Confidential

Characterization of *Arabidopsis thaliana S*-gene orthologs in *Solanum tuberosum* for disease resistance to *Phytophthora infestans*

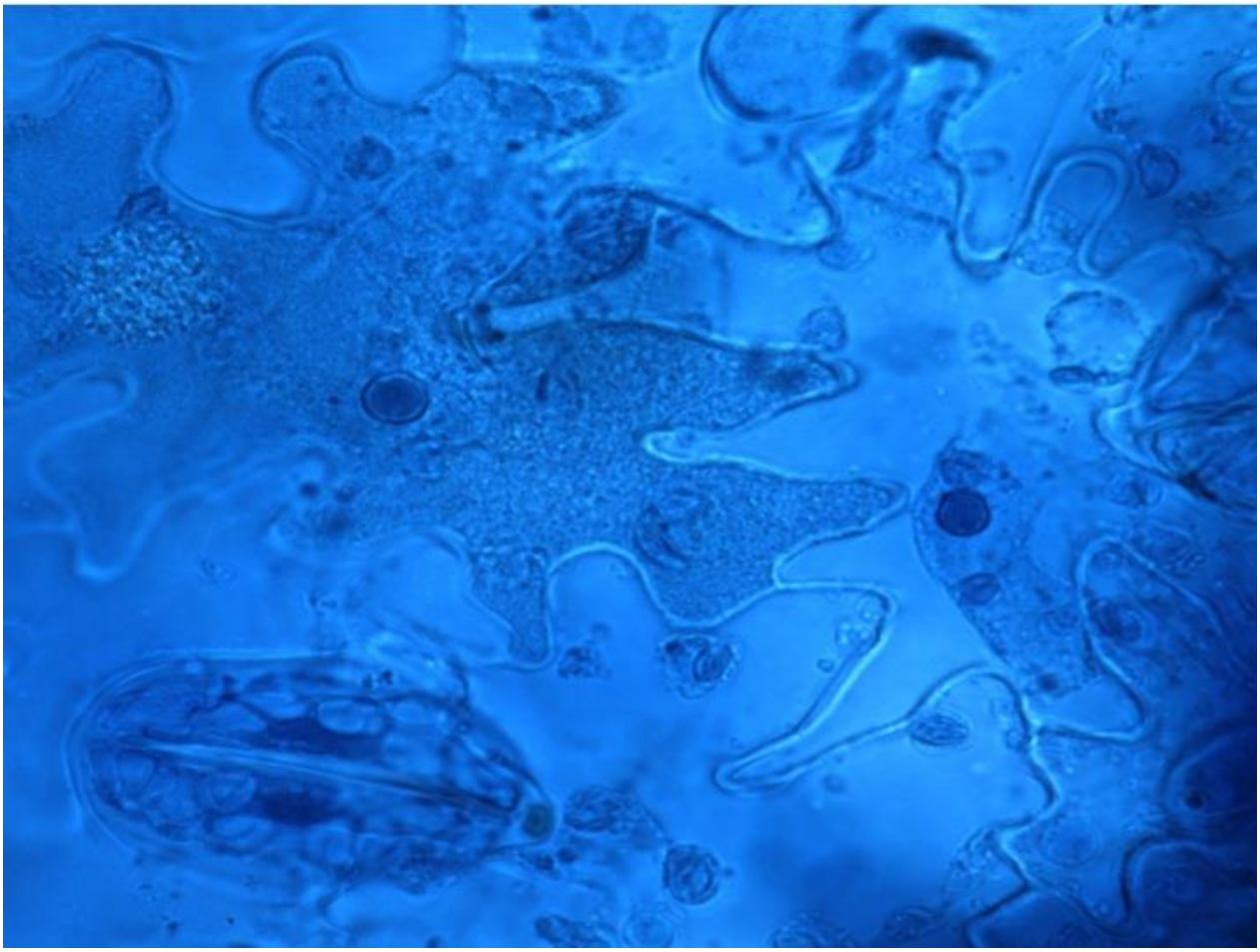

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Table of contents

Abstract
Breeding for late blight resistance in potato via *R*-gene introgression from wild potato species is an important breeding goal. However, resistance is lost in the field by breakage of *R*-genes due to rapid adaptation of *Phytophthora infestans,* the causal agent of late blight. A novel, alternative strategy to combat this destructive pathogen of potato is the impairment of susceptibility genes (*S*-genes); *S*-genes are genes that upon impairment result in disease resistance. Although several *S*-genes have been characterized in *Arabidopsis thaliana* unknown is whether or not the disease susceptibility function is conserved in different plant species. Here we report the existence of candidate *S*-genes orthologs of *Importin beta-3* and *PLC2* in potato, and conserved *S*-gene function of Arabidopsis genes *SR1* and *PMR4,* but not *PMR5* in potato. The impairment of *StSR1* by RNA interference resulted in partial resistance to *P. infestans* in the foliar part of potato. The impairment of *StPMR4* and *StPMR5* by RNAi induced partial resistance in the tubers of *StPMR4* silenced transformants but not in the tubers *StPMR5* silenced transformants. In histological and expression studies we elucidated the mechanism of resistance to *P. infestans* infection in RNAi::*StDND1* silenced transformants and confirmed that *S*-gene *StDND1* is a negative regulator of defense in potato.

Chapter 1 – General introduction

Breeding for resistance in potato (*Solanum tuberosum*) has been an on going challenge for breeders. Potato breeding is complicated as potato cultivars are highly heterozygous autotetraploid plants. In particular, breeding efforts for resistance to late blight caused by the oomycete *Phytophthora infestans* have been tremendous, but mostly unsuccessful in past decades. In the 1840s *P. infestans* triggered the renowned Irish potato famine and is considered as the most destructive pathogen to cultivated potato.

P. infestans, a filamentous water-mold, is able to invade, colonize and to complete its hemibiotrophic life cycle within several days to weeks on susceptible potato plants (Eschen-lippold et al., 2012). The life cycle of hemibiotrophs has two distinct phases, bio- and necrotrophic. The biotrophic phase of *P. infestans* is required for survival as well as sexual or asexual reproduction (Clément et al., 2010). In both phases *P. infestans* obtains nutrients from plants by formation of feeding structures called haustoria in host plant cells. For a pathogen to exploit plants it first has to overcome innate mechanisms of immunity consisting of preformed passive barriers and inducible active mechanisms.

Passive mechanisms are constitutively present in the plant irrespective of the presence or absence of the pathogen and consist of preformed mechanical barriers such as wax layers or antimicrobial compounds called phytoanticipins (VanEtten et al., 1994).

Pathogens that are not troubled by, or escape, preformed passive barriers can be recognized by plant receptors. During the course of evolution plants have adapted two inducible response mechanisms (defences) that stops pathogens infecting their living cells, termed PTI and ETI (Jones & Dangl, 2006). Recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) activates PAMP-triggered immunity (PTI). Active defense responses such as production of reactive oxygen species (ROS), phytolalexins and pathogenesis related (PR) proteins, and callose deposition to reinforce cell walls can be induced by PTI. Phytoalexins are similar antimicrobial compounds as phytoanticipins, the difference in terminology is based on the presence before or production after infection by a pathogen (VanEtten et al., 1994). However, pathogens have developed the means to supress PTI by interfering with the perception of the plant receptors or by secreting effectors protein in the plant cell that manipulate resistance signalling, Jones & Dangl (2006) classified this as effector-triggered susceptibility (ETS). ETS is an important theme throughout the coming chapters of this thesis and will be elaborated on in the next section of this introduction.

The ability of pathogens to overcome primary defences led to the adaption of a specialized mechanism in plants able to detect the pathogen effector proteins (Chisholm et al., 2006). Plant immune receptors recognize either directly or indirectly pathogen effector proteins resulting in effector-triggered immunity (ETI), upon which a hypersensitivity response (HR) signalling cascade is activated (Dangl & Jones, 2001). HR is a form of programmed cell death that acts within hours at the infection site and restricts pathogen growth (Goodman & Novacky, 1994). Upon recognition, phytohormones play a key role in the defense signalling to pathogens with different lifestyles. Salicylic acid (SA) is involved in defense signalling towards biotrophic pathogens, whereas jasmonic acid (JA) and ethylene (ET) are induced by recognition of necrotrophic pathogens (Glazebrook, 2005; Thomma et al., 2001). In the defense model of Jones and Dangl (2006) PTI precedes ETI, and ETI is believed to be more robust than the PTI. Recently nuances have been suggested for this generalizing model, indicating that both mechanisms can be equally robust depending on the specific interaction of molecules present.

Furthermore, the distinction between PAMPs and effectors seems not that black and white (Thomma et al., 2011).

_R_-genes

Resistance that is HR related is often associated with the gene-for-gene model of Flor. For every dominant resistance gene (*R*-gene) there is a matching dominant avirulence gene (Avr-gene)(Flor, 1971). Although, *R*-genes provide full resistances (narrow-spectrum) to pathogens in possession of the corresponding *Avr*-genes, meanwhile this interaction opposes a selection pressure on the pathogen population. Shifting the population towards individuals that posses the (loss of function) recessive avirulence gene (*avr*-gene), in this case the *R*-gene has been broken and resistance is lost. An additional factor contributing to the breakage of *R*-genes occurs when *P. infestans* reproduces sexually. *P. infestans* is a heterothallic (i.e. the sexes reside in different individuals) species of the genus *Phytophthora spp.* Therefore, sexual reproduction of *P. infestans* (oospore production) only occurs when both mating types, A1 and A2, are present as well as the ability to form antheridia and oogonia (gametangia). Sexual reproduction is believed to maintain the genomic integrity within *P. infestans* (Lamour & Kamoun, 2009, p124-128). As a consequence recombination occurs leading to novel genotypes that might overthrow *R*-gene resistances.

_S_-genes

In the field of resistance breeding a new strategy has been proposed by De Almeida Engler et al. (2005) and Pavan et al. (2010). This strategy is based on plant disease susceptibility genes (*S*-genes)(Eckardt, 2002). *S*-genes encode proteins that can be manipulated by pathogen effector proteins to facilitate their proliferation thereby enhancing infection (de Almeida Engler et al., 2005; Gawehns et al., 2013; Gust et al., 2010; Hückelhoven et al., 2013; Pavan et al., 2010). When thinking about *R*-gene functioning in resistance breeding, it is not hard to imagine that the impairment of a *S*-gene diminishes the ability of the pathogen to cause infection. From a genetic point of view *S*-genes are dominant genes that upon impairment lead to recessive resistances (Pavan et al., 2010). A well-known example of durable broad-spectrum recessively inherited resistance is the loss of function locus *mlo* (Mildew locus 0) (Bai et al., 2008; Büschges et al., 1997; Humphry et al., 2006). *MLO* is a negative regulator of the plant immune system, supressing PTI upon activation by pathogen effectors (Büschges et al., 1997). Besides negative defense regulators, another type of recessive resistance is conferred by susceptibility factors. Pathogen effectors activate host genes that are required for the proliferation of the pathogen (Pavan et al., 2010). For instance, isoforms of eIF4E and eIF4G that function as translation factors for potyvirus replication and infection (Diaz-Pendon et al., 2004; Robaglia & Caranta, 2006).

Pavan et al. (2010) showed that there are around 30 *S*-genes identified, mostly in the model organism *Arabidopsis thaliana* but also in crops like barley, pepper, tomato and rice. Since 2010 till present (august 2014), we have identified seven additional novel *S*-genes reported in literature (Table 1.1.1) but it is likely even more *S*-genes have been found. Even though these *S*-genes promote pathogen proliferation, in terms of evolution it is suggested they are beneficial for plant fitness, or at least neutral. This idea arose from the pleiotropic effects caused by the impairment of some *S*-genes (Pavan et al., 2010; Table 1.1.1). Albeit these pleiotropic effects such as dwarfed plant growth or auto necrotic lesions, efforts have been made to explore if the *S*-gene function, found in *A. thaliana*, is conserved in commercial crops (Table 1.1.2).

Table 1.1.1. Conserved *S*-gene functions between Arabidopsis and several commercial crops, asterisk indicate conserved *S*-gene function in commercial crops. Adapted from Pavan et al. (2010).

Role as an effector target	Gene	Plant species	Encoded protein	Pathogen	Reported pleiotropic phenotype	References
Negative regulator	*DND1*	Arabidopsis	Cyclic nucleotide-gated ion channel	*Pseudomonas syringae* *Xanthomonas campestris* Tobacco ringspot virus *Hyaloperonospora parasitica* *	Dwarf	Yu et al. (*1998*) Clough et al. (*2000*) Genger et al. (2008)
	LSD1	Arabidopsis Wheat	Zinc finger protein	*H. parasitica* *P. syringae* *Puccinia striiformis* f.sp. *tritici* *	Lesion formation	Dietrich et al. (*1994*) Kaminaka et al. (*2006*) Guo et al. (2013)
	MLO orthologues	Arabidopsis Barley Tomato/Pepper Pepper	Transmembrane protein	*Golovinomyces orontii* *G. cichoracearum* *Blumeria graminis* *Oidium neolycopersici* *X. campestris (virulent, not avirulent)* *	Early senescence and axenic cell death Not reported	Consonni et al. (*2006*) Bai et al. (2008); Zheng et al. (2013) Kim and Hwang (2012)
	MPK4	Arabidopsis Soybean	Mitogen-activated protein kinase	*H. parasitica* *P. syringae* *Peronospora manschurica* * *Soybean mosaic virus* *	Dwarf, curled leaves and reduced fertility	Petersen et al. (*2000*) Liu et al. (2011)
	SSI2	Arabidopsis Rice	Stearoyl-acyl carrier protein desaturase	*H. parasitica* *P. syringae* *G. cichoracearum* * *Magnaporthe grisea* * *X. oryzae pv. Oryzae* *	Small rosette, curled leaves, lesions (Not reported) Retarded growth	Kachroo et al. (*2001*) Shah et al. (*2001*) Song et al. (2013) Jiang et al. (2009)
	TOM1 and TOM3	Arabidopsis Tobacco	Transmembrane proteins	Tobacco mosaic virus *	Not reported	Diaz-Pendon et al. (*2004*) Kumar et al. (2012)
	EDR1	Arabidopsis	Mitogen-activated protein kinase	*P. syringae* *G. cichoracearum* *O. neolycopersici* *	Stunted plants with spontaneous lesions under drought conditions	Asai et al. (2002) Frye et al. (2001) Gao et al. (2014)
	PMR4	Arabidopsis Tomato	Callose synthase	*G. orontii* *G. cichoracearum* *H. parasitica* *O. neolycopersici* *	Epinastic leaves	Vogel and Somerville (2000) Nishimura et al. (2003) Huibers et al. (2013)
Susceptibility factor	DMR1	Arabidopsis Tomato	Homoserine kinase	*H. parasitica* *O. neolycopersici* *	No effect or slightly smaller size, depending on the *dmr1* allele	van Damme et al. (2005) Van Damme (2007) Huibers et al. (2013)

Table 1.1.2. Novel *S*-genes found in literature since the paper of Pavan et al. (2010).

Role as an effector target	Gene	Plant species	Encoded protein	Pathogen	Reported pleiotropic phenotype	References
Negative def. reg.	*TaS3*	Wheat	Ubiquitin-like protease 1 peptidase	*O. neolycopersici*	Not reported	Li et al. (2013)
	PAPP2C	Arabidopsis	Phosphatase type 2C	*Golovinomyces* spp.	Leaf cell death and stunted growth	Wang et al. (2012)
	UBC2	Nicotiana benthamiana	Ubiquitin-conjugating enzyme	Pto DC3000		Unver et al. (2012)
	RWA2	Arabidopsis	Homolog of protein CAS1	*Botrytis cinerea*		Manebe et al. (2011)
Susceptibility factor	*WAT1*	Arabidopsis	Predicted integral membrane protein	*Ralstonia solanacearum* *Xanthomonas campestris pv. campestris* *Verticillium dahliae* *Verticillium albo-atrum*	Not reported	Denancé et al. (2013)
	COI1	Arabidopsis	F-box protein	Fusarium oxysporum *Verticillium longisporum*	Not reported	Thatcher et al. (2009) Ralhan et al. (2012)
	RFC3	Arabidopsis	Replication factor C	*Hyaloperonospora arabidopsidis*	Dwarfed	Xia et al. (2009)

Knocking out *S*-genes by RNA interference in potato

Candidate *S*-genes in potato that upon impairment would provide resistance to *P. infestans* are scarce. Only one gene, *CPMG1*, is considered a *S*-gene for *P. infestans* pathogenicity in potato (Gawehns et al., 2013). *CPMG1* however, is necessary for basal immunity and acts both as a positive and negative regulator of defense. Silencing *CMPG1* renders the plant susceptible to a range of other pathogens by supressing PTI (Bos et al., 2010).

There is a clear gap in literature concerning candidate *S*-genes in potato, but two candidate S-genes were found in an internship report and in a MSc thesis report (unpublished data). From the internship report candidate *S*-gene *importin beta-3* for resistance to *P. infestans* is considered, and from the MSc thesis report *PLC2* is considered as a candidate S-gene.

Once candidate *S*-genes have been identified, their stable inheritable silencing can be achieved by RNA interference (RNAi) techniques, such as hairpin RNAi (hpRNAi)(Fire et al., 1998; Wesley et al., 2001) or loss of function alleles can be obtained by mutagenesis. Although both techniques are effective, in this thesis hpRNAi was used for silencing target genes in potato (Figure 1.1.1). RNAi is a posttranscriptional gene silencing process that occurs in many eukaryotes including plants, and is part of the plant immune system against viruses (Voinnet, 2005). An enzyme called Dicer cleaves double stranded RNA (dsRNA) molecules into short interfering RNA (siRNA) fragments around 20 nucleotides in length. Each siRNA fragment existing of a sense and anti-sense strand is unwound upon which the sense strand is degraded and the antisense is incorporated in a RNA-induced silencing complex (RISC). When the antisense strand of the RISC pairs with complementary sense mRNA, the mRNA is cleaved by the catalytic domain of RISC and preventing translation into proteins. Via the pHellsgate vector target fragments that form the hairpin (dsRNA) can be introduced into the potato genome by *Agrobacterium tumefaciens* mediated plant transformations (An et al., 1986; Wesley et al., 2001). The pHellsgate vector contains the cauliflower mosaic virus (CaMV) 35S promoter that regulates expression of the inserted fragments in transformed plants carrying the vector. The CaMV 35S promoter acts as a strong constitutive promoter in most organs of transgenic plants (Fang et al., 1989; Kay et al., 1987). This strong promoter is used to obtain stable silencing of the target genes. Compared to mutagenesis, RNAi might be less stable, however, loss of function after a mutation is inherited recessively whereas after RNAi it is inherited dominantly. For tetraploid potato this is important to know.

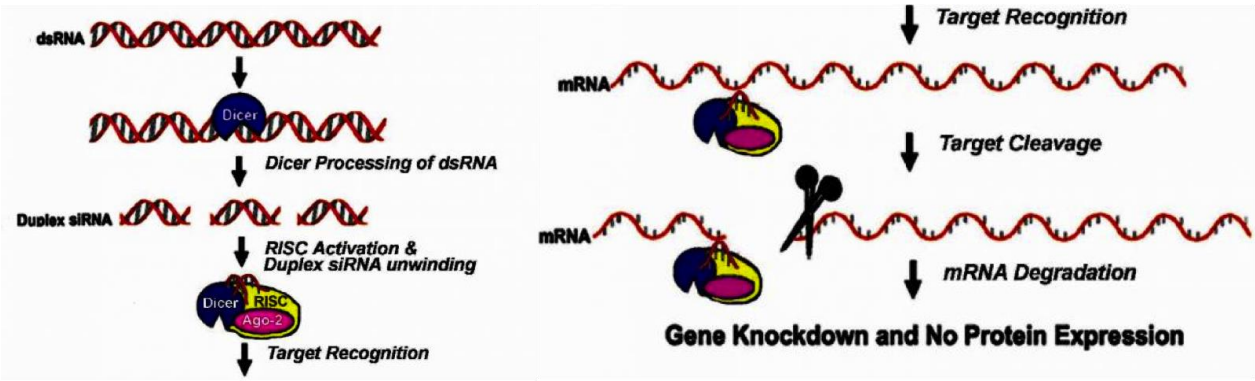

Figure 1.1.1. *Graphical representation of RNAi pathway. Adapted from Chen & Evans (2012).*

S-gene strategy and thesis outline

The aim of this thesis was to identify novel *S*-genes in potato that upon impairment by RNAi induce durable broad-spectrum resistance to *P. infestans*. The experimental duration of all steps in the impairment of a gene by RNAi and disease assessment of transformed plantlets that

belongs to the *S*-gene strategy is labour intensive and testing a single gene takes longer than the amount of time that was available for this MSc thesis period. Therefore, the different steps that the *S*-gene strategy comprises have been performed for different genes at different phases of the RNAi process (Figure 1.1.2). Steps 1 to 3 were performed for gene *Importin beta-3* and partly for *PLC2* as described in Chapter 2 of this thesis. The study of four *S*-genes *SR1, PMR4, PMR5* and *DND1* was continued at different succeeding steps of the *S*-gene strategy, these genes are identified as *S*-genes by Pavan et al. (2010). Resistance to *P. infestans* was assessed for RNAi::*SR1* silencing potato transformants with a detached leaf assay and effectiveness of silencing was quantified by expression studies (Step 4 and 5; Chapter 3). For RNAi::*PMR4* and RNAi::*PMR5* silencing potato transformants a detached leaf assay has been performed (Kaile Sun, unpublished data) therefore, a tuber assay (TA) was performed to determine tuber resistance to *P. infestans* (Chapter 4). Finally, histological and expression studies were performed to characterize the resistance mechanism in RNAi::*DND1* silencing potato transformants (Chapter 5).

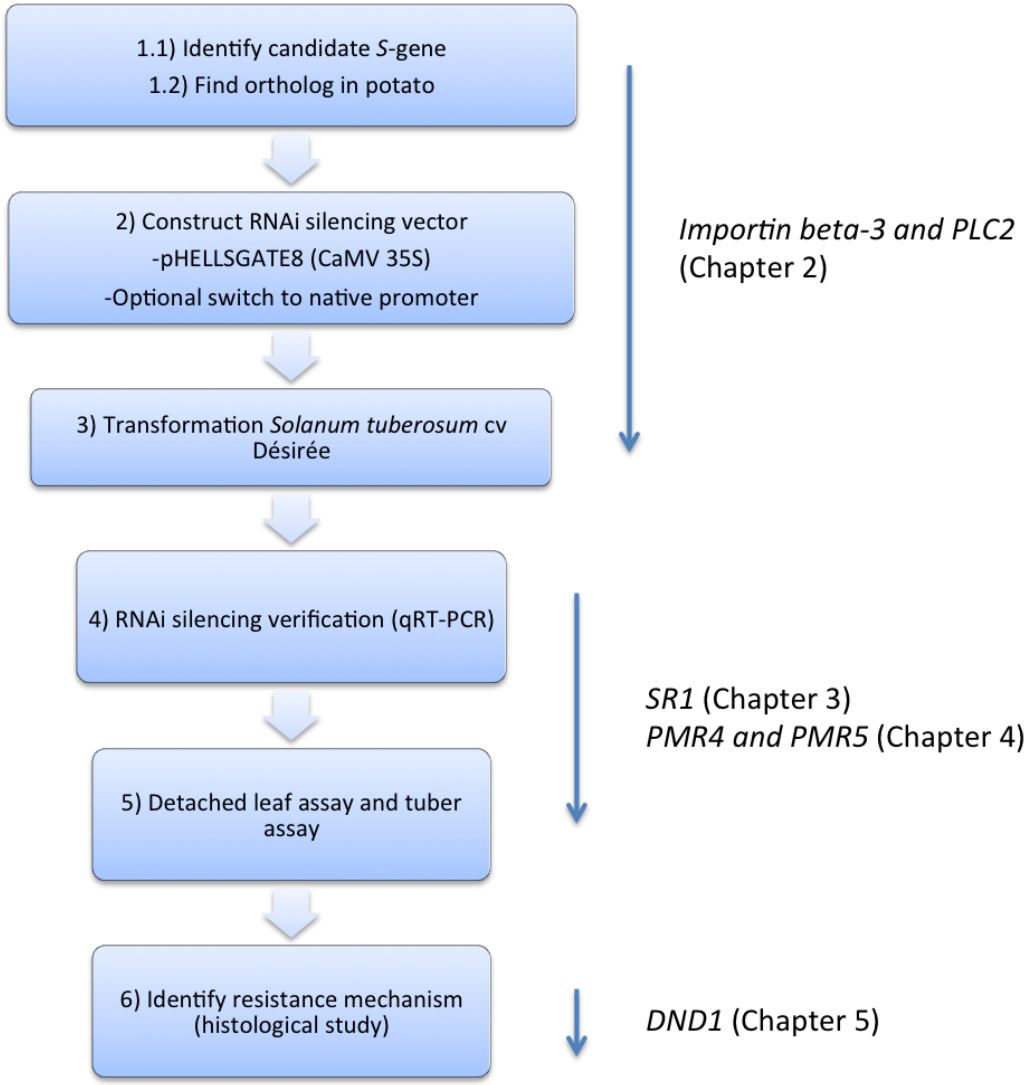

Figure 1.1.2. *S-gene strategy. Steps 1 to 3 are described in Chapter 2. Disease assay and silencing verification of the foliar part of RNA::SR1 silenced plants (steps 4 and 5) is discussed in Chapter 3. Similar steps as in Chapter 3 were performed for the tubers of RNAi silencing transformants (Chapter 4). The resistance mechanism of RNAi::DND1 transformants is described in Chapter 5.*

The objectives and research questions for this thesis were:

- To perform a literature study that gathered information on novel *S*-genes identified since the review paper of Pavan et al. (2010), and detect candidate *S*-genes for durable resistance to *P. infestans* in potato (Chapter 1 and 2).
- Study if the *S*-gene function of *AtSR1* is conserved in the foliar part of potato (Chapter 3).
- Study if *S*-gene function of *StPMR4* is conserved in the tubers of RNAi::*StPMR4* transformants (Chapter 4).
- Study if *S*-gene function of *StPMR5* is conserved in the tubers of RNAi::*StPMR5* transformants (Chapter 4).
- What are the physiological responses of RNAi::*StDND1* transformants upon exposure to *P. infestans* zoospores? (Chapter 5, a histological and expression studies).

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Chapter 2 – Identification of candidate *S*-genes *Importin beta-3* and *PLC2* in potato

Transformation of Désirée plants with a RNAi silencing construct coding for candidate *S*-gene *Importin beta-3* and cloning of a *PLC2* silencing fragment

Abstract – In this chapter we report the identification of candidate *S*-genes *Importin beta-3* and *PLC2* in potato. For *Importin beta-3* primers for silencing fragments were designed to silence all three homologs separately, and one primer pair to silence all homologs simultaneously. For the ortholog of *Importin beta-3* a silencing vector was generated and potato explants were transformed via Agrobacterium mediated transformation, about 200 RNAi::*Importin beta-3* transformants were obtained. For *PLC2* we were successful in cloning a silencing fragment into an entry vector but unable to recombine the silencing fragment into the pHellsgate 8 silencing vector.

2.1 Introduction

This methodological study describes the first three steps of the *S*-gene strategy that are prerequisites for characterization of candidate *S*-genes in potato. First a literature is performed to identify candidate *S*-genes in potato. When candidates are identified verification of the existence of orthologs in potato is needed. If the ortholog indeed exists, in the second step the predicted coding sequence of the ortholog is used to design a silencing fragment that can be inserted into the silencing vector pHellsgate 8. The final step of this chapter is potato explant transformation by *Agrobacterium tumefaciens* carrying the pHellsgate 8 vector with the silencing fragment.

The aim of this study was to silence candidate *S*-genes *Importin beta-3* and *PLC2* in potato. Enhanced resistance is described to *Phytophtora infestans* by virus induced gene silencing (VIGS) of *Importin beta-3* in *Nicotiana benthamiana* (AFO internship report). The exact gene function of *Importin beta-3* in potato is unkown (www.potato.plantbiology.msu.edu, 2014). In contrast, *PLC2* is a gene that has been widely studied. The discovery of the phosphoinositide/phospholipase C (PI/PLC) signalling in animals systems was an important step towards identification of PI/PLC signalling also occurring in plants (Munnik & Testerink, 2009). Activation of PLC signalling in animals systems leads to formation of second messenger IP_3 and DAG which are involved in Ca^{2+} signalling. In plants second messengers IP_6 and PA are formed, of which the latter has been reported to be involved in disease resistance signalling (Vossen et al., 2010). In the recent MSc thesis report of Ernest Beckee Aliche overexpression of *StPLC2* is suggested to enhance resistance to *P. infestans* and increase susceptibility to *Botrytis cinerea*. This suggests that upon silencing of *StPLC2* susceptibility towards *B. cinerea* should decrease, therefore *PLC2* is considered as a candidate S-gene of *B. cinerea* in potato.

The necrotrophic fungal pathogen *B. cinerea*, also known as grey mould, is capable of infecting over 200 different plant species. Necrotic areas with extensive fungal growth characterize the manifestation of the disease. In contrast with biotrophic pathogens, *B. cinerea* is able to benefit from necrotic tissues that follow from a hypersensitivity response (Govrin & Levine, 2000).

2.2 Material & methods

Potato ortholog identification

By blasting the primer sequences of the VIGS construct the coding sequence of *Importin beta-3* in *N*. *benthamiana* was retrieved. The found coding sequence of *Importin beta-3* was blasted on the ncbi website with the nucleotide blast tool. The predicted protein sequence of the potato and *Arabidopsis* gene with the highest matching coding sequence (% identity) was taken. This sequence was blasted by using the blast tool on Solgenomics against the Potato PGSC DM v3.4 protein sequences database (Solgenomics, 2013). This was performed in order to find homologous amino-acid sequences of the *Importin beta-3* gene in *S. tuberosum*.

In addition to the *S. tuberosum* homologs, the found sequence of *Importin beta-3* in *N. benthamiana* was blasted on solgenomics to identify orthologs in *S. lycopersicum.* Also a gene search was performed on the Arabidopsis website tair (Arabidopsis, 2013) for *Importin beta-3*. In potato, *A. thaliana*, tobacco, and tomato candidate orthologs were found, four, two, one and one, respectively. Using the protein sequences a phylogenetic tree was constructed (figure 2.2.1). Using shortest distances, PGSC0003DMP400007618 of *S. tuberosum* is considered the true ortholog of the *N. benthamiana Importin beta-3* gene. There is a possibility that the three potato homologs in the same clade are functionally redundant. Therefore, silencing fragments were designed that are (theoretically) able to silence the three different homologs separately. In addition, a fourth silencing fragment was designed that can silence three homologs.

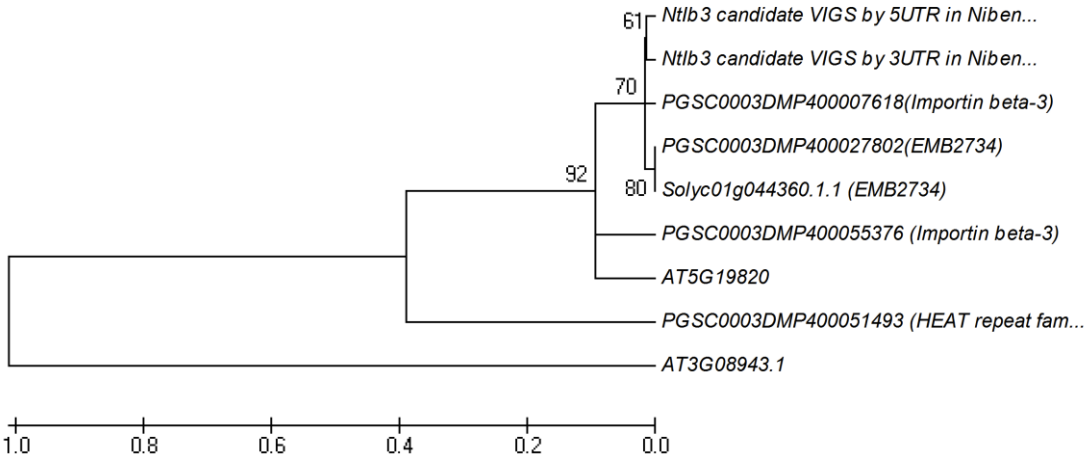

Figure 2.2.1. *Phylogenetic tree of Importin beta-3 familiy members. PGSC0003DMP400007618 is considered the potato ortholog of N. benthamiana Importin beta-3 (Ntib 3 candidate VIGS).*

Primer design, construct generation and explant transformation

Primers were designed with the online programme Primer3. The 3' UTR of the gene was selected and inserted into the primer3 programme. Out of the four options given by primer3, the most optimal one was chosen according to GC content (40-60%) and tm (±60). To all forward primers four bases CACC were added, this in order for the connection of the PCR product to the pENTR^{tm}/D-TOPO entry vector (invitrogen). All primers for this gene are listed in table 2.1.1. In this thesis only a silencing construct was made for the potato PGSC0003DMP400007618 gene, which is closest to the *N. benthamiana* ortholog (the other three silencing constructs are made by Marc Hendriks).

Table 2.2.1. Primers for the silencing fragments of the *Importin beta-3 homologs. Both the protein and transcript ID are listed.*

Gene ID	Primers	'UTR
PGSC0003DMP400007618 PGSC0003DMT400010931	F - CACCCCCCGTGCTATGTCAGCTAT R - GCCATTGATTTTCAGGGAGA	3
PGSC0003DMP400027802 PGSC0003DMT400041005	F - CACCTCATTTGGGCTTTTGAGGTC R - CAAACCGTGATCACCAACAG	3
PGSC0003DMP400007376 PGSC0003DMT400081965	F - CACCGCATGCTGATCGAAACATTC R - TTGCCCTTAACAGACAAGCA	3
All three homologs	F - CACCAAGGATAAAACAGCTGAAGAAAGGAG R - CTCCTTTCTTCAGCTGTTTTATCCTT	-

The 208 bp *Importin beta-3* fragment was amplified by using a Phusion PCR kit (Invitrogen); the polymerase chain reaction (PCR) program is listed in table 2.2.2. The product was run on a 1% agarose gel to confirm PCR production and total length of the product by using a one kb ladder. The *Importin beta-3* fragment was recombined into the pENTR^{tm}/D-TOPO entry vector, containing attL1 and attL2 sites, by using the pENTR^{tm}/D-TOPO cloning kit (invitrogen). The construct with *Importin beta-3* fragment was recombined into *Escheria coli* DH5α competent cells (Table S2.2.1). The heat shocked DH5α competent cells were spread out on kanamycin containing solid LB medium (1:1000), and grown overnight at 37° C. All obtained colonies were transferred to LB + kanamycin containing petridishes, by using a sterile pipet point and softly making a stripe on the medium. These petridishes were stored at 4° C. Several of those colonies were grown in liquid LB medium with kanamycin by inserting the pipet point that was used to draw the line. The bacterial culture was grown for overnight at 37° C after which the construct was extracted with the Plasmid extraction kit 27106 (Qiagen, Germany). Plasmids were sequenced to confirm insertion and no sequence alteration of the *Importin beta-3* fragment.

The silencing construct was recombined into the pHellsgate 8 vector using the LR clonase enzyme mix from Invitrogen and transformed to *E. coli* DH5α competent cells by heat shock and grown on LB + Spectomycin plates (Table S2.2.1). pHellsgate 8 carries a Spectomycin resistance gene for selection of bacteria that carry this vector. The same steps were repeated as mentioned in the paragraph above to obtain plasmids from bacterial colonies. However, this time the plasmids were used for restriction enzyme digestion instead of sequencing. Enzymes XhoI and XbaI were used to confirm insertion of the intended insert and transformed to *Agrobacterium tumefaciens* strain AGL1+virG by electroporation.

Table 2.2.2. Settings PCR program, in red varying annealing temperatures according to primer design

Stage	Temperature °C	Time (seconds)	Cycles
1	98	30	1
2	98	20	35
2	52-62	30	35
2	72	30	35
3	72	600	1

For potato transformation four-week-old sterile *in vitro* propagated plants, cultivar Désirée, were used to obtain around 150 internodal cuttings. *A. tumefaciens* preparation and explant transformation was carried out as described by Heilersig et al. (2006).

For the gene *PLC2* the same methodology was used as described for *Importin beta-3*. One candidate ortholog was identified in potato (PGSC0003DMP400011713; figure 2.2.2), the homologs were identical in both 3 and 5 'UTR. For both 'UTR's primers were designed for a sequence fragment (table 2.2.3). Generation of the silencing construct was not completed for this gene. After successful recombination of the gene fragment into the pENTR^{tm}/D-TOPO entry vector, multiple LR reactions for recombination into silencing vector pHellsgate 8 did not result in the growth of bacterial colonies.

Figure 2.2.2. *Phylogenetic tree of PLC2 family members. The phylogenetic tree was constructed by using phylogeny.fr (www.phylogeny.fr ,2013).*

Table 2.2.3. Primers for the silencing fragments of the *PLC2 homologs.*

Primers	'UTR
Fw - CACCAGCTCCGGCAGACATTAAGA Rv - AGCTGGAGGCCTTTCTGATT	*3*
Fw - CACCTGGGAACAAAAGATGGGAAG Rv - AAAGCTTGGCTCAAATTGACA	*5*

2.3. Results

Verification of the *Importin beta-3* ortholog in potato, generation of the silencing fragment and potato explant transformation

The gene PGSC0003DMP400007618 was considered the potato ortholog of *N. benthamiana Importin beta-3* that was silenced by VIGS in the AFO internship report. The coding sequence of the potato ortholog (PGSC0003DMT400010931) consists of 3066 nucleotides, and the silencing fragment is based on 208 nucleotides near the 3' UTR (figure 2.3.1).

Figure 2.3.1. *Alignment of predicted coding sequence of PGSC0003DMP400007618 with the DNA fragment used for silencing.*

Visualisation of the DNA production showed a band between 200 and 300 bp (Figure 2.3.2A). The PCR product was inserted into the pENTR^{tm}/D-TOPO entry vector, which was used for transformation of *E. coli* DH5α competent cells. PCR amplification of transformed colonies with the primers of the *IB3* fragment showed that nine colonies potentially carry the proper insertion (Figure 2.3.2B). Plasmid isolation of the first four colonies and PCR amplification confirmed the presence of the target fragment in the construct (Figure 2.3.2.C). Sequencing of the four isolated plasmids with the primers of the silencing fragment and alignment with the predicted

nucleotide sequence showed that the first two plasmids matched for 100% (Figure S2.3.1). The LR reactions to insert the target fragment into silencing vector pHellsgate 8 succeeded for plasmid number one and two colonies were obtained. Again the construct was isolated and enzyme digestion performed with XhoI and XbaI, confirming the insertions of the DNA fragment into the pHellsgate 8 vector (Figure 2.3.2D).

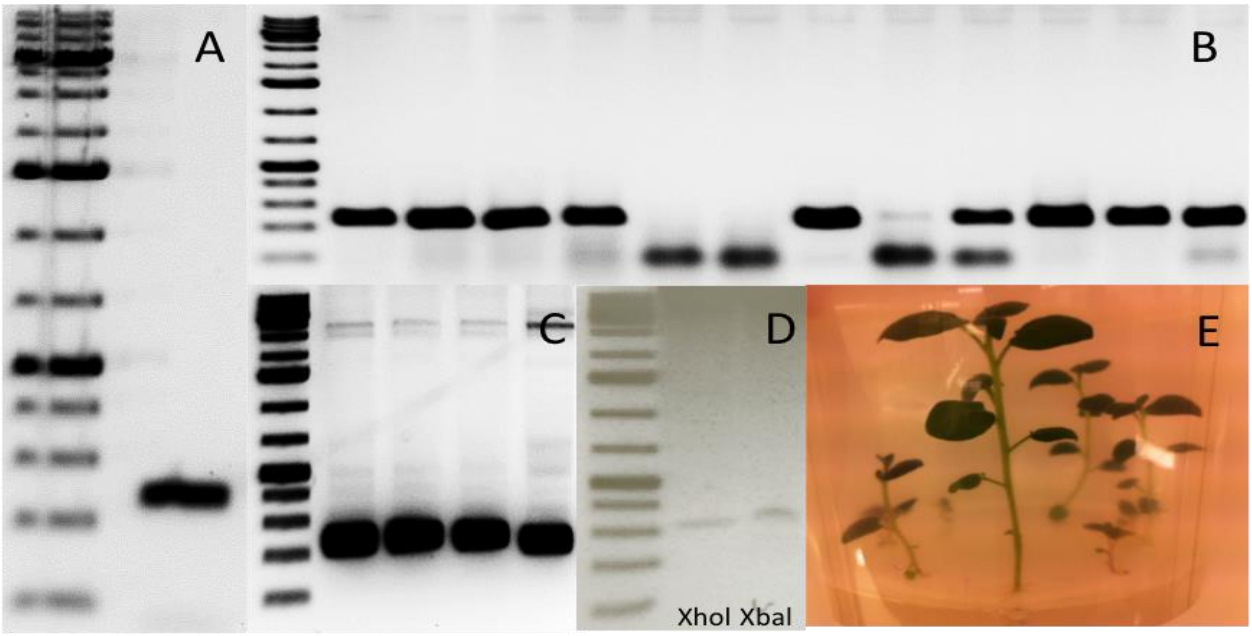

Figure 2.3.2. *PCR production of the Importin beta-3 target fragment (**A**). PCR amplification of the silencing fragment in bacterial colonies after recombination with pENTR^{tm}/D-TOPO (**B**) Amplification of silencing fragment in the entry vector of the four colonies (**C**) XhoI and XbaI enzyme digestion of plasmids after LR reaction with destination vector pHellsgate8 (**D**). Shoots grown from explant callus (**E**).*

The *Importin beta-3* silencing construct was transformed to *Agrobacterium* strain AGL1+virG by electroporation. This resulted in eight colonies of which one was used for potato explant transformation as described by Heilersig et al. (2006). From the explants callus about 200 shoots were excised, that were able to root on kanamycin (Figure 2.3.2E).

Cloning of *PLC2* into pENTR^{tm}/D-TOPO

Two primer pairs were designed based on the 3'UTR and 5'UTR of the predicted *St-PLC2* coding sequence. Visualisation of the PCR product of the 5'UTR primers indicated bright bands that were approximately 200 bp in size (Figure 2.3.3A). The PCR product amplified by the primers based on the 3'UTR was similar in size but amplification seemed lower (Figure 2.3.3B). The PCR product of the 5'UTR was chosen for recombination into pENTR^{tm}/D-TOPO (Invitrogen) and used for *E. coli* (DH5α competent cells) transformation. Thirteen colonies were obtained (Figure 2.3.2B) and the plasmids of six colonies were sequenced, the plasmid of colony number four matched the predicted sequence (Figure 2.3.2C or supplemental figure). Subsequently, the plasmids were used for recombination into vector pHellsgate 8 by using the LR reaction clonase enzyme mix (Invitrogen). However, multiple attempts using varying ratio's of plasmid to vector concentrations (ng/µL) did not result in any transformed *E. coli* bacterial colonies. In addition, minor changes in the protocol for the LR reaction were attempted, such as duration of the incubation time of the LR reaction or changing to pHellsgate 12. pHellsgate 12 has an extra intron site compared to pHellsgate 8.

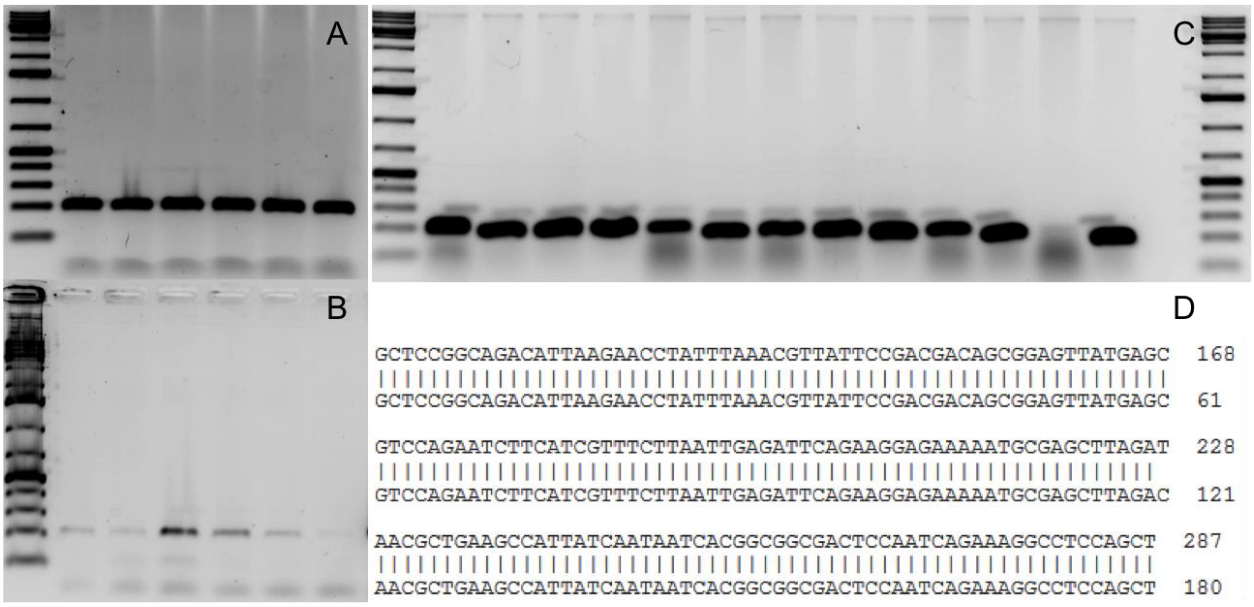

Figure 2.3.3. *PCR production of the PLC2 target fragments based on the 5'UTR (**A**) and 3'UTR (**B**) annealing temperatures ranged from 52° C (most left) to 62° C (most right), 54, 56, 58 and 60 were temperatures in between. PCR amplification of the 5'UTR based silencing fragment in bacterial colonies after recombination with pENTR^{tm}/D-TOPO (**C**). Alignment of sequence isolated plasmid (upper) and predicted sequence target fragment (lower)(**D**).*

After a series of unsuccessful attempts, we decided to design new primers for both 'UTR regions. PCR amplification with these new primers resulted in multiple products with varying product lengths (figure S2.3.2A). To obtain the band that had approximately the same size as the predicted target fragment, the gel was cut and purified. The obtained product was used for PCR production instead of the cDNA, however the result was similar as the normal PCR production (figure S2.3.2B). Due to time constraints of this thesis period further work on this gene was stopped.

2.4 Discussion

Successful transformation of Désirée explants now carrying a RNAi::*Importin-beta-3* silencing construct was performed as described. However, some steps were repeated, as they were unsuccessful in the first or even second attempt. As in the process of generating a vector for explant transformation there are numerous steps that are controllable to a limited extent. The PGSC0003DMP400007618 potato gene is considered the true ortholog of the *Importin beta-3* gene that was silenced by VIGS in the AFO internship. This is based on the shortest distance in the phylogenetic tree, despite the shortest distance it is possible that one of the other two homologs is the actual ortholog. In the case of functional redundancy between the homologs, silencing one might not alter the resistance phenotype in the transformed plants. Therefore, if the plants transformed in this chapter are equally susceptible to the control plant (Désirée), transformation can be performed with the sequence fragment that in theory is able to silence all the homologs.

The results of, or failure to generate a silencing construct for *PLC2* is a good illustration of this limited control. Changing steps in the protocol after unsuccessful attempts of the LR reaction is no guarantee for success. Even changing the individual that performed the procedure

did not work but at least gave piece of mind. The *in silico* search for the potato ortholog of *PLC2* also identified a tomato *PLC2* candidate ortholog (Solyc06g051620), silencing this gene in tomato should be performed as a follow up experiment. When silencing of this gene can be achieved in tomato, afterwards disease resistance can be assessed to *B. cinerea*. This will elucidate the possibility to silence *PLC2* and the role of *PLC2* as a candidate *S*-gene.

When designing the primers for a silencing fragment it is useful to design multiple primers based on different regions of the coding sequence. It is advisable to start with at least two primer pairs, but more raises the chance of successful cloning. From the practical point of view it is easier to handle four silencing fragments simultaneously, and continue with the successful fragments. Compared to starting over with primer design when the cloning or sequencing is unsuccessful.

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Méthodes et Algorithmes pour la Bio-informatique, from http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi

National Center for Biotechnology information, from http://blast.ncbi.nlm.nih.gov/Blast.cgi

Spud DB, Potato Genomics Resource, from http://potato.plantbiology.msu.edu/cgi-bin/annotation_report.cgi

Sol genomics network, from http://www.solgenomics.net/

The Arabidopsis Information Resource, from http://www.arabidopsis.org/

Supplemental figures

Score	Expect	Identities	Gaps	Strand
385 bits(208)	2e-111	208/208(100%)	0/208(0%)	Plus/Plus

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  105  CCCCGTGCTATGTCAGCTATACTCCTCCGAAAGTTGCTTACTCGGGATGACGACTTCATC  164

Query  61   TGGCCAAAACTCACTCACTCTACCCAGTCCTCTATTAAGTCACTCCTACTCACATGTATC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  225  CAACATGAACAATCTAAATCCATTATCAAAAAGTTATGTGACACCATTTCCGAACTCGCT  284

Query  181  TCATCTATTCTCCCTGAAAATCAATGGC  208
            ||||||||||||||||||||||||||||
Sbjct  285  TCATCTATTCTCCCTGAAAATCAATGGC  312
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Figure S2.3.1 *Alignment of sequence isolated plasmid (lower) and predicted sequence Importin beta-3 target fragment (upper).*

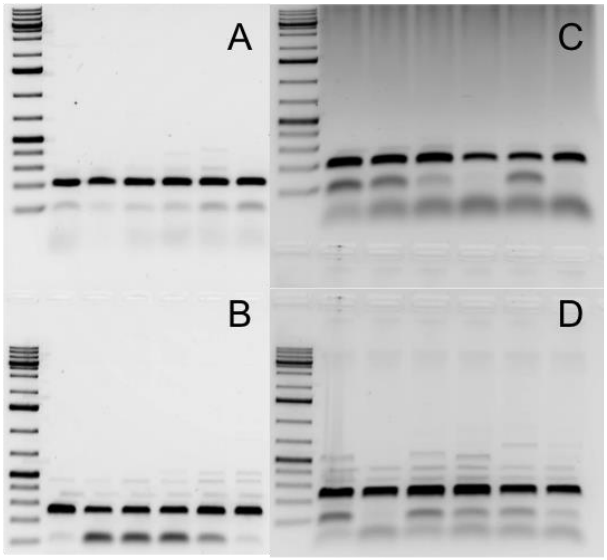

Figure S2.3.2. *PCR production of PLC2 target fragment based on 5'UTR (**A**) and 3'UTR (**B**) using cDNA. PCR production PLC2 target fragment using the gel cut and purified PCR product of **A** and **B** as template (**C** and **D**).*

Table S2.2.1. Adapted Invitrogen pENTR^{tm}/D-TOPO protocol

Step	Performed action(s)
1	2 µL PCR product, 0,5 µL Salt solution (Invitrogen), 0,5 µL Vector (Invotrogen) and 2 µL MilliQ was added to an eppendorf and incubated for 30 minutes at 25° C.
2	25 µL DH5α compentent cells (*E. coli*) was added to the solution, mixed gently and incubated on ice for 30 minutes.
3	The cells were heat shocked for 30 seconds in a 42° C water bath after which they were immediately placed on ice and 250 µL of room temperature S.O.C. medium (Invitrogen) was added. Mixed gently.
4	The cells were incubated at 37° C for 1 hour and shaken horizontally at 180 rpm.
5	50 µL of the transformation was spread on 50 µg/mL Kanamycin selective plate.
6	The left over 200 µL was spun down and most liquid discarded, the bacterial pellet re-suspended and spread on a second 50 µg/mL Kanamycin selective plate.
7	Both plates were incubated at 37° C overnight.

Chapter 3 – Partial late blight resistance by impairment of *StSR1* in potato

Characterization of *St-SR1-h3* silenced potato transformants for foliage resistance to *Phytophthora infestans* and endemic greenhouse powdery mildew species

Abstract – Loss of function alleles of Arabidopsis *Signal Responsive1* (*SR1*) provided constitutive resistance to virulent *Pseudomonas syringae* pv. *tomato* DC3000. Here we report the first indication that the *S*-gene function of *SR1* is conserved between Arabidopsis and potato. Impairment of the ortholog *StSR1* in potato conferred partial resistance to *Phytophthora infestans* in the foliar part. Furthermore, quantitative variation was found for resistance to endemic greenhouse powdery mildew. Knocking out of *StSR1* resulted in a change of the flower colour of two RNAi transformants, this appeared as the only pleiotropic effect.

3.1 Introduction

In the previous chapter we described the steps that are needed for silencing a gene by RNA interference in potato. This chapter describes one of the succeeding steps in the *S*-gene strategy. In this master thesis period, it is a constraint to complete the full *S*-gene strategy for a single gene. From finding the ortholog to rooting of transformed shoots grown from callus alone, it takes about four months. These shoots harvested from explant callus are tested for their rooting ability in medium with kanamycin. In this way false positives, which are not transformed, are discarded. This is needed to confirm that these plants are true transformants, at least for the presence of the nptII gene. When a sufficient number of rooting transformants is obtained from different explants and multiplied to acquire biological replicates, these plants can be used in experiments. To complete the next step that is part of the *S*-gene strategy RNAi::*St-SR1-h3* silenced potato plants were provided by Kaile Sun, and prepared to test foliage resistance to *Phytopthora infestans.*

Quantification of foliage resistance

The assessment of foliage resistance to late blight caused by *P. infestans* can be pursued in a multitude of ways. Whole plant assays in the field or greenhouse are important to screen resistance to late blight in plants while resembling natural conditions (Colon & Budding, 1988; Fry, 1978). However, testing in the field can only be done once in a growing season and for genetically modified plants special applications have to be admitted due to legislation concerning genetic modification of organisms in Europe. In addition to the whole plant assay, estimation of foliage resistance to late blight can also be obtained in laboratory tests. Amongst others (leaflets and leaf disks) the detached leaf assay is a good test to quantify partial resistance (Hodgson, 1961; Vleeshouwers et al., 1999). In a comparison of foliage resistance between the detached leaf assay (DLA) and the whole plant assay for cultivars Bintje and Robijn, foliage resistance is estimated to be lower in DLA tests than for intact plants. The difference is suggested to depend on environmental conditions: a higher relative humidity by experimental design, rather than by the detachment of leaves (Vleeshouwers et al., 1999).

***AtSR1* is a negative defense regulator**

AtSR1 (Signal Responsive 1) encodes a Ca^{2+}/calmodulin-binding transcription factor that is involved in salicylic acid-mediated immune response (Du et al., 2009). Loss of function alleles of *AtSR1* provides constitutive resistance to virulent *Pseudomonas syringae* pv. *tomato* DC3000

(Pst DC3000). AtSR1 binds to the promoter of *EDS1* (Enhanced Disease Susceptibility1), a known regulator of salicylic acid level, and supresses its expression (Wiermer, Feys, & Parker, 2005). Furthermore, binding of Ca^{2+}/calmodulin to AtSR1 is required for defense suppression linking Ca^{2+} signalling to SA level (Du et al., 2009). Suggesting that *AtSR1* is a negative defense regulator of the plant innate immunity, it can be classified as a susceptibility gene. In potato we found three candidate orthologs of *AtSR1* (Figure 3.1.1), of which *St-SR1-h1* did not induce resistance to *P. infestans* (Kaile Sun, unpublished data). But this study is a first indication that the *S*-gene function is conserved between *AtSR1* and *St-SR1-h3* (PGSC0003DMP400026345) for resistance to *P. infestans* isolate PIC99177.

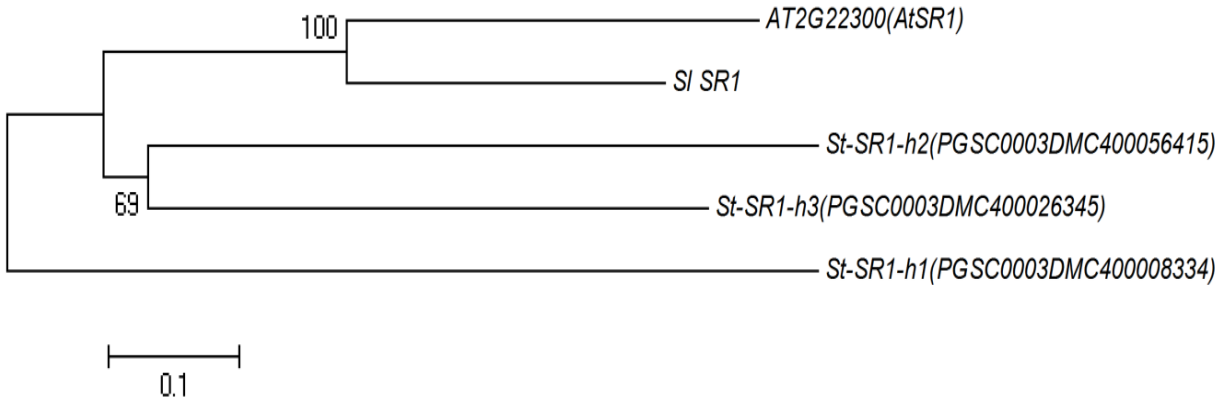

Figure 3.1.1. *Phylogenetic tree of Arabidopsis SR1 plus candidates of tomato (Sl) and potato (St) orthologs. St-SR1-h3 has been studied in this chapter.*

3.2 Material & Methods

Growth and experimental conditions

In vitro propagated *St-SR1-h3* (PGSC0003DMP400026345) silenced transformants of Désirée were kindly provided by Kaile Sun. Plant transformation was similar as the procedure described in the material and methods of chapter 2. The transformants were transferred from MS medium (Murashige & Skoog, 1962) supplemented with vitamins, sucrose 30 g L^{-1} and $Kanamycin^{+}$ 100 mg L^{-1} to similar fresh MS medium without Kanamycin. After three weeks of growth at 24° C, relative humidity of 70% and light intensity 100 W/m^2, the rooted transformants were relocated to the greenhouse (compartment 12.8, Unifarm, Wageningen). Three biological replicates were grown for each transformant in plastic pots in potting soil (Horticoop, Lentse potgrond). After five and seven weeks of greenhouse growth the fourth or fifth fully developed leave (counted from the top) were used for the detached leaf assays, DLA1 (five weeks) and DLA2 (seven weeks). The compound leaves consisting of three leaves, the two small leaves closest to the stem were cut and removed, were placed in distilled water saturated florists foam (Oasis®). The two cut leaves were immediately wrapped in aluminium foil and frozen in liquid nitrogen, all samples were stored at -80° C and used for RNA isolation. The three leaflets of the compound leave were inoculated by pipetting four 10-µL droplets to the abaxial side of each leaflet, avoiding direct contact with the veins. During the experiment up to three compound leaves were placed in a plastic box by randomization. The boxes were covered with transparent plastic bags to maintain 100% relative humidity inside the box and placed in a climate chamber at 15° C in order to provide optimal growth conditions for *Phytophtora infestans*.

***Phytophthora infestans* culture**

For the preparation of the inoculum of *P. infestans,* isolate PIC99177 (race 1, 2, 3, 4, 7, 9 and 11) was transferred to rye sucrose agar (RSA), according to Caten & Jinks (1968) provided by Gert van Arkel. It was placed for 10-15 days at 15° C with high humidity to induce sporangia formation. Zoospore release by the sporangia was accomplished by adding five mL cold milliQ water to the Petridish and incubation for three hours at 4° C. The zoospore concentration was observed by bright field microscope using a Fuchs-Rosenthal counting chamber and adjusted to 5×10^5 spores per mL. The total zoospore number was obtained by counting all zoospores in the sixteen grids of the counting chamber and multiplying that number by 625 (http://www.celeromics.com, 2014). Isolate PIC99177 is mild in its aggressiveness to infect host plants, and hyphae growth is more visible compared to other isolates of *P. infestans*.

Infection measurements

Infection was assessed by using a disease score system (Figure 3.2.1) on the fifth and sixth days post inoculation (dpi) for DLA1. In DLA2 this incubation was extended with an extra day as infection in the susceptible control was slower compared to DLA1. The lesion diameter was measured from three to six dpi for DLA1 and from three to seven dpi for DLA2, by using an electronic calliper (Helios DIGI-MET®) attached to a computer with software programme Excel (Microsoft). The largest length and width (perpendicular to the length) of each lesion was measured and the average lesion size was calculated. On the fifth day post inoculation pictures of leafs were taken.

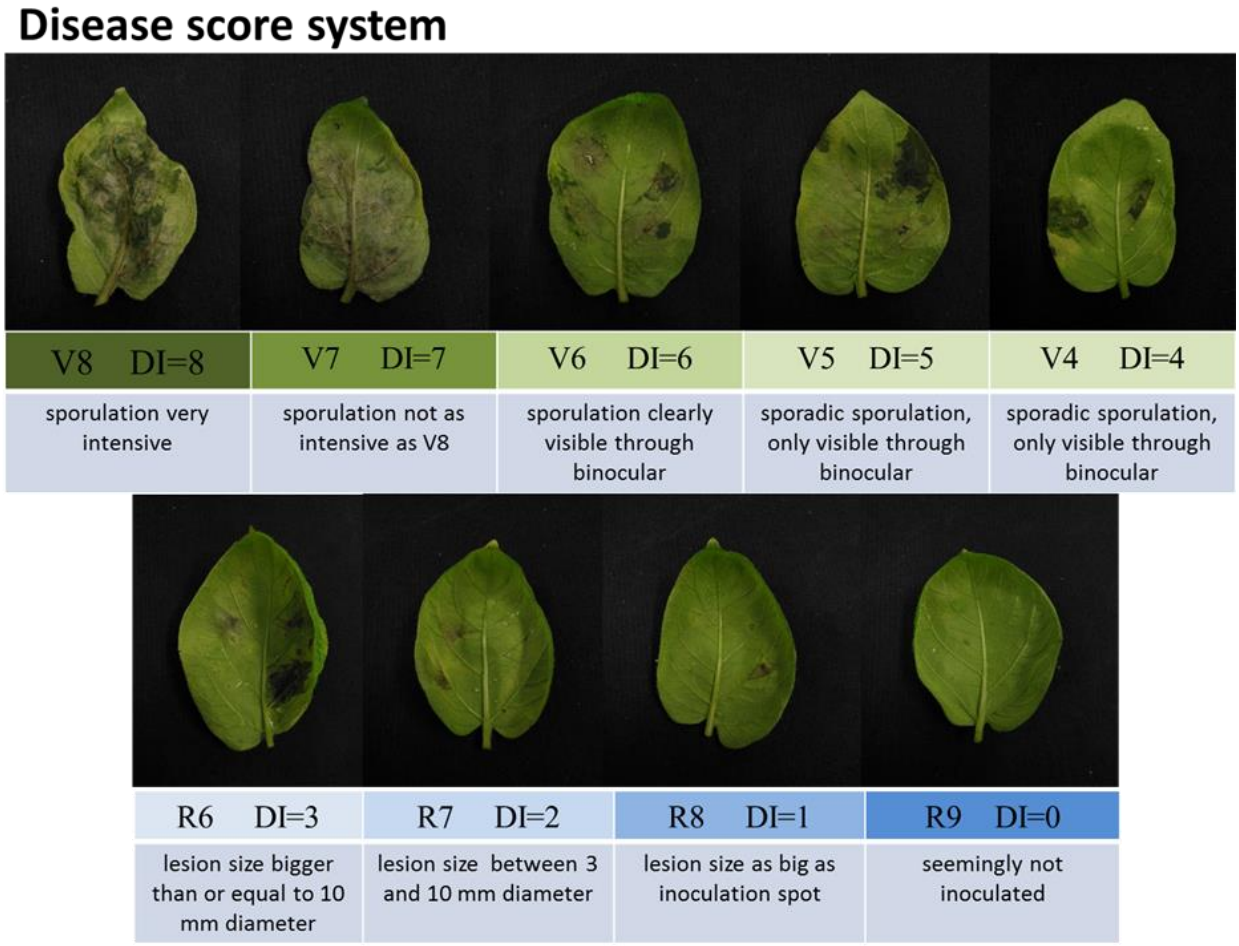

Figure 3.2.1. *General potato pictorial disease index (DI) score system for late blight infection (DI 0-8).*

When the plants had grown for eleven weeks in the greenhouse, spontaneous infection of the transformants and the untransformed controls by greenhouse endemic powdery mildew was assessed. Infection was scored using a disease score system (Figure 3.2.2).

Figure 3.2.2. *Pictorial powdery mildew score system (DI 0-3)*

Comparision of susceptibility

Cultivar Désirée was included in all experiments as the susceptible control. In addition, a positive control (A13-13) was included as a reference for HR-like (qualitative) resistance, A13-13 is a Désirée transformant carrying the *vnt1.1 R*-gene.

RNA isolation and (qRT)-PCR

Excised Leaf material was taken from the -80° C freezer and kept in liquid nitrogen until grinding by using mortal and pestle. Total RNA was extracted using the MagMAX-96 total RNA Isolation kit (Ambion). RNA was treated with RNAse free DNAse (Qiagen). The quantity of isolated RNA was measured by using the Isogen Nanodrop Spectrophotometer ND-1000. The results of the nanodrop were used to calculate dilutions of the RNA for the final concentration of 500 ng/µL in a final solution of 10 µL. The final solution was used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad). For the determination of relative transcript levels 40 ng cDNA was used together with the iQ SYBR Green supermix (Bio-Rad) and the C1000^{TM} Thermal Cycler PCR system (Bio-Rad). The annealing temperature of the program was set at 60° C, and repeated 40 cycles. For normalisation by the $2^{-\Delta\Delta Ct}$ method transcript levels of housekeeping gene *EF1α* were used (Livak & Schmittgen, 2001; Nicot et al., 2005). Primers used for determination of transcript levels were gene specific (Table 3.2.1.). Three technical replicates were used.

Table 3.2.1. Primer used for determination relative transcript levels of *St-SR1-h3, St-SR1-h2, EF1α PR1* and *Lox-D.*

Gene	Primer pair
St-SR1-h3	Fw - CTGGGAAGAGAAAGGAGGGG Rv - GCAGGAGTATTAACATTCGATGC
St-SR1-h2	Fw- GGAGCTTATGGAAGTACAGAGGA Rv - CAATGATCTGGGACCATGAA
EF1α	Fw - ATTGGAAACGGATATGCTCCA Rv - TCCTTACCTGAACGCCTGTCA
SlPR2/StPR1?	Fw - GCTACATACTCGGCCCTTGA Rv - TGTTGTAAGTCCTCGCGTTG
Lox-D	Fw - CCGTGGTTGACACATTATCG Rv - ACAGCAGTCCGCCCTATTTA

Statistical analysis

For comparisons of means Student's t-test was performed.

3.3 Results

RNAi::*St-SR1-h3* transformants had quantitative resistance to PIC99177

We screened 50 RNAi::*St-SR1-h3* (numbers 11-60) silenced potato transformants (cv Désirée) by a detached leaf assay (DLA) to asses foliage resistance for *Phytophthora infestans* isolate PIC99177. Out of these 50 transformants a selection was made (Figure S3.3.1), a criterion for the selection was based on uniform infection pattern over the biological replicates. In total 25 out of the 50 transformants were selected based on the first DLA experiment. The average disease index (DI) score for Désirée and A13-13 was 4,25 and 0,5, respectively. In this selection twelve transformants had lower average DI scores than cv Désirée, but higher DI scores than A13-13, and thirteen transformants had DI scores equal to Désirée (Figure 3.3.1).

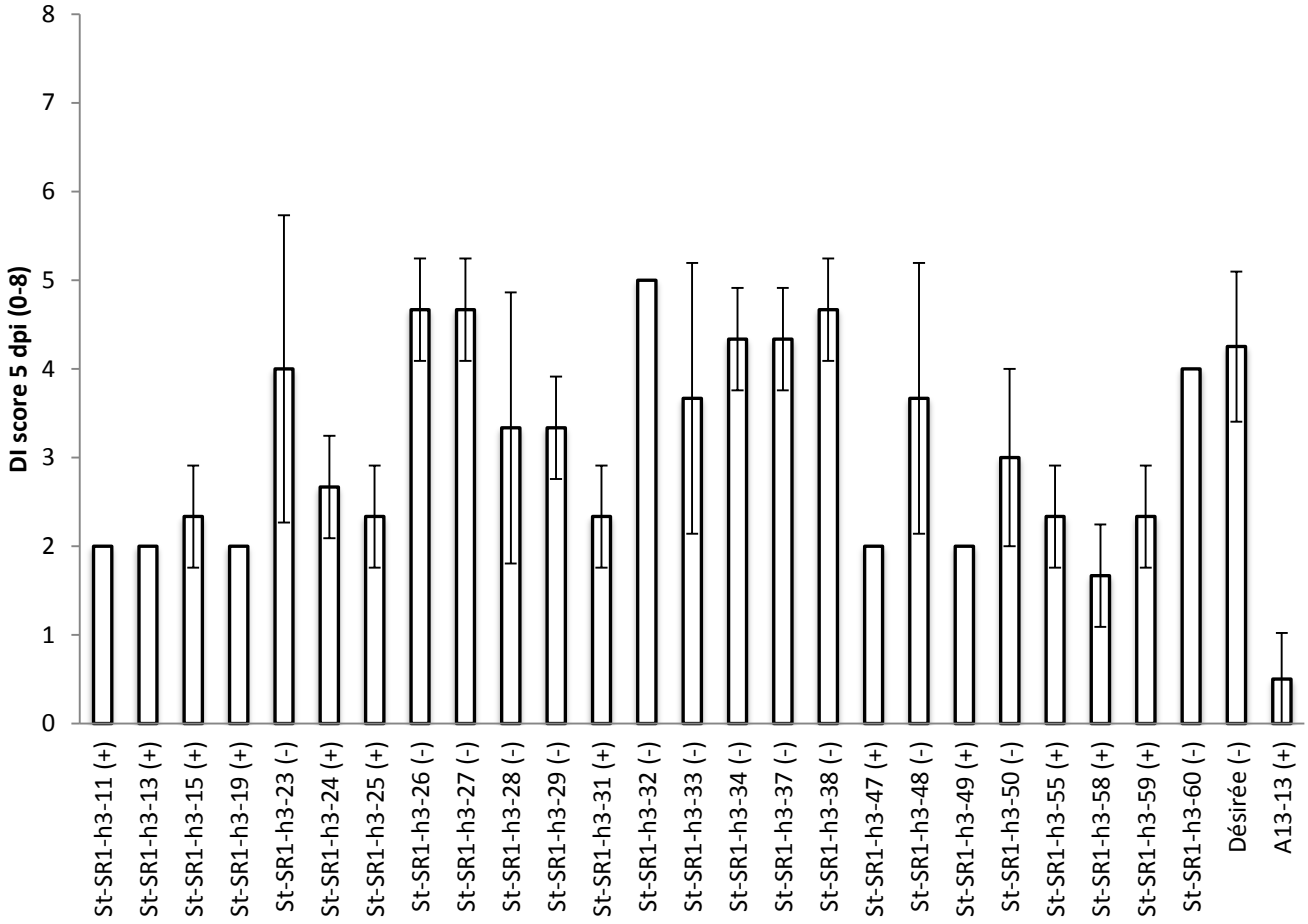

Figure 3.3.1. *Disease index (DI) score of 25 St-SR1-h3 transformants and their controlles for P. infestans isolate PIC99177 five days post inoculation (dpi). Minus signs behind the transformants names indicate no difference with Désirée. Plus signs indicate a lower disease index score than Désirée. Bars and error bars represent average ± standard deviation, respectively (n=3 for RNAi::St-SR1-h3 plants, n=12 for Désirée and A13-13 plants).*

To study the gene expression level of *St-SR1-h3* in the selected transformants a (qRT)-PCR was performed to quantify to what extent silencing occurred in the leaves prior to the inoculation step with PIC99177. In total eight transformants, showed an average relative expression level below 0.40 when compared to the relative expression of *St-SR1-h3* in Désirée (Kaile Sun et al.,

submitted) (Figure 3.3.2). In these eight transformants (19, 28, 33, 34, 49, 55, 58 and 59), silencing is considered as effective for inducing resistance.

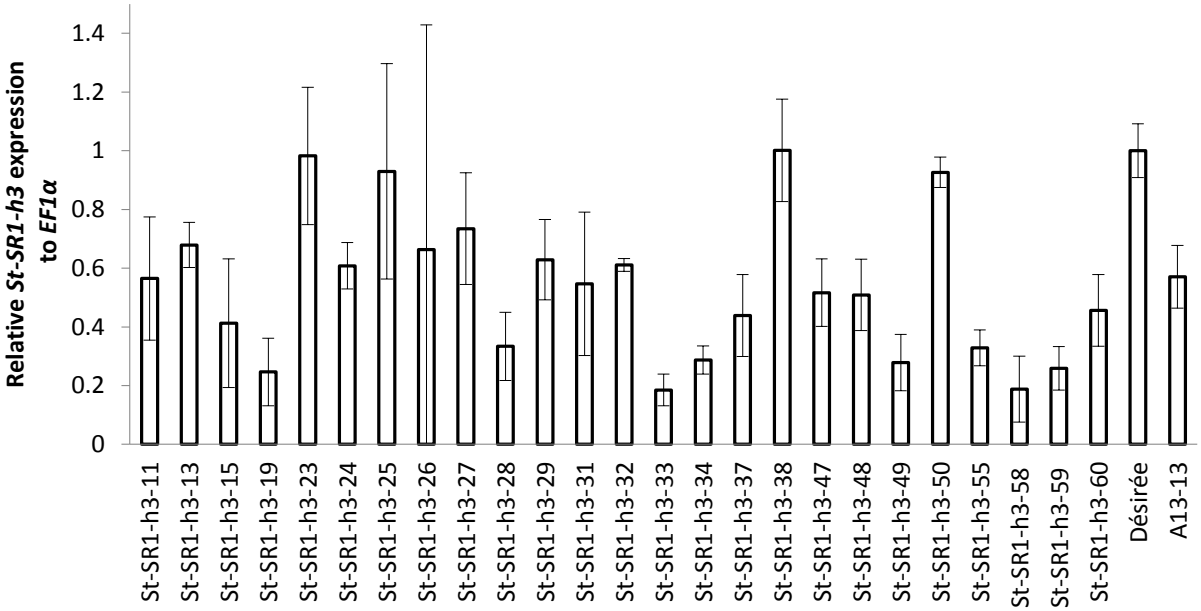

Figure 3.3.2. *Relative expression of St-SR1-h3 to EF1α in the leaves of 25 RNAi::St-SR1-h3 silenced Désirée transformants, A13-13 and cv Désirée. Average ± standard deviation, n=3 with three technical replications per biological replicate.*

For a better understanding of the relation between *St-SR1-h3* expression and *P. infestans* infection, the expression value was plotted against the lesion size at six dpi (Figure 3.3.3). The lesion size of six out of the eight transformants with a low *St-SR1-h3* transcript level was statistically lower compared to the lesion size of cv Désirée, the two others did not show statistical difference.

No HR-like resistance was observed in the first DLA experiment for the 50 *St-SR1-h3* silenced transformants compared to the qualitative resistance of the positive control plant carrying the *vnt1.1* R-gene (A13-13). Transformants 19, 28, 34, 49, 55, 58 and 59 had a low *St-SR1-h3* expression (<0,4) prior to inoculation and lower infection of PIC99177 than on Désirée in at least one of the two quantification methods: DI score, transformants 19, 49, 55, 58 and 59 (Figure 3.3.1) or lesion diameter, transformants 19, 28, 34, 55, 58 and 59 (Figure 3.3.4). Transformant 33 that had a low *St-SR1-h3* expression level did not differ in both lesion size (statistically) and disease index score (Figure 3.3.1, 3.3.2 and 3.3.4). To find an explanation for the variation in these results gene expression of the closest homolog of *St-SR1-h3*, named *St-SR1-h2* (PGSC0003DMP400056415) in the phylogenetic tree (figure 3.1.1), was studied to see if these homologs are functionally redundant.

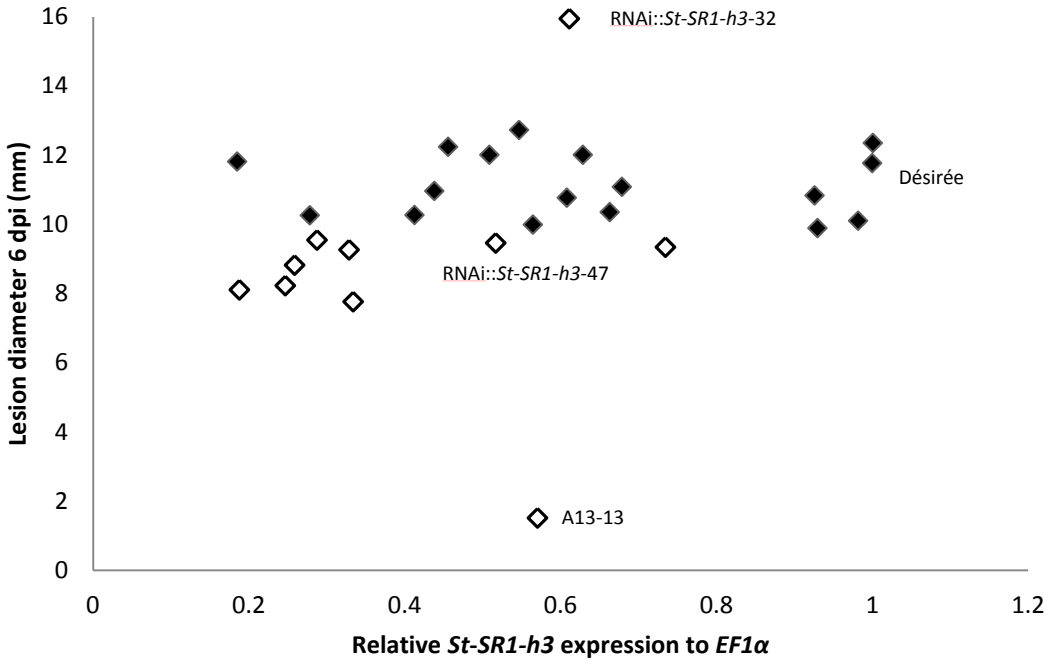

Figure 3.3.3. *Relative expression of St-SR1-h3 (x-axis) in the leaves prior to inoculation was plotted against the lesion diameter at six dpi (y-axis) of leaves from 25 RNAi::SR1-h3 potato transformants. Lesion diameter differs statistically from Désirée for the white diamonds but not for the black diamonds (Student's t-test, P<0,05).*

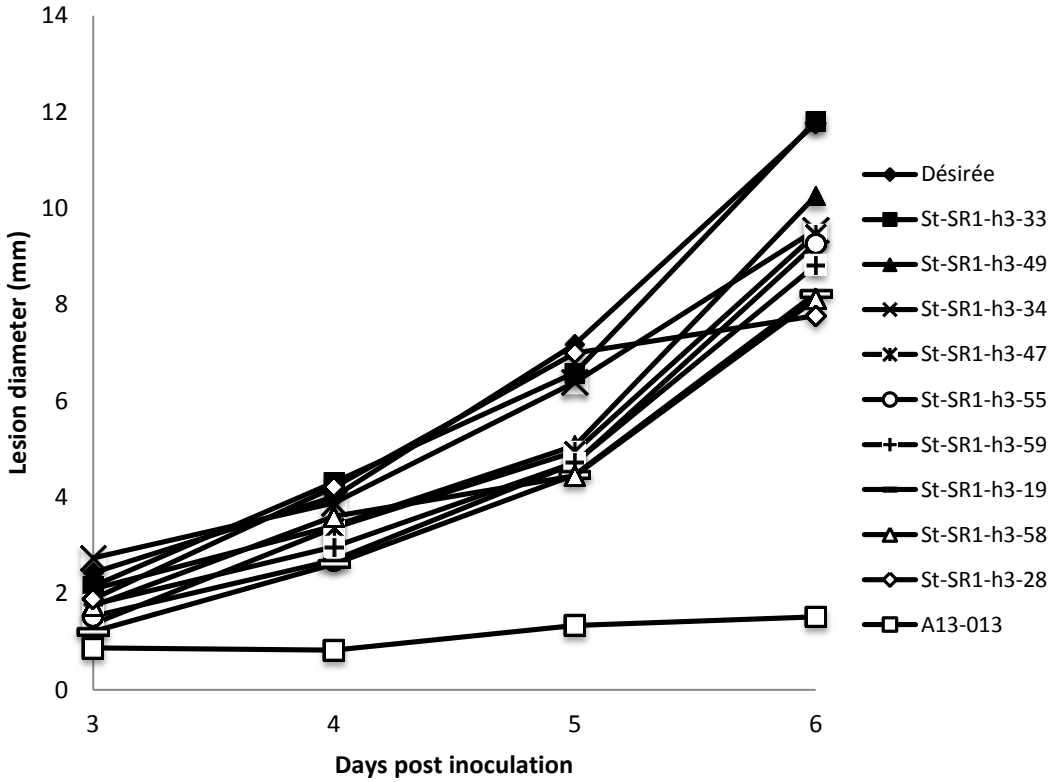

Figure 3.3.4. *Average lesion diameter (mm) of infection by P. infestans PIC99177 measured on three to six dpi for nine RNAi::St-SR1-h3 silenced plants, Désirée and A13-13. White markers indicate that the lesion size differed statistically from Désirée on six dpi (student's t-test, P<0,05). n=3 with 3 leaflets per compound leave, each leaflet was inoculated with four droplets of inoculum.*

Co-silencing of homolog *St-SR1-h2*

Unexpectedly the relative gene expression of the homolog *St-SR1-h2* is lower in all of the 25 selected RNAi::*St-SR1-h3* transformants, except for transformants 24, 26, 28, 49 and 59, compared to the relative expression in the reference cv Désirée. In transformants, 28, 49 and 59, that had a resistant phenotype and low *St-SR1-h3* transcript level, the relative expression of *St-SR1-h2* was equal or higher compared to Désirée. Another observation was the relative expression of *St-SR1-h2* and *St-SR1-h3* in the positive control A13-13. In this transformant the expression is around 55% and 57% compared to cv Désirée, respectively (Figure 3.3.5 and 3.3.2).

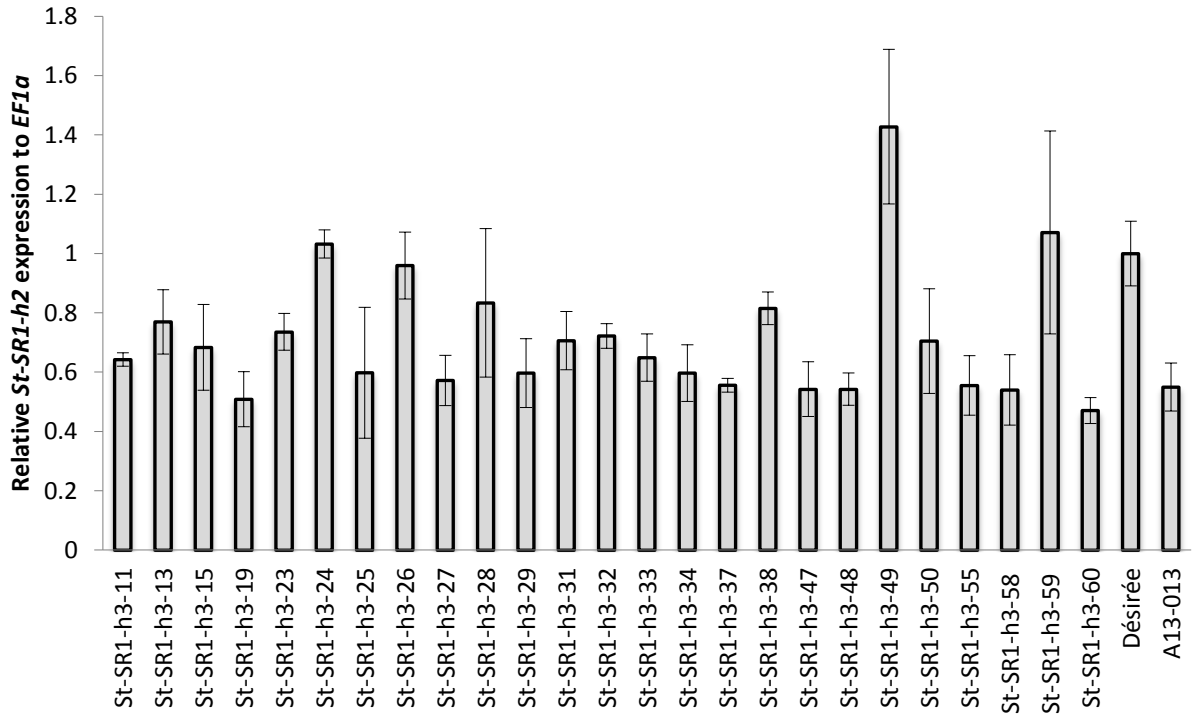

Figure 3.3.5. *Relative expression of St-SR1-h2 to EF1α in the leaves of 25 RNAi::St-SR1-h3 silenced Désirée transformants, A13-13 and cv Désirée. Values represent average ± standard deviation, n=3 with three technical replications per biological replicate.*

Two week older RNAi::*St-SR1-h3* transformants showed similar results

To study if the results from the first detached leaf assay are reproducible, at seven weeks of growth in the greenhouse, the selected 25 *St-SR1-h3* silenced potato plants were tested for a second time with a detached leaf assay. RNAi::*St-SR1-h3* silenced plants 19, 28, 49, 58 and 59 are transformants that had lower infection patterns in both detached leaf assays (Figure 3.3.6). An interesting transformant was number 47 that switched from quantitative resistance to qualitative resistance between DLA1 and DLA2 (Figure 3.3.4 and 3.3.6).

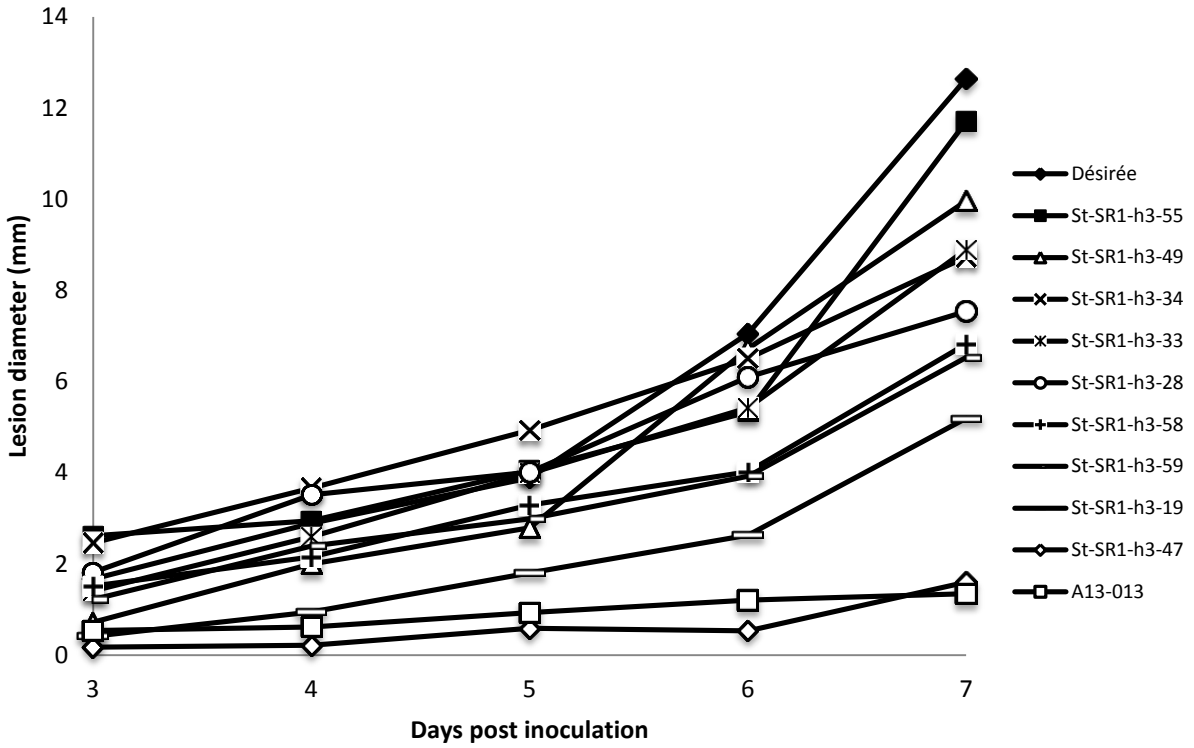

Figure 3.3.6. *Average lesion diameter (mm) on leaves upon infection with P. infestans PIC99177 measured on three to seven dpi. White markers indicate that the lesion size differed statistically from Désirée on seven dpi (student's t-test, P<0,05). n=3 with 3 leaflets per compound leave, each leaflet was inoculated with four droplets of inoculum.*

Transformant 47 was together with transformants 19, 49, 58 and 59 used to study *PR1* and *LoxD* transcript levels in the leaves prior to the second detached leaf assay. *PR1* is a marker gene for SA signalling and *LoxD* for JA signalling. In this small selection of plants *PR1* was only higher in the leaves of RNAi transformants 19 and 47 in the onset of DLA2 compared to the expression of *PR1* in Désirée (Figure 3.3.7A). Transcript level of *LoxD* was higher in transformants 19, 47 and 59, lower in transformant 49 and equal to the expression of *LoxD* in Désirée for transformants 58 and A13-13. In the clones of 19, 49 and A13-13 *St-SR1-h3* expression was low, but not in transformants 47, 58, and 59 when compared to the expression of *St-SR1-h3* in Désirée (Figure 3.3.7C). Transformant 47 had a five-fold increase in the relative expression of *St-SR1-h2* compared to cv Désirée, whereas for RNAi::*St-SR1-h3* no difference was observed (Figure 3.3.7C and D). Expression of *St-SR1-h2* was slightly higher in transformant 59, lower in A13-13 and similar in transformants 19, 49 and 58 when compared to the expression of *St-SR1-h2* in Désirée (Figure 3.3.7D).

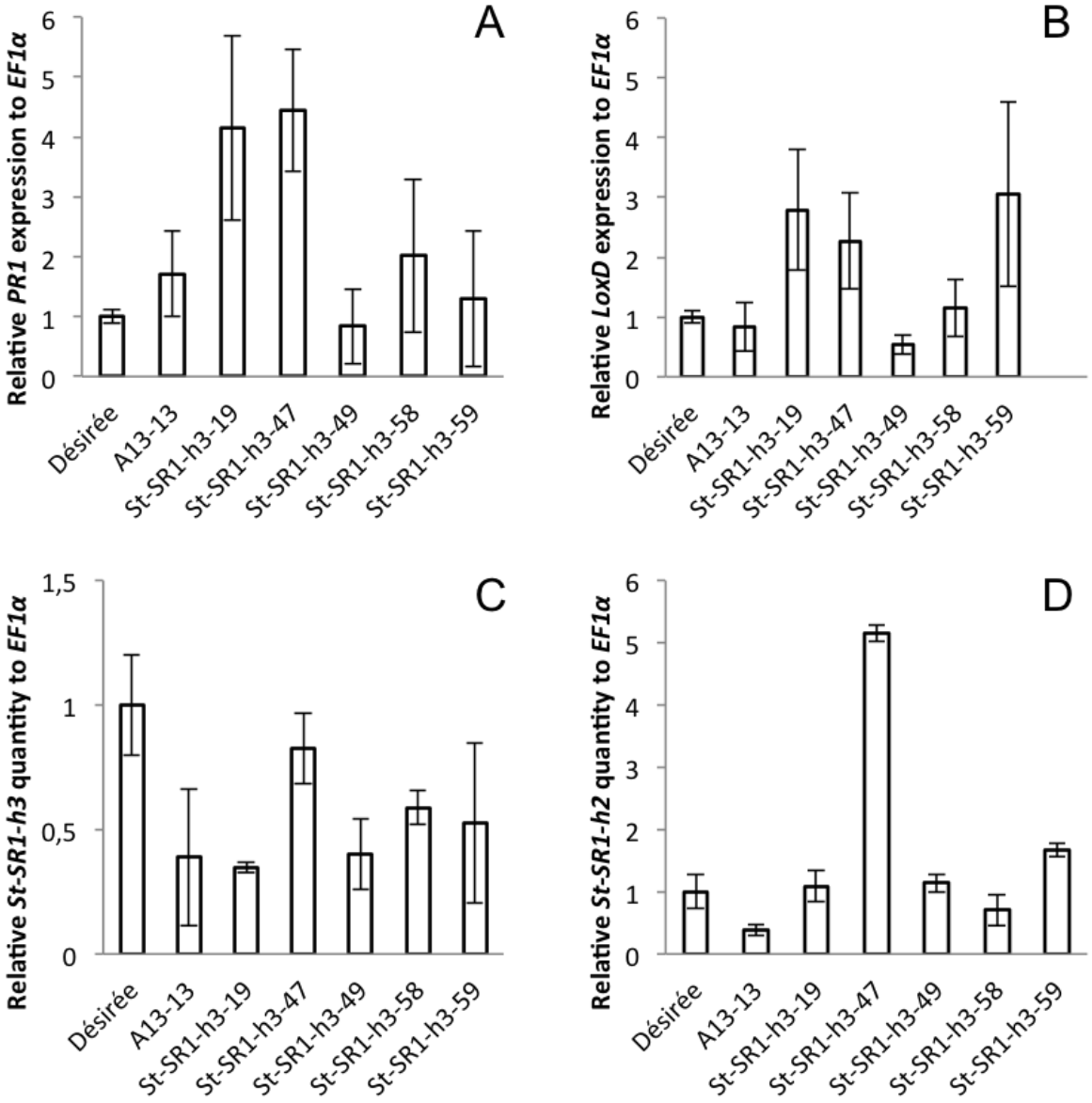

Figure 3.3.7. *Relative PR1 (**A**), LoxD (**B**), St-SR1-h3 (**C**) and St-SR1-h2 (**D**) expression to EF1α in seven week old greenhouse grown RNAi::St-SR1-h3 transformants, untransformed Désirée and Désirée carrying the vnt1.1 R-gene. Both PR1 and LoxD transcript level was higher in transformants 19 and 47 compared to Désirée. Average ± standard deviation, n=3 with three technical replications per biological replicate.*

RNAi transformants 19 and 47 are holding the attention

As the greenhouse grown plants aged towards flowering, most transformants had either the expected purple flowers (Figure 3.3.8A) or did not flower (Figure 3.3.8B). A striking observation was the white coloured flowers of transformants 19 and 47 (Figure 3.3.8A and C). Especially since in the second DLA, the detached leaves of these transformants had the highest quantitative resistance and qualitative resistance to *Phytophthora infestans* isolate PIC99177 (Figure 3.3.6). Interestingly, the tuber colour of these transformants with a deviating flower colour was similar to the tuber colour of Désirée (Figure 4.3.8D).

Figure 3.3.8. *Flowering of RNAi::St-SR1-h3 transformants. The white flowers of St-SR1-h3-47 next to a transformant with purple flowers (**A**). A non-flowering transformant (**B**). White flowers of RNAi transformant St-SR1-h3-19 (**C**). Similar coloured tubers of Désirée and St-SR1-h3-47 (**D**).*

RNAi::*St-SR1-h3* transformants had normal plant height

Plant height was measured prior to the first DLA experiment. The eight transformants in which *St-SR1-h3* expression was low, did not differ in plant height (cm) when compared to cv Désirée (Figure 3.3.9). Except for transformant 33 that was larger in length.

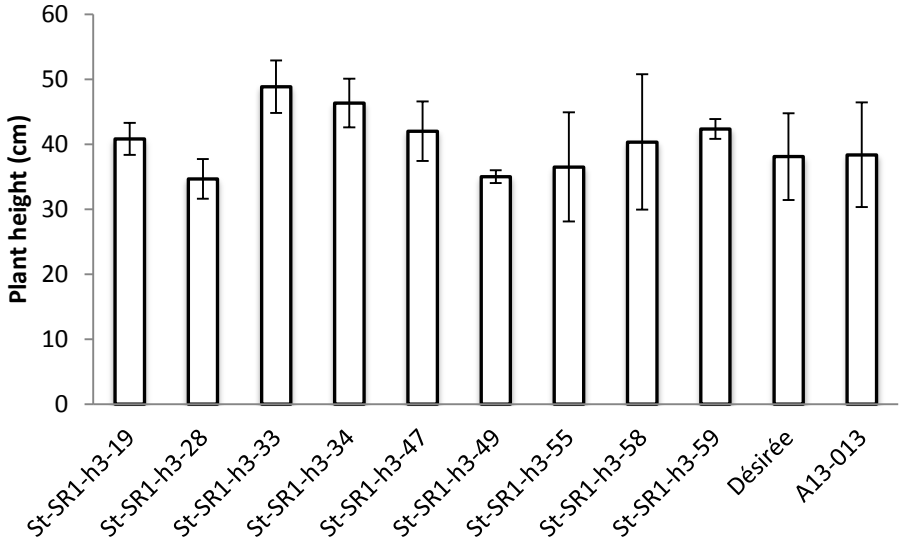

Figure 3.3.9. *Plant height in cm of Désirée, A13-013and RNAi::St-SR1-h3 transformants that had a low St-SR1-h3 expression in the leaves. The plantlets were eight to nine weeks old when measured.*

A pleiotropic effect of the *Arabidopsis Atsr1* mutant grown in the low temperature regime was reduced growth (Du et al., 2009). To study if this abnormal plant growth holds up in RNAi::*St-SR1-h3* potato transformants we performed an *in vitro* experiment. After 17 days of growth no such difference was found in the selected plants of this experiment (Figure S3.3.3). The only abnormal growth observed in our *in vitro* temperature experiment was reduced root size of transformant RNAi::*St-SR1-h3*-19, that was observed at both temperature regimes (Figure S3.3.4)

RNAi::*St-SR1-h3* transformants were susceptible to greenhouse endemic powdery mildew

After eleven weeks all plants were screened for natural powdery mildew infestation, caused by greenhouse endemic species. The plantlets were not inoculated but infection occurred spontaneously in the greenhouse. On all the 50 *St-SR1-h3* transformants, A13-13 and Désirée plantlets infection by endemic powdery mildew was observed. The average disease index score for powdery mildew on Désirée and A13-13 was 1,5 and 2,0, respectively, twelve RNAi::*St-SR1-*

h3 transformants had a lower disease index score than Désirée and A13-13 (Figure S3.3.5). For eight out of these twelve RNAi::*St-SR1-h3* transformants the expression of *St-SR1-h3* was quantified (13, 15, 33, 47, 48, 49, 55 and 59), only in transformants 33, 49, 55 and 59 *St-SR1-h3* expression was lower than 0,40 (Figure 3.3.2).

3.4 Discussion

Results of this study indicate that *St-SR1-h3* is likely to be the functional potato ortholog of the *Arabidopsis AtSR1* gene. As quantitative foliage resistance of RNAi::*St-SR1-h3* transformants 19, 28, 34, 49, 55, 58 and 59 to *Phytophthora infestans* isolate PIC99177 seemed related to low expression of *St-SR1-h3* in these seven transformants. A comparable, more qualitative, resistant phenotype was reported for the *Arabidopsis Atsr1* mutant challenged with *Pst* DC3000 (Du et al., 2009). RNAi::*St-SR1-h3* transformant 33, that also had a low *St-SR1-h3* expression, does not support the suggested hypothesis based on the result from the first DLA experiment. In the second DLA experiment this transformant had a resistant phenotype however it is not known whether or not the expression of *St-SR1-h3* was low at that time. In addition, quantitative variation for endemic powdery mildew resistance was found. In this study one isolate of *P. infestans* was tested with a DLA experiment that was repeated using the same plants. To identify if the quantitative resistance is broad spectrum, in future experiments isolates with different virulence spectrums should be tested. As well as testing for different pathogens or parasites, such as *Alternaria solani* (early blight) or nematodes. Besides different pathogens, the tubers of the resistance candidates should be tested for resistance to late blight.

Differences in lesion were small between the RNAi::*St-SR1-h3* plants and the susceptible control Désirée in the first detached leaf assay. It even occurred that the lesion diameter was equal to Désirée on three to five dpi but on six dpi the average lesion size was significantly smaller (Figure 3.3.4). A good method to measure partial resistance is to calculate the lesion growth rate (LGR) and arrested lesions can be used to obtain the infection efficiency (IE) as described by Vleeshouwers et al. (1999). Using these methods might give a more accurate indication of partial resistance.

The expression studies of *PR1* and *LoxD* in a selection of plants prior to the second DLA clarified the lower susceptibility of transformants 19 and 47 for *P. infestans* in the second DLA to some extent. Both *PR1* and *LoxD* seemed to be up regulated prior to inoculation with *P. infestans*. This suggests that in these transformants defense mechanisms were more active at that moment, potentially resulting in partial- and qualitative resistance to *P. infestans* for transformants 19 and 47, respectively.

It appears that expression of the homolog *St-SR1-h2* was co-silenced by the RNAi::*St-SR1-h3* silencing vector. The coding sequence used for the *St-SR1-h3* silencing vector has an overlap of eleven nucleotides with the coding sequence of *St-SR1-h2* (Figure S3.3.2). Despite that the vector contains a stretch of only eleven nucleotides homology with *St-SR1-h2*. It has been shown that short homologous RNA sequences of 23 nucleotides were effective for post-transcriptional gene silencing (Thomas et al., 2001). In several transformants, that showed quantitative resistance to *P. infestans*, the expression of *St-SR1-h2* was equal to the expression of this homolog in Désirée. Suggesting that the homologs *St-SR1-h2* and *St-SR1-h3* are not functionally redundant. To test this hypothesis, re-transformation of RNAi::*St-SR1-h3* transformants that had a resistant phenotype with a *St-SR1-h2* silencing vector should elucidate the function of this homolog regarding resistance to *P. infestans.*

The remarkable white flower phenotype of RNAi::*St-SR1-h3* transformants 19 and 47 was unexpected and a very rare combination with the expected pink tuber colour. It is common for white flowered potato plants to have yellow coloured tubers. It would be interesting to grow another batch to identify if this is a stable phenotype. It appears that these transformants were the only ones that showed pleiotropic effects as a result from the transformation.

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Supplemental (S) figures

	Name	5 dpi	6 dpi		Name	5 dpi	6 dpi
	SR1C-11 a	R7	V5		SR1C-36 a	V5	V7
	SR1C-11 b	R7	R6		SR1C-36 b	V4	V7
	SR1C-11 C	R7	R6		SR1C-36 C	R7	V5
	SR1C-12 a	V5	V7		SR1C-37 a	V5	V7
	SR1C-12 b	V5	V7		SR1C-37 b	V4	V7
	SR1C-12 C	V5	V5		SR1C-37 C	V4	V7
	SR1C-13 a	R7	V6		SR1C-38 a	V4	V8
	SR1C-13 b	R7	V5		SR1C-38 b	V5	V7
	SR1C-13 C	R7	V5		SR1C-38 C	V5	V7
	SR1C-14 a	V4	V6		SR1C-39 a	V4	V5
	SR1C-14 b	V4	V6		SR1C-39 b	R6	V7
	SR1C-14 C	R6	V6		SR1C-39 C	R7	V6
	SR1C-15 a	R6	V6		SR1C-40 a	V4	V6
	SR1C-15 b	R7	V4		SR1C-40 b	V4	V8
	SR1C-15 C	R7	R6		SR1C-40 C	R6	V6
	SR1C-16 a	V4	V7		SR1C-41 a	V5	V8
	SR1C-16 b	V4	V5		SR1C-41 b	V4	V6
	SR1C-16 C	V4	V5		SR1C-41 C	R6	V5
	SR1C-17 a	R7	V6		SR1C-42 a	V4	V6
	SR1C-17 b	V6	V8		SR1C-42 b	V4	V8
	SR1C-17 C	V4	V6		SR1C-42 C	V4	V6
	SR1C-18 a	R7	V4		SR1C-43 a	V4	V6
	SR1C-18 b	V4	V8		SR1C-43 b	R6	V7
	SR1C-18 C	V5	V7		SR1C-43 C	R7	V6
	SR1C-19 a	R7	V5		SR1C-44 a	V4	V7
	SR1C-19 b	R7	V6		SR1C-44 b	V4	V7
	SR1C-19 C	R7	V5		SR1C-44 C	R6	V5
	SR1C-20 a	R6	V5		SR1C-45 a	V4	V8
	SR1C-20 b	V4	V7		SR1C-45 b	R7	V4
	SR1C-20 C	R7	V5		SR1C-45 C	V4	V7
	SR1C-21 a	R6	V5		SR1C-46 a	V4	V8
	SR1C-21 b	R6	V4		SR1C-46 b	V5	V7
	SR1C-21 C	V4	V7		SR1C-46 C	R7	V6
	SR1C-22 a	V4	V6		SR1C-47 a	R7	V4
	SR1C-22 b	V4	V5		SR1C-47 b	R7	V5
	SR1C-22 C	R7	V4		SR1C-47 C	R7	V5
	SR1C-23 a	V5	V8		SR1C-48 a	R7	V7
	SR1C-23 b	R7	V7		SR1C-48 b	V4	V6
	SR1C-23 C	V5	V7		SR1C-48 C	V5	V7
	SR1C-24 a	R6	V8		SR1C-49 a	R7	V6
	SR1C-24 b	R7	V5		SR1C-49 b	R7	V6
	SR1C-24 C	R6	V6		SR1C-49 C	R7	V6

	SR1C-25 a	R6	V6		SR1C-50 a	R6	V7
	SR1C-25 b	R7	V5		SR1C-50 b	R7	V6
	SR1C-25 C	R7	V5		SR1C-50 C	V4	V7
	SR1C-26 a	V5	V7		SR1C-51 a	V4	V6
	SR1C-26 b	V5	V7		SR1C-51 b	R6	V4
	SR1C-26 C	V4	V7		SR1C-51 C	V4	V7
	SR1C-27 a	V4	V7		SR1C-52 a	V4	V7
	SR1C-27 b	V5	V7		SR1C-52 b	R7	V5
	SR1C-27 C	V5	V7		SR1C-52 C	R6	V6
	SR1C-28 a	V5	V7		SR1C-53 a	R7	V6
	SR1C-28 b	R6	V5		SR1C-53 b	R7	R6
	SR1C-28 C	R7	V4		SR1C-53 C	V4	V7
	SR1C-29 a	R6	V5		SR1C-54 a	V5	V7
	SR1C-29 b	V4	V7		SR1C-54 b	V5	V5
	SR1C-29 C	R6	V6		SR1C-54 C	V4	V7
	SR1C-30 a	V5	V7		SR1C-55 a	R7	V5
	SR1C-30 b	R6	V5		SR1C-55 b	R7	R6
	SR1C-30 C	V5	V7		SR1C-55 C	R6	V6
	SR1C-31 a	R7	V6		SR1C-56 a	R7	R6
	SR1C-31 b	R7	V5		SR1C-56 b	V4	V5
	SR1C-31 C	R6	V6		SR1C-56 C	V5	V7
	SR1C-32 a	V5	V7		SR1C-57 a	R7	V4
	SR1C-32 b	V5	V7		SR1C-57 b	R7	V6
	SR1C-32 C	V5	V7		SR1C-57 C	V4	V6
	SR1C-33 a	V5	V5		SR1C-58 a	R7	V4
	SR1C-33 b	V4	V7		SR1C-58 b	R8	R6
	SR1C-33 C	R7	V5		SR1C-58 C	R7	R6
	SR1C-34 a	V5	V7		SR1C-59 a	R6	V6
	SR1C-34 b	V4	V7		SR1C-59 b	R7	R6
	SR1C-34 C	V4	V7		SR1C-59 C	R7	V5
	SR1C-35 a	V4	V6		SR1C-60 a	V4	V7
	SR1C-35 b	V4	V7		SR1C-60 b	V4	V7
	SR1C-35 C	R7	V4		SR1C-60 C	V4	V7
	Desiree-1	V5	V7		Desiree-13	V5	V7
	Desiree-2	V4	V6		Desiree-14	V5	V7
	Desiree-3	V5	V7		Desiree-15	V5	V7
	Desiree-4	V4	V7		Desiree-16	V4	V6
	Desiree-5	V4	V7		Desiree-17	V4	V7
	Desiree-6	R7	V4		Desiree-18	V4	V8
	Desiree-7	V4	V6		Desiree-19	V5	V8
	Desiree-8	V4	V7		Desiree-20	V5	V7
	Desiree-9	V4	V7		Desiree-21	R7	V6
	Desiree-10	V5	V7		Desiree-22	V4	V6
	Desiree-11	V5	V7		Desiree-23	V4	V7
	Desiree-12	V5	V7		Desiree-24	V4	V7

	A13-013-1	R8	R8		A13-013-7	R8	R8
	A13-013-2	R9	R8		A13-013-8	R8	R8
	A13-013-3	R8	R8		A13-013-9	R9	R9
	A13-013-4	R9	R9		A13-013-10	R9	R8
	A13-013-5	R9	R9		A13-013-11	R9	R8
	A13-013-6	R8	R8		A13-013-12	R8	R8

resistance candidates
A13-013 lines
susceptible lines

Figure S3.3.1. *Selected 25 RNAi::transformants for (qRT)-PCR, selection was based on uniformity of infection scored using the disease score system.*

St-SR1-h2(PGSC0003DMC400056415)

ATGTGGAAGGATATGCTGGATCACTATGGGGTTTCTGCATCTGCTGAATCACAAACCAAATATTTGCACA
AGTTGGATGAGAATGCAATGCTTCAAACCTCATCAGAGAGAAGGGCCATAGAAGCATATGAAAGTTACA
AGTGGTGTGATTTCAGTGACAGGGAGGCTCAGACGGCTCCTGTACCAGCTTTTAAGCAACTCGAAGATTT
CAAGTATACTACATATCCTCCTGCTATCACTACTTTTGGATCCAACCCTGACGAATATACAACAATATTTGA
CCAAGATCAGATTGGTACTTCTCTTGAAGATGAGATGAGCTTAACTATTGCTCAGACGCAGAAATTTACTA
TTCGACATATATCCCCTGATTGGGGGTATTCATCTGAGGCAACAAAGATTGTTATCATTGGATCTTTTCTTT
GTAATCCTTCAGAGTGCACGTGGACTTGCATGTTCGGTGATATTGAAGTTCCTGTTCAGATCATTCAAGAA
GGTGTCATCTGTTGTCAAGCTCCTCGTCACTTGCCTGGTAAGGTCACCCTCTGTGTTACTAGCGGCAATCG
GGAGTCGTGCAGTGAGGTGAGGGAGTTTGAATACCGGGTAAAGCCTGATGATTGTGCTCGAAATAATCA
GCCTGATGTAGAAGGAGCTTATGGAAGTACAGAGGAACTGTTGTTACTTGTCAGGTTTGTGCAGCTGCTT
TTATCAGACTTATCTGTCCAAAAGGGAGAAAGCTCTGAATTAGGCAATGATTTCTTGGAAAAATCCAAAG
CAAGTGAAGATTCATGGTCCCAGATCATTGAATCTCTTTTGTTTGGATCTTCAATGCCAATGGTAACCATT
GATTGGCTTCTTCAAGAGCTTTTGAAAGACAAGTTCCAGCAGTGGCTTTCTTGTAAATTGCAACAAAAAG
ATAATCAGATAGGCTGTTCTTTATCTAAGAAAGAGCAAGGAGTAATTCACATGGTTGCTGGATTGGGATT
TGAGTGGGCGCTGCACCCAATTCTAAATGCTGGAGTCAGTGTTAACTTCCGTGATATTAACGGCTGGACG
GCCCTGCACTGGGCTGCACGCTTTGGGAGGGAAAAAATGGTTGCTTCGCTCATTGCATCTGGGGCATCG
GCTGGAGCTGTTACAGATCCCTCTTCACGAGATCCAGTTGGTAAAACTGCTGCATCAATTGCTTCCAGCTG
TGATCATAAGGGACTTGCAGGATATCTTTCAGAGGTAGCTCTCACCAGTCATCTTTCATCCCTCACTTTAG
AGGAGAGTGAGCTCTCAAAAGGTACTGCTGATGTGGAAGCAGAGAGAACTATTAGTAGCATATCAAACA
CAAGTGCCACCATAAATGAGGATCAGCGTTCTTTAAACGACACGTTAGCTGCAGTCCGTAATGCAGCTCA
GGCTGCTGCTCGTATACAGTCTGCATTCCGAGCACATTCGTTCCGCAAAAGACAAGAGAGAGAATTTGGT
GTCTCTGCTAGTGGAGATGAATATGGTATCCTCTCAAACGATATTCAGGGGCTTTCAGCTGCATCAAAGTT
GGCATTCCGCAACCCGCGAGACTACAACTCAGCAGCCTTAGCTATTCAGAAGAAATATCGAGGATGGAA
AGGCCGGAAAGATTTCCTTGCATTCCGCCAGAAAGTAGTGAAGATACAGGCTCATGTACGAGGCTATCA
GGTTAGAAAGCAATACAAGGTATGTTGGGCTGTTGGAATCTTGGAGAAGGTGGTGCTAAGGTGGCGTC
*GACGAGGTGTTGGTCTCCGGGGATTTCGACATGACACAGAATCAATTGATGAAATTGA**GGATGAAGACA***
TTCTAAAGGTGTTCCGCAAACAAAAAGTTGATGCTGCTCTTGACGAGGCTGTCTCGAGAGTTCTATCAAT
GGTTGAATCTCCAGGGGCACGTCAGCAATATCATCGGATTCTTGAGAAGTATCGGCAAGCTAAGGCTGA
ACTTGAAGGAGCGGACAGTGAAACAGCATCAACTGCTCATGGAGACATGTCTAACATGGAAAATGATGA
CATATACCAGTTCCCTAGTTATTAA

Figure S3.3.2. *Transcript sequence of St-SR1-h2, in red the nucleotides that are homologous to the silencing vector of St-SR1-h3.*

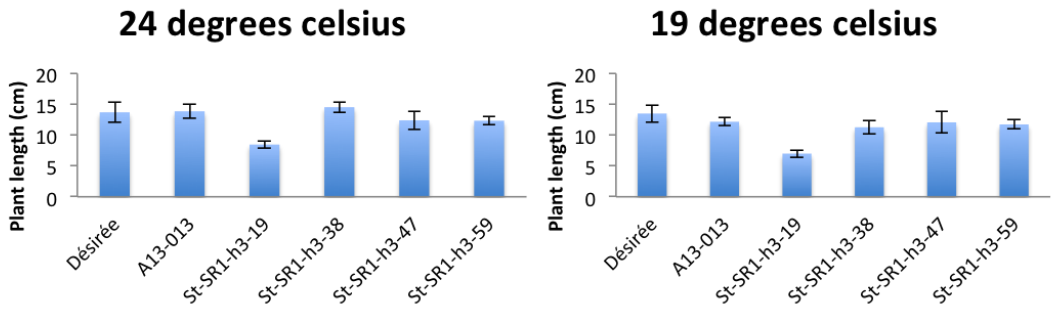

Figure S3.3.3. *Plant length (root + shoot) of seventeen days old in vitro grown RNAi::St-SR1-h3 transformants under two temperature regimes. No difference was observed between the different temperatures.*

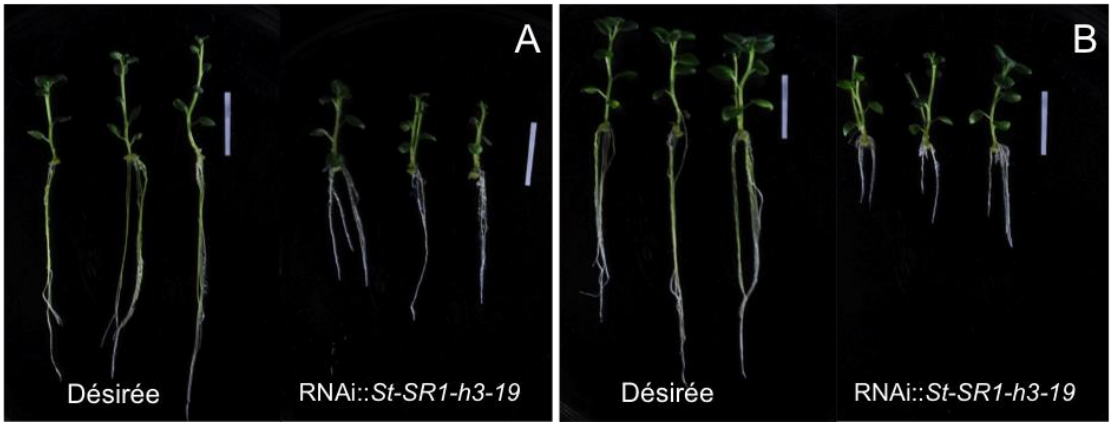

Figure S3.3.4. *Seventeen days old in vitro grown Désirée and RNAi::St-SR1-h3-19 plantlets. Plants were grown at 24° Celsius (**A**) and 19° Celsius (**B**). For both temperature regimes the root length of RNAi::St-SR1-h3-19 transformants were shorter than Désirée.*

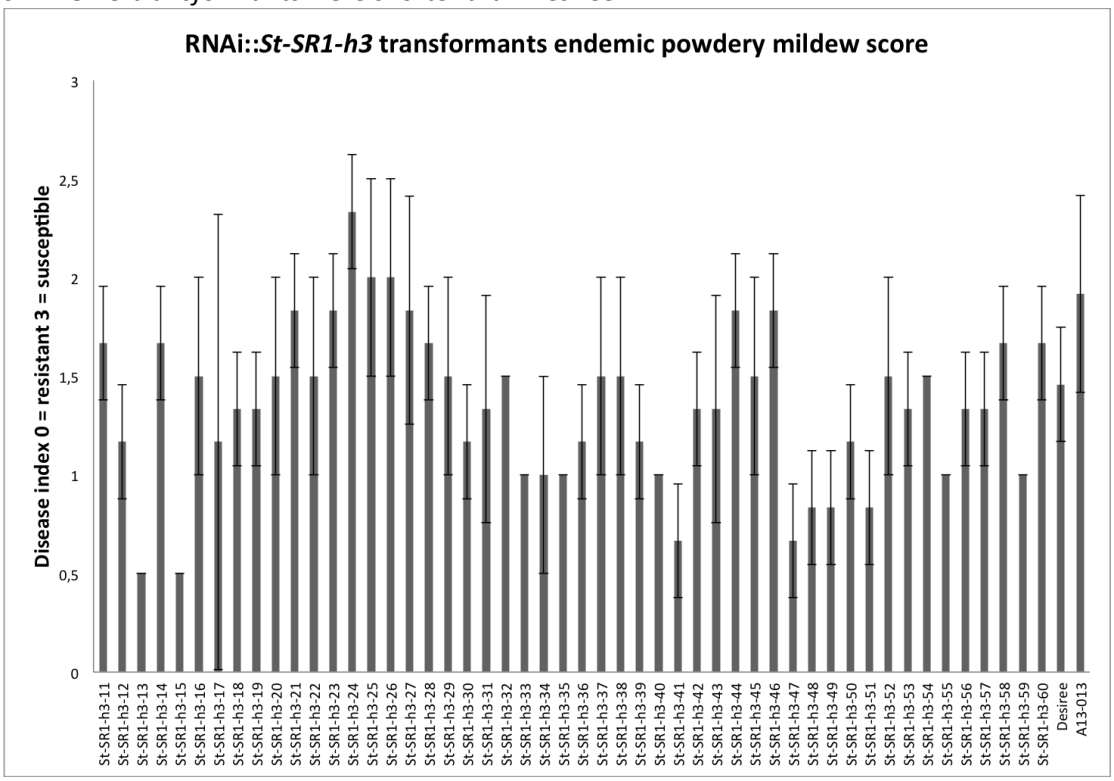

Figure S3.3.5. *Greenhouse endemic powdery mildew infection on eleven weeks old RNAi::St-SR1-h3 transformants.*

Chapter 4 – Partial late blight resistance in potato by impairment of *StPMR4*, but not by impairment of *StPMR5*

Characterization of tuber resistance to *Phytophthora infestans* in *StPMR4* and *StPMR5* RNAi silencing transformants

Abstract – Here we show partial resistance in potato tubers to *P. infestans* induced by impairment of the ortholog *StPMR4-h1*. The *S*-gene function of a homolog of *StPMR4-h1,* named *StPMR4-h3,* appeared not to be conserved in the tubers of *StPMR4-h3* silenced potato plants for resistance to *P*. *infestans.* Impairment of *StPMR5* in potato did not enhance disease resistance to *P. infestans* in the tubers, indicating that the *S*-gene function is not conserved between Arabidopsis and potato for *PMR5*.

4.1 Introduction

Successful colonisation of potato plants in the field by *Phytophthora infestans* will lead to diseased leaf material to die off and fall to the ground. This allows the mixture of soil and *P. infestans* infected organic material. Three sources of asexual soilborne inoculum have been identified: spores produced on foliage, saprophytic survival of mycelium, and mycelium and sporangia that survive on infected potato tubers (Atidrivon, 1995). Indicating that testing tuber resistance to *P. infestans* is an important aspect when plant resistance is assessed. In this chapter we report the identification of potato candidate orthologs of susceptibility genes *AtPMR4* and *AtPMR5* for disease resistance to *P. infestans* in tubers of RNAi silencing transformants.

PMR4 also known as *GSL5* (Dong et al., 2008; Huibers et al., 2013; Jacobs et al., 2003) encodes a gene with a glucan synthase domain. *PMR4* originally was identified by mutant screening for loss of susceptibility to *Erysiphe cichoracearum* in *Arabidopsis thaliana* (Vogel & Somerville, 2000). Recently, the functional ortholog (*SlPMR4-h1, Solyc07g053980),* was identified in tomato and impairment of *SlPMR4* enhanced resistance to the powdery mildew fungus *Oidium neolycopersici*. The same was observed in the *Atpmr4* mutant (Huibers et al., 2013), indicating that the *S*-gene function across these plant species with respect to powdery mildew fungus *O. neolycopersici* is conserved. Silencing the potato ortholog *St-PMR4-h1* of *AtPMR4* and its homolog *StPMR4-h3* (Figure 4.1.1) resulted in enhanced foliar resistance to virulent *P. infestans* isolates PIC99189 and PIC99177 (Kaile Sun, unpublished data). This is implying that the *S*-gene function of *PMR4* is conserved between different plant-pathogen-systems. A tuber assay can confirm if the *S*-gene function for this gene is similar in both the tubers as in the leaves.

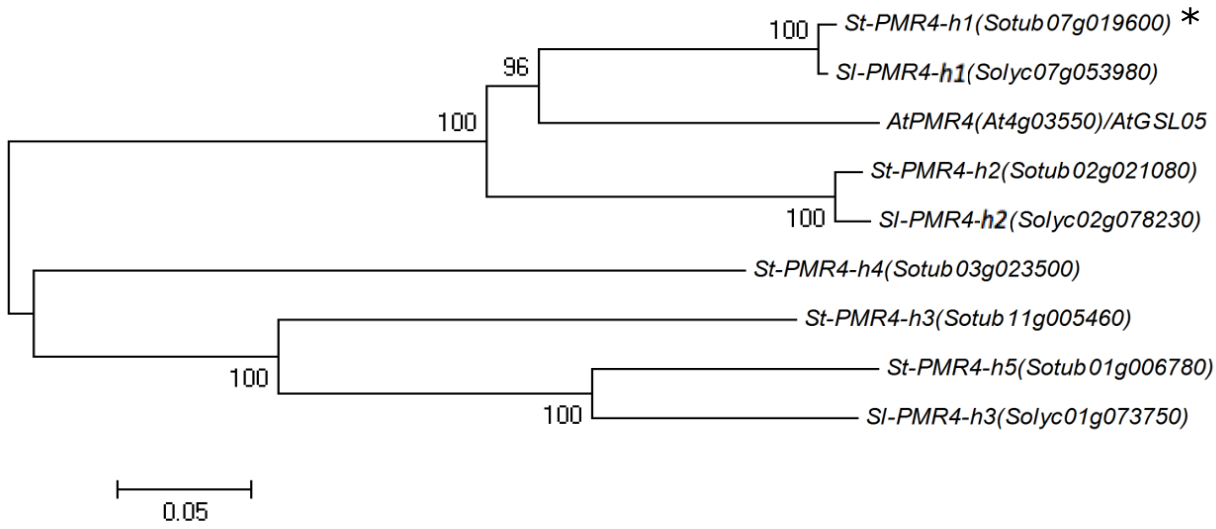

Figure 4.1.1. *Phylogenetic tree of Arabidopsis PMR4 and PMR4 family orthologs of both potato (St) and tomato (Sl). The protein marked by the asterisk is considered to be the potato ortholog of AtPMR4 based on shortest distances.*

Powdery Mildew Resistant 5 (*PMR5*) belongs to a large family of plant specific genes of which its function remains unknown. The *Arabidopsis thialiana* mutant *pmr5* has enhanced resistance to Powdery mildew disease caused by species *E. cichoracearum* and *E. orontii* (Vogel et al., 2004). Phylogenetic analysis identified two possible orthologs of *AtPMR5* in potato and tomato (Figure 4.1.2). Different relative levels of *St-PMR5-h1* expression had no effect on the growth of PIC99177 in a detached leaf assay (Kaile Sun, unpublished data).

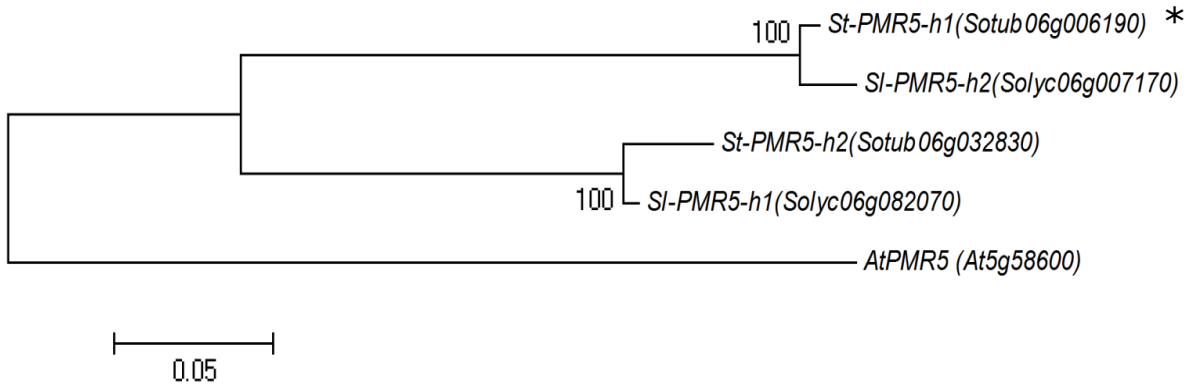

Figure 4.1.2. *Phylogenetic tree of Arabidopsis PMR5 and PMR5 family orthologs of both potato (St) and tomato (Sl). The protein marked by the asterisk was studied as a potato candidate ortholog of AtPMR5.*

4.2 Materials and Methods

Plant material

Tubers were selected from greenhouse pot-grown RNAi *St-PMR4-h1, St-PMR4-h3* and *St-PMR5-h1* silencing transformants. This selection was based on a low expression of the gene that was silenced by the RNAi vector in the foliage of the RNAi transformants. Expression and disease index score information, and tubers were kindly provided by Kaile Sun. RNAi:: *St-PMR4-h1* transformants 8, 10 and 19, RNAi::*St-PMR4-h3* transformants 9, 11 and 12 and RNAi::*St-PMR5-*

h1 9, 24 and 30 were selected as transformants with effective gene silencing for inducing resistance (Kaile Sun et al., submitted; Figure S4.2.1A, B and C). Transformant RNAi::*St-PMR4-h1*-7 was included as susceptible control as expression of *St-PMR4-h1* was similar compared to Désirée in this transformant (Figure S4.2.1A). RNAi::*St-PMR5-h1-19* was used in a similar way (Figure S4.2.1C) no such transformant was available for the *St-PMR4-h3* gene.

Tuber assay

Tubers from pot grown greenhouse plants were collected and rinsed with 5% sodium hypochlorite for five minutes and hereafter rinsed three times with tap water. Four slices of one centimetre were cut from two tubers of different biological replicates, i.e. two biological replicates and two technical replicates. The slices were placed in a tray with a wet paper on the bottom. Each slice was inoculated with five droplets of five µL inoculum ($5x10^4$ zoospores mL^{-1}). Four droplets were placed along the side of the tuber slide and one in the middle, in a plus (+) sign shape. As in the detached leaf assay, PIC99177 was used and isolate preparation is as similar as written in the materials and methods of chapter 3. The boxes were covered with a transparent plastic bag and the paper was regularly checked for wetness to keep humidity at 100%. The boxes were placed in 15° Celsius climate chamber and covered with a box placed up side down on top to keep the inoculated slices in a dark environment.

After six days sporangia numbers were counted and the length and width of the tubers measured. The sporangia were washed of the tubers slices and diluted into five mL miliQ water. Sporangia number was counted (as described in chapter three of this thesis) four times by pipetting a droplet to a Fuchs-Rosenthal counting chamber (Zeiss, Germany), before pipetting the five mL wash water was mixed thoroughly. Data shown in the figures was based on the average of sixteen counted squares of the counting chamber, two droplets were counted per sample.

Gene expression

The ends of the tuber cuttings without inoculation were saved in liquid nitrogen and grinded for RNA isolation. The protocol used for RNA isolation is given in table 4.2.1, RNA was reverse transcribed with the iScript^{tm} cDNA Synthesis Kit (BIORAD) as described in chapter 3. The primers used to quantify gene expression are listed in table 4.2.2.

Table 4.2.1. Protocol RNA isolation for grinded tuber material.

Step	Performed action(s)
1	1 mL Trizol was added to grinded tuber material and samples were incubated for 5 minutes (min.) at room temperature. After which samples were centrifuged for 15 min. at 12.000 g at 4° C, supernatant was pipetted to a new eppendorf.
2	0,2 mL chloroform was added and shaken vigorously for 15 seconds and incubated for 15 min. at room temperature.
3	The samples were centrifuged for 15 min. at 12.000 g at 4° C.
4	Supernatant was taken and 0,5 mL isopropanol was added to the supernatant. The solution was mixed gently by inverting, after which it was incubated for 10 min. at room temperature.
5	Samples were centrifuged for 10 min. at 12.000 g at 4° C.
6	Supernatant was removed and RNA pellet was washed with 1 mL 70% Ethanol followed by centrifugation for 5 min. at 12.000 g at 4° C.
7	Pellets were dried by air-drying in a fume hood.
8	RNA (pellet) was dissolved in 100 µL TE.

9	RNA concentration was measured by pipetting 1,5 µL to the Isogen Nanodrop Spectrophotometer ND-1000.

Table 4.2.2. Primers *St-PMR4-h1*, *St-PMR4-h3* and *St-PMR5-h1* and *EF1α*

Gene	Primer pair
EF1α	Fw - ATTGGAAACGGATATGCTCCA Rv - TCCTTACCTGAACGCCTGTCA
St-PMR4-h1	Fw - GGGGTGAGTCAGCGAATCTA Rv - GGGCAGTACCATTCCGACTA
St-PMR4-h3	Fw - CCCATGGTTATGGAGATTGG Rv - TATTTTGCGCCACCATGTAA
St-PMR5-h1	Fw - TGAAATTCCAAGGTTCAATGG Rv - GAGGGTCACCCGTGATGTAT

4.3 Results

Quantitative resistance that was found in the leaves of RNAi::*St-PMR4-h1* transformants was similar in the tubers of these transformants. The sporangia number showed a three-fold difference between the repeated experiments (Figure 4.3.1A and B). Interesting is that the transformant St-PMR4-h1-7, the susceptible control, had normal *St-PMR4-h1* expression in the leaves and was susceptible to PIC99177 (Figure S4.2.1A; Figure S4.2.2A), but it shows to be partially resistant in the tubers (Figure 4.3.1A and B). Quantification of the transcript level of the ortholog could not be performed as the C(t) values of the qRT-PCR exceeded beyond 30 or were not available. In addition, the quantification of the housekeeping gene *EF1α* was not stable between the different clones (Supplemental document S4, qRT-PCR data).

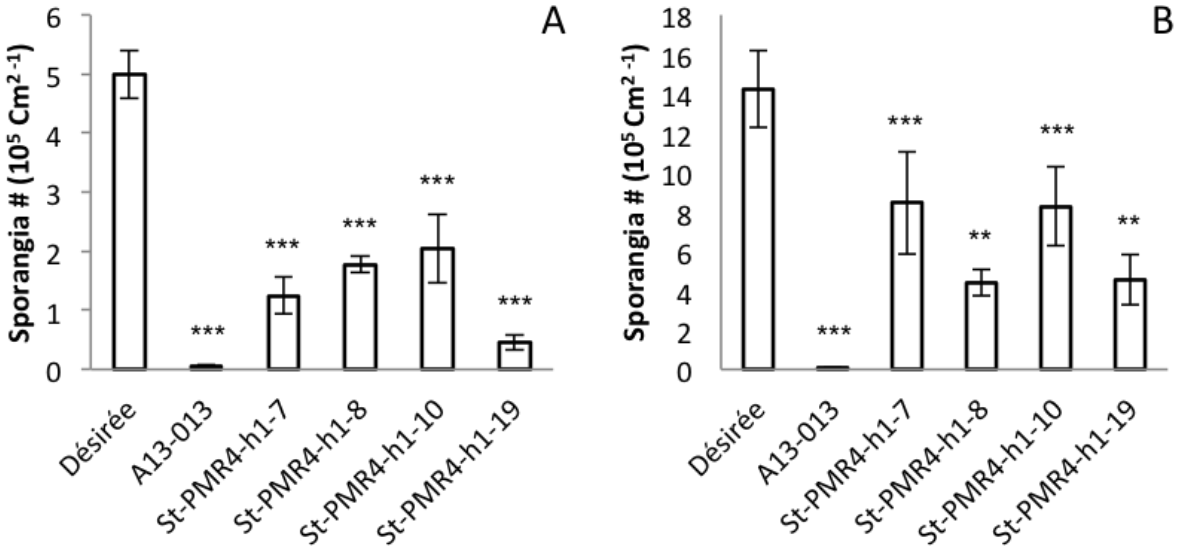

Figure 4.3.1. *Sporangia number per square centimetre on tuber slices. The experiment was performed twice using potatoes from the same harvest (**A** and **B**). Asterisks indicate significant differences as tested by left bounded two samples t-test CI 95%. ***<0.001 **<0.01.*

Silencing the homolog of *St-PMR4-h1,* named *St-PMR4-h3,* enhanced resistance in the foliar part of the clones to *P. infestans* isolate 99177 (Kaile Sun, unpublished data). Infection of this isolate on tuber slices did not differ compared to unaltered Désirée tubers, except for St-PMR4-h3-11

(Figure 4.3.2A), or was even higher in the repeated experiment for transformants 9 and 11 (Figure 4.3.2B).

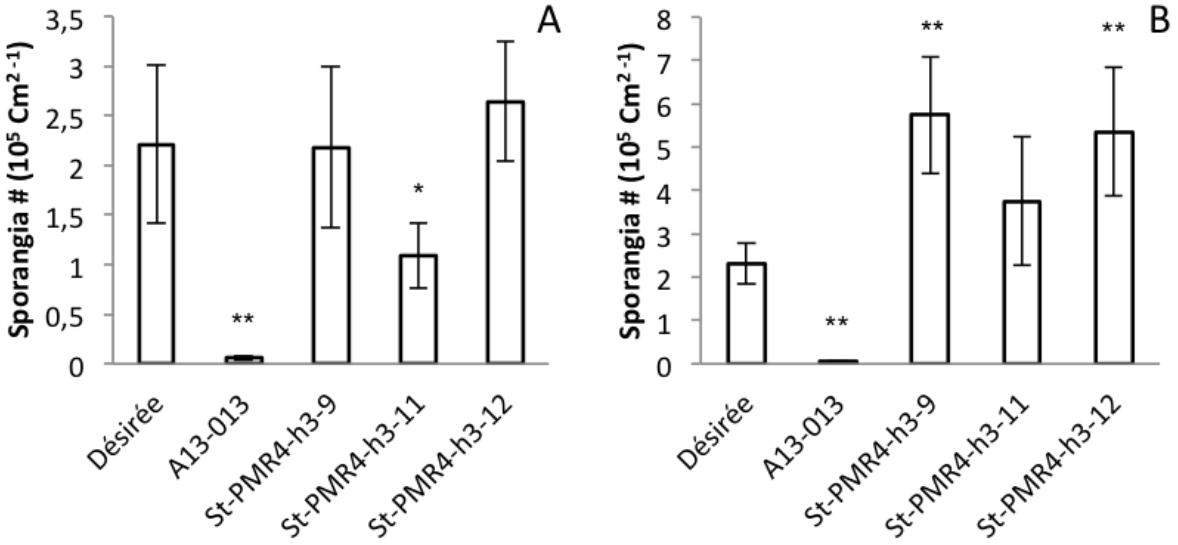

Figure 4.3.2. *Sporangia number per square centimetre on tuber slices. The experiment was performed twice using potatoes from the same harvest (**A** and **B**). Asterisks indicate significant differences as tested by left bounded two samples t-test CI 95%. ** <0.01 *<0.05.*

Sporangia number per square centimetre on RNAi::*PMR5-h1* transformants of PIC99177 was higher compared to tubers of Désirée (Figure 4.3.3A and B). Except for St-PMR5-h1-9 in the first tuber assay and St-PMR5-h1-19 in the second tuber assay, in these two cases no statistical difference was observed. In the second experiment sporangia numbers per cm^2 showed a two-fold increase compared to the first experiment in the RNAi::*St-PMR5-h1* clones.

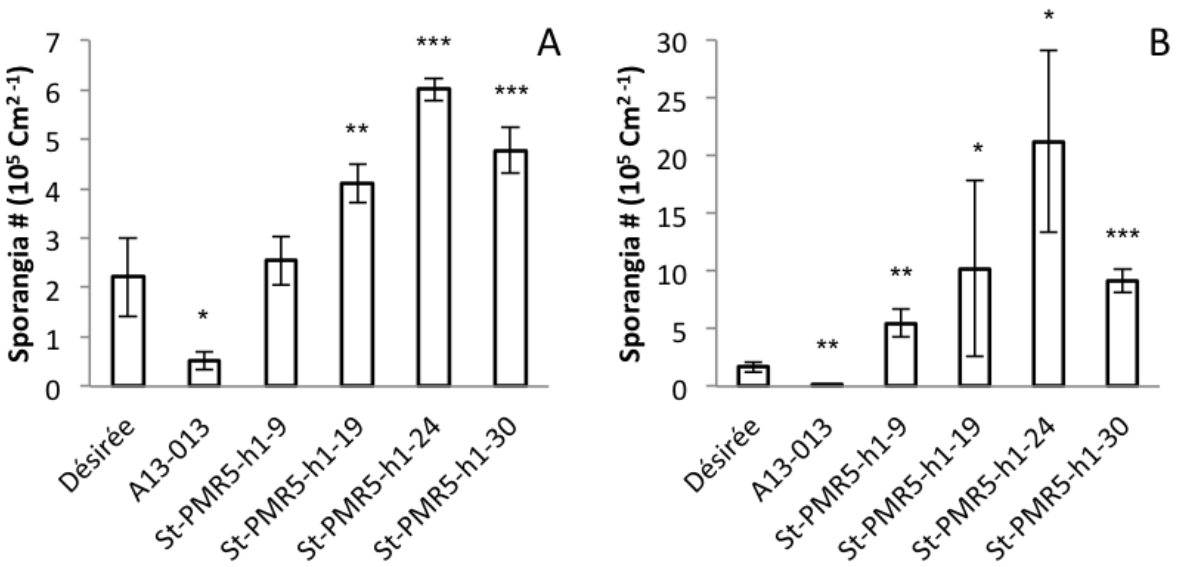

Figure 4.3.3. *Sporangia number per squared centimetre on tuber slices. The experiment was performed twice using potatoes from the same harvest (**A** and **B**). Asterisks indicate significant differences as tested by left bounded two samples t-test CI 95%.*

4.4 Discussion

RNA from tubers was isolated and qRT-PCR performed after cDNA synthesis, results from the Nanodrop led to no suspicion that the RNA isolation was unsuccessful. However, relative expression could not be calculated with the $2^{-\Delta\Delta Ct}$ method. As C(t) values were above 30 or not available for *St-PMR4-h1, St-PMR4-h3* and *St-PMR5-h1* expression including control plants (Désirée and A13-013). In tubers of the control and transformed plants the expression of *EF1α* showed normal values (Supplemental document S4; Nicot et al., 2005). Seemingly, *St-PMR4-h1, St-PMR-h3* and *St-PMR5-h1* expression in all tubers of this study were expressed in a minimal amount, including the control plants. It could be that there are small differences between the control plants and the transformants that logically explain the quantitative resistance in RNAi::*St-PMR4-h1* tubers.

PMR4 is a qualitative *S*-gene in the foliar part of potato to *P. infestans* isolates 99177 and 99189 (Kaile Sun, unpublished data). Here we show that quantitative resistance to PIC99177 is indeed found in the tubers of the selected RNAi::*PMR4-h1* plants. However, the tubers from the RNAi::*PMR4-h3* transformants are amenable to this isolate. The difference in resistance found between the tubers, but not in the foliar part of the potato plantlets suggests a different function for the *PMR4* homologs in potato. In *Arabidopsis* it has been shown that there are at least twelve *GSL* genes (Jacobs et al., 2003). Furthermore, expression of these *GSL* genes differs between plant organs (Dong et al., 2008) and between developmental stages (Verma and Hong, 2001). Interestingly, in the *Arabidopsis thaliana* RNAi::*GSL5* line aniline blue fluorochrome staining revealed reduced callose staining at wounded sites. Whereas in lines RNAi::*GSL6* and RNAi::*GSL11* callose accumulation at wounded sites is similar as in the wild type (Jacobs et al., 2003). Therefore it is recommended to perform aniline blue staining of both leaves and tubers of RNAi::*StPMR4-h1* and RNAi::*StPMR4-h3*. This experiment could elucidate three missing links: i) identification of the true *Arabidopsis PMR4* ortholog(s) in potato ii) possible functional redundancy between homolog one and homolog three iii) identify if the mechanism between organs of potato is identical. It is recommended to repeat the DLA and tuber assay together with the staining experiment, to avoid misinterpretation of the staining results.

Callose deposition at the penetration sites is part of the defense response of potato plants to *P. infestans* infection (Halim et al., 2007; Vleeshouwers et al., 2000). Interestingly, papilla size and shape is unaffected upon fungal infection in the absence of callose deposition in *Arabidopsis Atpmr4* mutants (Nishimura et al., 2003). In addition, RNAi::*StSYR1* potato plants have an enhanced resistance phenotype to *P. infestans*, in which callose deposition in papilla is reduced near the penetration site (Eschen-lippold et al., 2012). It seems that absence of callose enhances resistance, however, impairment of syntaxin or callose synthase leads to activation of salicylic acid pathways and spontaneous necrosis (Nishimura et al., 2003; Eschen-lippold et al., 2012), suggesting that these genes are negative regulators of SA signalling. SAR is responsible for the enhanced resistant phenotypes.

In contrast to *PMR4* it is clear that *St-PMR5-h1* is not an *S*-gene of *S. tuberosum* cv Désirée for *P. infestans* isolate PIC99177. In both leaf and tuber of RNAi::*StPMR5-h1* plants infection of *Phytophthora* is similar to that of cv Désirée (Kaile Sun, unpublished data; this thesis). In the tubers of the *PMR5* transformants even an increase in sporangia number is observed compared to cv Désirée. Suggesting that silencing of *St-PMR5-h1* in potato cv Désirée negatively regulates the defense of tubers. The sporangia number on RNAi::*St-PMR5-h1*-24 in the repeated tuber assay is not representative. Since for this experiment only small pot grown tubers were left for testing. The small size might have had an effect on the formation of

sporangia. Although the pattern of all transformants is similar over the two experiments (Figure 4.3.3A and B). Characterization of the *Arabidopsis Atpmr5* mutant revealed that the resistance to two *Erysiphe* species did not require the SA or JA/ethylene pathway. The *Arabidopsis Atpmr5* mutant is susceptible to *Pst* pv tomato DC3000 and to *Peronospora parasitica* EMCO5 an obligate biotrophic pathogen. Suggested is that the resistance mechanism acts through an aberrant enriched pectin phenotype or other cell wall alterations (Vogel et al., 2004). The absence of elevated SA signalling in *Arabidopsis Atpmr5* mutant could clarify, if the mechanism is the same in potato, the susceptible phenotype to *P. infestans* of the selected RNAi::*St-PMR5-h1* transformants. It cannot be excluded that in fact *St-PMR5-h2* is the functional ortholog of *AtPMR5*. This idea is supported by a disease assay for endemic greenhouse powdery mildew infection wherein almost all *St-PMR5-h1* were equally susceptible as Désirée (Kaile Sun, unpublished data; figure S4.4.1). Generation of RNAi::*St-PMR5-h2* potato plants is needed to study if *StPMR5* is truly not involved in susceptibility to *P. infestans.*

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Supplemental figures (all information in the figures were provided by Kaile Sun)

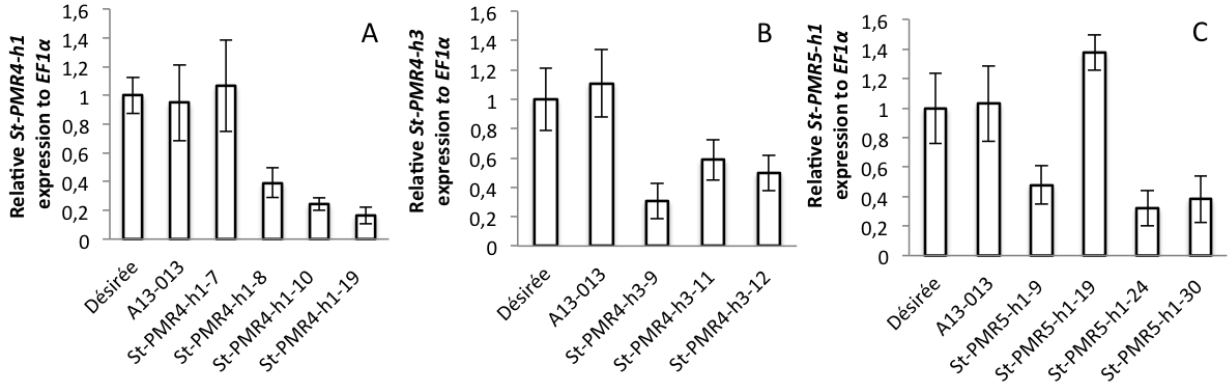

Figure S4.2.1. *Relative expression of St-PMR4-h1 in the leafs of RNAi::St-PMR4-h1 transformants 7, 8, 10 and 19 (**A**). Relative expression of St-PMR4-h3 in the leafs of RNAi::St-PMR4-h3 transformants 9, 11 and 12 (**B**). Relative expression of St-PMR5-h1 in the leafs of RNAi::St-PMR5-h1 transformants 9, 19, 24 and 30 (**C**).*

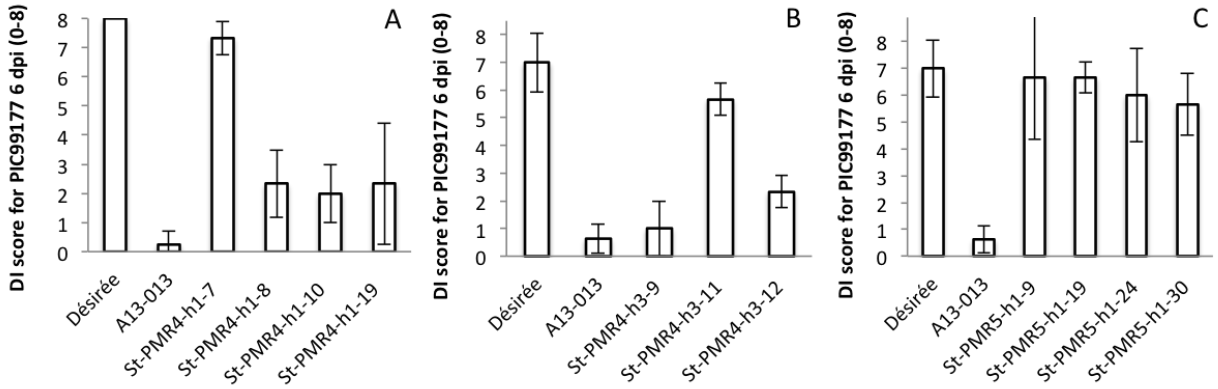

Figure S4.2.2. *Disease Index (DI) score at 6 days post inoculation for PIC99177 for foliage of selected RNAi::St-PMR4-h1 transformants (**A**), RNAi::St-PMR4-h3 transformants (**B**) and RNAi::St-PMR5-h1 transformants (**C**).*

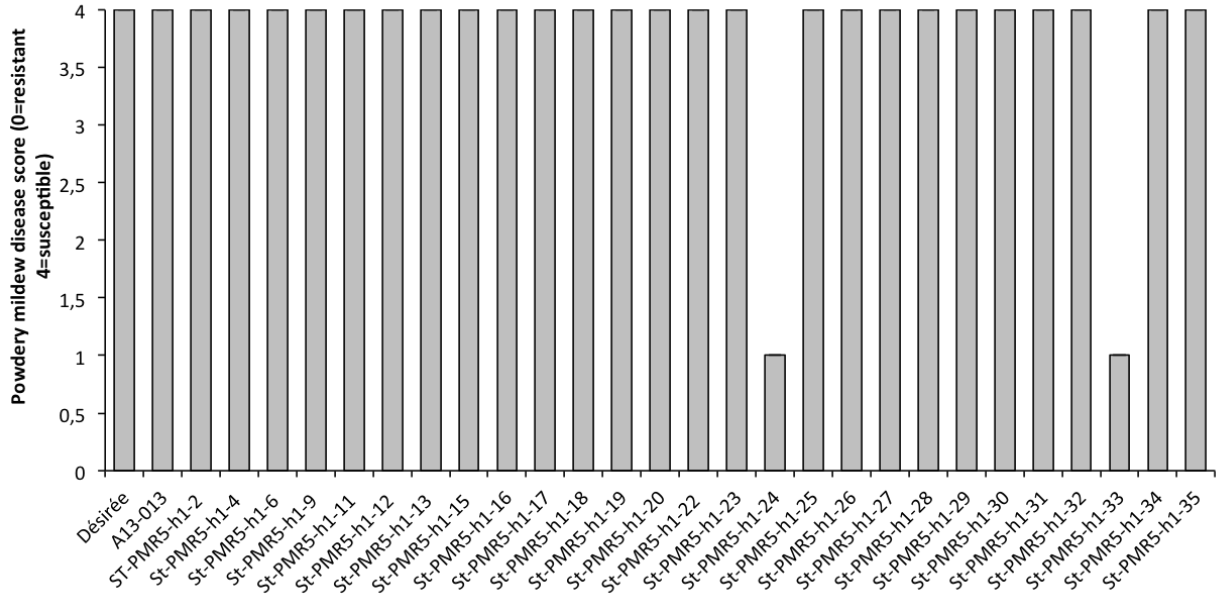

Figure S4.4.1. *Endemic greenhouse powdery mildew infection of St-PMR5-h1 RNAi silencing transformants. Only transformants 24 and 33 had lower DI scores for powdery mildew infection.*

Chapter 5 – *S*-gene *StDND1* is a negative defense regulator

Histological and expression studies of resistance to *Phytophthora infestans* (PIC99189) in RNAi::*DND1-h1* silenced potato plants of cv Désirée

Abstract – To unravel the defense mechanism of disease resistance to *P. infestans* in *StDND1* impaired potato plants, histological and expression studies were performed at different time points post inoculation. DAB staining revealed absence of hydrogen peroxide generation (indicator for plant stress responses) and trypan blue staining indicated that there was no growth of *P. infestans* hyphae in potato plants with low *StDND1* expression. The expression study confirmed that *S*-gene *StDND1* is negative regulator of defense as *PR1* expression was highly up regulated in RNAi::*StDND1* transformants.

5.1 Introduction

In potato breeding, introgression of *R*-genes to improve durable resistance against *Phytopthora infestans* appeared as a promising strategy. Introgression of eleven *R*-genes (*R1-R11*), identified by the Mastenbroek differential potato set, from the Mexican wild species *Solanum demissum* initiated this strategy (Black et al. 1953; Malcolmson & Black, 1966). However, resistance of cultivar Pentland Dell conferred by *R1* and *R3* got broken six years after market release (Shattock et al., 1977). Recently, cultivar Sarpo Mira has been identified as a good candidate for durable late blight resistance, and is suggested to harbor at least five known *R*-genes (Rietman et al., 2012). Stacking of *R*-genes by genetic modification might also produce improved candidate varieties for durable late blight resistance (Zhu et al., 2012). Unknown is how long these resistances will last to this pathogen that is also known as the destroyer of *R*-genes (Fry, 2008). This stresses the importance of exploring other strategies that provide durable broad-spectrum resistance, like the impairment of *S*-genes that is reported in the previous chapters of this thesis.

It is worth knowing that *S*-gene functions are conserved between plant species. Another interesting step of the *S*-gene strategy is to study the mechanism of resistance induced by posttranscriptional gene silencing. *Arabidopsis* mutant *Atdnd1-1* is suggested to restrict pathogen growth in a gene-for-gene way without hypersensitive response (HR) like cell death and has a constitutive systematic acquired resistance phenotype (Yu et al., 1998). Hence the name, defense no death 1 (*DND1*), indicates the phenotypic reaction of the loss of function mutant in Arabidopsis after inoculation with *P. syringae*. The aim of our study is to identify if the resistance mechanism in RNAi::*DND1-h1* potato transformants to *P. infestans* is the same or not.

5.2 Material and methods

Experimental design

Three Désirée transformants carrying *DND1-h1* silencing constructs were kindly provided by Kaile Sun and were addressed as DND1-h1-5, DND1-h1-6 and DND1-h1-17. Preliminary results showed that *DND1-h1* expression in transformants 1-5 and 1-17 was silenced effectively showing a resistance to multiple isolates of *P. infestans.* The resistance phenotype was accompanied by plants spontaneously occurring auto-necrotic lesions (Kaile Sun, submitted).

Transformant DND1-h1-6 without silencing was considered as a susceptible control: relative gene expression of *DND1-h1* did not differ from the expression in Désirée, no pleiotropic effects were found and plants were found susceptible to *P. infestans.* A fourth transformant that was included in this experiment carries the *vnt1.1 R*-gene in background Désirée. This transformant was referred to as A13-13 and is the positive, resistant, control. Cultivar Désirée was included as the non-transformed susceptible control. From these five genotypes ten replicates were grown *in vitro* for two weeks on Murashige and Skoog medium with addition of 3 g L^{-1} sucrose in a growth chamber at 24°C with 16 h day and 8 hour night periods, a relative humidity of around 70% and a light intensity of 100 W/m^2. Subsequently, the plants were transferred to a greenhouse (compartment 12.4, Unifarm, Wageningen, The Netherlands) and grown for four to five weeks, at about 25°C during a 16 h day and 8 h night period. The relative humidity was around 70% and light intensity was supplemented with 100 W/m^2 when light intensity in the greenhouse dropped below 150 W/m^2. Thus, conditions that varied were light intensities and temperatures according to the local climate in February and March 2014.

The fourth or fifth fully developed compound leaf (counted from the top) was used in the experiments. The two smallest leaflets of each compound leaf were removed. Four out of the ten collected compound leaves were randomly selected for mock inoculation with four droplets of 10 µL H_2O (Milli-Q) per leaflet. The leaflets of the other six compound leaves were inoculated with four droplets of 10 µL PIC99189 zoospores, 5×10^5 zoospores mL^{-1}. Per compound leaf (replicate), one leaflet was used for *3,3-Diaminobenzidine* (DAB; Sigma-Aldrich) staining, one for trypan blue staining and one for RNA isolation.

Detection of H_2O_2

In order to detect H_2O_2 generation in detached plant leaves DAB staining was performed. Leaves were placed in a solution of 1 mg/mL *3,3-Diaminobenzidine* dissolved in 0.2 M PBS (phosphate buffer) and a pH of 3.8. Concentrated HCL was used to adjust the pH. The samples were placed in light overnight to optimize the staining reaction (as DAB is light sensitive). The following day the samples were cleared by boiling with 96% ethanol until the leaves were transparent, after which they were stored in fresh 70% ethanol. H_2O_2 is visualized as a brown coloration (Thordal-Christensen et al., 1997).

Trypan blue staining (detection of hyphae and cell death)

Per leaflet two samples were cut for trypan blue staining, each halve of the leaflet was cut to a piece of 1 cm by 3 cm that included the two incolution spots. This was done to obtain a suitable sample size for slides that could be observed by microscope. The cut samples were immediately fixed and bleached by boiling for 1,5 minutes in a water bath in lactophenol/ethanol (1:2 v/v) and left to rest overnight. Subsequently the following washing steps were performed: 30 minutes in ethanol 50%, 30 minutes in 0.05 N NaOH, rinsed 3 times with water and soaked for 30 minutes in 0.1 M Tris/HCL buffer pH 8.5. The fixed and bleached samples were placed for at least 30 minutes in acetic acid/96% ethanol solution (1:3 v/v). After which the samples were boiled in 4 hours in lactophenol/96% ethanol (1:2 v/v) with 0.01% trypan blue, the goal was to obtain dark velvet blue stained samples. The boiled samples were transferred to saturated chloral hydrate (5:2 w/v) for about 7 days to clear the samples. Cleared samples were embedded in glycerol/H_2O (1:1 v/v) on glass slide covered with cover slide and sealed with transparent nail polish. Preparations were observed by bright field microscope (Zeiss, Germany), pictures were taken with a Canon powershot A620 mounted on the microscope.

Expression studies
RNA isolation and qRT-PCR was performed as described in Chapter 3, primer sequences of *PR1* and *EF1α* can also be found in the material and methods of Chapter 3. Primer sequences for *StDND1-h1* were: Fw - GCACCTCTTCCATTTGTGGT and Rv – GAACGGGAAGGATGACAAAA.

Statistical analysis
For comparisons of means of the expression data Student's test was performed

5.3 Results

Absence of both macroscopic H_2O_2 generation and microscopic hyphae growth in *DND1-h1* silenced transformants
To study if hydrogen peroxide generation in RNAi::*DND1-h1* silenced transformants upon infection with *P. infestans* zoospores, we performed DAB (3,3-diaminobenzedine) staining at five different time points after inoculation (0, 24, 48, 72, and 96 hours). Near the inoculation sites no macroscopic difference was observed in H_2O_2 levels between mock (H_2O) inoculation and inoculated leaves of all plants zero hours post inoculation (hpi) (Figure 5.3.1A and B). *DND1-h1* silenced transformants 1-5 and 1-17 have a qualitative resistant phenotype to *P. infestans* accompanied with pleiotropic effects such as dwarfing and auto-necrotic lesions (Kaile Sun, submitted). Auto-necrotic lesions were visible in both mock and inoculated leaves of RNAi::*DND1-h1* transformants 1-5 and 1-17 but not 1-6 (Figure 5.3.1A and B).

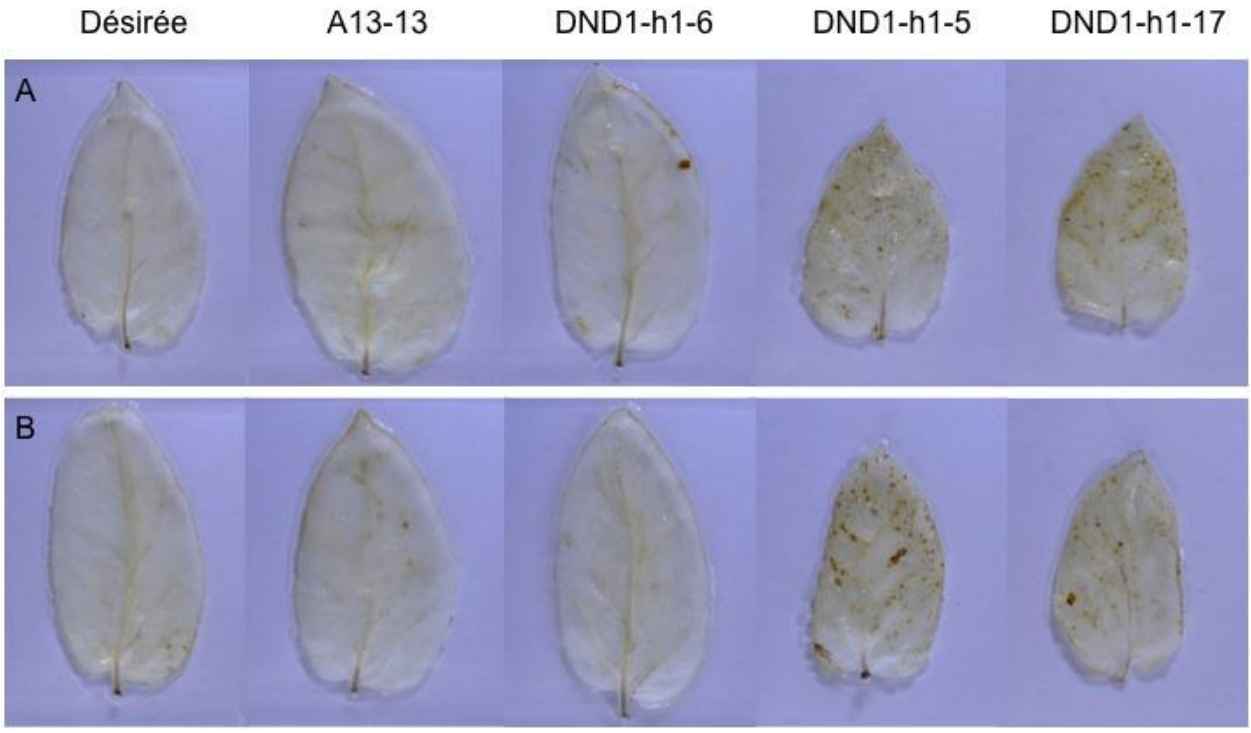

***Figure 5.3.1**. 3,3-Diaminobenzidine (DAB) staining of detached leaves from Désirée, A13-13 and RNAi::DND1-h1 silencing construct carrying transformants (background Désirée). No H_2O_2 generation was detected at the inoculation sites. Zero hours post mock (H_2O) inoculation (**A**) and inoculation with PIC99189 zoospores, 5 × 10^5 zoospores mL^{-1} (**B**). Auto-necrotic lesions of RNA::DND1-h1 transformants 1-5 and 1-17 were stained by DAB in both mock inoculation and inoculation.*

At 22 hpi observations were similar for A13-13 and *DND1-h1* silenced transformants 1-5 and 1-17 between inoculation and mock treatment (Figure 5.3.2A and B). This was not the case for the inoculated leaves of Désirée and transformant 1-6 (susceptible controls), on these leaves small brown spots were visible indicating H_2O_2 generation (Figure 5.3.2B). Microscopic observation with trypan blue staining at 22 hpi showed the presence of zoospores on DND1-h1-17, and penetration sites in the susceptible control Désirée. In the other plants these phenomenons were not observed (Figure 5.3.2C). These microscopic observations were based on one biological replicate with two inoculation sites.

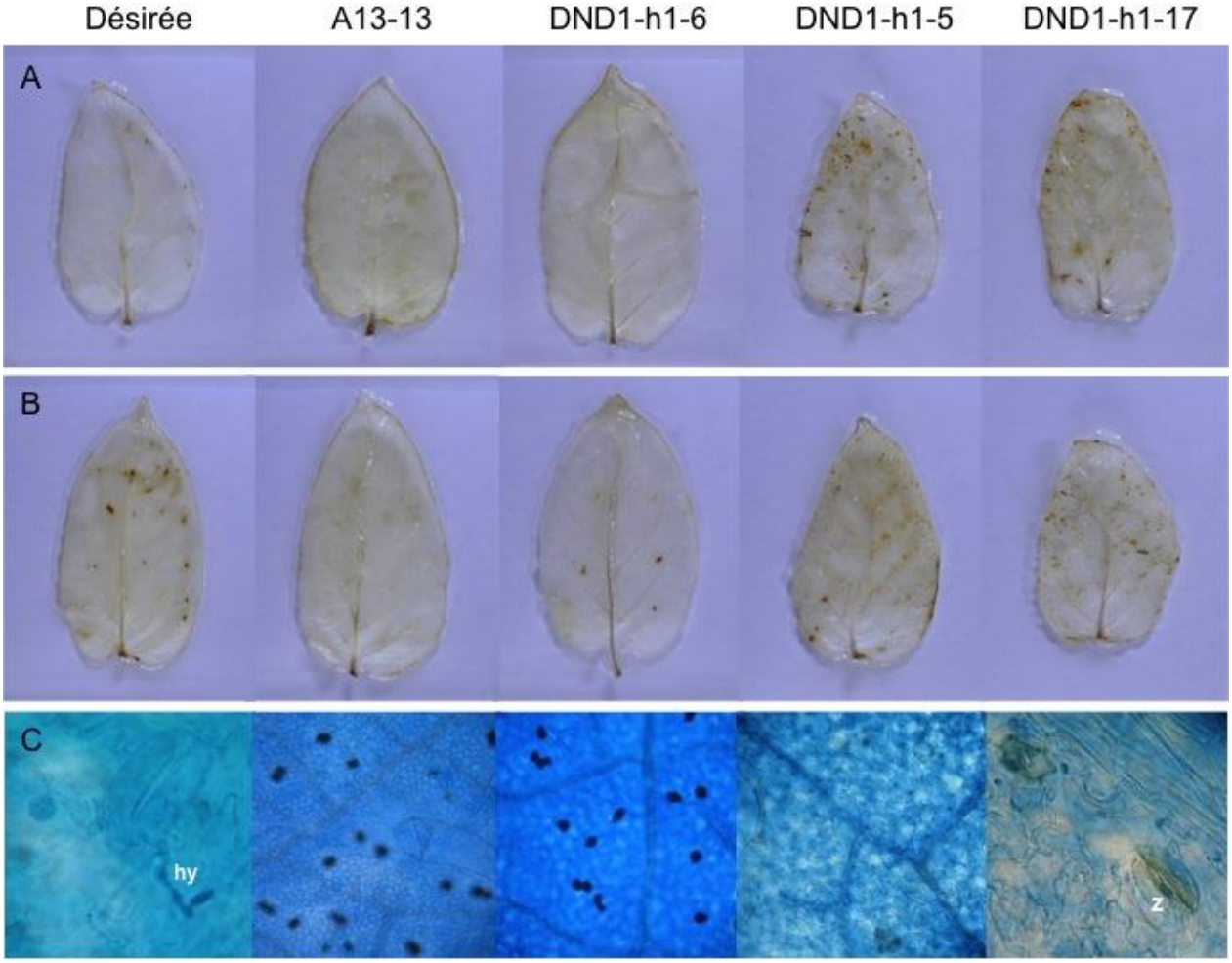

Figure 5.3.2. *3,3-Diaminobenzidine (DAB) (**A** and **B**) and Trypan Blue (**C**) staining of detached leaves from Désirée, A13-13 and RNAi::DND1-h1 silencing construct carrying transformants (background Désirée). No macroscopic H_2O_2 generation was detected at the inoculation sites, twenty-two hours post mock (H_2O) inoculation (**A**) and inoculation with PIC99189 zoospores, 5 × 10^5 zoospores mL $^{-1}$, except for Désirée and RNA::DND1-h1-6 (**B**). Auto-necrotic lesions of RNA::DND1-h1 transformants 1-5 and 1-17 were stained by DAB in both mock treatment and inoculation. Cyst and hyphae were stained on Désirée, a zoospore and cell death were observed on RNA::DND1-h1-17, 80× magnification (**C**). No staining of PIC99189 was observed on A13-13 and DND1-h1 transformants 1-5 and 1-6, 20× magnification (**C**).*

Pathogen inoculated and mock-inoculated leaves of *DND1-h1* silenced plants looked alike for both 48 hpi and 72 hpi, no clear brown spots were visible at inoculation sites except for the auto-necrotic lesions (Figure 5.3.3A, B, C and D). For these two time points small brown spots were visible on the inoculated leaves of resistant control A13-13, the leaves of the mock inoculation are without these spots (Figure 5.3.3A, B, C and D). In the two susceptible controls, the size of DAB stained spots increased between 48 and 72 hpi (Figure 5.3.3B and D).

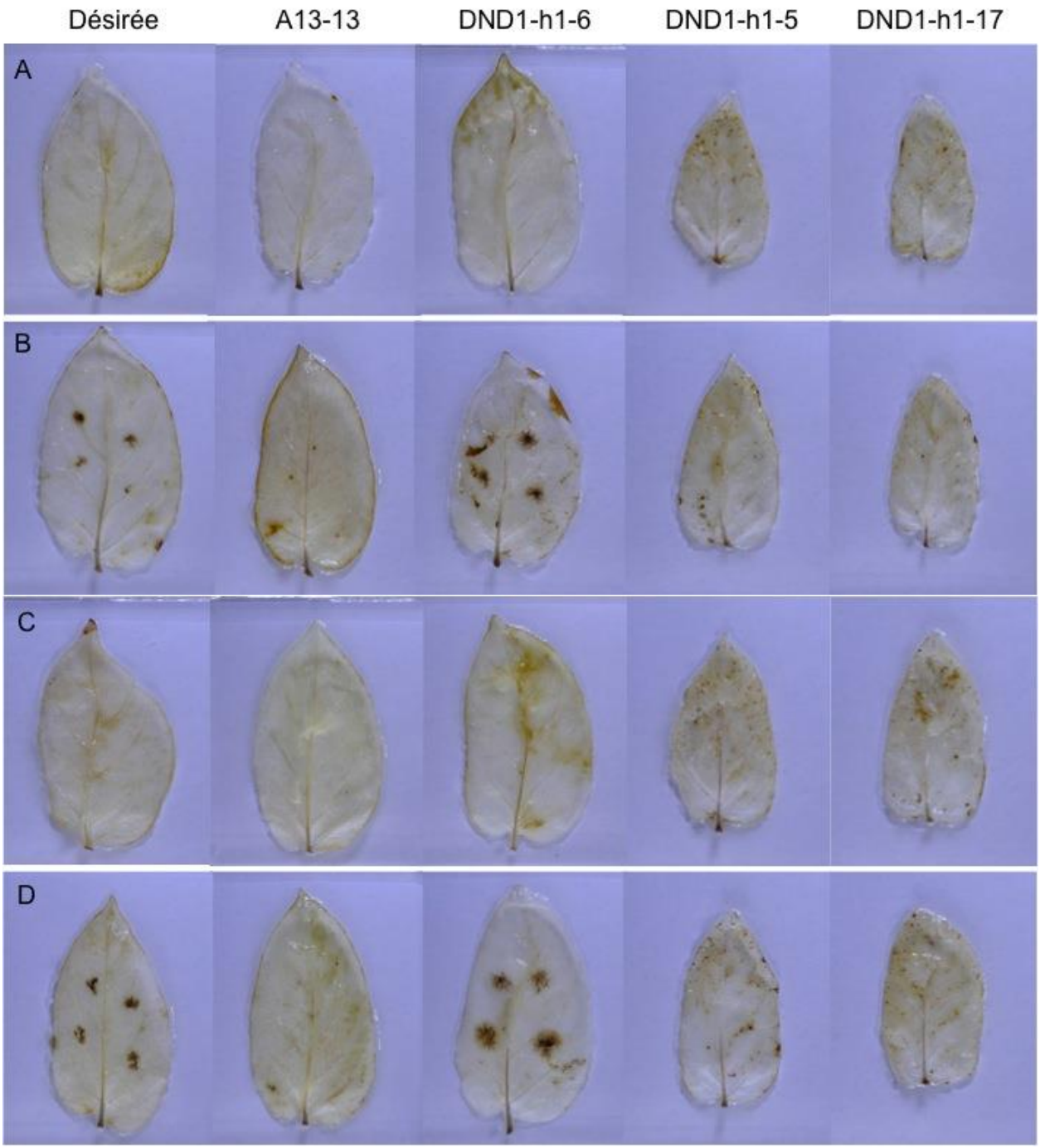

Figure 5.3.3. *3,3-Diaminobenzidine (DAB) staining of detached leaves from Désirée, A13-13 and RNAi::DND1-h1 silencing construct carrying transformants (background Désirée). No macroscopic H_2O_2 generation was detected at the inoculation sites 48 and 72 hours post mock (H_2O) inoculation (**A** and **C**) and inoculation of RNA::DND1-h1 transformants 1-5 and 1-17 with PIC99189 zoospores, 5 × 10^5 zoospores mL $^{-1}$ (**B** and **D**). Inoculated leaves of Désirée, A13-13 and RNAi::DND1-h1-6 differed in H_2O_2 generation (**B** and **D**). Auto-necrotic lesions of RNA::DND1-h1 transformants 1-5 and 1-17 were stained by DAB in both mock and after inoculation (**A**, **B**, **C**, and **D**).*

DAB stained more than 50% of the leave area at 96 hpi in the susceptible controls (Désirée and RNAi::*DND1-h1-6*), while in *DND1-h1* RNAi silenced plants H_2O_2 generation did not increase on the macroscopic level (Figure 5.3.4A and B). Besides the brown coloring of the main vein in A13-13 there were also small brown spots visible between the vein and the edge of the leaf (Figure 5.3.4B).

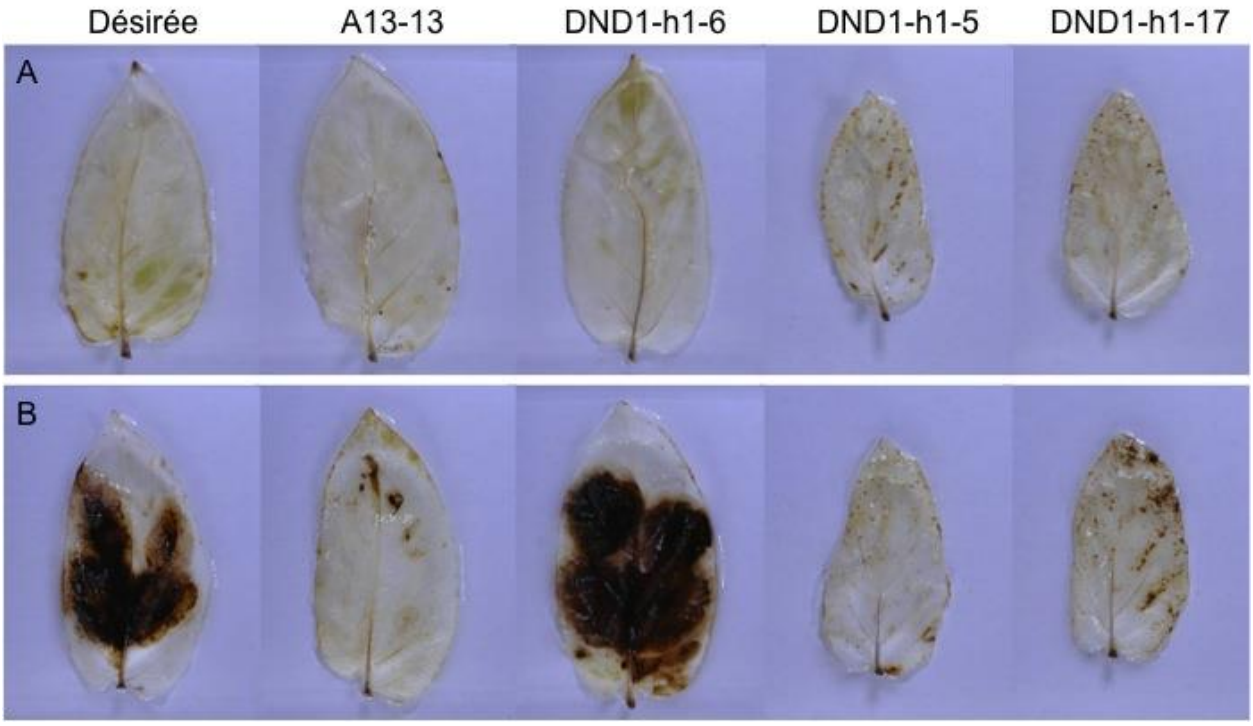

Figure 5.3.4. *3,3-Diaminobenzidine (DAB) staining performed on Désirée, A13-13 and RNAi::DND1-h1 transformants to detect macroscopic H_2O_2 generation in detached leaves 96 hours after inoculation. No H_2O_2 was detected on the mock inoculated leaves, except for auto-necrotic lesions in RNAi::DND1-h1 transformants 1-5 and 1-17 (**A**). H_2O_2 was generated in inoculated leaves of Désirée, A13-13 and RNAi::DND1-h1-6 with PIC99189 zoospores, 5×10^5 mL $^{-1}$. But not in RNAi::DND1-h1 transformants 1-5 and 1-17, except for autonecrotic lesions (**B**).*

Microscopic observation with trypan blue staining of the susceptible controls (Désirée and DND1-h1-6) showed hyphae growth at 48, 72 and 96 hpi (Figure 5.3.5). Extensive hyphae growth was visible at 72 and 96 hpi (Figure 5.3.5B and C) indicating that these plants were unable to stop *P. infestans* growth. In DND1-h1 transformants 1-5 and 1-17 no hyphae growth was observed, moreover zoospores or cysts appeared to be absent at 48, 72 and 96 hpi (Figure 5.3.5). Zoospore induced programmed cell death (or HR?) was observed for A13-13 48 hours after inoculation (Figure 5.3.5A). No such site, including zoospores, was found on leaves of A13-13 at 72 and 96 hpi and hyphae growth was absent (Figure 5.3.5B and C).

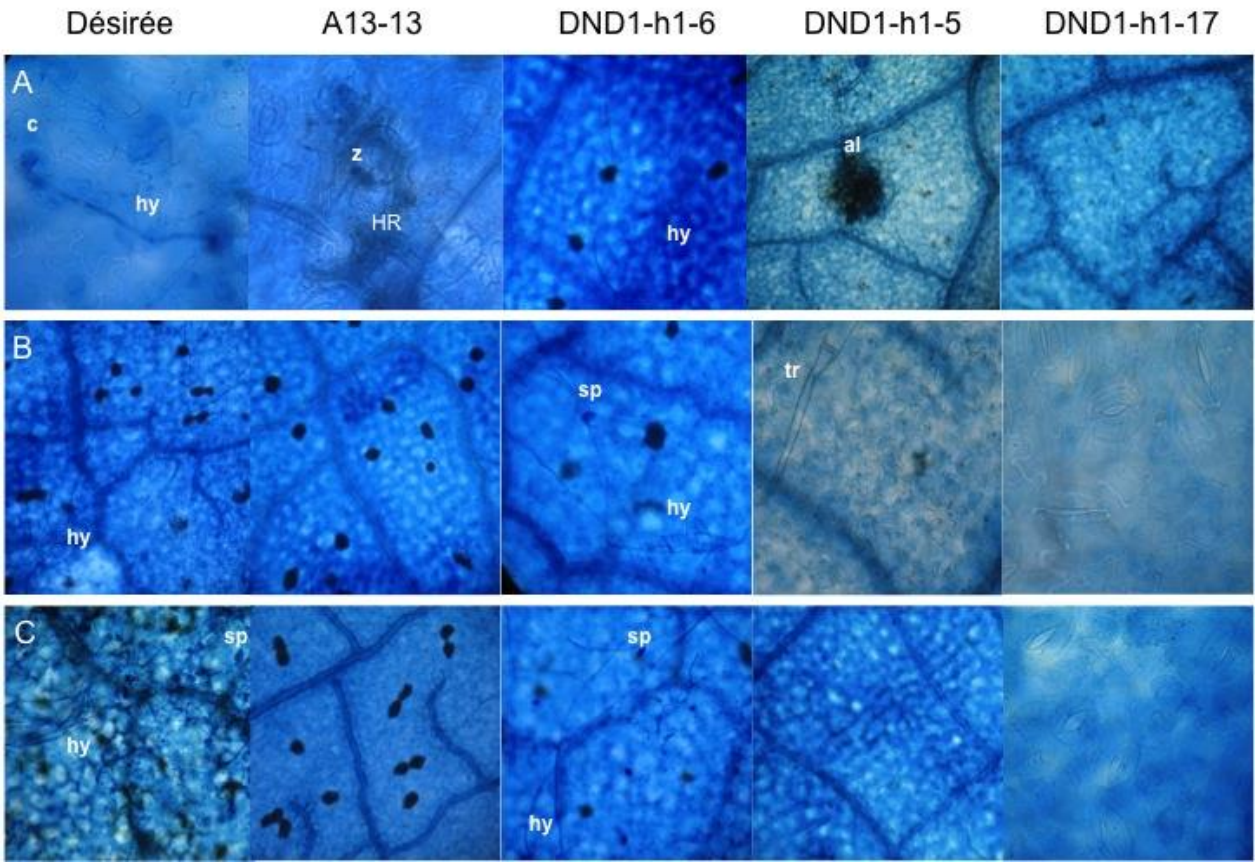

Figure 5.3.5. *Microscopic analysis of inoculated detached leaves stained by trypan blue of Désirée, A13-13 and RNAi::DND1-h1 transformants, 48 (**A**), 72 (**B**) and 96 (**C**) hours post inoculation. al, auto-necrotic lesion; c, cyst; HR, hypersensitive respons; hy, hyphae; sp, sporangia; tr, trichome. All observations were made with 20× magnification, except for Désirée and A13-13 at 48 hpi, and DND1-h1-17 at 72 and 96 hpi for which 80× magnification was used.*

So far two out of three leaflets of the compound leave have been used for a macro- and microscopic studies. The third leaflet was used to study gene expression of *DND1-h1* and *PR1*.

Expression of *DND1-h1* and *PR1*

Quantification of *DND1-h1* transcript levels in the two *DND1-h1* silenced transformants was equal for time points 0, 22, 48 and 72 hpi. *DND1-h1* expression 96 hours after inoculation showed a two-fold increase compared to 0 hpi and was just above 0.40 (Figure 5.3.6). In control plants expression of *DND1-h1* was slightly higher at time point 22 hpi, but was lower at time points 48, 72 and 96 hpi, when compared to time point 0 hpi (Figure 5.3.6). In all plants there was a gradual increase of *PR1* expression as time after inoculation increased, except for A13-13 at 72 hpi (Figure 5.3.7). However, *PR1* up-regulation in *DND1-h1* silenced transformants compared to Désirée decreased over time. Relative *PR1* expression was increased 194-fold in the DND1-h1-5 transformant at 0 hpi, 70, 22, 4 and 5-fold at 22, 48, 72 and 96 hpi, respectively. In RNAi transformant DND1-h1-17 a similar trend was observed but the increase of *PR1* was higher on average (Figure 5.3.7).

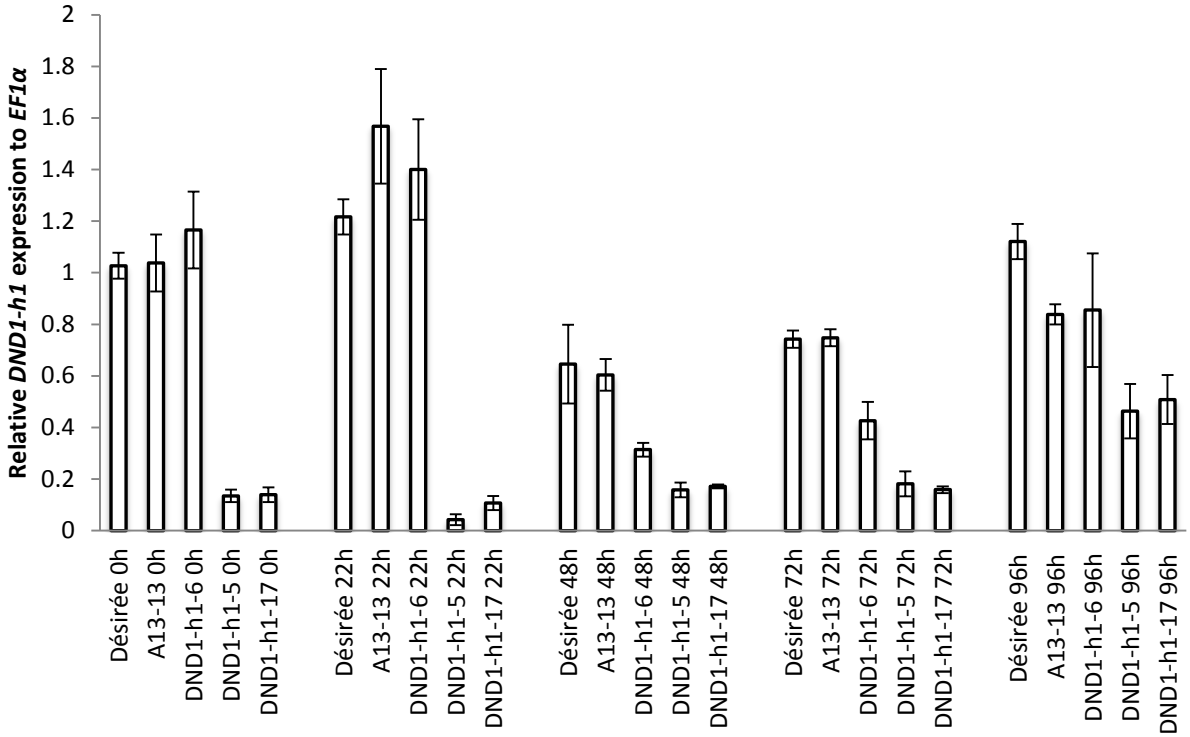

Figure 5.3.6. *Relative DND1-h1 expression to EF1α in Désirée, A13-13 and RNAi::DND1-h1 transformants 1-5, 1-6 and 1-17 at time points 0, 22, 48, 72 and 96 hours post inoculation with isolate PIC99189.*

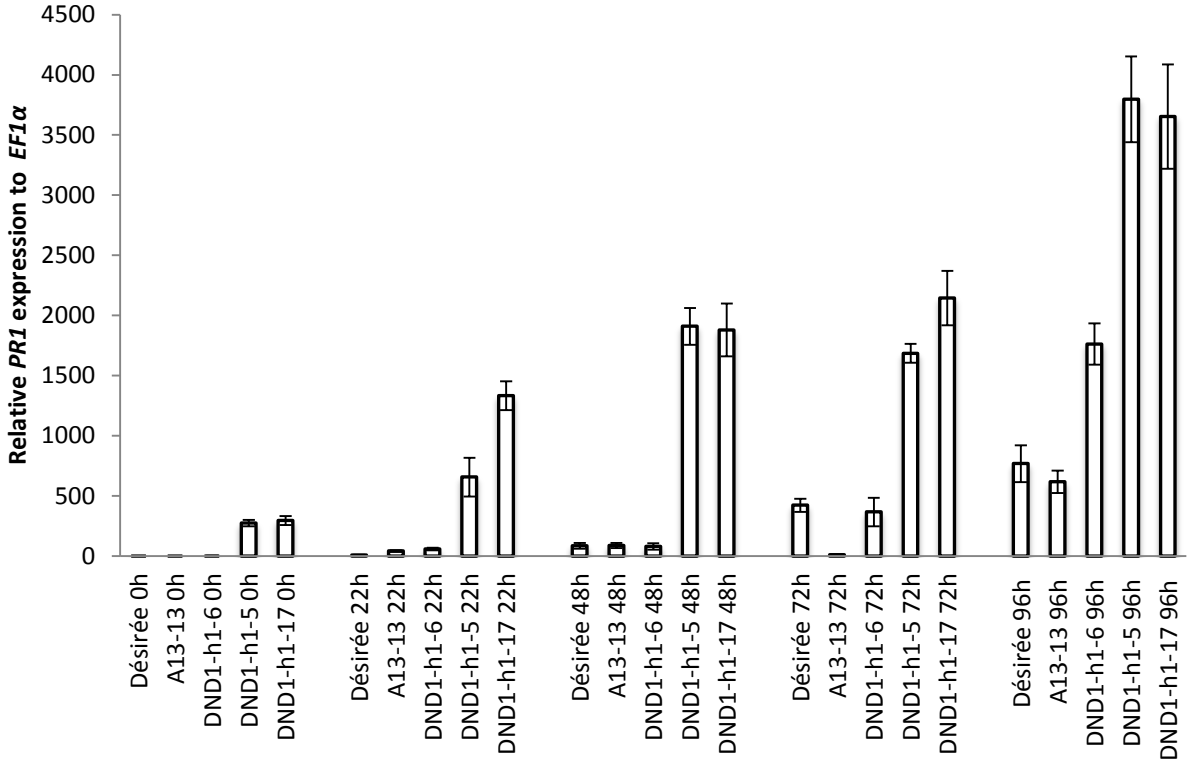

Figure 5.3.7. *Relative PR1 expression to EF1α in Désirée, A13-13 and RNAi::DND1-h1 transformants 1-5,1-6 and 1-17 at time points 0, 22, 48, 72 and 96 hours post inoculation with isolate PIC99189.*

5.4 Discussion

Generation of H_2O_2 is involved in stress and programmed cell death signalling (Gechev & Hille, 2005). In the susceptible controls (Désirée and DND1-h1-6) low H_2O_2 generation was visible 22 hpi, and this was more substantial 48 and 72 hpi. This is suggesting transition between the initial biotrophic phase of *P. infestans* and the following necrotrophic phase of its life cycle. In the latest time point after inoculation H_2O_2 levels covered over 50% of the leave area. Microscopic observation with trypan blue staining showed that in susceptible controls indeed hyphae and sporangia were present at 48, 72 and 96 hpi.

We showed that macroscopic H_2O_2 generation appears to be absent in two RNAi::*DND1-h1* potato transformants after inoculation with *P. infestans.* In addition, trypan blue staining did not reveal any growth of *P. infestans*. Still sometimes cell death and a zoospore were found at 22 hpi (Figure 5.3.2C). It seems that zoospores were unable to establish infection bodies on the leaves of RNAi::*DND1-h1* in which expression of *DND1-h1* was silenced effectively. In these potato RNAi::*DND1-h1* silenced transformants transcript levels of SA defense related gene *PR1* was highly up regulated. Increased *PR1* expression was also observed in the *Arabidopsis Atdnd1-1* mutant (Yu *et al.,* 1998), but *PR1* up regulation reported here was extensive. Increased *PR1* expression is often associated with elevated resistance and systematic acquired resistance (SAR), suggesting constitutive activation of defense responses in RNAi::*DND1-h1* silenced plants (Durrant & Dong, 2004; Ryals et al., 1994). This indicates that *DND1-h1* can be categorized as a *S*-gene that negatively regulates defense in potato.

The observations made in our study indicate that the infection events were similar in the susceptible controls Désirée and RNAi::*DND-h1-6* (Figure 5.4.1A), and resembled the infection events as suggested by Vleeshouwers et al. (2000). In the *R*-gene carrying control plant cell death was observed that could be HR related and hindered infection and proliferation of the pathogen (Figure 5.4.1B). Interestingly, in the RNAi::*DND1-h1* transformants (1-5 and 1-17) it seemed that zoospores were unable to infect the plants. (Figure 5.4.1C).

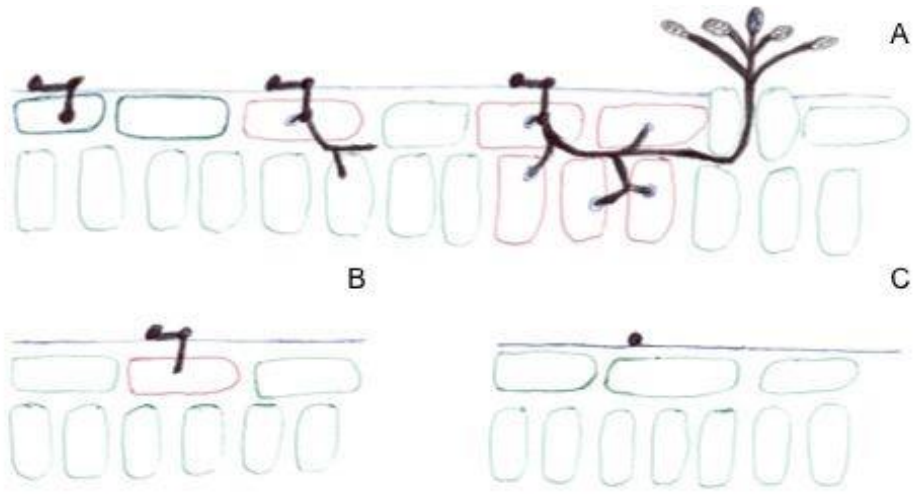

Figure 5.4.1. *Schematic overview of susceptible and resistance interactions between potato plants and P. infestans, green cells represent living cells and red cells represent cell death. Suggested infection interaction between susceptible plants and P. infestans (**A**). Infection interaction between a resistant R-gene carrying plant and P. infestans, infection is stopped after rapid cell death (**B**). Suggested infection interaction between RNAi::DND1-h1 transformants and P. infestans, it seemed that zoospores were unable to infect plant tissue (**C**). This schematic is based on observing two inoculated sites on the leaf of biological replicate.*

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Chapter 6 – General discussion

Usability of the *S*-gene strategy in potato breeding

Since the term "plant susceptibility gene" was first coined by Eckardt (2002) and suggested as a novel breeding strategy for disease resistance (de Almeida Engler et al., 2005; Pavan et al., 2010), functional orthologs of Arabidopsis *S*-genes have been identified in several commercial crops (Gao et al., 2014; Genger et al., 2008; Guo et al., 2013; Huibers et al., 2013; Jiang et al., 2009; Kim & Hwang, 2012; Kumar et al., 2012; Song et al., 2013; Zheng et al., 2013). In this thesis we reported the existence of candidate *S*-genes *Importin beta-3* and *PLC2* in potato (Chapter 2) and characterized *S*-genes *StSR1*, *StPMR4* and *StDND1* for disease resistance to *Phytophthora infestans* in potato (Chapter 3, 4 and 5, respectively). However, we have also seen the example of *StPMR5,* wherein the closest homolog that was considered the true ortholog, which did not function as a *S*-gene for disease resistance to *P. infestans* in potato. Or the example of a homolog of *StPMR4* that upon silencing resulted conferred partial resistance in the foliar part but not in the tubers of potato (Chapter 4). Indicating that functions of *S*-genes are not always conserved between species or between different plant organs.

Although *S*-gene function might be conserved between different plant species, the induced resistance by loss of function alleles or impairment by RNAi is sometimes not that black and white. The impairment of *StPMR4* in potato conferred partial resistance to *P. infestans* (Chapter 4; Kaile Sun, unpublished data); partial resistance to *P. infestans* was also found in potato plants wherein *StSR1* was impaired (Chapter 3). Both resistances can be classified as partial, but in contrast, the partial resistance conferred by *S*-gene *StPMR4* is more convincing for exploitation in breeding programmes than the resistance provided by the impairment of *S*-gene *StSR1*. Partial resistances discovered in lab settings could prove to be valuable for breeding as they are often related to field resistances. In addition, the impairment of *S*-genes can induce qualitative broad-spectrum resistance to *P. infestans* in potato. An example is *S*-gene *StDND1* (Kaile Sun, submitted). In our histological and expression studies we showed that *StDND1* is a negative regulator of defense and the resistance mechanism is suggesting a nonhost type of resistance.

During the course of evolution *S*-genes have not been excluded, this suggests that despite their role in susceptibility to pathogens they increase the plants general fitness as functional *S*-genes dominate over their loss of function alleles. Indeed, it has been shown that loss of function alleles of *S*-genes are often accompanied by deleterious pleiotropic effects. For example, the impairment of the *StDND1* gene in potato results in spontaneous autonecrotic lesion on the leaves and smaller plant size (Kaile Sun; submitted). However, there are also examples wherein knocking out *S*-genes provides resistance while plant growth is unaffected, such as *SlPMR4* in tomato, *StPMR4* and *StSR1* in potato (Huibers et al., 2013; Kaile Sun, unpublished data; Chapter 3 of this thesis).

S-genes and *R*-genes for durable broad-spectrum resistance

For plant transformations cultivar Désirée was used, Désirée does not possess any known *R*-genes and is considered as a susceptible potato cultivar, using Désirée as a control provides a good contrast to characterize disease resistance to *P. infestans* of candidate *S*-genes in potato. Once a *S*-gene is identified it would be interesting to study the combination of an impaired *S*-gene with a *R*-gene to identify which defense mechanism acts first. Recently, major *R*-genes have been stacked by genetic modification conferring broad spectrum resistance to potato light

blight (Zhu et al., 2012), this approach can be combined with the *S*-gene strategy to prevent the breaking down of *R*-genes by *P*. *infestans.*

Future perspectives

The research described in this thesis is part of novel strategy to obtain durable late blight resistance in potato. The loss of susceptibility strategy is an alternative to the classical *R*-gene resistance breeding strategy. The impairment of *S*-genes by RNAi proves to be a promising strategy in potato, even though posttranscriptional gene silencing was not always effective (Chapter 3) and obtaining RNAi transformants can be labour intensive (Chapter 2). An advantage of using RNAi is that gene silencing is inherited dominantly and is stable, therefore, directly useable to improve disease resistance in existing potato cultivars. However, the RNAi vector in this thesis has properties that would classify such an improved cultivar as a genetically modified (GM) crop. This is due to the CaMV 35S promoter and selectable marker genes. The CaMV 35S is strong promoter with the efficacy to obtain effective gene silencing, an alternative is to use the native promoter. In this way the crop can be classified as an intragenic GM crop, only endogenous genetic material is transferred that originates from the species itself or a crossable plant species (Rommens et al., 2007). Illustrating the potential of the RNAi technique as an extension to traditional potato breeding in the future. Currently, intragenesis is still classified as transgenic modification.

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