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Resistance against *Dickeya solani* in potato with the help of a susceptibility gene

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Dickeya solani-resistens i Potatis med hjälp av en suseptibilitetsgen

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Abstract

Potato (*Solanum tuberosum*) is a staple crop across Europe, including Sweden. Among the reasons why it is so ubiquitous is its fairly easy cultivation, good adaptability to various climates, and high nutritional value. Today's potato production is based on disease-free seed tubers. However, this technology often proves to be insufficient. Seed tubers serve as a target for accumulation of pests and pathogens, and one such pathogen is the necrotrophic bacterium *Dickeya solani*, which causes blackleg and soft rot. Due to its ability to macerate plant tissue and cause severe damages in the field, it is responsible for substantial yield losses across Europe. Consequentially, *D. solani* is treated as a quarantine organism in some countries. More importantly for this study, its presence has been reported in Sweden.

In the present study, a new approach to potentially offer a durable and broad-spectrum disease resistance towards *D. solani* and some other pathogens is explored.

Susceptibility genes encode products that are required for the pathogen's survival or proliferation, thus making a plant more susceptible to disease development. By silencing the homologs of the susceptibility gene *Downy Mildew Resistant 6 (DMR6)* in diploid DM1-3516 R44 and tetraploid Desirée background using RNA interference, an enhanced resistance was anticipated. Pleiotropic growth effects of *DMR6* silencing were investigated and greenhouse-based infection assays were carried out. Two silenced RNAi silenced Desirée lines were tested, however, only one (dmr6-6) showed promising results as it repeatedly had smaller blackleg symptoms, high constitutive *PR-1* expression, and showed no developmental and growth impairments compared to the corresponding wild type. Four diploid DM1-3516 R44 *DMR6* silenced lines exhibited no growth impairments. This study indicates a potential of *DMR6* for the further research in potato as an interesting target in potato breeding programs.

Keywords: potato, *Dickeya solani*, susceptibility gene, blackleg

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Abbreviations

Avr	Avirulence protein
BABA	β – amino butyric acid
CABI	Centre for Agriculture and Biosciences International
CFU	Colony forming unit
CIP	The International Potato Center
Ct	Cycle threshold
DAMP	Damage-associated molecular patterns
DM	Downy mildew resistance 6 gene
DMR6	Downy mildew resistance 6 gene
DPI	Days post infection
DTI	DAMP-triggered immunity
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
FAO	Food and Agriculture Organization
HR	Hypersensitive response
IPM	Integrated Pest Management
JA	Jasmonic acid
LPS	Lipopolysaccharide
MAMP	Microbial-associated molecular patterns
MLO	Mildew Locus O gene
NB-LRR	Nucleotide binding leucine-rich repeat domains
OD	Optical density
PAMP	Pathogen-associated molecular patterns
PCD	Programmed cell death
PCR	Polymerase Chain Reaction
PGSC	The Potato Genome Sequencing Consortium
Phi	Phosphite
PM	Powdery mildew
PR-gene	Pathogenicity-related genes
PRR	Pattern recognition receptors
PTI	PAMP-triggered immunity
qPCR	Quantitative PCR
R-gene	Resistance gene
RH	Relative humidity
RNAi	RNA interference
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SD	Standard deviation
S-gene	Susceptibility gene
SRE	Soft Rot Enterobacteriaceae
T2SS	Type II Secretion System

1 Introduction

1.1 Potato (*Solanum tuberosum*)

Solanum wild species: originate mainly from Andean highlands of Peru and serve as an important diverse gene pool of various traits (e.g. disease resistance) for potato breeding.

It was the year of 1532 when potato was first collected in Peru by Francisco Pizarro and his conquistadors (Hawkes and Francisco-Ortega, 1993). Since then, cultivation of potato (*Solanum tuberosum*) rapidly spread all around the world (**Fig. 1**), and it is currently the fourth largest cultivated crop, after rice (*Oryza sativa*), maize (*Zea mays*) and wheat (*Triticum aestivum*) (Mullins *et al.*, 2006; fao.org, 2008). Potato is a tuber-bearing crop and a member of the economically important Solanaceae family, which also includes tomato, pepper, eggplant, petunia, and tobacco. There are approximately 5000 varieties of potatoes known, and about 200 wild *Solanum* species have been recorded (Burlingame *et al.*, 2009; Watanabe, 2015). Potato is considered an “easy-to-grow” plant with excellent water-use efficiency that produces a lot of biomass per area compared to other crops (Mullins *et al.*, 2006). It is valued for balanced nutritional content consisting of starch, protein, antioxidants and vitamins. Moreover, potato accounts for about 2% of the world’s dietary energy/caloric intake (Burlingame *et al.*, 2009). Therefore, potato is one of the ideally suited crops for feeding large populations (Burra, 2016). In 2014, the potato was grown on more than 19 million ha with a total yearly production estimated up to 380 million tons, with Germany being the biggest producer in Europe (Ce Cicco and Jeanty, 2017; fao.org, 2014). Potato consumption is still more abundant in developed countries compared to developing countries. A large proportion of the potato production is used in processing industries for the production of starch, alcohol, snack foods, frozen fries, and cooked preserved foods. According to the International Potato Center (CIP, 2017), the potato is currently the third most consumed crop in the world.

Fennoscandian region: countries of Sweden, Norway and Finland

Potato was first introduced in Sweden by Olof Rudbeck in Uppsala in 1658, and 350 years later it is an irreplaceable component of the Swedish diet. In 2014, 2929 registered farms grew commercial table potato on 23,779 ha across Sweden with an average yield of 31.3 t ha⁻¹ and a total market value between 5,000 and 6000 million SEK (approx. € 600 million) (Eriksson *et al.*, 2016). However, due to the relatively small market size in the

Fennoscandian region, there has been little interest by companies in potato breeding in the past two decades. The potato breeding activities in Sweden are only carried out at SLU in Alnarp, where the focus mainly lies on developing late blight resistant and high yielding table potatoes (Eriksson *et al.*, 2016).



Figure 1: World potato production in 2010. (Obtained from http://www.targetmap.com/ThumbnailsReports/17619_THUMB_IPAD.jpg)

Locus: specific position of gene/allele on a chromosome

Allele: alternative form of a gene on the same locus

Ploidy level: number of sets of chromosomes in a cell; potato has four sets (4n) of chromosomes with 24 being a haploid number (somatic polyploidy)

Somatic ploidy: an organism contains multiple copies of the basic set of chromosomes of the same genome

Inbreeding depression: reduction of fitness as a result of inbreeding with increased genome-wide homozygosity in offspring

Cultivated potato varieties include ploidy levels between diploid and pentaploid. The commonly cultivated potato is a self-compatible polysomic tetraploid ($2n=4x=48$) with a basic number of 12 chromosomes, which makes breeding complicated. The complexity of polyploidy genetics is associated with (1) genotypic variation at a locus, (2) multiple alleles with different functions at the same locus, (3) allelic interactions with multiple alleles, and (4) complicated chromatid/chromosome segregation (Watanabe, 2015). For this reason, diploid and F1 hybrid breeding in potato could simplify breeding obstacles (see www.solynta.com) and break down major in-crossability problems with many promising diploid wild *Solanum* species (Shelley *et al.*, 2005; Watanabe, 2015). To ensure genetic uniformity and a relatively short cultivation period, most tetraploid potato cultivars are

propagated vegetatively by tubers, but also with tissue culture, or by cuttings (Watanabe, 2015). This is not only a costly process, but it often does not ensure disease-free potato tubers, which results in reduced yield or quality. Moreover, a seed potato tuber degradation, caused by an accumulation of pathogens, is transferred to successive generations, and stands as the number one problem for potato vulnerability (Thomas-Sharma *et al.*, 2015).

Multiple-layer analysis:
combining of the data from
different “-omics” fields to
better predict genome-
environment interactions

According to The Potato Genome Sequencing Consortium (PGSC, 2011), potato is highly heterozygous, however, it suffers from acute inbreeding depression which is another reason that makes it vulnerable to many pests and pathogens (Xu *et al.*, 2011). In 1996, CIP published an overview of common potato diseases consisting of a number of bacterial, fungal, viral diseases, and mycoplasmas. Between 1996 and 1998, total potato losses were estimated to vary between 24% in Northwest Europe and 50 % in Central Africa (Oerke and Dehne, 2004). Furthermore, in 2013 in Sweden, 21 % of all fungicides used, were used for potato production (Eriksson *et al.*, 2016). With a help of recently emerging fields of “-omics” and genome-wide approaches that enable us to do a “multiple-layer analysis”, we are one step closer to a better understanding of plant defences and pathogen etiology (Burra, 2016).

1.2 *Dickeya solani*

There are approximately 150 bacterial species that cause plant diseases known to man. Some of them are responsible for serious economic losses in potato such as bacterial wilt caused by *Ralstonia solanaeum*, ring rot caused by *Clavibacter michiganensis* subsp. *Sepedonicus*, and blackleg and tuber soft rot caused by genus *Pectobacterium* (Czajkowski *et al.*, 2011; Xun *et al.*, 2011). In early 2000's, a novel pectinolytic blackleg and tuber soft rot causing bacteria emerged, now known as *Dickeya solani*. The bacteria has been considered as one of the ten most scientifically and economically important bacterial plant pathogens (Masfield *et al.*, 2012), and is the focus of my master thesis.

The earliest strains of *D. solani* were isolated from hyacinth, but have since been found in many other ornamental plants, as well as the crop plants maize, rice, and pineapple (Golanowska, 2016). Relevant for my thesis, *Dickeya solani* has also caused a substantial economic damage in potato in many European countries including Sweden (**Fig. 2**). In the

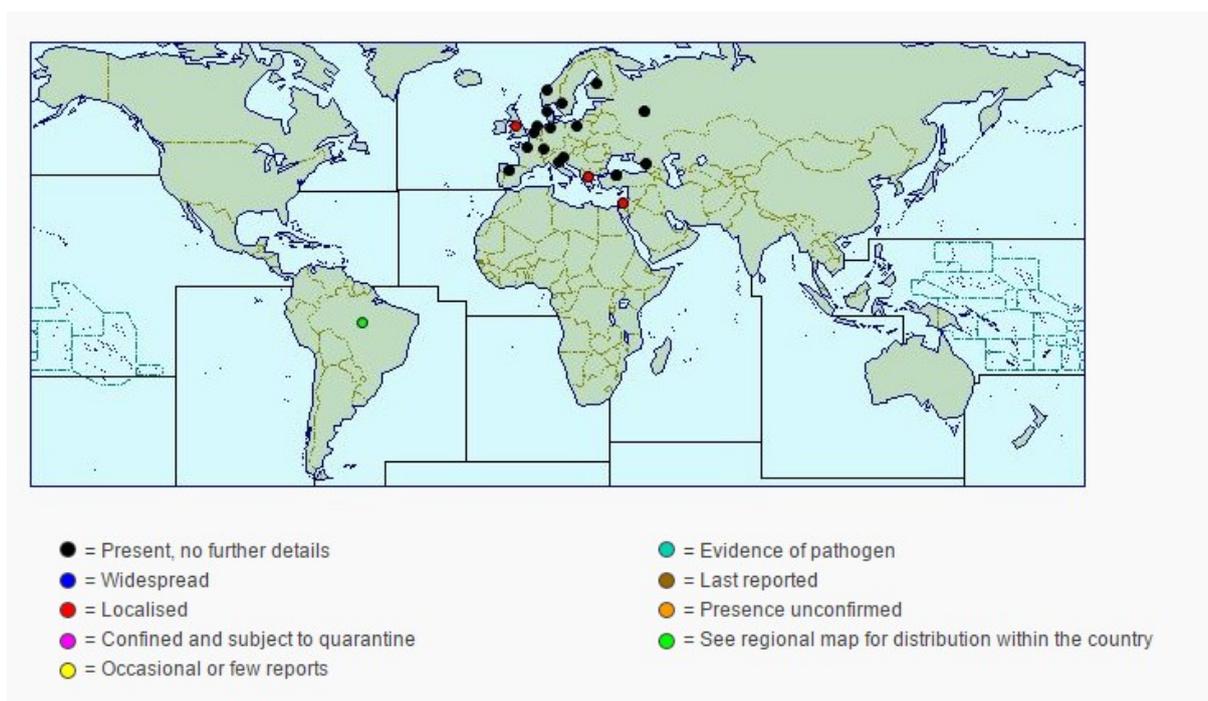


Figure 2: Worldwide distribution of *Dickeya solani*. <http://www.cabi.org/isc/datasheet/120278>

Netherlands, stringent policies of rejecting blackleg-affected seed tuber stocks led to increased annual losses estimated up to €30 million (Toth *et al.*, 2011). Moreover, yield reductions of 20-25% due to *D. solani* infections have been recorded for various potato cultivars in Israel, and *D. solani* is now considered to be a quarantine organism in Israel (Toth *et al.*, 2011). *D. solani* presence in Sweden has been reported (CABI, 2017; Rölin and Nilsson, 2011; Rölin and Persson, 2013), however, no current data or the degree of severity can be found. Also, outbreak incidences have been reported in Finland (Degefu *et al.* 2013).

Lesion: symptoms (cell death/necrosis) caused by pathogen (and not by hypersensitive response of plant)

Dickeya solani, previously known as *Erwinia chrysanthemi*, is a necrotrophic, Gram-negative, facultatively anaerobic, non-sporulating, motile, rod-shaped bacterium (0.9x2.0 µm) with peritrichous flagella (Czajkowski *et al.*, 2012; van der Wolf *et al.*). Among seven representatives of the highly diverse genus *Dickeya*, *D. solani* is considered to be the most aggressive and prevalent in Europe (Golanowska M., 2016). Toth *et al.* (2011) reported that little as 10 cells of *D. solani* inoculated into susceptible tubers was enough for disease development under optimal temperature conditions.

Dickeya solani can be found on roots, in tuber lenticels, and inside the xylem of the potato plant. It enters the plant via natural openings (lenticels, stolon ends or wounds), spreads

Quarantine organism:

policy to prevent spreading of harmful plant pathogens that are not yet present in EU, or are present but not widely distributed

Necrotroph: pathogens that rapidly kill plants to obtain nutrients (e.g. rotting bacteria)

Anaerobe: an organism that does not require oxygen for growth

through the vascular tissue and thrives under humid and broad-range temperature conditions (Toth *et al.*, 2011). Bacteria are often present in the latent state, however, a bacterial multiplication is initiated when the environmental conditions are favourable (Pérombelon, 2002). Moreover, in his overview, Pérombelon (2002) emphasizes on multiple occasions that presence of water is an essential factor.

The genera *Pectobacterium* and *Dickeya* both belong to the Enterobacteriaceae family and cause blackleg, stem rot, and soft rot symptoms. They are often phenotypically indistinguishable (Charkowski, 2015) and are collectively grouped as Soft Rot Enterobacteriaceae (SRE) as they both produce plant cell wall degrading enzymes that are required for the infiltration and maceration of plants (Czajkowski *et al.*, 2014). *Dickeya* spp. in particular, produce extracellular enzymes called pectinases which cleave glycosidic linkages or the methyl-ester bonds of the pectic polymers (Golanowska M., 2016). Additionally, the type II secretion system (T2SS) is required for delivery of enzymes to the plant cell wall (Abramovitch *et al.*, 2006). The disease first appears on leaves, stems, and/or underground parts as small, water-soaked, translucent lesions that rapidly enlarge both in diameter and depth. Under optimal wet conditions, plant tissues are left cream-coloured gray, brown or black, and usually release a putrid odour (Golanowska M., 2016). Wilting, chlorosis, and stunted growth are caused by restricted water flow in the xylem vessels due to infection, resulting in plant collapse and death (Pérombelon, 2002) (Fig. 3).

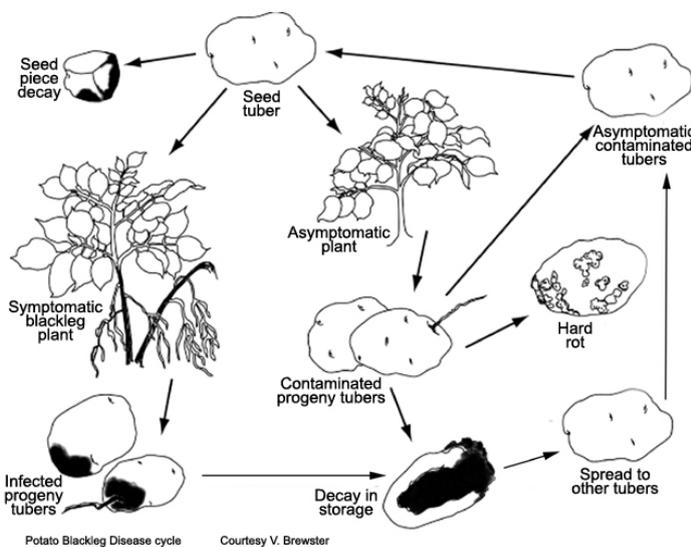


Figure 3: Disease cycle of *Pectobacterium* and *Dickeya*. (Reproduced from De Boer, S. H. and Rubio, I. 2004. Blackleg of potato. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2004-0712-01)

Zero tolerance policy: strict regulation policy, which entails that if a single positive/infected plant is found in a field, a whole seed stock can be rejected

Some of the many reasons for bacterial disease damage are inadequate hygiene at grading, poor soil drainage, presence and increasing levels of the pathogen on seed tubers, over-irrigation, wet spring weather, damage at harvest, and absence of adequate ventilation at storage (Toth *et al.*, 2011). Much of the disease spread of *D. solani* occurs by trade and movement of latently infected vegetative propagating material (**Fig. 3**) (Czajkowski *et al.*, 2014). Chemical treatments are often applied on the surface of potatoes, however, if the bacteria reside inside the tubers, surface sterilization is ineffective. In order to prevent the occurrence and spread of blackleg, Scotland has introduced a zero tolerance policy for blackleg caused by *Dickeya* spp. (CABI, 2017).

Many diagnostic tools are applied in order to identify the presence of *Dickeya* spp. efficiently. For that reason, multiple epidemiological studies are employed such as biological control and serological methods, and molecular assays with multiplex real-time PCR being the most prominent (Czajkowski *et al.*, 2014).

Abiotic factors temperature and humidity play a major role in disease development. Studies show that *D. solani* aggressiveness increases as the temperature increases (Toth *et al.*, 2011). Moreover, its temperature tolerance level has been reported to be as high as 39°C, consequently raising many concerns for its spreading in the future as a result of climate change (Toth *et al.*, 2011). In 2006, the warmest summer in Finland recorded in 100 years, concurred with a series of blackleg outbreaks on potato (Degefu *et al.* 2013). The same group isolated bacterial strains from diseased samples collected in North Finland and confirmed that *D. solani* was the main causative.

Epidemic: outbreak of disease characterized by an infection that starts from a low level and then progresses to a high one

Etiology: study of the cause of disease

The lack of successful solutions is mainly due to the complex etiology, lack of knowledge about the ecology of this genus, and the possible emergence of new *Dickeya* pathogen strains (Charkowski A.O., 2015). However, an advantage is the availability of a complete assembled genome of *D. solani* IPO 2222, which could contribute to further understanding of the *Dickeya* spp. infection mechanisms and epidemiology in order to better control disease occurrence (Khayati *et al.*, 2016).

1.3 Resistance mechanisms and breeding for disease resistance

Close to 800 million humans are suffering from malnutrition (worldhunger.org, 2017). The reason behind this is complex and is the result of several factors such as poverty, pre- and post-harvesting losses as well as the wastage of 1.3 billion tons of food a year (FAO, 2011).

The first use of disease prevention treatment took place in the middle of the 17th century when salt water was used to control bunt on wheat. Up until 1940, sulfur-, copper- and mercury-based, often homemade, products were used in excessive amounts to treat fields. By the end of the 1970s, the crop protection industry was flourishing with many chemical products offering a broad range of effective bactericidal, fungicidal, and antibiotic treatments. However, the agrochemical industry has been challenged by resistance development of pathogens and a trend towards a more judicious use of chemicals (Morton and Staub, 2008). New alternatives are being explored, and new laws such as the European Union IPM (Integrated Pest Management) directive (Directive 2009/128/EC) are being implemented to limit chemical based disease control, thus restraining undesirable consequences and concerns raised by scientists and society (Burra, 2016; Alexandersson *et al.*, 2016). In our group, the potential of induced resistance, with β - amino butyric acid (BABA) and phosphite (Phi) based salts, is being explored. By applying such compounds, we aim to stimulate the induction of defense responses in the plant prior to infection by a plant pathogen (Burra, 2016). Other strategies such as biological control, transgenic plants, and the use of germplasm of wild relatives of potato showing resistance are also employed to limit disease outbreaks.

PAMPs/MAMPs: highly conserved microbial factors recognized by a host plant

Effectors: molecules that act inside the cell and contribute to pathogen virulence, by mimicking or inhibiting eukaryotic functions

Biotroph: a pathogen that is fully dependent on a living tissue to survive and cause infection (e.g. fungal mildews)

Another aspect to improve control of emerging plant diseases is to study plant innate immunity. Due to their immobility, plants have evolved a robust immune system which enables them to compete with certain types of biotic stress. The plant immune system consists of two branches. The first one, PAMP-triggered immunity (PTI), consists of transmembrane pattern recognition receptors (PRRs) that respond to microbial- or pathogen-associated molecular

Resistance (R-) genes: genes in plant genomes which are translated into R-proteins, crucially needed for resistance against pathogens; they usually consist of a NB and LRR domains

Hypersensitive response (HR): rapid programmed cell death (PCD) induced by plant to prevent spreading of infection of a pathogen

Hemibiotroph: a pathogen that is only partially dependent on living tissue and continues to live in dead tissue to complete its cycle (e.g. oomycetes)

Pathogenesis related (PR-) genes: defense-related genes often induced by SA as a part of SAR, and possess antimicrobial or antifungal properties

Programmed cell death (PCD): genetically encoded, active process which results in death of individual cells, tissues or whole organs to prevent spreading of disease

Pattern recognition receptors (PRR): proteins that are part of plant innate immunity, and are responsible for recognizing PAMPs

patterns (MAMPs or PAMPs) such as bacterial lipopolysaccharides (LPS) and flagellin. The second branch, effector-triggered immunity (ETI), generally present inside the cell, relies on nucleotide binding leucine-rich repeat domains (NB-LRR) proteins encoded by disease resistance (*R*-) genes (Jones and Dangl, 2006). NB-LRRs recognize effector proteins, which are secreted by pathogens to overcome PTI, and activate defense responses. The recognized effectors are termed avirulence (*Avr*) proteins. Both PTI and ETI result in activating defense responses, whereof ETI is faster and stronger and results in a hypersensitive response (HR) at and surrounding infection sites, which is often associated with programmed cell death (PCD), but may also trigger secondary immune responses collectively known as SAR in distal uninfected tissues (Huot *et al.*, 2014). ETI is generally only considered to be effective towards biotrophs and hemibiotrophs, and ineffective towards necrotrophic organisms such as soft rot bacteria. PTI, on the other hand, induces down-stream signaling cascades of defense-related genes (e.g. *PR*-genes, phytoalexins), activates ROS production, and is associated

with callose deposition (Jones and Dangl, 2006). The constant coevolution between newly encoded plant *R*-genes and pathogen effector-based evasion is termed an evolutionary arms race.

In the past few decades, conventional breeding techniques were used to introgress usually a single dominant monogenic resistance gene into well-established potato cultivars. However, this approach proved to be laborious and inefficient (Vleeshouwers *et al.*, 2011). Quintessential examples are potato cultivars Bionica and Toluca, into which a durable late blight resistance gene *Rpi-blb2* has been introgressed from a diploid wild species *Solanum bulbocastanum*. The process of introgression took almost 50 years, yet the resistance was broken down within three years (Vleeshouwers *et al.*, 2011). Dominant *R*-gene-mediated resistance works by recognition of a single elicitor, therefore, a

Monogenic dominant resistance/major gene resistance: resistance in which host's major gene interacts with specific major gene for avirulence in pathogens

Polygenic: a trait controlled by two or several genes, each with minor effect

frequency of overcoming resistance is relatively high (Adolfo *et al.*, 2016). As a result, breeding for single dominant *R*-gene resistance is not considered practical anymore. Their durability solely can depend on a simple point mutation of a pathogen effector to be sufficient to overcome the host plant resistance (van Schie and Takken, 2014). Instead, genetic modifications for broad-spectrum disease resistance are being explored. Combining and transferring of multiple *R*-genes into a plant is known as *R*-gene pyramiding or stacking. With the presence of multiple *R*-genes, it is expected to lessen the selection pressure on each individual gene, thus making the resistance more durable (Jones *et al.*, 2014). Moreover, it would require the pathogen to have multiple adaptations to circumvent recognition by the plant.

Up to now, far too little attention has been paid to blackleg and soft rot causing bacteria such as *D. solani*. There are no commercial potato cultivars available that show resistance to *Pectobacterium* or *Dickeya* spp. (Czajkowski *et al.*, 2011; Degefu *et al.*, 2012). Additionally, no major resistance genes have been identified, to my knowledge. Besides, Burra (2016) speculated, on the basis of results from a segregating population, that resistance towards *D. solani* is multigenic. For now, the main strategies to control the disease spread are avoidance of contamination, monitoring sources of irrigation water, chemical treatments, and biocontrol. As the screening for blackleg and soft rot resistance is not straightforward, it has not been given a priority in most breeding programs, or is sometimes done only at advanced selection stages (Czajkowski *et al.*, 2011). Some promising studies have shown that the utilization of wild *Solanum* species increased the resistance to both *Pectobacterium* and *Dickeya* spp., but have not been employed in breeding programs (Czajkowski *et al.*, 2011).

1.4 Susceptibility genes

Vertical resistance: inheritance based on a major gene resistance that results in hypersensitive reaction towards race- or pathotype-specific pathogens, which is usually considered as non-durable resistance

As described above, vertical resistance has had a fundamental role in breeding for resistance, however, not for necrotrophic *D. solani*. Additionally, narrow-spectrum resistance of a single *R*-gene is usually quickly overcome by pathogens. Therefore,

Susceptibility (S) gene:
encode proteins that are required by pathogens either for their growth process on the parasitized plant or for negative regulation of plant defense responses

different approaches that could possibly offer durable resistance must be explored.

May it be a successful entry of a pathogen into a host plant such as formation of fungal feeding structures (i.e. haustoria), or as

simple as entry of bacteria through a stomatal opening – the host plant-pathogen interaction is crucial. Genes, which are involved in facilitating and supporting of host-pathogen compatibility can be considered to be susceptibility (S-) genes (van Schie and Takken, 2014). In the review, Van Schie and Takken (2014) classify three different mechanisms of S-genes, depending on how they contribute to the infection: (Class 1) genes allow the entrance of a pathogen by facilitating host recognition and penetration, (Class 2) genes act as negative regulators of immune signalling, and (Class 3) genes that encode essential substrates paramount for pathogen proliferation. Employing them for resistance breeding has been one of the newly used strategies, and it appears to have a great potential (Pavan et al. 2009).

Durable resistance:
resistance, often of quantitative nature, that continues during its prolonged and widespread use in an environment favourable to the disease

Mutating or silencing a susceptibility gene can result in impeding a pathogen to cause disease. As a result, a pathogen would have to overcome a dependency on a host's susceptibility factor (e.g. essential metabolites that cannot be produced by a pathogen), which would require a substantial

reprogramming from the pathogen's side. It would probably mean reversing a long evolutionary process or finding a new compatible host to regain the advantage (van Schie and Takken, 2014). This principle makes susceptibility genes highly interesting targets for resistance breeding as they might provide plants with a broad-spectrum and durable resistance. So far, the use of S-genes in breeding has been limited due to their recessive nature, which makes the identification and breeding fairly complicated. According to Pavan *et al.* (2009), recessive resistance is explained as "*Genetically, S-genes can be defined as dominant genes whose impairment will lead to recessive resistance.*" Up to recently, more than 200 susceptibility genes have been identified, and most of them are of monogenic recessive nature (van Schie and Takken, 2014). The clearest example of durable S-gene utilization is the Mildew Locus O (*MLO*) gene family in barley against powdery mildew (PM) (Büschges *et al.*, 1997). *MLO* encodes for a plant-specific transmembrane protein, and is required for the successful entry of the PM pathogen *Blumeria graminis f. sp. Hordei*

(van Damme *et al.*, 2008). Its loss-of-function mutation also resulted in PM resistance in *Arabidopsis*, pea, tomato, wheat and strawberry (reviewed by van Schie and Takken, 2014). *Mlo* mutants display broad-spectrum (non-race-specific) and durable resistance against all isolates of the fungus (reviewed by Eckardt N., 2002; van Schie and Takken, 2014). On the other hand, mutations of the classical dominant race-specific “gene-for-gene” *Mla-1* and *Mla-32* locuses conferred the resistance only against PM caused by *Blumeria graminis* f. sp. *Hordei*. Moreover, *Mlo* has been used for many decades, and resistance breaking has not been recorded yet. Besides *Mlo* archetype, not many successful implementations of *S*-genes have been reported.

1.5 Susceptibility gene *DMR6*

Salicylic acid (SA): one of defense-associated phytohormones (besides jasmonic acid and ethylene), which is involved in SAR and orchestrates immune response against biotrophic and hemibiotrophic pathogens

Another promising *S*-gene, which has received much attention lately is *Downy Mildew-Resistant (DMR6)* gene.

Van Damme *et al.* (2005) were the first ones to have come across *DMR6* while studying the interactions between the oomycete *Hyaloperonospora parasitica*, the causal agent of downy mildew, and its natural host *Arabidopsis thaliana*. *Dmr6* mutants were one of eight different downy mildew-resistant (*dmr*) mutants analysed, linked to six loci. In the study, *dmr6* mutants showed an increased resistance towards *H. parasitica* without enhanced defense responses, thus suggesting that corresponding gene/loci plays an important role in the *H. parasitica* infection process. A subsequent study from van Damme *et al.* (2008), a characterization of *Downy Mildew Resistant 6 (DMR6)* was performed. They found that *DMR6 (At5g24530)* encodes an oxidoreductase, a member of 2-oxoglutarate (2OG)-Fe(II) oxygenase superfamily, with no described biological function (Zeilmaker *et al.*, 2014). GUS expression analysis showed that *DMR6* was highly induced and strictly localized at sites in direct contact with *H. parasitica*, and is independent of salicylic acid (SA) and pathogenesis-related (*PR*) genes during the early transcriptional activation (van Damme *et al.* 2008). They also indicated that loss-of-function *DMR6* leads to elevated constitutive expression levels of a number of tested defense-related genes (*ACD6*, *PR-1*, *PR-2*, *PR-4*, and *PR-5*). *PR* genes are mainly induced by SA; Zeilmaker and colleagues (2015) proposed that *DMR6* might have a role in regulating SA levels, thus controlling over-activation of defense responses which could result in

impaired plant growth and development (e.g. dwarfism). According to van Schie and Takken (2014), the *DMR6* is considered as a suppressor of immunity and belongs to Class 2 susceptibility genes (Fig. 4). To prevent an over-activation of defense responses in a host plant, many genes encoding negative regulators of immunity are activated during pathogen infection (Zeilmaker *et al.*, 2015).

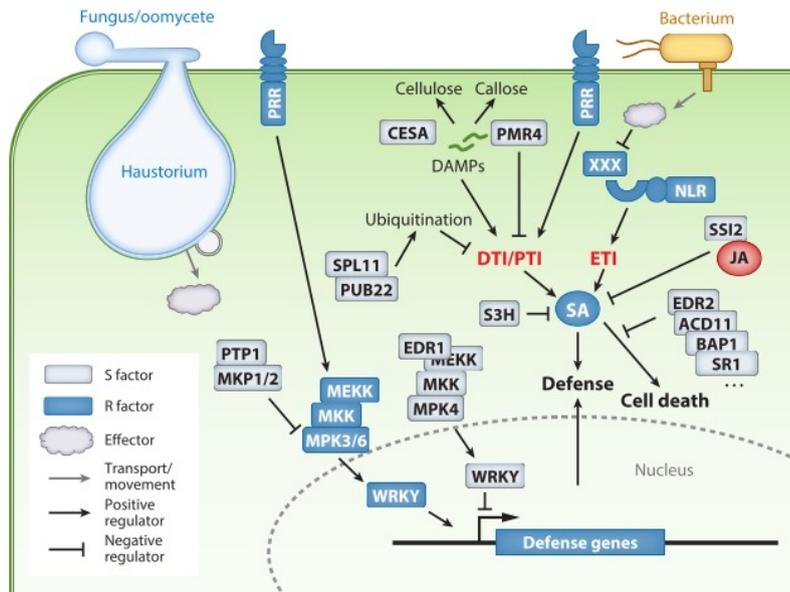


Figure 4: Presentation of some known susceptibility genes involved in suppression of host defense. Class 2 S-genes may interfere with DTI and ETI pathways, effect the transcription of WRKY transcription factors, or control salicylic acid (SA) levels such (e.g. *DMR6*). (Taken from van Schie, C. C. N., & Takken, F. L. W. (2014). Susceptibility Genes 101: How to Be a Good Host. *Annual Review of Phytopathology*, (June), 1–31.)

Until now, *A. thaliana dmr6* mutants have been described to exhibit complete or partial resistance against obligate biotrophic oomycetes *H. parasitica* and *H. arabidopsis*, and hemibiotrophs oomycete *Phytophthora capsici* and bacterium *Pseudomonas syringae*, (van Damme *et al.* 2005, 2008; Zeilmaker *et al.*, 2015; Thomazella *et al.*, 2016). Recently, the first use of *DMR6* in tetraploid potato cv. Desirée (susceptible to late blight) showed promising results as RNAi silenced lines displayed no symptoms seven days after inoculation with the hemibiotrophic oomycete *Phytophthora infestans* on detached leaves (Sun *et al.*, 2016). No clear analysis of growth phenotypes has been described in potato. Evidently, silencing or mutation of *DMR6* seems to provide resistance to a broad range of pathogens, however, necrotrophic pathogens have not been tested. Few susceptibility genes have been linked to soft rot causing bacteria such as *pectin methylesterase 3 PME3* in *Arabidopsis* and Tobacco, ABA aldehyde oxidase (*Sit*) in tomato, and CNGC2/4 cyclic nucleotide gated channel (*DND1/2*) in *Arabidopsis* (supplementary table, van Schie and Takken, 2014), but none for the blackleg causing *D. solani*.

2 Hypotheses

Blackleg disease caused by *Dickeya solani* has led to considerable economic losses in many European countries, and its presence has already been reported in Sweden. New approaches and other alternatives to provide plants with enhanced resistance must be explored before bacterial outbreaks start emerging. My aim was to test a possible applicability of silencing of susceptibility gene *DMR6* to increase resistance towards *D. solani*. Characterization of RNAi silenced *DMR6* potato lines, greenhouse infection assays, and quantitative PCR analysis were employed to test the hypotheses listed below:

- 1 The *DMR6* homologue in potato is a susceptibility factor towards *Dickeya solani*.
- 2 *DMR6* silenced lines do not have developmental impairments in potato.
- 3 The *DMR6* gene is involved in regulation of *PR-1* in potato.

3 Materials and methods

Plant material and pathogens

Potato plants

Potato plant material (**Tab. 1**) was kindly provided to me by Dr. Nawaporn Onkokesung in prof. Erik Andreasson's group. RNAi silencing was previously done to silence the *DMR6* gene. The lines with the lowest expression, in diploid DM1-3516 R44 and in tetraploid Desirée potato background, were identified.

Table 1: List of potato RNAi silenced lines used in the project.

Lines	Gene	Expression	Plant background genotype
DM1-3516-R44	/	/	/
DM-DMR6-5	StDMR6	RNAi	DM1-3516-R44
DM-DMR6-10	StDMR6	RNAi	DM1-3516-R44
DM-DMR6-11	StDMR6	RNAi	DM1-3516-R44
DM-DMR6-17	StDMR6	RNAi	DM1-3516-R44
Desirée	/	/	/
Des-DMR6-6	StDMR6	RNAi	Desirée
Des-DMR6-7.1	StDMR6	RNAi	Desirée

Dickeya solani

The Finish *Dickeya solani* isolate Ds 0432-1 (Laurila *et al.* 2008) was used throughout the study. Vials containing bacterial on glass beads were stored at -80°C.

Growth conditions and preparation

In vitro plantlets were grown on shoot-inducing medium containing 0.5x Murashige and Skoog medium (Murashige and Skoog, 1962; Duchefa), sucrose (20 g/L; Duchefa), Phytoagar (8 g/L; Duchefa), IBA (500 mg/L), and adjusted to pH 5.8. An *in vitro* climate chamber was set on long day conditions at 23°C and 18°C during 16-h light and 8-h darkness, respectively, with a relative humidity (RH) of 60 % and a light intensity of 80 $\mu\text{mol m}^{-2}\text{s}^{-1}$. 24 plants per line always served as a "stock" from which three weeks before the start of each experiment top shoots were excised with surgical scissors and transferred to fresh media (9 cuttings/box). Every three months, stock plants were also refreshed on a new medium.

For greenhouse and climate chamber experiments, 3-week-old *in vitro* plants were planted in separate 0.6 L pots filled with a soil substrate (Blom och Plantjord; Emaljunga

Torvmull AB, Sweden), and cultured for two weeks. The first week, plants were covered with transparent plastic cups to acclimatize to the environment. After two weeks, plants were transferred to either 3.5 L (phenotype evaluation and infection assays) or 5 L (tuber growth) pots, and were fixated with bamboo sticks and plastic coated garden wire. From the fifth week on, all plants were supplied with fertilizer WH-BOUYANT Rika S (7-1-5+mikro; Weibulls Horto, Sweden) on a weekly basis. Growth conditions in the climate chamber were: 20°C and 65% RH with 16-h light (intensity 160 $\mu\text{mol m}^{-2}\text{s}^{-1}$) and 8-h dark period. Growth conditions in the greenhouse chamber were: between 18 and 21°C in the winter and spring time, with light reflectors turned on 16-h per day if the radiation outside was below 200 W/m².

Ten glass beads with *D. solani* were transferred into conical tube with 20 ml of sterile high salt LB broth (Duchefa Biochemie, Haarlem, the Netherlands). The bacteria were cultured on a shaker at 220 rpm at 27°C for 16–24 h. The overnight culture was centrifuged at 4000 g for 10 min (Rotina 380R; Hettich Zentrifugen, Germany), washed with sterile tap water and re-centrifuged at the same speed. Bacteria were re-suspended in sterile tap water and adjusted to OD600 0.2 (5×10^9 CFU*ml⁻¹) using a Thermo Scientific MultiskanGo.

Phenotypic performance and data analysis

Upon transferring the plants from media to soil, stem width of the *in vitro*-grown plantlets was measured with a calliper. After two weeks of acclimatization period, phenotypic evaluation was performed during three consecutive weeks, with measurements taken once per week. Stem width was measured between the 3rd and 4th leaf from the top leaf down, and height was measured from the soil surface up to the highest apical meristem. In total, three separate experiments were carried out with generally four plants per genotype up to 12, depending on what was to be tested. Experiments lasted between 8 and 12 weeks, depending on whether flowering and tuber setting were to be investigated. Weekly plant development and the rest of photographic material presented in the Results and Appendices sections were recorded using a Sony Cyber-shot DSC-RX100 camera. Simple statistics such as calculation of mean values, Student's t-test, and standard deviation were performed in Microsoft Excel. The acceptance level of statistical significance was $p < 0.05$.

Greenhouse *Dickeya solani* inoculation and infection assay

For each experiment ten *in vitro* plants of each line (Desirée, dmr6-6, and dmr6-7.1), nine for infection and one as negative control, were grown in soil for five weeks as described above. Maximally two stems per pot were left to grow and the rest were cut off after 1-2 weeks. Five-week-old greenhouse plants were injected with bacterial solution at the stem base (one injection per stem), approximately 10 cm above the soil (above the slim stem region) with 20 µl bacterial solution adjusted to OD600 0.2 (5×10^9 CFU*ml⁻¹) (**Fig. 10 A**). To ensure that all plants receive the same volume, the stem was first pricked using a 0.8 mm syringe needle and the bacterial solution was subsequently injected with a 20 µl pipette tip directly into the puncture wound. Genotypes were randomly inoculated and injecting air bubbles was carefully avoided. As a negative control, autoclaved tap water was injected into control plants. The infected wound was covered with Nescofilm (Nesco, Greenfield, MA, USA) and sprayed with distilled water (**Fig. 10 B**). To get representative blackleg symptoms, without excessively stressing the plants, the greenhouse assay (Burra *et al*, 2015), was optimized by creating a climate chamber-like environment tunnel by covering a prebuilt frame with transparent plastic foil (**Fig. 10 C**). This arrangement allowed to retain the humidity (RH 60-80%) and temperature (24-28°C). Each plant was put on an inverted plant pot and the bench was filled with water. The chamber was sprayed once a day to ensure high humidity. Symptoms were scored 7 days post inoculation (7 DPI) by measuring the length of the black lesion, both on the surface of the stem and by cutting the stem open lengthwise, to measure the rate of bacterial spread outside and within the plant's vascular system (**Fig. 11**).

A two-way ANOVA combined with a posthoc test (Tukey) was used to compare the performance of three genotypes over three experiments. Type III sum of squares was used in the model. The measurements were log transformed, and validation of the assumption of normally distributed residuals of the model was done. SPSS Statistics Software was used (IBM).

RNA extraction, cDNA synthesis and quantitative RT-PCR analysis

Total RNA was isolated from 8-week-old non-treated and healthy plants using the RNeasy kit from Qiagen (Germany, <http://www.qiagen.com>) following the RNeasy Mini Handbook (Qiagen).

RNA samples were treated with RNase-free DNase (Qiagen). For the cDNA synthesis, SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR kit (11752-050; ThermoFisher) was used, following the steps according to the instructions. Quantification and quality of the RNA was determined using a NanoDrop™ ND-2000 (ThermoFisher). cDNA samples were diluted 10x and stored at -20°C. Reagents used and steps taken are presented in the **Table 2** and **3** below.

Table 2: cDNA reaction system

Step	Reagent Name	Volume (µl)
DNAase treatment	RNA	Calculate (500 ng)
	MiliQ water	Up to 7
	DNAase (Thermo Fisher)	8
	Ribolock	1
	50mM EDTA	1
First strand cDNA synthesis	DNase treated RNA	8
	2x SuperScript® III Reaction Mix	10
	SuperScript III Reverse Transcriptase Mix	2
	MiliQ water	Up to 20
RNAse treatment	<i>E.coli</i> RNAase H	1

Table 3: cDNA synthesis program

Steps	Temperature (° C)	Time (min)
DNAase treatment	37	30
+ EDTA	75	10
Annealing	25	5
cDNA synthesis	42	30
Termination	85	5
Cooling	0	5
RNAase treatment	37	20

For quantitative RT-PCR analysis Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen by Thermo Fisher Scientific) was used. Master Mix for all samples was prepared according to volumes presented in the **Table 4**. Additionally, 2 µl of cDNA template were added into each well. A negative control without cDNA template was included. Cycle thresholds were determined using three technical replicates of each

sample with at least three biological replicates of each line. Five-step three-fold serial dilution of with wild type RNA template was performed to check the reaction efficiency of all primer pairs. Each dilution was tested in triplicates. The data was normalized to the housekeeping gene *elongation factor 1- α* (*ef1 α*) using the Pfaffl method (Pfaffl M.V., 2001). The primers used are listed in the Appendices (**Tab. 9**).

Table 4: Master Mix for one quantitative RT-PCR reaction

Reagent Name	Volume (μ l)
Platinum [®] SYBR [®] Green	10
Primer Forward (10 μ M)	0.4
Primer Reverse (10 μ M)	0.4
MiliQ	7.2

Program for real-time qPCR was run for 40 cycles (Biorad CFX96™ Real Time) as presented in **Tab. 5**.

Table 5: Quantitative RT-PCR program

Step	Temperature ($^{\circ}$ C)	Time	
Initial Denaturation	95	5 min	
Denaturation	95	15 s	} 39 x
Annealing and elongation	60	25 s	
Final Elongation	72	30 min	
Melting curve	50-95 with 0.5 $^{\circ}$ C increment	Every 5 s	

PCR analysis of RNAi insert

DNA isolation was performed according to Edwards *et al.* (1992) with the following adjustments. One small leaf (approximately 30 mg) of each genotype was collected in 1.5 ml Eppendorf vials separately. Samples were processed fresh instead of being flash-frozen. Isolated genomic DNA was stored at -20oC. Quantification and quality of DNA was measured with by NanoDrop™ and on a 1.5% agarose gel electrophoresis.

Master Mix for all samples was prepared according to volumes presented in **Table 6**. Additionally, 2 μ l of DNA template was added to each tube. Two separate pK7GWIWG2 (II) vectors were used as positive controls. A negative control without genomic template was included. Program for PCR was run for 35 cycles (Biorad S1000™ Thermal Cycler) as presented in **Table 6**. The PCR products were run on 1.5% (300-1000 bp amplicons) agarose electrophoresis gel stained with SYPRO® Orange Protein Gel Stain (1 ml/50 ml gel; S6650, ThermoFisher) at 90 V for 30 min (FMSMINIDUO, Fisher Scientific). The 1 kb

GeneRuler (SM0313; ThermoFisher) was used as a size marker. Transilluminator BioDoc-It™ 220 Imaging System (UVP) was used to capture the images of DNA agarose gels.

Table 6: Master Mix for one PCR reaction

Reagent Name	Volume (μl)
10X DreamTaq Buffer™	2
dNTP	2
Primer Forward (5 μM)	1
Primer Reverse (5 μM)	1
Mili-Q	11.8
Taq Polymerase	0.2

Table 7: PCR amplification program

Step	Temperature (° C)	Time	
Initial Denaturation	94	5 min	} 34 x
Denaturation	94	15 s	
Annealing	60	15 s	
Elongation	72	20-120 s	
Final Elongation	72	10 min	
Rest/Cooling	4	∞	

4 Results

4.1 Growth phenotypes

The first part of my thesis was to examine the pleiotropic effect of silencing of *DMR6* gene. A number of developmental traits were recorded: plant height, stem width, number of flowers and tubers, tuber setting, side shoot formation, senescence, and leaf colour.

Neither Desirée nor DM1-3516 R44 *DMR6* RNAi silenced lines showed any significant ($p < 0.05$) growth and stem width impairments compared to non-silenced lines throughout five weeks. The results presented below (**Fig. 5**) were obtained by joining all three experiments together. For DM1-3516 R44 lines, plant structure in the climate chamber appeared to be more condensed and resulted in wider stems and shorter internodes,

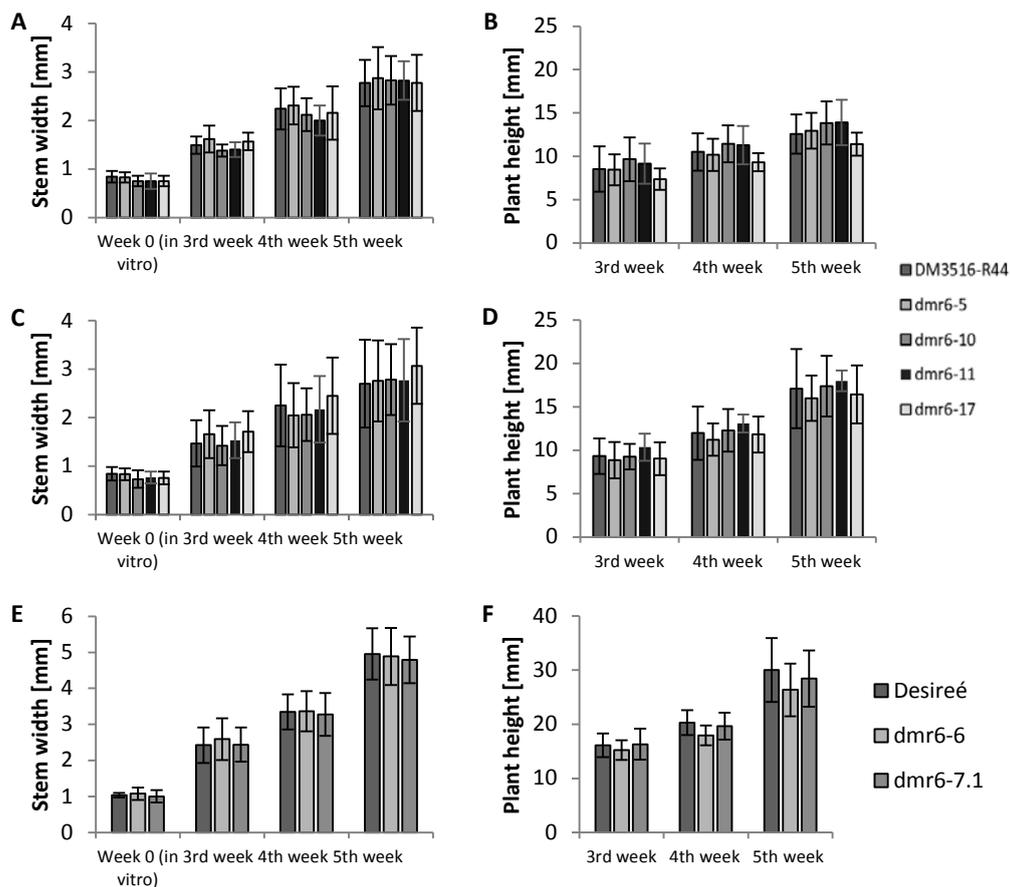


Figure 5: Measurement of stem width and height on all potato genotypes. DM1-3516 R44 lines were screened under controlled climate (A, B) and greenhouse (C, D) conditions, and Desirée *DMR6* lines under greenhouse conditions (E, F). Data are presented as mean trait size \pm SD from three different experimental repeats. Student's t-test ($p < 0.05$). DM1-3516 R44 lines (N=12), Desirée lines (N=20).

while the greenhouse-grown plants ended up growing slightly taller with thinner stems. Eight-week development of all genotypes was visually recorded (**Appendices, Fig. 15, 16, 17**). Desirée lines performed much better than DM1-3516 R44 lines and appeared to be healthier, despite trips and aphid infestation in the greenhouse. Starting from the fifth week, DM1-3516 R44 lines, including wild type, displayed wilting/senescing of the lower leaves (**Appendices, Fig. 18**) and started growing auxiliary shoots that resembled stolons. Leaf discolorations were not present on any of the plants, except on wilting/senescing leaves, and side shoots appeared to be more present on transgenic lines than the wild type (**Fig. 6**). On the other hand, Desirée and *DMR6* RNAi silenced lines showed no visible impairments or unusual growths. Noteworthy to mention, all transformed plants survived and developed new leaves up to the 12th week.

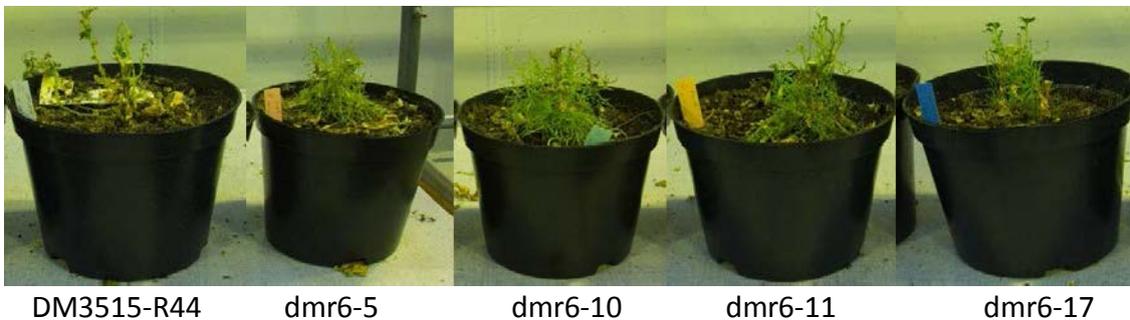


Figure 6: Formation of auxiliary shoots on DM1-3516 R44 lines. Wild type displayed less side shoots than silenced lines.

In order to determine whether the flower production was affected by the silencing of *DMR6*, two scorings were performed. Desirée lines were scored for flowering in the 9th week, while the scoring of DM1-3516 R44 lines was performed in a climate chamber on the 11th week. In average, DM1-3516 R44 lines produced more than 20 flowers per plant, and Desirée lines never produced more than 10 flowers per plant (**Fig. 7**). However, both DM1-3516 R44 and Desirée *DMR6* RNAi silenced lines showed an increased production of flowers. Lines dmr6-5, dmr6-10, dmr6-11, and dmr6-6 had a significantly higher ($p < 0.05$) production of flowers than corresponding wild types. The results presented below (**Fig. 7**) were obtained by joining two separate experiments together for climate chamber grown DM1-3516 R44 lines (N=8), whereas the counting of greenhouse grown Desirée lines (N=12) was performed only once. Flowering in the climate chamber was delayed for two weeks, therefore, screening in the greenhouse was done on the 9th week and in the climate chamber on the 11th week. Flowers appeared to be uniform in all silenced lines compared to their corresponding wild types.

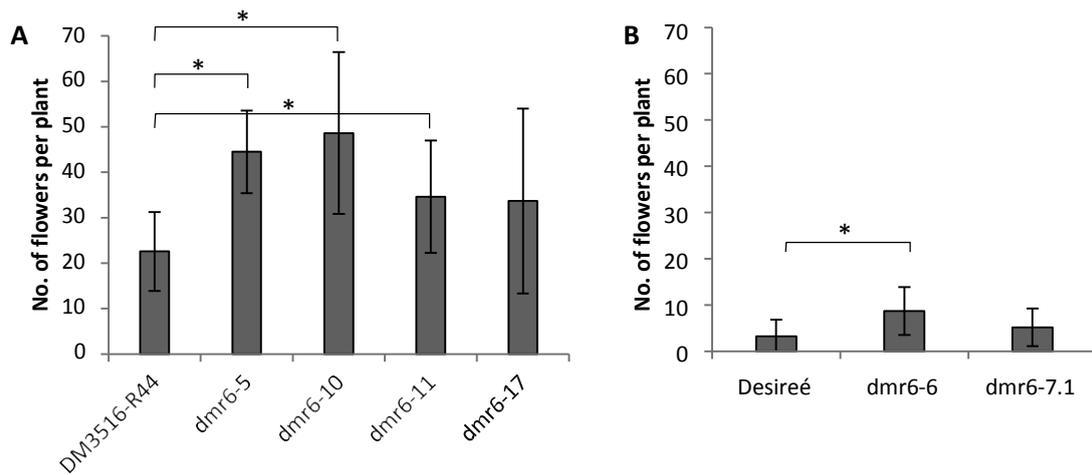


Figure 8: Flower production on all potato genotypes. 11-week-old climate chamber grown DM1-3516 R44 lines (A) and on 9-week-old greenhouse Desiree lines (B). Results are presented as mean \pm SD. Lines represent pairwise comparison with Student's t-test ($p < 0.05$). DM1-3516 R44 lines (N=8), Desiree lines (N=12).

Above ground tuber growth in the *DMR6* silenced lines was already reported in our group (data not published). For that reason, tuber setting was followed to confirm this occurrence. *In vitro* potato cultures of all genotypes were kept in *in vitro* climate chambers for approximately 5-7 months - all produced microtubers high above media (**Appendices, Fig. 14**). Tubers penetrating the soil or that set above ground in 12-week-old plants were observed on DM1-3516 R44 RNAi silenced lines as well as wild type plants (**Fig. 8 A, Appendices, Fig. 19**), whereas Desiree nor Desiree-*DMR6*-RNAi lines did not display this (**Fig. 8 B**). The phenotype in DM1-3516 R44 lines was more apparent under climate chamber conditions compared to greenhouse conditions. Moreover, in more than one case DM3515-R44 plants did not produce any tubers both under climate chamber and

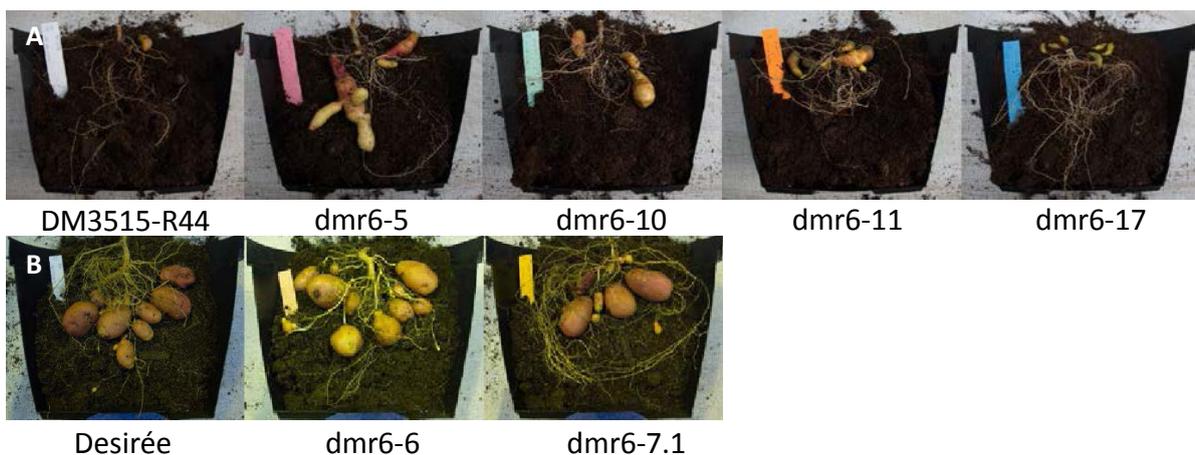


Figure 7: Tuber setting and production on *DMR6* RNAi silenced lines. Representative pictures of potato profiles of 12-week old (A) Desiree and (B) DM1-3516 R44 lines under greenhouse conditions.

greenhouse conditions, while all the climate chamber grown DM3515-R44 lines rarely produced tubers bigger than 1 cm. Therefore, only greenhouse evaluation results are presented above in the **Figure 8**. Shapes of tubers from Desirée lines resembled wild type, and were round shaped and pink-coloured, while DM3515-R44 genotypes produced tubers with irregular shapes, containing a lot of lateral buds (eyes).

A similar tendency as in flower production was observed for tuber production. Some Desirée and DM1-3516 R44 *DMR6* RNAi silenced lines seemed to produce more tubers and had a greater yield per plant compared to their respective wild types (**Fig. 9 A,B,C,D**). The average number of tubers per plant was significantly higher only in line dmr6-7.1 ($p < 0.05$), while dmr6-5 and dmr6-6 had a higher ($p < 0.05$) production of tuber biomass per plant. The data was obtained by joining two separate experiments together for DM1-3516 R44 lines ($N=8$). Again, Desirée lines were analyzed only once ($N=12$).

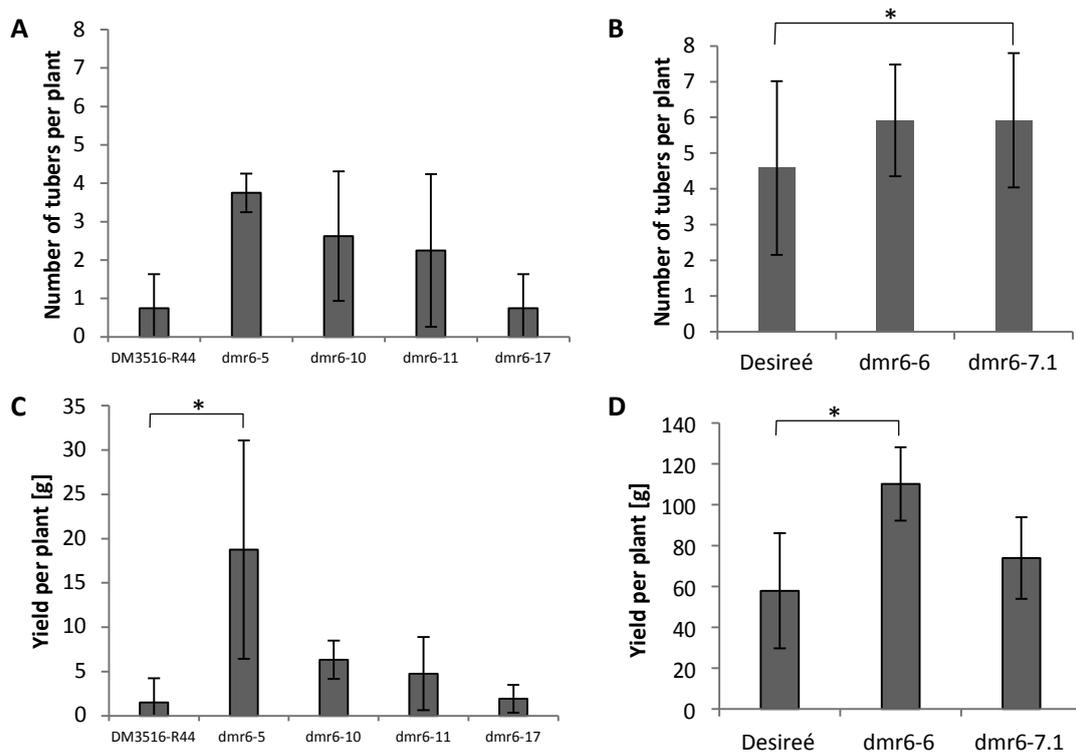


Figure 9: Tuber production on all potato genotypes. Bar charts show number of tubers produced per plant (**A, B**) and yield per plant (**C, D**) for both Desirée and DM1-3516 R44 lines. Results are expressed as mean trait value \pm SD. Asterisks represent significance with a two-way Student's test compared to wild type ($p < 0.05$). DM1-3516 R44 lines ($N=8$), Desirée lines ($N=12$).

Overall, both Desirée and DM1-3516 R44 RNAi silenced lines did not appear to be negatively affected by silencing our gene of interest. On the contrary, some lines showed higher production of flowers, and both Desirée and DM1-3516 R44 RNAi silenced lines produced a higher tuber biomass.

4.2 *Dickeya solani* greenhouse infection assay

To quantify the level of resistance of *DMR6* RNAi silenced lines, a bacterial solution of *D. solani* was applied at the base of the stem on 5-week-old greenhouse-grown plants (**Fig. 10**).



Figure 10: Representative pictures of greenhouse-based *Dickeya solani* infection assay. (A) Stem inoculation was done approximately 5 cm above the slim stem region (approx. 10-15 cm above the soil). (B) Plants were put on inverted plant pots in order to flood the bench with water to maintain humidity levels. (C) To retain humidity (RH 60-80%) and temperature (24-28°C) sufficiently high, transparent plastic foil was put over the plants on a prebuilt frame to create a climate chamber-like environment.

Seven days after infection, developed symptoms on the plants were distinct. Both wild type and *DMR6* RNAi silenced lines showed clear signs of infection on the surface as well inside the vascular tissue (**Fig. 11**). Black lesions were observed on the surface, and vascular tissue appeared damaged with signs of maceration of black and brown colour, which expanded up- and down-wards. The damage was substantially bigger on the vascular system compared to the surface of the stem. Control plants showed only a small brown wound spot (**Fig. 11 D, E**). Additionally, another negative control was used where only puncturing without water was done, which showed the same results.

In total, three separate experiments with 6, 9 and 9 infected lines per genotype, respectively, were performed. A two-way ANOVA was applied to test all three experiments combined for each genotype. The applied model revealed a significant difference ($p < 0.05$) for vascular lesions in the *dmr6-6* *DMR6* RNAi silenced line compared



Figure 11: Blackleg symptoms on surface and within the plant 7 DPI. (A, B, C) Pictures represent surface (left) and vascular lesions (right) for Desirée, dmr6-6 and dmr6-7.1, respectively, (D), puncturing with a sterile tap water, and (E) puncturing only with a syringe needle.

to Desirée (Table 8). Contrary to dmr6-6, line dmr6-7.1 did not show any differences. Moreover, it can be clearly seen from **Table 8** that dmr6-6 exhibited lower infection rates in all three experiments, for vascular measurements. Although the combined data shows lower surface infection size in dmr6-6, no significant differences were observed. No differences comparing RNAi silenced lines to Desirée were observed when the two-way ANOVA was applied for surface lesions in both RNAi silenced lines.

Table 8: Scoring results of *Dickeya solani* infection for three separate experiments. They are presented as mean lesion size values \pm SD for each experiment separately. Asterisk represents a significant difference according to ANOVA test ($p < 0.05$).

	Lesion size (in mm)			
	I. Experiment (N=6)	II. Experiment (N=9)	III. Experiment (N=9)	Combined
Surface lesion				
Desirée	0.80 \pm 0.47	2.65 \pm 0.99	2.51 \pm 1.76	2.13 \pm 1.39
dmr6-6	0.60 \pm 0.42	2.33 \pm 0.78	2.66 \pm 0.47	2.02 \pm 0.64
dmr6-7.1	0.43 \pm 0.15	2.67 \pm 0.66	2.40 \pm 1.13	2.15 \pm 0.91
Vascular lesion				
Desirée	3.18 \pm 1.73	5.92 \pm 3.10	4.73 \pm 1.91	4.79 \pm 2.53
dmr6-6	1.04 \pm 0.65	5.52 \pm 2.05	3.32 \pm 0.61	3.58 \pm 2.21*
dmr6-7.1	0.38 \pm 0.17	6.77 \pm 3.71	3.92 \pm 1.91	4.44 \pm 3.50

The biggest difference was observed on sagittal sections as these lesions were much bigger, more distinctive and easier to measure. All infected plants survived, nonetheless, wilting of the leaves close to infection site was observed (**Fig. 10 A**). All plants also produced new leaves at the end of the experiment.

4.3 Gene expression analysis

To investigate the expression levels of defense-related gene *PR-1* in Desirée DMR6 RNAi silenced lines, a quantitative PCR analysis was performed. At first, the expression of *EF1 α* was analysed, since this gene serves as a standard housekeeping gene. The primers had good efficiency and little variation in the *EF1 α* expression was detected.

The expression of *PR-1*, often used as a salicylic acid responsive gene, was analysed. *EF1 α* was used as a reference gene and for normalization of the *PR-1* expression values. Only dmr6-6, showed 5-fold expression levels compared to Desirée, while dmr6-7.1 showed only a slight increase. These results suggest that RNAi silencing was not effective in line dmr6-7.1.

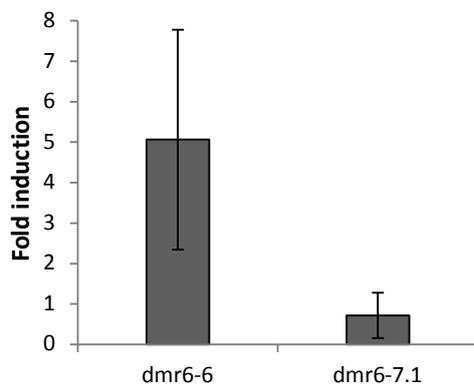


Figure 12: Relative transcript levels of *PR-1* in *DMR6* RNAi silenced lines dmr6-6 and dmr6-7.1. Expression levels were normalised to the reference gene *EF1 α* .

A preliminary test showed no differences in gene expression between the three Desirée lines, with all Ct values below 25 for the plant defense associated genes *PR-5*, HRmarker, ABA marker, *ACC* marker, *Lox3*. Therefore, a more thorough analysis of these genes was not followed.

4.4 PCR analysis of RNA interference insert

To investigate the insert in dmr6-6 and dmr6-7.1, DNA was extracted from the three Desirée lines. The integrity of the extracted DNA was tested by running it on a 1% agarose gel (**Fig. 13 A**). Additionally to the gel, *EF1 α* primer set was used to confirm integrity by PCR (**Fig. 13 B**). Both tests showed that extracted genomic DNA was intact.

Different primer sets were then used to investigate the presence of T-DNA in dmr6-6 and dmr6-7.1. NosT primers were used to confirm the presence of the nopaline synthase terminator located close to the left border – only dmr6-6 showed a band (**Fig. 13 C**). Similar results were obtained when using the primers CM R1 and int R3 located in the middle of the T-DNA (**Fig. 13 D**). When 35S Prom 3'F2 and 35S Term 5'R primers were used to check the integrity of a whole insert, only fragments of considerable smaller sizes than the expected 2339 bp were visible on the gel after PCR amplification (**Fig. 13 E**).

Despite genomic DNA being intact and previous information that plant lines have been silenced, only line dmr6-6 seems to have the parts of *DMR6* silencing construct, while this cannot be confirmed on dmr6-7.1.

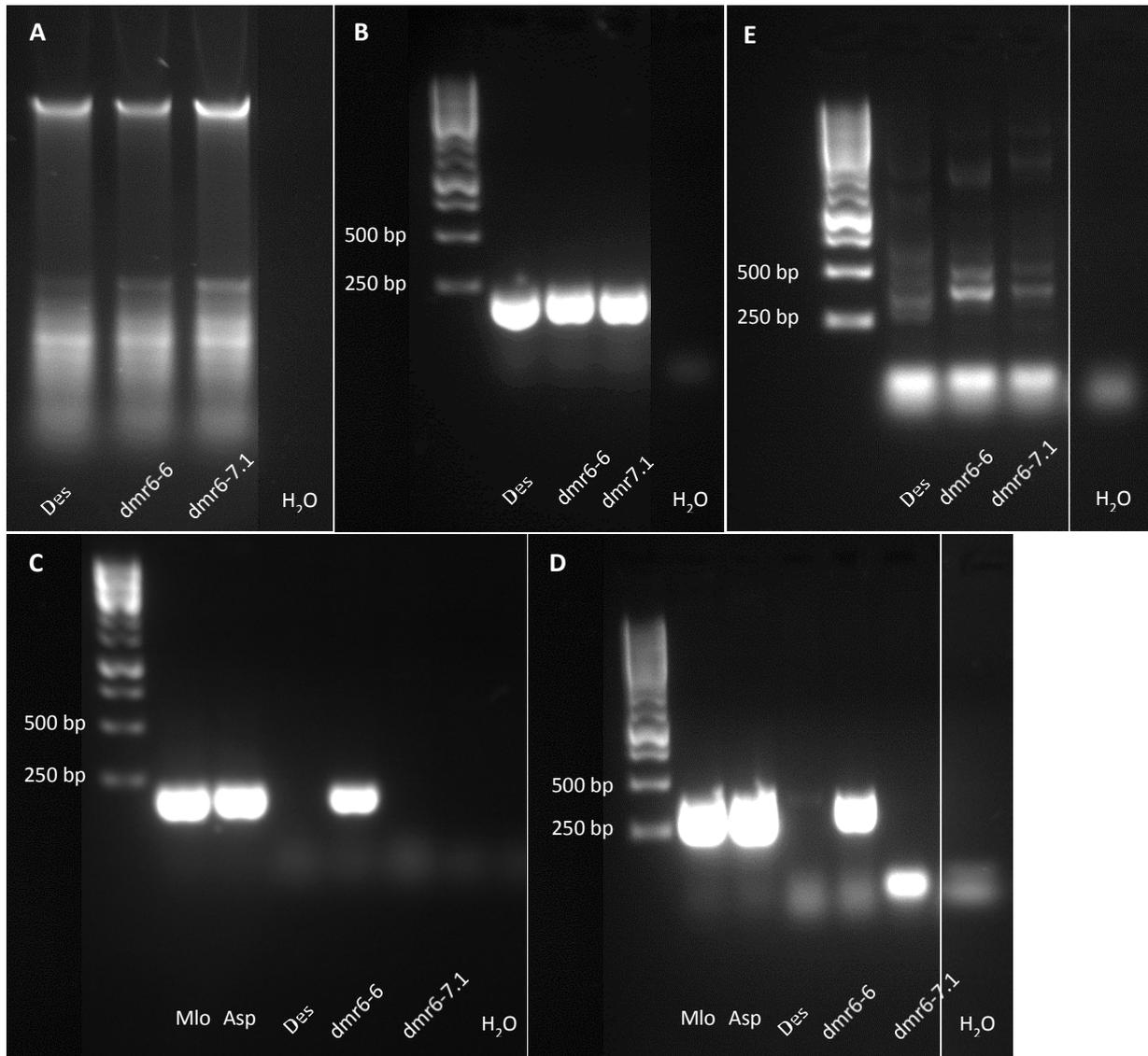


Figure 13: Agarose gel electrophoresis of PCR products to test the presence of the *DMR6* RNAi construct in *Desirée* silenced lines. (A) Integrity of genomic DNA was tested directly on 1% agarose gel. (B) Integrity was also checked with by amplifying 219 bp sequence of *EF1α* gene. (C) NosT primers were checked for nopal synthase terminator gene close to the left border (223bp). (D) Primers Cm R1 and int R3 were used to check the construct in between RNAi repeats (396 bp). (E) Primers 35S Prom 3'F2 and 35S Term 5'R were used to test the completeness of the construct of 2339 bp in length. pK7GWIWG2(II) containing two different RNAi constructs (MLO or Asp) were used as positive controls in C and D.

5 Discussion

The aim of the study was to investigate 1) if *DMR6* homologues in potato are a susceptibility factor towards *D. solani*, 2) if silencing of *DMR6* leads to developmental impairments in potato, and 3) if the *DMR6* gene is involved in regulation of *PR-1* in potato.

5.1 Growth phenotypes

Mutating susceptibility genes may have unwanted and detrimental consequences for a plant. Often these genes carry out functions other than being compatibility factors such as the control of other metabolic and physiological functions (van Schie and Takken, 2014). Therefore, a plant with a potential silenced *S*-gene must be first tested for pleiotropic effects before it can be considered for practical use. This can be achieved either by analysing plants that have been modified either by knocking out the gene of interest using genome editing (Xie and Yang, 2013) and other mutation techniques, or by silencing it with techniques such as RNAi silencing (Waterhouse and Helliwell, 2003). The latter method was used in our study. Development of several important morphological traits was followed and the RNAi silenced lines appeared to be largely unaffected. The evidence presented here, supports what has already been shown by Sun *et al.* (2016). Their RNAi silenced potato lines did not show signs of dwarfing, autonecrosis and chlorosis in greenhouse, however, they did not investigate tuber setting and flowering. Likewise, *DMR6* impaired *Arabidopsis thaliana* (van Damme *et al.*, 2005, 2008) and tomato (Thomazella *et al.*, 2016) showed no detrimental effects on development in greenhouse.

A higher production of flowers and tubers was observed in some RNAi silenced potato lines. These findings might serve as an advantage when considering applicability of *DMR6* for future crop resistance improvement programs. Frequently crop improvement scientists run into a bottleneck as their main goal is to maximize growth-related traits, but the consequences often end up compromising plant's defense mechanisms. Hindering plant hormone levels has been implicated to have an intrinsic role in growth-defense processes. This phenomenon also known as 'growth-defense trade-off' was described in the review by Huot *et al.* in 2014. Salicylic acid has been shown to be involved in many developmental and defense processes, including senescence and cell growth (An and Mou,

2011; Huot *et al.*, 2014). Hence, disrupting the balance of important hormones, a trade-off could result in low-yielding plants. *DMR6*'s speculated involvement in down-regulation of salicylic acid responses (Zeilmaker *et al.* 2015), is supported by our data.

Above ground tuber setting was observed on soil-grown DM1-3516 R44 lines, and was present on all *in vitro* grown potato genotypes. First above ground tuber setting reports date back to 1950s. In an extensive report about tuberization physiology by Okazawa (1967), suggestions like blockage of downward movement of nutrients due to girdling or injury by some fungus can be found. Later reports also suggested that production of aerial tubers is associated with stress, injury or disease on a plant (Ewing, 1977; Percival and Dixon, 1995). Since the phenotype occurred in wild type plants as well, one can assume that this off-type phenotype was not a result of *DMR6* RNAi silencing, but rather a result of suboptimal environmental conditions or due to some form of stress. Plants upon the transferring to the soil were supported by tying them to a stick with a garden wire – it is possible that the wire slightly disrupted the transfer of nutrients, which stimulated the above ground tuber formation. However, aerial tubers under *in vitro* conditions might have been a result of many factors. Plants being subjected to environmental stress might have also resulted in early senescing of lower leaves on DM1-3516 R44 lines, both under climate chamber and greenhouse conditions. Senescence is an adaptive strategy present in all living organisms, and is usually stress- or age-related (Schippers *et al.*, 2015). As already mentioned, salicylic acid is known senescing factor, whose levels are disrupted by RNAi silencing of *DMR6* (Schippers *et al.*, 2015). However, wild type DM1-3516 R44 plants exhibited the same senescing of lower leaves, which thus can again be a result of stress and not RNAi silencing. Another possible explanation for tuber setting and early senescing leaves might be the weak nature of these diploid (doubled-monoploid) potato plants.

By confirming that silencing of *DMR6*, supposedly, does not cause any developmental impairments to the plant and might even positively affect the tuber production, the utilization of this gene in breeding programs could be possible. However, separate experiments varied between one another to some degree. Ultimately, field experiments should be performed to investigate how these plants react under conditions relevant to agricultural practices.

5.2 *Dickeya* resistance

Over the years, a number of publications have demonstrated new methods to assess resistance to bacterial stem rot on plants under either ‘controlled’ greenhouse or field conditions (Lojkowska and Kelman, 1989; Allefs *et al.*, 1996; Zimnoch-Guzowska *et al.*, 1999; Burra *et al.*, 2015; El-Hendawy and Abo Elyousr, 2016). Finding an efficient method to ensure optimal conditions needed for development of disease of interest often requires a lot of attention and optimization. Well-established infection assays can improve the producibility, and lower the costs of prebreeding programs. In this study, an optimization of greenhouse-based infection assay was executed to test the performance of *DMR6* RNAi silenced lines.

Several studies have demonstrated that the mutation of *DMR6* has resulted in either partial or complete resistance towards several hemi- or biotrophic pathogens (van Damme *et al.* 2005, 2008; Zeilmaker *et al.*, 2015; Sun *et al.*, 2016; Thomazella *et al.*, 2016). In this study, I tested if the broad-spectrum-like resistance can be extended to necrotrophic pathogens, such as *D. solani*. Only the line dmr6-6 gave promising results as it repeatedly showed significantly lower infection spread on the vascular tissue compared to moderately resistant potato cultivar Desirée (reported in Burra *et al.*, 2015). This line also showed elevated PR1 levels.

Two studies have showed that the silencing of *DMR6* leads to increased SA-related gene expression levels in *A. thaliana* (van Damme *et al.* 2008; Zeilmaker *et al.*, 2015). A crosstalk between two defense-related phytohormones SA and JA has been well documented in the past 10 years. It has been shown more than once, that SA and JA act antagonistically, and their fine-tuned interaction is needed for defense against a pathogen (Spoel *et al.*, 2003; Beckers and Spoel, 2006; Thaler *et al.*, 2012). According to El Rahman *et al.* (2012), biotrophic *Pseudomonas syringae* induces SA-mediated defense which leads to more susceptible plants towards necrotrophic pathogen *Alternaria brassicicola* due to suppression of the JA-signalling pathway. JA plays an important role in defense against necrotrophic organisms (Thomma *et al.*, 2001). If SA levels are increased in *DMR6* RNAi silenced lines, one could therefore assume that JA related gene expression would be down-regulated. Hence, RNAi silencing of *DMR6* would lead to plants that are more susceptible to necrotrophic organisms. If that is the case, it is reasonable to speculate that

silencing of *DMR6* would not render the plants with lowered susceptibility against necrotrophic pathogens. Nonetheless, the role of *DMR6* is still not fully understood, and data now suggest that SA is also important for the necrotroph *D. solani* (Burra *et al.*, 2015). Additionally, a study showed that SA application on tomato plants resulted in increased resistance to necrotrophic *Alternaria solani* (Spletzer and Enyedi, 1999). Davidsson *et al.* (2013) further confirmed that the activation of innate immunity against soft rot *Pectobacterium* requires both SA- and JA-mediated defences. Indicating, that SA plays a more important role in necrotroph defence than previously thought.

In this study, an improved greenhouse-based infection assay and a new way to measure blackleg infection were introduced. Wrapping of each plant with a plastic bag, as described by Burra *et al.* (2015), is rather laborious and damaging for a plant. Instead, a plastic tunnel to keep high relative humidity up was used. This rendered green but symptomatic plants at the end of each experiment. As *D. solani* spreads through the vascular system, measuring of vascular lesions was tested. It gave statistically clearer results in this investigation, which could possibly lower the amount of required repetitions of experiments if a higher number of plants was to be used. Nevertheless, a combination of both types of measurements is still recommendable.

Still, greenhouse-based experiments are time- (e.g. 9 weeks for one experiment used in this study) and space-consuming. Ensuring constant environmental conditions proved to be rather hard, which is why the infection results varied. It has commonly been assumed (Franklin *et al.*, 2014) that expression of many genes is critically dependent on environmental cues. For example, Zhu *et al.* (2010) published a study where they describe that NB-LRR type of *R*-gene proteins might be responsible for temperature sensitivity. Moreover, aggressiveness of *D. solani* is also dependent on temperature and humidity levels (Golanowska, 2016). For this reason, constant and repeatable environmental conditions should be a priority when establishing an optimal infection assay. A possible solution to provide sterile and more stable environment could be of the use of an *in vitro* assay. Moreover, a possibility to test more plants in a shorter time could provide more consistent results with higher statistical power. In our group, Burra *et al.* (2015) developed such an *in vitro*-based disease screening assay, especially adapted to blackleg causing *D. solani*. The use of an *in vitro* assay could also serve as a better alternative for

diploid DM1-3516 R44 lines due to their observed phenotype, which made stem inoculations problematic.

Similarly, as for the growth phenotype analysis, infection assays should also be carried out in the field. According to Allefs *et al.* (1996), field experiments tend to be more reliable when evaluating the resistance of potato lines against blackleg and soft rot. In our study, only stem base resistance was tested. In the future, it would be interesting to test soft rot resistance against *D. solani* and correlate it with blackleg resistance. It is known that blackleg resistance is not necessarily correlated with soft rot resistance (Czajkowski, 2011). Accordingly, field trials will be carried out this year to acquire a large number of potatoes, which will be used for soft rot infections.

5.3 Gene expression and DNA analyses

Many well-characterized enhanced disease susceptibility mutants are associated with SA and JA/ethylene signal transduction pathways (van Schie and Takken, 2014). Moreover, mutant phenotypes are often associated with cell death and/or the constitutive expression of downstream response genes such as *PR-1* (Eckardt N., 2002). Similarly, *DMR6* has been implicated to have a role in regulating the expression levels of salicylic acid (Zeilmaker *et al.*, 2015). As noted in the results section, *PR-1* expression showed 5-fold increase in *dmr6-6* compared to wild type Desirée (**Fig. 12**), which is similar to what van Damme *et al.* (2008) reported in *A. thaliana*. However, *dmr6-7.1* lines showed no difference.

Moreover, a limited amount of biological replicates was tested in a preliminary experiment to check the expression levels of five defense-related genes. They seemed to be unaffected in both lines.

It is reasonable to assume that RNAi silencing construct in line *dmr6-7.1* has been either lost, fragmented, or that the silencing was not efficient. It seems that silencing in *dmr6-6* did work, especially as the RNA expression and the greenhouse infection assay results show some promising outcomes. Regardless, a subsequent study to test the presence of the *DMR6* silencing construct was carried out. Primer sets, which were used for PCR amplification of specific regions of the construct, suggested that the presence of construct is only in *dmr6-6*. Per contra, *dmr6-7.1* did not show corresponding bands on the gel after

PCR amplification which led us to conclude that at least some of the insertion (T-DNA) of the silencing construct was incomplete or unsuccessful. It is important to keep in mind that PCR methods might give unreliable results due to methylation for example (Waterhouse and Helliwell, 2002), therefore, sequencing could be employed to give more conclusive information about the presence of the insert.

Technologies that result in knocking out of the gene of interest should be considered in the future. For example, Thomazella *et al.* (2016) used CRISPR-Cas9 technology to inactivate *DMR6* gene in tomato, resulting in mutants displaying resistance against several different pathogens, including *Pseudomonas syringae*, *Phytophthora capsici* and *Xantomonas* spp.

6 Conclusion and future prospects

In the future, it is not unlikely that the severity and occurrence of *D. solani* outbreaks will increase, due to more favourable temperatures as a result of global warming. Therefore, it is important to stem the tide before the potential outbreaks arise.

Here, I presented phenotypic evaluation of *DMR6* RNAi silenced lines with DM1-3516 R44 and Desirée backgrounds which did not show any developmental impairments. Furthermore, I showed that silenced Desirée line dmr6-6 shows high *PR1* expression levels and a tendency to have lower infection sizes against *D. solani* compared to moderately resistant Desirée.

In the future, more confirmed *DMR6* RNAi silenced lines should be used to make a full conclusion, including field trials. In addition, a more cautious expression analysis of targeted silenced gene should be conducted. In advent of new genome editing technologies, employment of methods such as CRISPR-Cas9 to deliver targeted mutations at the site might be more reliable. Still, current results indicate that *DMR6* susceptibility gene could be a useful target not only for biotrophic and hemibiotrophic pathogen resistance but also for necrotrophic pathogens.

DM1-3516 R44 lines proved to be susceptible to biotic as well as abiotic stresses which made the investigations somewhat troublesome. The use of high performing diploid potatoes could be advantageous in many different ways as it could enable us to more effectively and quickly introduce new characteristics using traditional or novel breeding

techniques. Moreover, avoiding tedious traditional practices in potato breeding that require a multiplication of often contaminated tetraploid seed-tubers and replacing them with true seeds could start a new chapter for, already in a lot of countries, staple crop potato. Therefore, further efforts should be directed towards diploid potato research and development.

All in all, this study shows promising results for applicability of *DMR6* susceptibility gene towards necrotrophic pathogens. Moreover, a relatively reliable modified greenhouse-based blackleg infection assay, and a new way to score stem-inoculated plants are presented here.

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Appendices

Table 9: Primers used in this study

Primer name	Sequence (5' to 3')	Used for	Gene
St Efl α F2	GAACTGTCCCTGTTGGTCGT	Expression analysis/ Insert analysis	Elongation factor 1- α 1 (eEF1 α 1)
St Efl α R2	GGGTCATCCTTGGAGTTTGA		
StPR1F	AACACTCTGGTGGCCCTTAC	Expression analysis	Pathogenesis-related protein 1 (AT261410)
StPR1R	AGCACAACCGAGACGTA CTG	Expression analysis	
St LX-3F	ATCCCTCCAAAAAGCCAGT	Expression analysis	Lipoxygenase 3
St LX-3R	GGCTTCATTGTTCCATCGTC	Expression analysis	
StPR5-F	GCATAAGAGATTACGACACC	Expression analysis	Pathogenesis-related protein 5
StPR5-R	TCCACCCAACACTTTAGC	Expression analysis	
StHsr203J_HR_F	CGTCAAAGATGTAGTCGCCG	Expression analysis	Hypersensitive response marker
StHsr203J_HR_R	GGGAGGAAGACGGAGACAAT	Expression analysis	
StGHB20_ABA_F2	CCAGTTCCAATCCACACACA	Expression analysis	ABA receptor 1
StGHB20_ABA_R1	ACCTCCTCGCGTTGTTATTG	Expression analysis	
StOsmotin2_ACC_F	CTGCCCTACACCGTTTG	Expression analysis	Osmotin2 gene
StOsmotin2_ACC_R	CACCAACTCTGACCTCTCTCG	Expression analysis	
StNosT F	GAATCCTGTTGCCGGTCTT	Insert analysis	T-DNA
StNosT R	TGCCGGTTCGATCTAGTAACA	Insert analysis	
StCm R1	GCCGGATAAACTTGTGCTT	Insert analysis	T-DNA
StInt R3	GAATAAGCTGTAAATAACCGAGT	Insert analysis	
35SProm 3'F2	CTTCGCAAGACCCTTCCTCT	Insert analysis	T-DNA
35STerm 5'R	CTGGTGATTTTTGCGGACTCT	Insert analysis	

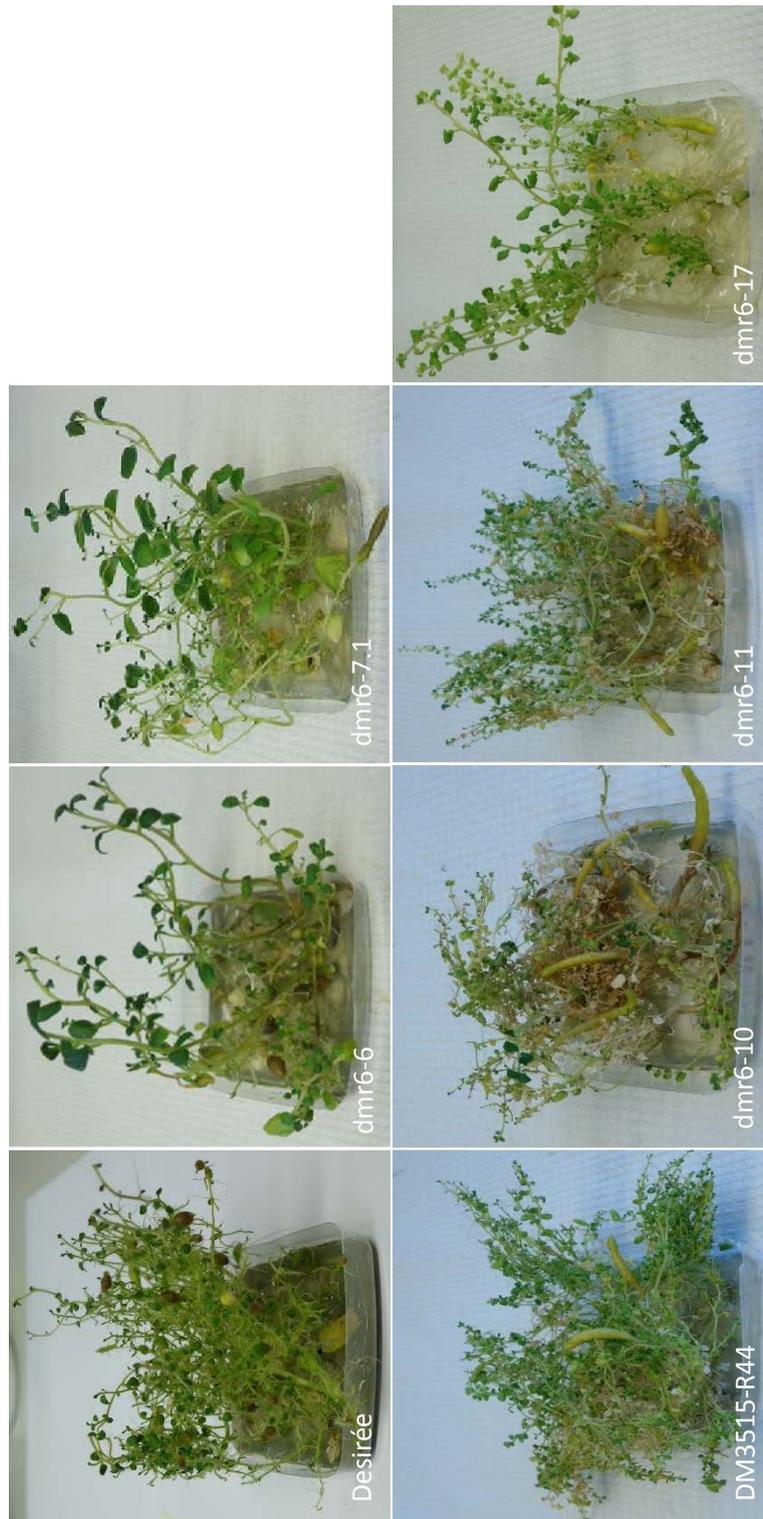


Figure 14: Occurrence of aerial tubers on 5-month-old *in vitro* plants on Desirée and DM1-3516 R44 lines. Dmr6-5 line is missing due to problems with bacterial contamination, however, the same phenotype was observed.

Climate chamber



Figure 15: Eight week plant development progress of climate chamber grown DM1-3516 R44 lines.

Greenhouse



Figure 16: Eight week plant development progress of greenhouse grown DM1-3516 R44 lines.

Greenhouse



Desirée

dmr6-6

dmr6-7.1

Figure 17: Eight week plant development progress of greenhouse grown Desirée lines.



Figure 18: Senescing of lower leaves on 5-week-old DM1-3516 R44 lines.



Figure 19: Side shoot and aerial tuber formation on DM1-3516 R44 lines started on the fifth week.

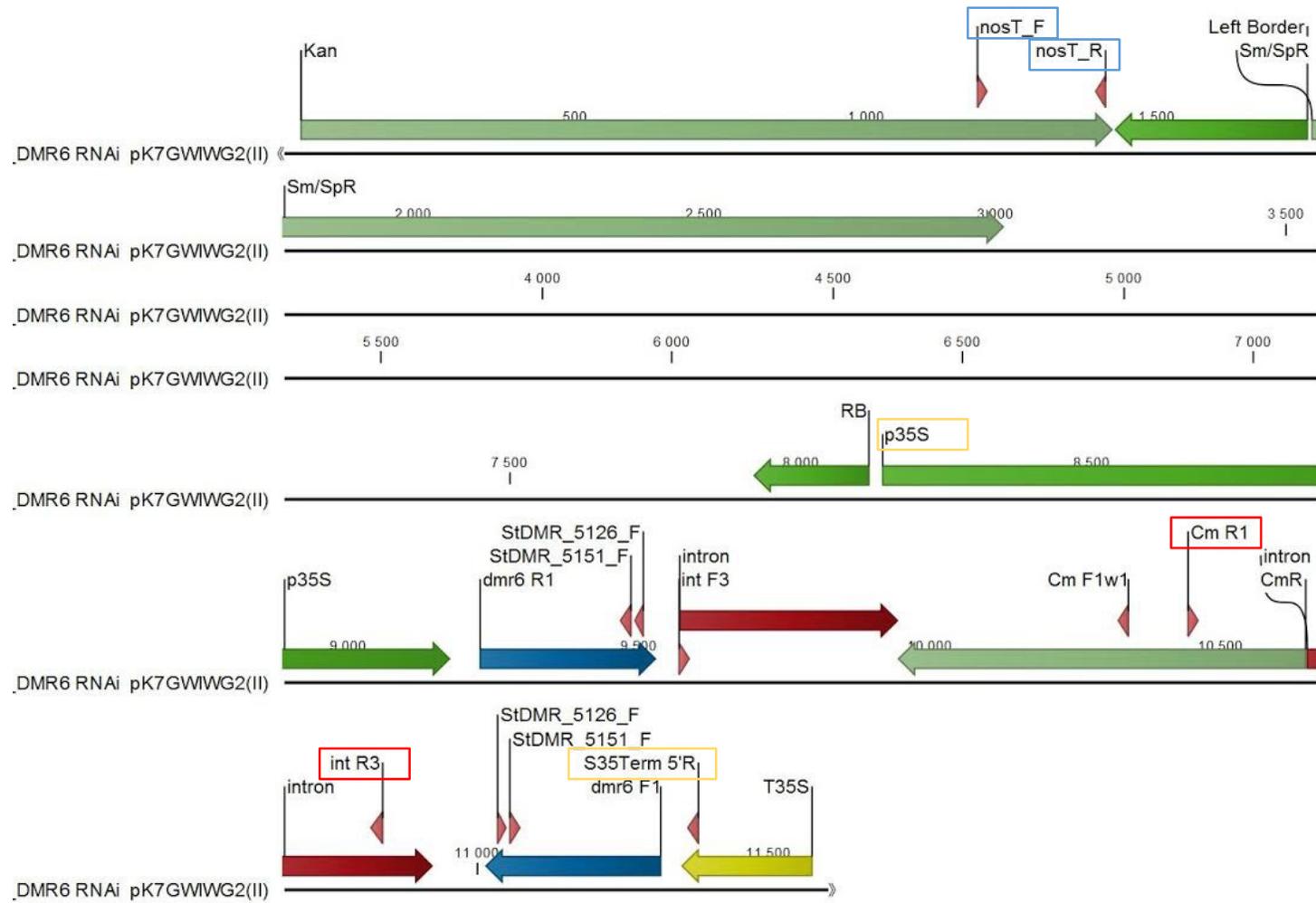


Figure 20: RNAi silencing construct of *DMR6* gene. Squares with a matching colour represent some of the primer sets which were used for testing of the presence of construct.