks old *N. bentamiana* leaves. Photos were taken at 5 days after ATTA. The left column image displays the picture of DAB stained *N. bentamiana* leaves 5 days after ATTA. The right column are normal pictures taken under florescent and UV light 2 days after ATTA.

Virulence level of *P. infestans* strains

Effect of Scr74 on *P. infestans* virulence during infection of potato

Virulence assays were performed to determine whether Scr74 protein variants, Scr74-G1 and Scr74-B3B, play a role in the virulence of *P. infestans* during potato infection. Two isolates, NL07434 and IPO-C were used in this virulence assay. Potato cultivars, Désirée and Bintje, that are susceptible to *P. infestans* were used for this experiment. The virulence assay performed using IPO-C isolate were conducted twice (with two biological repeats). While, the virulence assay of NL07434 isolate was performed only once. For virulence assay, pGWB14-Scr74-G1-3HA and pGWB14-Scr74-B3B-3HA, Avr3a^{KI} (pK7WG2-Avr3a^{KI}) and EV (pGWB14) were agroinfiltrated in every four middle leaves of 10 plants of each cultivars. Afterwards, leaves of the cv. Désirée were detached two days after ATTA in the first virulence assay and one day later in the second biological repeat. Whereas, in cv. Bintje, the leaves were detached one day after ATTA for *P.*

infestans inoculation. It was performed using the isolates mentioned above. First, the presence of Scr74 effector proteins was examined using western blotting, but only Scr74-G1 was detected with an approximate size of 10 kDa (Figure 7). Since, we could not detect Scr74- B3b construct in the western blotting analysis, only plants infiltrated with construct Scr74-G1 were considered in the virulence assay.

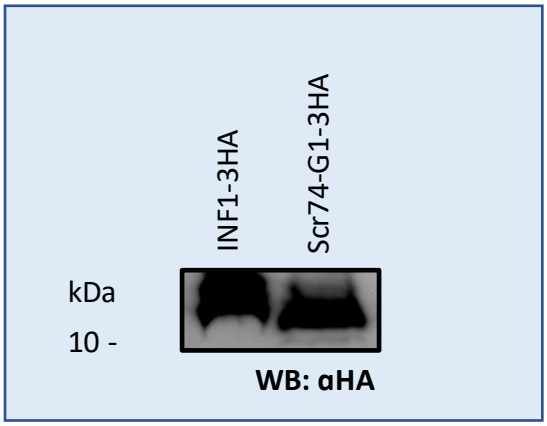

Figure 7. Western-blot analysis of protein extracts from *N. benthamiana* leaf cells transiently expressing INF1-3HA and Scr74-G1-3HA. An anti-HA antibody was used for detection; sizes of protein markers are given in kDa.

Transient expression of Scr74-G1 in detached potato leaves of cv. Désirée significantly increased (LSD $P \leq 0.05$ Annex 1) *P. infestans* lesion growth compared to the positive and negative controls at 5 and 6 days post inoculation (Figure 8A). While, significant difference in lesion growth between the negative control and Scr74-G1 was only observed at 4 dpi (Figure 8A). This implies that the Scr74 enhances the virulence of *P. infestans* isolate IPO-C. In addition, the infection efficiency of Scr74-G1 (100 %) was higher than in the positive control Avr3a ^{KI} (55.6 %, 66.7 % & 77 %) and negative control empty vector (EV) (66.7 %, 88.9 % & 88.9 %) at 4, 5 and 6 days post inoculation (Figure 8B). However, in the second biological repeat there was no statistical difference among the treatments (Figure 8C). Despite of the differences in infection efficiency (Figure 8B and D), the treatments showed similar pattern of infection in both biological repeats (Figures 8A & C). The quantitative data from the first biological repeat support the phenotypic observation where the lesion size was larger in Scr74-G1 than in Avr3a^{KI} and EV, indicating possible role of the effector protein in enhancing virulence of *P. infestans* IPO-C isolate.

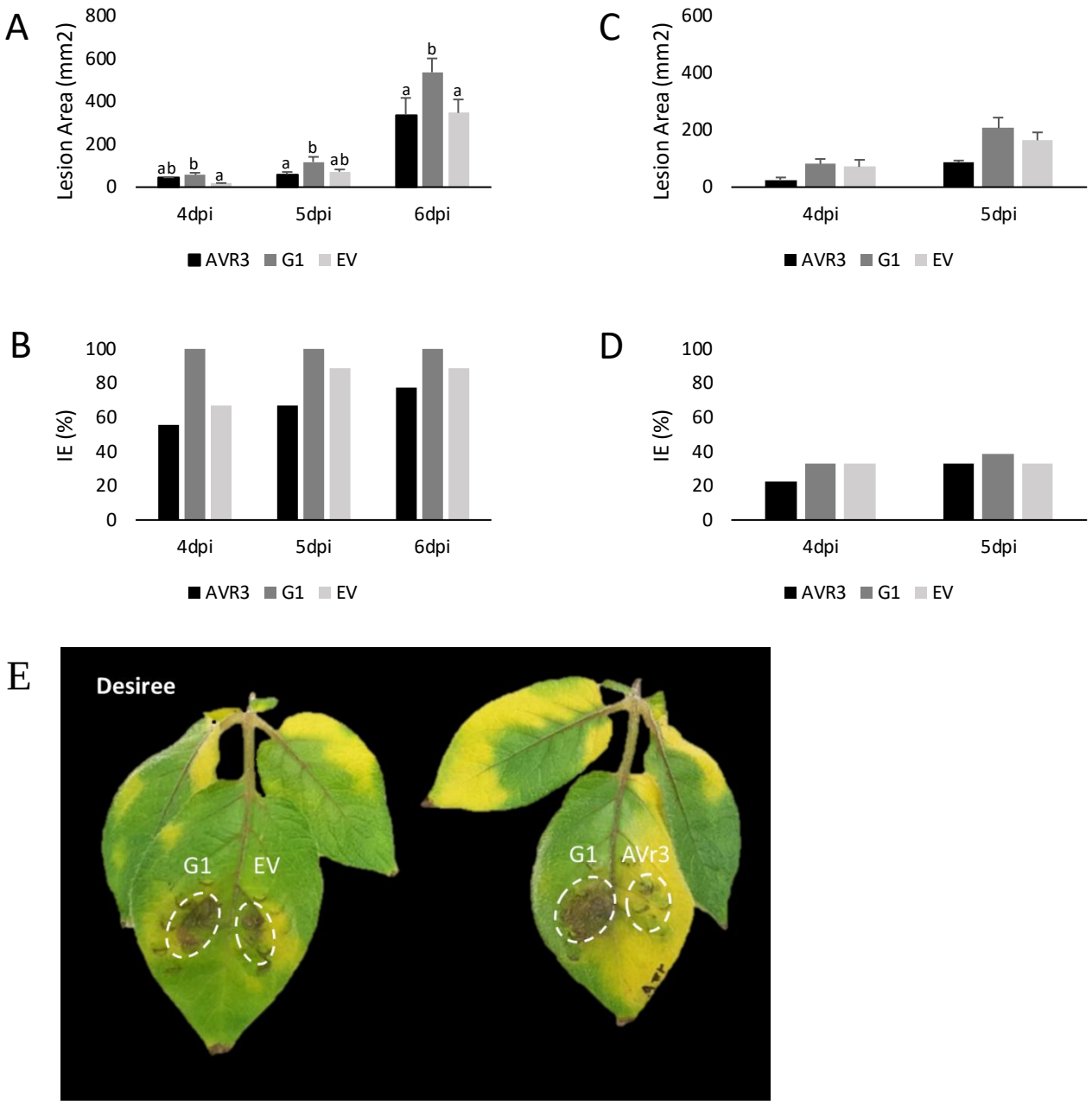

Figure 8. Virulence assay of *Phytophthora infestans* strain IPO-C on detached leaves of potato cv. Désirée upon transient Scr74-G1 expression. (A) Average lesion area of leaf agroinfiltrated with Scr74-G1-3HA after 4, 5 and 6 dpi of first biological repeat. Error bar represents standard error of the mean (n = 1). (B) Percentage of infection efficiency up on Scr74-G1 treatment 4, 5 and 6 days after inoculation. (C) Average lesion area of leaf agroinfiltrated with Scr74-G1 variant 4 and 5 dpi of second biological repeat. Error bar represents standard error of the mean (n = 1). (D) Percentage of infection efficiency upon Scr74-G1 treatment 4 and 5 dpi. (E) Image of cv. Désirée detached leaf agro infiltrated with Scr74-G1, Avr3a^{KI} and empty vector (EV) that are also inoculated with IPO-C isolate of *P. infestans*. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Fisher's LSD test; letters a, b and c represent P≤0.05.

Likewise, we conducted the same experiment on potato cultivar Bintje. However, in the first and second biological repeats, the *P. infestans* lesion area was not statistically different (P≥0.05)

among the effector treatments and the negative control (Figure 9). Generally, the infection efficiency of *P. infestans* IPO-C strain after introduction of the effectors into Bintje was lower than in cultivar Désirée. Briefly, in the first virulence assay experiment the infection efficiency of Scr74-G1 was greater than 60% (Figure 9B). This infection efficiency was higher than the Avr3a^{KI} and empty vector. However, the infection efficiency due to Scr74-G1 in the second virulence assay was less than in the first virulence assay (Figure 9D). In the second virulence assay, at 6dpi, the lesion area in Avr3a^{KI} treated plants was statically larger than the lesion area on plants treated with Scr74-G1 ($p \leq 0.05$) or EV ($p \leq 0.05$) (Figure 9C) (Appendix B). There was no statistically significant lesion area difference between EV and Scr74-G1 (Figure 9C).

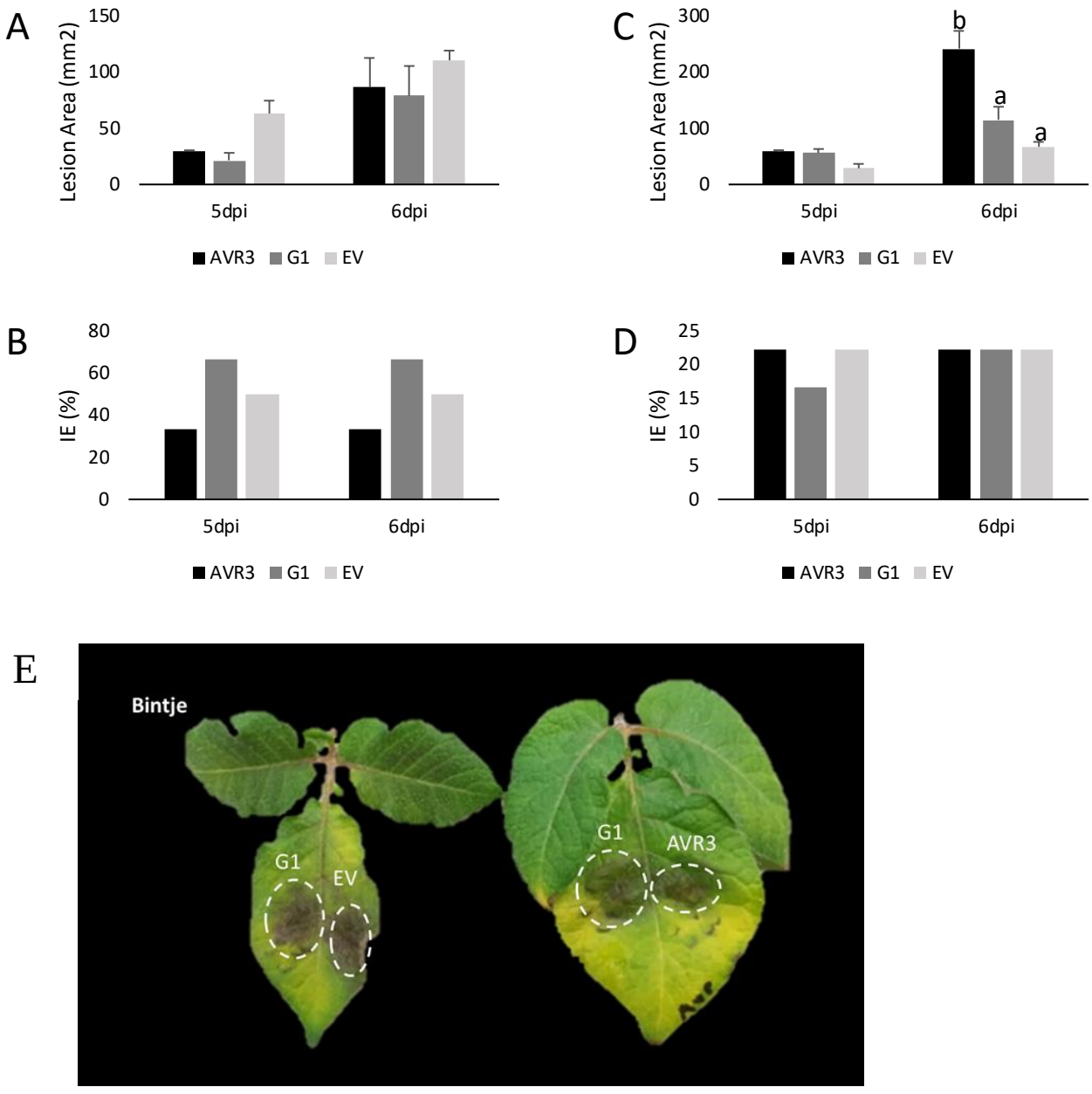

Figure 9. Virulence assay of *Phytophthora infestans* strain IPO-C on detached leaves of potato cv. Bintje upon transient Scr74-G1 expression. (A) Average lesion area of leaf infiltrated with Scr74-G1 after 5 and 6 days post infiltration (dpi) of first biological repeat. Error bar represents standard error of the mean. (B) Percentage of infection efficiency up on Scr74-G1 treatment 5 and 6 days after inoculation. (C) Average lesion area of leaf infiltrated with Scr74-G1 variant 5 and 6 dpi of second biological repeat. Error bar represents standard error of the mean. (D) Percentage of infection efficiency up on Scr74-G1 treatment 5 and 6 dpi. (E) Image of cv. Bintje detached leaf agroinfiltrated with Scr74-G1, $Avr3a^{KI}$ and empty vector (EV) that are also inoculated with IPO-C isolate of *P. infestans*. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Fisher's LSD test; letters a, b and c represent $P \leq 0.05$.

To further investigate if there is a role of Scr74 in *P. infestans* virulence, we tested another, more virulent, isolate NL07434. As illustrated in Figure 10, a virulence assay on the potato cultivars Bintje and Désirée using NL07434 strain of *P. infestans* was performed to understand the effects of effectors Avr3a^{KI} and Scr74-G1 on the pathogen's virulence. The lesion area from the Scr74-G1 treated Désirée plants was not significantly different to the lesion in Avr3a^{KI} and EV treatments (Figure 10A). The infection efficiency was 100% in all leaves that are treated by Avr3a^{KI}, Scr74-G1 and EV at 4 days after inoculation (Figure 10B). The Bintje leaves treated with Scr74-G1 showed a statistically different lesion area compared to the leaves treated with Avr3a^{KI} at five days after inoculation (Figure 10D). Meanwhile, the infection efficiency of strain NL07434 reached 100% right after four days post to infection.

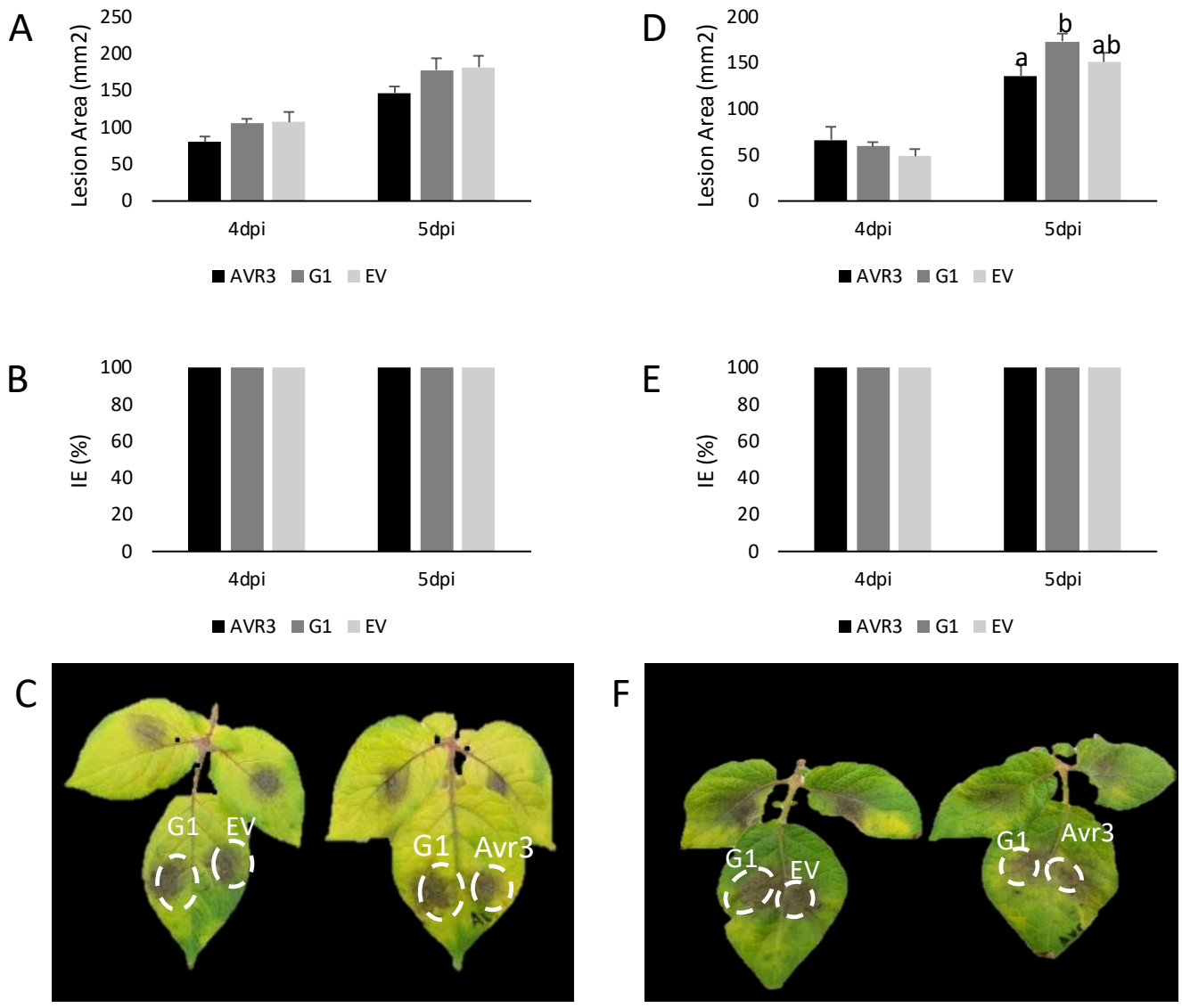

Figure 10. Virulence assay of *Phytophthora infestans* strain NL07434 on detached leaves of potato cv. Bintje upon transient Scr74-G1 expression. (A) Average lesion area of detached leaf of cv. Désirée agroinfiltrated with Scr74-G1-3HA at 4 and 5 days post inoculation (dpi). Error bar represents standard error of the mean. (B) Percentage of infection efficiency up on Scr74-G1 treatment 4 and 5 dpi. (C) Image of cultivar Désirée detached leaf agroinfiltrated with Scr74-G1, Avr3a^{KI} and empty vector (EV) and inoculated with NL07434 isolate of *P. infestans*. (D) Average lesion area of cv. Bintje leaf agroinfiltrated with Scr74-G1 variant 4 and 5 dpi. Error bar represents standard error of the mean. (E) Percentage of infection efficiency up on Scr74-G1 treatment 4 and 5 dpi. (F) Image of cv. Bintje detached leaf agroinfiltrated with Scr74-G1, Avr3a^{KI} and empty vector (EV) that are also inoculated with NL07434 isolate of *P. infestans*. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Fisher's LSD test; letters a, b and c represents $P \leq 0.05$.

Virulence assay on *N. benthamiana*

To further understand the extent of virulence efficiency upon transient expression of Scr74 variants, we performed disease assay on *N. benthamiana* leaves using IPO-C strain of *P. infestans*. Western blot analysis was used to detect the transient expression of a newly cloned Scr74-B3b

effector protein which was not detected at the beginning of our virulence study (Figure 7). The newly cloned C-terminal HA-tagged Scr74-B3b-3HA effector protein was found stable, similar to Scr74-G1-3HA (Figure 11). Afterwards, we proceed with the disease assay on *N. benthamiana* detached leaves as described for potato.

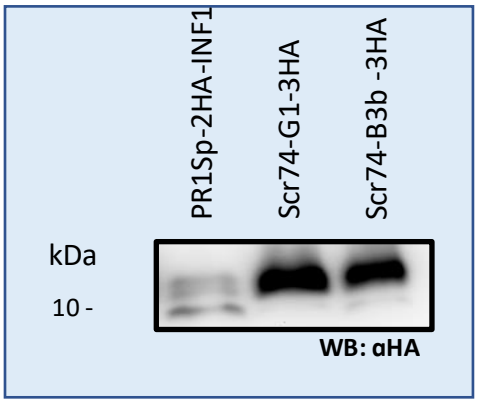

Figure 11. Western blots probed with a αHA antibody showing stable protein fusions of PR1Sp-2HA-INF1, Scr74-G1-3HA and Scr74-B3b-3HA of the expected size.

At 4 and 5 days post inoculation, there was no significant difference ($P \geq 0.05$) in lesion area on leaves between empty vector and Scr74-B3b treated plants, when infected with *P. infestans* isolate IPO-C (Figure 12A). However, infection efficiency of *P. infestans* was relatively higher in Scr74-B3b treated leaves of *N. benthamiana* than on leaves treated with EV (Figure 12B). Similarly, F-test between the means of Scr74-G1 and EV revealed no statistical difference ($P=0.484$) (Figure 12D). Unlike Scr74-B3b, lower infection efficiency of *P. infestans* (maximum 69 % at 6 dpi) was scored in leaves treated with Scr74-G1 comparing to empty vector (Figure 12E).

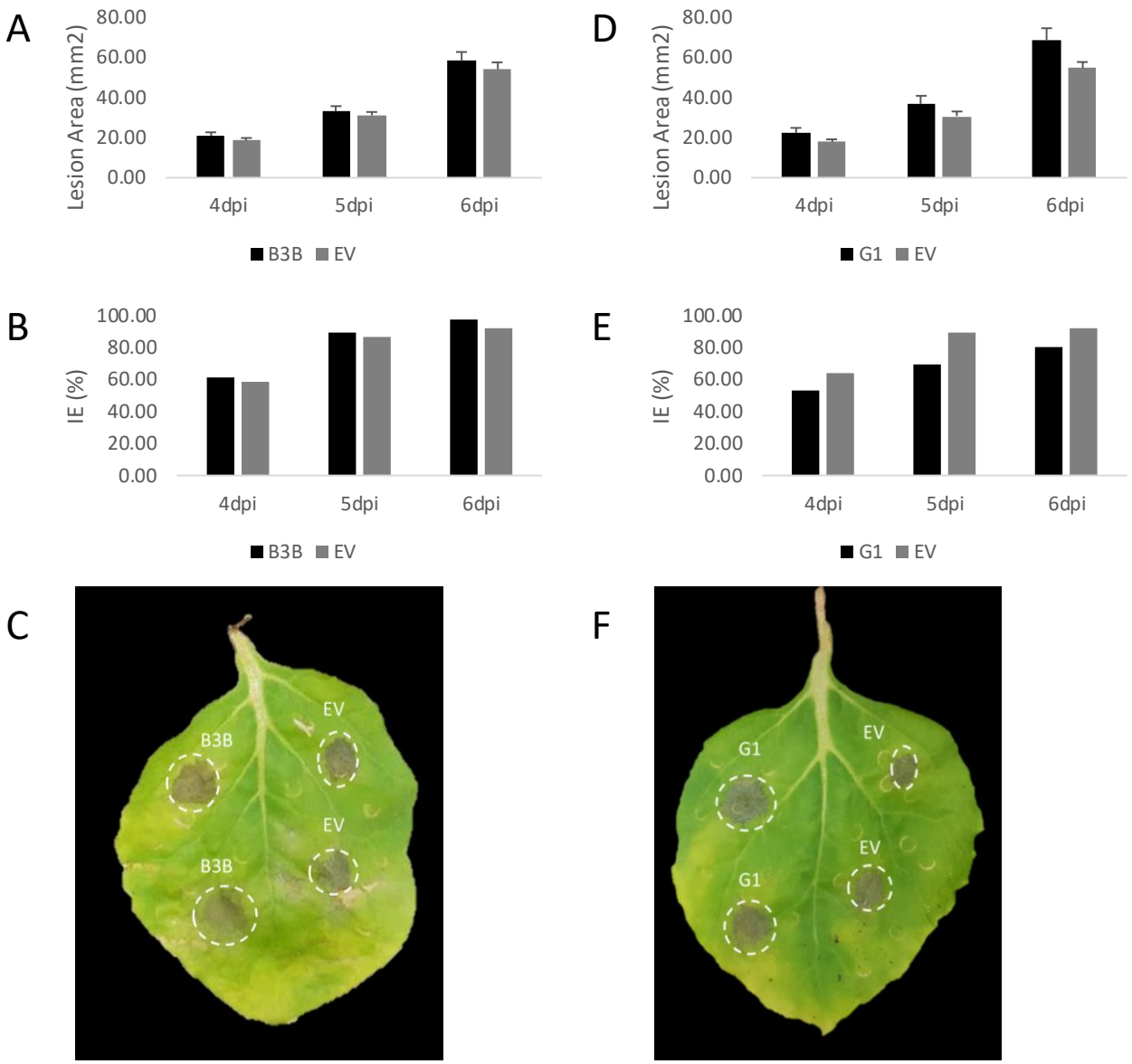

Figure 12. *P. infestans* IPO-C strain virulence assay on *N. benthamiana* plants upon transient expression of Scr74-B3b and Scr74-G1. (A) Average lesion area of leaf infiltrated with Scr74-B3b after 4, 5 and 6 days post inoculation. Error bar represents standard error of the mean. (B) Percentage of infection efficiency upon Scr74-B3b treatment 4, 5 and 6 days after inoculation. (C) Image of *N. benthamiana* detached leaf agroinfiltrated with Scr74-B3b and empty vector (EV) and inoculated with IPO-C isolate of *P. infestans*. (D) Average lesion area of leaf infiltrated with Scr74-G1 variant 4, 5 and 6 dpi. Error bar represents standard error of the mean. (E) Percentage of infection efficiency upon Scr74-G1 treatment 4, 5 and 6 dpi. (F) Image of *N. benthamiana* detached leaf agroinfiltrated with Scr74-G1 and empty vector (EV) that are also inoculated with IPO-C isolate of *P. infestans*. Statistical analysis was carried out using ANOVA with pairwise comparisons performed with a Fisher's LSD test; letters a, b and c represent $P \leq 0.05$.

Discussion

Plant pathogens have a range of mechanisms to invade hosts and cause a disease. A very sophisticated interaction is made during the host colonization process to modify the plant's metabolic activities by the pathogen to promote disease development. Meanwhile, the plant's immune system begins to function against potential pathogen effectors by recognizing conserved molecular patterns (PAMPs) through pattern recognition receptors (PRRs). Because of the importance in the plant-pathogen interactions, pathogen effectors and the plant receptors are increasingly being studied. Studies on oomycete effector proteins can be used to identify plant receptors that target the PAMP's (Dodds and Rathjen, 2010). In most recent years, several elicitors that trigger plant immunity and host genes that intervene in susceptibility to oomycete pathogens were identified (Fawke et al., 2015). The PcF/Scr family, an effector protein family originally discovered in the oomycete plant pathogen *Phythophthora catorum*, gained attention for investigating its effect on host plants (Liu et al., 2005). Scr74 effectors, which resemble PcF, are a secreted small cysteine rich and highly polymorphic effector gene family of *P. infestans* that encodes a 74-amino acid protein (Liu et al., 2005). The function of this effector protein family on *P. infestans* virulence and the host plant recognition is not known. Therefore, in this study, we have studied the role of Scr74 effector, which is under diversifying selection and highly polymorphic gene family, in the virulence of *P. infestans*.

This study demonstrates that there is recognition of Scr74 variants, Scr74-B3b and Scr74-G1, in wild potato species using protein infiltration assay. The results of protein infiltration experiment revealed that the potato wild species *Solanum microdontum* subsp. *gigantophyllum* (GIG362-6), showed a consistent and strong cell death reaction against Scr74-B3b at 10 µM protein concentration. Similarly, the wild potato species *Solanum verrucosum* (VER910-5) and *Solanum hougassi* (HCB353-8) also tested at 1 µM, 5 µM and 10 µM protein concentrations, but it only responds to Scr74-G1 10 µM protein concentration. In previous studies (Emmanouil Domazakis, Xiao Lin, unpublished) the recognition of different variants of Scr74 effectors on wild potato species was screened through PVX agroinfection method. Their result revealed that wild potato species *Solanum microdontum* subsp. *gigantophyllum* (GIG362-6) and *Solanum verrucosum* (VER910-5) have a cell death response against Scr74-B3b and Scr74-G1, respectively. This result has a similar trend to what we have displayed in our protein infiltration screening study. Although, *Solanum hougassi* (HCB353-8) demonstrates a cell death response against effector protein Scr74-

G1 in our experiment, but it didn't show any response to any Scr74 variants in the PVX agroinfection study. On the other hand, a cell death response against Scr74-B3b effector was shown on wild potato species *Solanum hougassi* (HOU655-1) in PVX agroinfection that didn't appear in our experiment. This could be possibly that utilisation of potato virus X (PVX) as a vector for effectoromics might prompt a cell death. However, previously, Orsomando et al. (2001), demonstrated that purified PcF effector elicits a cell death on tomato and strawberry. Moreover, infiltration of Scr74 and Scr91, homologues of PcF from *P. infestans,* and PcF can cause a leaf withering and induce phenylalanine ammonia lyase activity on tomato (Orsomando et al. 2011). The main factor why some of the wild potato species reacting against the *P. infestans* effectors is due to various metabolic changes in the host plant when they come into contact with those proteins or by their recognition from plant PRRs. In more detail, the pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) are exposed to the extracellular receptors or PRRs. Therefore, this indicates that the wild potatoes can recognise the apoplastic effectors which initiated the first reaction of defence or PAMP-triggered immunity (PTI) lead to cell death response (Jones and Dangl, 2006). Furthermore, Scr74 effector variants were agroinfiltrated to investigate reactive oxygen species accumulation on *N. benthamiana*. We observed that, except for the N' and C'-HA-tagged INF1, the *N. benthamiana* leaves do not shown the production of ROS against Scr74 variants. This might be possibly due to the absence of receptors which can recognise Scr74 variants in *N. benthamiana*.

Besides to protein infiltration, we additionally studied the effect of Scr74 on the virulence levels of *P. infestans* through *Agrobacterium* mediated transient expression of Scr74 variants on potato cultivars Bintije and Désirée as well as on *N. benthamiana* plants. *P. infestans* inoculation after two days of ATTA were the only observation that displayed significant involvement of Scr74-G1 in enhancing the virulence level of isolate IPO-C on cultivar Désirée. Inconsistent result was found among the different biological repeats of the disease assay which might be due to the differences in the growth of the two batches of *P. infestans* inoculum. The lesion area scored in both cultivars Désirée and Bintje at different time points do not show a similar pattern which might be due to season differences. We also observed a difference in phenotypes of lesion growth between one and two days of ATTA. The leaves of Désirée, that shown enhancement of virulence at number one of the two biological repeats were performed after two days of ATTA of Scr74-G1. The disease assay using NL07434 strain of *P. infestans* was performed only once but the result showed that virulence

of the pathogen enhanced when plants were treated with the Scr74 variants on cv. Bintje but not on Désirée. The NL07434 strain is relatively more aggressive compared to IPO-C strain, because a higher infection efficiency was recorded four days post inoculation. Similarly, the same experiment was done on *N. benthamiana* but differences were not clearly shown between the Scr74 variants and the empty vector. The $Avr3a^{KI}$, an RxLR effector of *P. infestans* that targets a host ubiquitin ligase was used as a positive control (Stassen & Van den Ackerveken, 2011). Despite of the inconsistency, infiltration of $Avr3a^{KI}$ sometimes results a lesser lesion area phenotype than the empty vector on potato cultivar cv. Bintje but not on Désirée which was unexpected. This suggests that $Avr3a^{KI}$ effector is not a right choice to consider it as a positive control in all cultivars since its effect on PTI suppression might not be required for our tested genotypes. In addition, other studies on Avr3a by Bos et al. (2009) and Bos et al. (2010) were mainly focused on a *P. infestans* Avr3a knock-down in order to investigate its role on virulence of *P. infestans*. However, these studies did not use a similar setup to our experiments and therefore it is hard to compare our results to those.

It is well known that *P. infestans* can manipulate host plants' biochemical, physiological, and morphological processes through a wide array of virulence molecules or effectors (Kamoun et al, 2006). In susceptible host plants, mainly some cultivars, pathogen effectors are used to stimulate infestation by suppressing the immune responses of host plants (Jones & Dangl, 2006). Our expectation was to see a consistent promotion of susceptibility due to Scr74 infiltration on both cultivars. But, there was an irregular trend on virulence stimulation and infection efficiency. Possibly, several factors which stimulate or hinder the virulence ability of *P. infestans* strains together with the agroinfiltrated Scr74 effectors might be involved. For example, the effector proteins and the agrobacterium used for transient transformation might induce a defence response.

Generally, from our studies we conclude that some wild potato species have shown a cell death response against the Scr74 effector proteins. The result will give insights into investigating the genes which are responsible for the response and could help in breeding for resistant cultivars. Though, the result from the virulence assay looks varying but still virulence is enhanced in the cultivars that have been tested in this study. Meanwhile, we recommend this virulence assay experiment should be repeated more times, at different seasons of the year using additional strains of *P. infestans*. In addition, it is also essential to investigate the function of untagged Scr74 effectors or purified protein.

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Appendices

Appendix A: ANOVA and pair wise comparison of virulence assay on cv. Désirée at 4, 5 and 6 dpi

ANOVA

Lesion area 4 dpi

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6013.807	2	3006.903	6.131	.011
Within Groups	7847.352	16	490.460		
Total	13861.159	18			

Multiple Comparisons

Dependent Variable: Lesion

LSD 4 dpi

(I) Desireel	POC4d	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
AVR3a	EV	28.202	14.295	.066	-2.10	58.51
	G1	-12.517	13.308	.361	-40.73	15.70
EV	AVR3a	-28.202	14.295	.066	-58.51	2.10
	G1	-40.719*	11.672	.003	-65.46	-15.98
G1	AVR3a	12.517	13.308	.361	-15.70	40.73
	EV	40.719*	11.672	.003	15.98	65.46

*. The mean difference is significant at the 0.05 level.

ANOVA

Lesion area 5 dpi

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	15876.218	2	7938.109	2.794	.084
Within Groups	59659.487	21	2840.928		
Total	75535.705	23			

Multiple Comparisons

Dependent Variable: Lesion

LSD 5 dpi

(I) Desireel	POC5d	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
AVR3a	EV	-13.948	27.586	.618	-71.32	43.42
	G1	-59.336*	26.861	.038	-115.20	-3.48
EV	AVR3a	13.948	27.586	.618	-43.42	71.32
	G1	-45.388	25.899	.094	-99.25	8.47
G1	AVR3a	59.336*	26.861	.038	3.48	115.20
	EV	45.388	25.899	.094	-8.47	99.25

*. The mean difference is significant at the 0.05 level.

ANOVA

Lesion area 6 dpi

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	264567.350	2	132283.675	3.476	.050
Within Groups	799174.410	21	38055.924		
Total	1063741.759	23			

Multiple Comparisons

Dependent Variable: Lesion

LSD 6 dpi

(I) DesireelPOC6d		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
AVR3a	EV	-10.051	100.963	.922	-220.02	199.91
	G1	-222.079*	98.311	.035	-426.53	-17.63
EV	AVR3a	10.051	100.963	.922	-199.91	220.02
	G1	-212.029*	94.791	.036	-409.16	-14.90
G1	AVR3a	222.079*	98.311	.035	17.63	426.53
	EV	212.029*	94.791	.036	14.90	409.16

*. The mean difference is significant at the 0.05 level.

Appendix B: ANOVA and pair wise comparison of virulence assay on cv. Bintje at 6 dpi

ANOVA

Lesion area 6 dpi

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	33652.528	2	16826.264	6.529	.040
Within Groups	12885.794	5	2577.159		
Total	46538.322	7			

Multiple Comparisons

Dependent Variable: Lesion
LSD 6 dpi

(I) Bintje2ndIPOC6d		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
AVR3a	EV	174.784*	50.766	.018	44.29	305.28
	G1	126.781*	43.964	.034	13.77	239.80
EV	AVR3a	-174.784*	50.766	.018	-305.28	-44.29
	G1	-48.003	43.964	.325	-161.02	65.01
G1	AVR3a	-126.781*	43.964	.034	-239.80	-13.77
	EV	48.003	43.964	.325	-65.01	161.02

*. The mean difference is significant at the 0.05 level.