Susceptibility genes: an additional source for improved resistance

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This thesis is dedicated to my family.
And in memory of Prof. Anne van den Ban.

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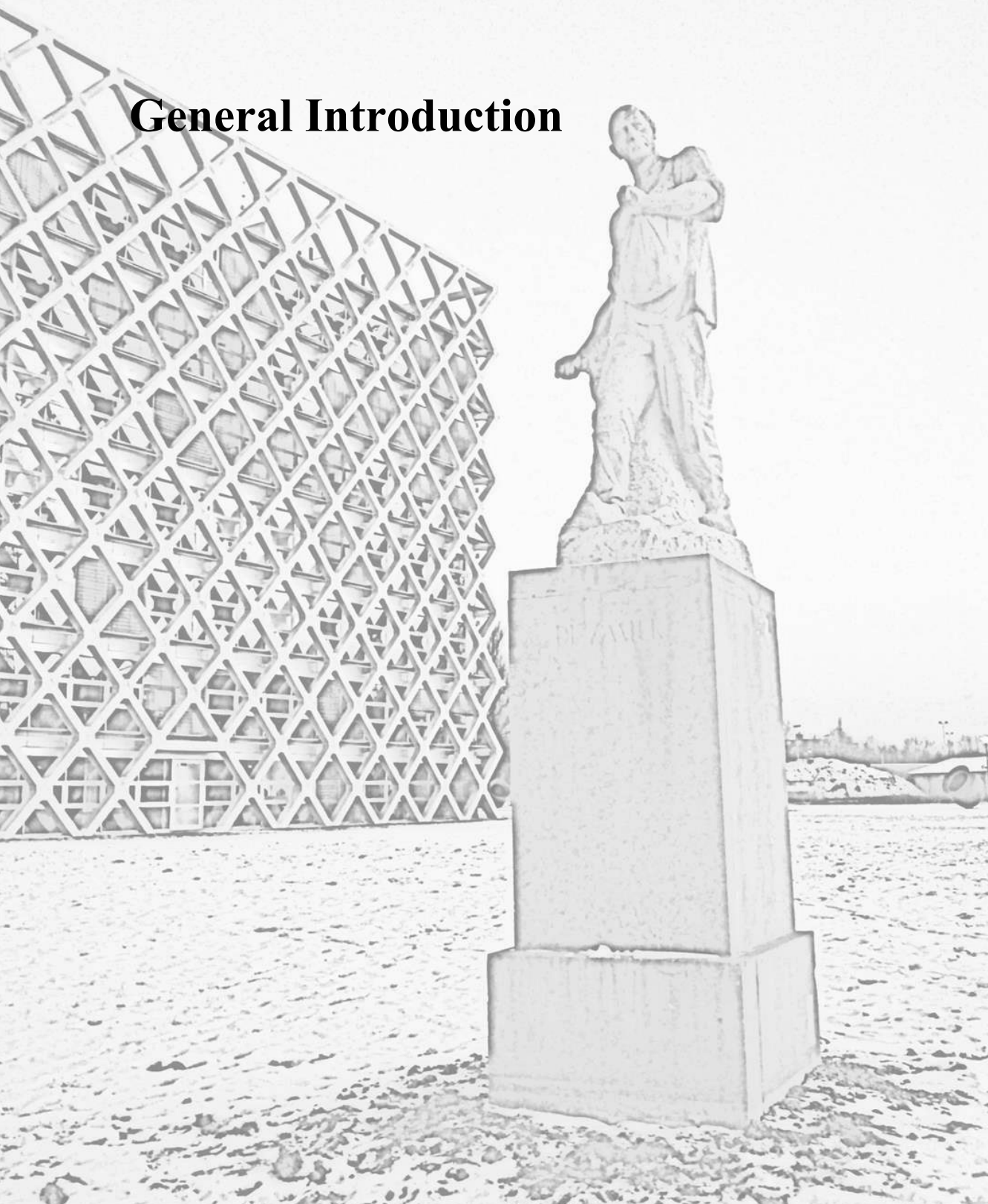
CHAPTER 1

General Introduction



The importance of potato

The potato (*Solanum tuberosum* L.), a tetraploid (2n=4x=48) herbaceous annual, is grown as a tuber producing crop world-wide. Potato is one of the world's favourite crops. It is a member of the nightshade family, like the vegetables tomato, bell pepper and eggplant or the ornamental Petunia. As a food crop, it originated about 8,000 years ago in the Andean mountains of South America and has been grown in Europe since the 16th century (Kiple 2000). Potato varieties have since than been developed and subsequently spread to all parts of the world. There are more than 200 wild species of potato considered to be taxonomically distinct (Huamán 1986), and it is believed that *Solanum tuberosum* was domesticated from *S. stenotomum*, a wild diploid species which hybridized with *S. sparsipilum*, another wild species to form *S. tuberosum* (Grun 1990; Spooner 1990).

Potato is the world's third most important crop after wheat and rice and it is cultivated for food and feed applications (chips, French fries, and table potatoes), but also important for non-food applications as starch supplier for the paper industry and pharmaceutical industry and for bio-ethanol production by the fermentation of waste potatoes (Liimatainen et al. 2004). The latest available information shows that the world-wide average potato production was more than 360 million tonnes in 2014, with 123 million tonnes in Europe and 221 million tonnes in Asia and Oceania (Actualitix: FAO 2014).

In the EU, the five main potato producers (EU-5) are Germany, UK, France, Belgium and the Netherlands with an output of 26 -30 million tonnes ware and seed potatoes, respectively per year (Faulkner 2012). In the Netherlands, an area of about 120.000 ha is under potato cultivation as ware potato and seed potato, and this results in 5.015 million tonnes tubers per year (Faulkner 2012). In addition 50.000 hectares of starch potatoes are grown with 2.5 million tonnes of tubers per year. Faulkner (2012) reported that the potato production by the EU-5 in 2003 and 2005 was around 25 million tonnes, but it reached more than 29.5 million tonnes in 2004 like it was in 2009. The threat of unstable production is real because potato yield is adversely affected by different stress factors in the environment, both biotic and abiotic. The biotic stress factors cause diseases like late blight, early blight, wart disease, virus disease and nematode infections (Rich 2013).

Biotic stress and plant immunity

Not only potato, but all plants have evolved to live in environments where they are often exposed to different stress factors. Generally, biotic stress organisms are

represented by pathogenic oomycetes, bacteria, viruses, fungi, pest insects and weed plants. Each year, biotic stress results in big economic losses within all cash crops, including potato. In the 1840s in potato fields the oomycete *Phytophthora infestans*, causing late blight, resulted in a widespread famine in England, Ireland and Belgium (Bourke 1964; Bourke 1993; Large 1940). Although there are many kinds of biotic stress, the majority of plant diseases are caused by fungi and oomycetes. Based on their lifestyles on plants, phyto-pathogenic microbes can be divided into biotrophs, hemi-biotrophs, and necrotrophs (Fig. 1) (Glazebrook 2005). While biotrophs (e.g. powdery mildew) feed on living cells and actively maintain host cell viability, necrotrophs (e.g. *Botrytis cinerea*) kill host cells first before feeding starts on dead tissue. Hemi-biotrophs (e.g. *Phytophthora infestans*) start with an early biotrophic phase followed later on by a necrotrophic phase (Glazebrook 2005).

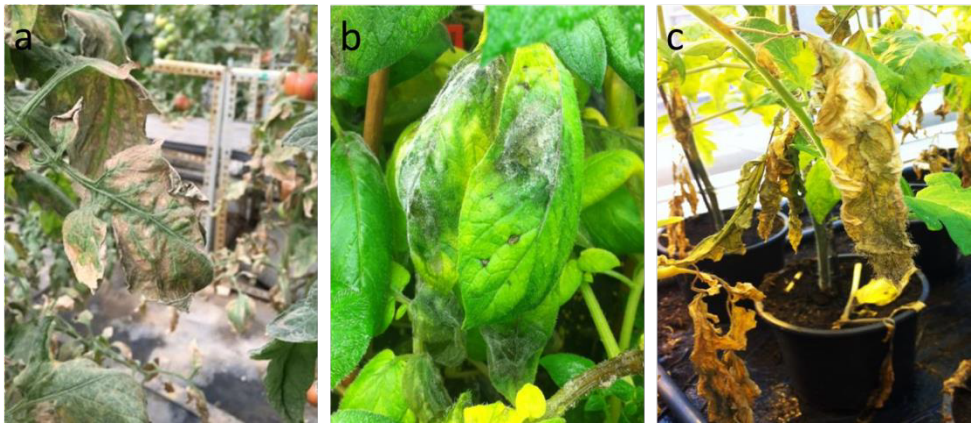


Fig.1. The symptoms of diseases on different plants. **a** Tomato leaves infected by the biotrophic pathogen *Oidium neolycopersici*; **b** Potato leaves infected by the hemi-biotrophic pathogen *Phytophthora infestans*; **c** Tomato leaves infected by the necrotrophic pathogen *Botrytis cinerea*.

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 (defenses) that stop pathogens infecting their living cells, termed PTI (PAMP-triggered immunity) and ETI (Effector-triggered immunity; Jones & Dangl 2006). Recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) activates PAMP-triggered immunity. 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 (Glazebrook, 2005). 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 plant receptors or by secreting effector proteins in the plant cell that manipulate resistance signalling. Jones & Dangl (2006) classified this as effector-triggered susceptibility (ETS).

The ability of pathogens to overcome primary defenses led to the adaption of a specialized mechanism in plants able to detect the pathogen effector proteins (Chisholm et al. 2006). Plant immune receptors (*R* genes) recognize either directly or indirectly pathogen effector proteins (*Avr* genes) 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 general 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). In resistance breeding of potato, the ETI based immunity system with plant encoded major *R* genes and *Avr* genes from the pathogen, has been deployed in the field against *Phytophthora infestans.*

Many plant pathogens can spread rapidly over great distances, vectored by water, wind, insects, and/or humans as indicated by CIMMYT for stem rust and yellow rust infection in wheat (http://rusttracker.cimmyt.org/). Across large regions and many crop species, it is estimated that diseases typically reduce plant yields by 10% every year in more developed countries or agricultural systems, but yield losses to diseases often exceeds 20% in less developed settings. Global crop production reduction by pathogens is estimated to be at least 15% (Dangl et al. 2013).

Potato breeding for late blight resistance

In potato, the hemi-biotrophic oomycete *Phytophthora infestans*, is causing late blight disease with very extensive yield losses up to 70% (Sedláková et al. 2011). Worldwide the financial deficit is estimated to be up to 10 billion Euro (Haverkort et al. 2008) every year. The costs in the Netherlands to protect the crop against late blight have been estimated to be 160 million Euro. This estimation is containing all kind of costs like the fungicides used, labour to spray up to 15 times, land damage during treatments with spraying machines which is influencing tuber yield, etc. (Haverkort et al. 2008).

These two aspects: 1. No or inadequate amounts of fungicides available in many lesser developed countries where late blight is one of the big risk factors, and 2. Extensive use of chemicals in developed countries with all their costs and risks, are the main reasons that a better solution is needed. This was already realised well before the second world-war when resistance research started by searching for sources of resistance. A few pioneers in this field were Dr. Black in Scotland and Dr. Mastenbroek from CEBECO in the Netherlands (Black et al. 1953). This early resistance research delivered plant breeders many *R* genes from the hexaploid wild species *S. demissum* which were introduced by introgression breeding via interspecific hybridisation followed by backcrosses with cultivated potato, into new late blight resistant cultivars. However, the resistance of these new varieties was broken quickly by this pathogen. A first step to stack different *R* genes, in order to avoid breakage, was made possible by the differential set of resistant plants with 11 *R* genes allowing differentiation of virulent and a-virulent isolates (Black et al. 1953). Recently this classical differential set was updated and a new GM set is in development with *R* genes inserted in the background of cv. Desiree (Zhu et al. 2015). In the eighties and nineties of the last century, more and more pressure came from society to reduce the use of pesticides in agriculture and to improve breeding methods to be able to use multiple resistance genes in one variety. Big projects like CBSG (Centre for BioSystems Genomics; http://www.cbsg.nl/; partly focused on quality in tomato and on late blight resistance in potato) and DURPh (Durable Resistance against *Phytophthora infestans*; http://www.wur.nl/en/Expertise-Services/Research-Institutes/plant-research/DuRPh.htm; which focused on cisgenic late blight resistance in potato) are examples in the early 2000s of how the Dutch government was stimulating sustainability by resistance breeding both in a classical way via advanced selection of *R* genes or by using cloned *R* genes which are inserted in a susceptible variety by genetic modification. In the CBSG project, many accessions from *Solanum* wild species have been tested for the presence of resistance to late blight and many different *R* genes have been identified which were localised in the potato genome for the development of marker assisted selection (MAS), which is nowadays applied in larger potato breeding companies. MAS allows functional stacking of different *R* genes efficiently. Vleeshouwers et al. (2011) published "SolRgene", which is an online database to explore disease resistance genes in tuber-bearing *Solanum s*pecies. The first potato varieties with (stacked) *R* genes are close to be released (unpublished data). Part of the mentioned *R* genes have been cloned and are available for genetic modification of existing susceptible varieties. At this moment, more than 20 *R* genes are available for transformation (Haverkort et al. 2016). In the DURPh project these *R* genes have been named "cisgenes" as they are natural genes obtained from the potato plant itself or from crossable wild species. Among the EU consumers, the acceptance level of the cisgenic approach appeared to be much higher than that of the transgenic approach (Gaskell et al. 2011). In Brussels, the regulators are still busy to decide how to handle the cisgenesis technology together with other technologies such as Grafting, Reverse breeding, Intragenesis, Zinc-finger-nuclease technology (ZFN), Oligonucleotide directed mutagenesis (ODM), RNA-dependent DNA methylation (RdDM) and Agro-infiltration (Lusser et al. 2012). Another reason why functional *R* genes are easy to determine is the phenomenon that from most important *R* genes also the

complementing pathogenic *Avr* gene has been isolated (Vleeshouwers et al. 2008). In 2014, Du and Vleeshouwers (2014) described how effectoromics can be used as a powerful tool to identify new *R* genes or to test whether an (introduced) *R* gene is biological active. When both genes, *R and Avr*, are brought together by agro-infiltration a hypersensitivity reaction (HR) is triggered, indicating that the inserted *R* gene in the susceptible variety is biological active. This HR-test can be made in the same plant for multiple *R* genes. Scientifically it can be said that at this moment breeding for sustainable resistance against *P. infestans* is possible in two ways: 1. MAS of one or more *R* genes in offspring of crosses directly leading to a new variety. Such a new variety has to pass all channels in agricultural practise in order to show its value not only in the field but also its quality traits and its behaviour during processing. It is expected that all detected *R* genes will be used and that they will be inserted in new varieties with one or more *R* genes. This brings the risk that all these *R* genes could be broken at a certain moment and that the expected increased durability of varieties with stacked *R* genes will decrease. The most well-known variety with sustainable late blight resistance at this moment is cv Sarpo Mira with at least 5 *R*-genes (Rietman et al. 2012) This cultivar was bred in the past without MAS by the Sárvári family in Eastern Hungary. However, the pedigree is not described in literature; 2. Insertion of multiple cisgenic *R* genes into existing susceptible varieties. This approach of improving existing varieties is very important. There are many susceptible varieties, with expired breeders rights, still on the market, like Bintje, Desiree, Russet Burbank, Atlantic and Spunta, which are very important in potato cultivation. Improvement of such a free variety with stacked *R* genes against late blight can have a big impact on reduction of the use of fungicides in the short run. Also during this approach the same problem with the uncontrolled use of *R* genes could also decrease the highly improved sustainability of resistance. In Belgium the free variety Bintje, which is still very popular in this country, is under investigation in order to come to an improved resistant variety "Bintje +" with multiple *R* genes, containing for example, *Rpi-sto1, Rpi-vnt1* and *Rpi-blb3* (Haesaert et al. 2015). At this moment cisgenic potato varieties are not commercially grown in the EU yet.

Resistance breeding by loss of function of *S* genes

From the above description, it is clear that disease control, except for chemical protection, is normally based on the immune system with plant encoded *R* genes, and pathogen encoded avirulence (Avr; Flor 1971) effector proteins of the RxLR class. As the pathogen populations are under selection pressure for increased virulence, new isolates and/or pathogens appear, and evolving cultivation practices and changing climate can reduce resistance and/or introduce new pathogens (Stuthman et al. 2007). Therefore, breeding for resistance has been an on-going challenge for breeders and additional sources with other mechanisms of resistance than obtained with classical *R* genes would be welcome. Resistance based on recessive genes is already known for a long time in, for example, barley against powdery mildew (*mlo*; Freisleben & Lein 1942; Jørgensen 1992) but also in many crop plants against virus diseases (Truniger & Aranda 2009). Such resistance appeared to be durable in barley already for more than 70 years. It appeared to be based on loss of function of the *Mlo* gene, which nowadays is indicated as *S* (disease-susceptibility) gene. An *S* gene is a plant gene required for

pathogen survival and proliferation (Pavan et al. 2011). The idea behind *S* gene resistance is that under normal conditions the pathogen is dependent on certain plant genes to be successful in the interaction. When one of these genes is not functioning, it will directly negatively influence the whole plant-pathogen interaction. It is, therefore, understandable that viruses, with their low number of genes are highly dependent on their host genes. This indicates that loss of function of several single plant genes would bring virus resistance which is inherited recessively. This recessive resistance is also even found in diploid potato *S. tuberosum subsp. andigena* (Hämäläinen et al. 2000). It is blocking vascular transport of potato virus *A* in the plant. Other examples in potato have not been found by us in literature. However, in *Lycopersicon hirsutum* and *Capsicum annuum* the recessive genes *pot-1* and *pvr*, respectively, control in those plants potato virus *Y* accumulation (Moury et al. 2004).

Research on mutation induction in Arabidopsis showed the occurrence of many examples of recessive genes which provide resistance to one or more diseases. These >180 *S* genes were placed in three different categories by van Schie and Takken (2014) which are acting during different stages of infection:1. Early phase of pathogen establishment; 2. Modulation of host defenses and 3. Pathogen sustenance. The first category comprises 47 genes allowing basic compatibility, facilitating host recognition and penetration. Cellulose is a major structural component of cell walls, and the cellulose synthase-like gene *CSLA9* (*rat4*) is required for susceptibility to *Agrobacterium* infection/transformation. *Agrobacterium* attachment to the root surface of *csla9*/*rat4* mutants is strongly reduced, indicating that the CSLA9 product play an essential role in the stage of recognition of pathogen and host plant (Zhu et al. 2003 a & b). The second category with 87 *S* genes encodes negative regulators of plant immune signalling, such as *EDR1*, suppressor of ethylene response. *EDR1* encodes a kinase with similarity to *CTR1* (Constitutive Triple Response), a negative regulator of the ethylene pathway (Frye & Innes 1998; Tang et al. 2005). The *edr1* mutant was identified to be resistant both to the bacterial pathogen *Pseudomonas syringea* pv. *tomato*, and to the fungus *Erysiphe cichoracearum* causing powdery mildew (Frye & Innes 1998). The third category with 48 *S* genes includes genes involved in pathogen sustenance, fulfilling metabolic or structural needs, and pathogen proliferation. For example, pathogens need nutrients from the host for further growth and propagation. The class of sugar transporters from several plant species, named SWEETs, has been identified showing that fungal and bacterial pathogens induce the expression of such SWEET genes (Antony et al. 2010; Jiang et al. 2013; Römer et al. 2010; Streubel et al. 2013). In our study, 11 *S* genes (chapter 3) were selected for investigation before this categorisation was published. Afterwards they belonged only to the second (*BIK1*, *CESA3*, *CPR5*, *DMR6*, *DND1*, *SR1*, and *PMR4*) and the third (*DMR1*, *PMR5*, and *PMR6*) category according to the review of van Schie and Takken (2014).

In chapter 2 we describe the impact of "Breeding for disease resistance by editing plant susceptibility genes" and will not repeat that here. Here we will indicate, how at the beginning of our project, when gene editing was not established, especially not for tetraploid crops, we proposed to investigate the presence of orthologues of a number of *S* genes from Arabidopsis in potato and that the orthologue with the highest homology would be used for gene silencing via RNAi in potato cv Desiree. From literature no *S*

gene was known which after loss of function would bring resistance to late blight but, for example, *dnd1* in Arabidopsis was able to reduce spore production not only from the bacterium *Pseudomonas syringea,* but also from the hemibiotrophic oomycete *Peronospora parasitica* (Yu et al. 1998) and later also from the necrotrophic fungus *Botrytis cinerea* (Govrin & Levine 2000). These important observations were stimulating us to investigate loss of function of *S* genes in potato by RNAi against *Phytophthora infestans.* What is the expected durability of resistance based on mutated or silenced *S* genes? In theory it can be said that in the *R-Avr* immunity system a simple mutation in the *Avr* gene is sufficient to alter recognition so that the HR reaction does not occur. In the *S* gene based resistance the pathogen has to overcome a dependency on a host factor (van Schie & Takken 2014). This is perhaps more difficult and an important difference between both types of resistance.

Scope of the thesis

The main aims of this thesis were 1) to identify *S* genes in potato that upon impairment by RNAi induce resistance to *P. infestans*; 2) to achieve broad-spectrum resistance to *P. infestans* by silencing *S* genes; 3) to determine, at cellular level, the primary resistance mechanism in *S* gene silenced potato (and tomato) plants to *P. infestans* and *Botrytis cinerea.*

Chapter 2 provides a comprehensive overview on how to impair plant *S* genes, complementary to the introgression of *R* genes, to achieve more durable and broad-spectrum resistance. In this review, *Mlo* is being used as an example to show that the disease-susceptibility function of many *S* genes is conserved not only in Arabidopsis but also in crop plants. Furthermore, it is discussed how to exploit genetic loss of susceptibility by RNAi and/or mutant selection to achieve durable and broad-spectrum resistance which can be exploited. In **chapter 3**, we selected 11 *A. thaliana S* genes and silenced orthologous genes in the potato cultivar Desiree, which is highly susceptible to late blight. The silencing of five potato genes (*CESA3, DMR1, DMR6, DND1, SR4*) resulted in complete resistance to the *P. infestans* isolate Pic99189, and the silencing of a sixth *S* gene (*PMR4*) resulted in reduced susceptibility. The application of *S* genes to potato breeding for resistance to late blight is further discussed. In **chapter 4**, we silenced *AtDND1* orthologs in potato and tomato by using RNAi. Our results showed that silencing of the *DND1* ortholog in both crops resulted in resistance to *P. infestans* and to two powdery mildew species, *Oidium neolycopersici* and *Golovinomyces orontii*. The resistance to *P. infestans* in potato was effective to four different isolates although the level of resistance (complete or partial) was dependent on the aggressiveness of the isolate. Our results indicate that *S* gene function of *DND1* is conserved in tomato and potato. We discuss the possibilities of using RNAi silencing or loss-of-function mutations of *DND1* orthologues, as well as additional *S* gene orthologues from Arabidopsis, to breed for resistance to pathogens in crop plants. In **chapter 5**, we monitor microscopically the infection process of *P. infestans* on *StDND1-, StDMR1-* or *StDMR6-* silenced potato plants. The results showed that the infection process of *P. infestans* was hindered at different stages on *StDND1-, StDMR1-* or *StDMR6-* silenced potato plants. These microscopic results were compared with the loss of function mutants of Arabidopsis for *dnd1, dmr1* and

dmr6. In **chapter 6**, microscopic analysis was performed to study the mechanism that confers reduced susceptibility *to B. cinerea* to *DND1*-silenced potato and tomato plants. A significantly lower number of *B. cinerea* conidia were attached to the leaf surface of *DND1* silenced potato and tomato plants and hyphal growth was hampered. The potential mechanisms associated with the reduced susceptibility to *B. cinerea* in *DND1* silenced potato plants as well as breeding aspects are further discussed. Finally, in **chapter 7**, the results obtained from the experimental chapters are discussed with reference to the advancement in our understanding of resistance mechanisms in *S* gene silenced plants and their gene functions. Additionally, the practical use of the knowledge to achieve disease resistance by using *S* genes is discussed, including the durability aspect.

CHAPTER 2

Breeding for disease resistance by editing plant susceptibility genes

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Abstract

Plants are continuously attacked by a broad range of pathogens. World-wide farmers use large amounts of chemicals to secure crop yield. Breeding for disease resistance is a major objective of breeding activities in order to minimize the yield and quality loss associated with diseases. Although, resistance can often be obtained by introgression of major resistance genes (*R* genes) from wild crop relatives, resistance conferred by *R* genes is rarely durable. Recently, increased research with a focus on suppression of plant immunity has led to the identification of (potential) disease-susceptibility genes (*S* genes) in Arabidopsis. Taking a few examples of conserved *S* gene function between Arabidopsis and crops, this review demonstrates how to impair plant *S* genes, complementary to the introgression of *R* genes, to achieve durable and broad-spectrum resistance.

Keywords: Effector target, Gene editing, Mutagenesis, Plant innate immunity, Recessive resistance, Resistance breeding, Susceptibility factor

Introduction

High quality and productive crops are often susceptible to a multitude of different pathogens and pests. In practice, disease-resistant crop varieties are commonly bred by the introgression of resistance (*R*) genes derived from wild crop relatives. This so-called "introgression breeding" is an important strategy to broaden the genetic basis of highly inbred crops like tomato and allowing access to the variation present in wild relatives. Introgression of wanted small DNA fragments from wild donor species to the crop is often a longstanding procedure starting with an interspecific hybridisation, followed by backcrossing with the recipient crop. Unfortunately, resistance offered by the introgressed *R* genes is rarely durable since its race-specificity asserts selective pressure on pathogen populations which in turn promotes the appearance of new races rendering the introgressed *R* gene ineffective (Jacobsen et al. 2013). Pyramiding of *R* genes has been proposed as a solution to this problem, but has so far not actively been deployed in varieties, although from natural populations results are known which would suggest that this might be an effective form of resistance. Based on studies on effector-triggered susceptibility and by looking from a different point of view into host and non-host resistance, we have proposed a new breeding strategy by disabling plant disease-susceptibility (*S*) genes to achieve durable and broad-spectrum resistance in crops (Pavan et al. 2010).

The *S* gene is a plant gene required for pathogen survival and proliferation, which was first highlighted by Eckardt (2002) when *PMR6* was discovered in Arabidopsis as a gene coding for a susceptibility factor to promote growth of powdery mildew. From the viewpoint of plant innate immunity, plant *S* genes code for susceptibility factors that operate as a negative control of plant defense (e. g. the barley *MLO* gene) and therefore contribute to disease susceptibility (Pavan et al. 2013; Hückelhoven et al. 2013). These susceptibility factors can belong to effector targets manipulated by pathogen effectors to supress plant immunity (Hückelhoven et al. 2013; Gawehns et al. 2013). The best example is the rice *Os-8N3* gene, which is the host target of the TAL effector PthXol of *Xanthomonas oryzae* pv. *oryzae* (Yang et al. 2006). While, plant susceptibility factors can also be immunity unrelated, such as PMR6 (Vogel et al. 2000), which in most of the cases are demanded by biotrophic pathogens. From a breeding point of view, a plant gene is named *S* gene when its impairment leads to recessively inherited resistance (Pavan et al. 2010).

Several natural loss-of-function alleles of *S* genes are known in agriculture supporting the concept that impairment of specific host genes results in durable disease resistance, such as barley *mlo* mutants (Lyngkjaer et al. 2000) and the rice *pi21* mutant allele

(loss-of-function of a proline-containing protein conferring resistance to rice blast for over a century of cultivation) (Fukuoka et al. 2009). With increasing interest in the research topic on suppression of plant immunity, a considerable amount of potential *S* genes has been identified in Arabidopsis (Pavan et al. 2010; Hückelhoven et al. 2013; Gawehns et al. 2013; Lapin et al 2013). However, it is largely unknown whether orthologs of these Arabidopsis *S* genes in crop species exist which are functional to corresponding crop pathogens. Taking a few genes as examples, this review shows (1) that between Arabidopsis and crops the disease-susceptibility function of many *S* genes is conserved; and (2) how to exploit genetic loss of susceptibility to achieve durable and broad-spectrum resistance.

Well-characterized examples of plant *S* genes

***Mlo* orthologs function as plant *S* genes for powdery mildews in different plant species**

So far, the best-characterized example on durable and broad-spectrum resistance by disabling *S* genes is the *mlo*-based resistance to powdery mildew in barley. In barley, the MLO protein was identified as a plant susceptibility factor manipulated by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* to cause disease (Büschges et al. 1997). The naturally occurring and mutation-induced recessive alleles of the *MLO* locus confer a broad spectrum resistance against all known isolates of barley powdery mildew identified so far. The resistance conferred by the loss-of-function mutations in the *MLO* gene prevents the mildew fungus from penetrating host cells, and thus prevents fungal haustorium formation (Peterhansel et al. 1997; Piffanelli et al. 2002). Barley *mlo* mutants have been known and used for more than 70 years and have been wildly employed in European spring barley agriculture over the last two decades (Jørgensen et al. 1992; Dreiseitl et al. 2013), emphasizing the durability of *mlo*-mediated disease resistance under agricultural conditions.

The later identification of natural and mutation-induced *mlo*-mutants in Arabidopsis, tomato and pea for resistance to different powdery mildews demonstrates a conserved requirement for MLO proteins in powdery mildew pathogenesis in plants (Bai et al. 2008; Consonni et al. 2006; Humphry et al. 2011; Pavan et al. 2011). In pepper, it has been demonstrated that silencing of the pepper *MLO* ortholog, *CaMLO2,* resulted in resistance to the powdery mildew species *Leveillula tauria* (Zheng et al. 2013). With the available genome sequences of many plant species, such as grape (*Vitis vinifera*), apple (*Malus domestica)*, cucumber (*Cucumis sativus*), *Mlo* like sequences are being identified (Feechan et al. 2008; Pessina et al. 2013; Schouten et al. 2014). In breeding

practices, these findings imply the success in applying *mlo*-based resistance to combat powdery mildew disease in a wide range of crop species either by mining the natural variation in wild crossable species and/or by using conventional mutagenesis to generate mutations in the gene (Pavan et al. 2010).

Disease-susceptibility function of Arabidopsis *S* genes are conserved in crops

Genetic dissection of disease-susceptibility in Arabidopsis to powdery and downy mildew has identified multiple *S* genes whose impairment results in disease resistance (Yang et al. 2006; van Damme et al. 2005). Although several of these *S* genes have been cloned and characterized in more detail in Arabidopsis it is unknown to which degree their function in disease susceptibility is conserved among different plant species. As a proof-of-concept research, we have tested several tomato and potato orthologs of Arabidopsis *S* genes. Our recent results showed that silencing of the tomato orthologs *SlPMR4* and *SlDMR1* resulted in powdery mildew resistance in tomato (Huibers et al. 2013). Together with *MLO*, these results indicate that the susceptibility function of these *Arabidopsis S* genes is conserved across plant species.

More surprisingly, the susceptibility function of Arabidopsis *S* genes in most cases is effective to very different pathogens when tested in crops. For example, the Arabidopsis *DMR1* gene was originally identified as an *S* gene to downy mildew caused by *Hyaloperonospora parasitica,* while the fact that silencing the Arabidopsis ortholog from tomato (*SlDMR1)* led to resistance to tomato powdery mildew suggests that some *S* genes can be exploited by different pathogens. This is consistent with the finding that resistance to the hemibiotrophic bacterial pathogen *X. campestris* pv. *vesicatoria* was achieved by silencing the pepper *MLO* gene (*CaMLO2)* (Kim & Hwang, 2012). In potato, our unpublished results show that silencing of certain *S* genes gives rise to broad-spectrum resistance to late blight caused by the oömycete *Phytophthora infestans*.

Natural recessive resistances to viruses are caused by mutations in translation initiation factors

With small genomes, viruses encode a very limited number of proteins and thus depend on host genes to complete their life cycle. Therefore, mutations in plant genes required by viruses for their infection would lead to recessive resistance, which explains the fact that recessive resistance is prominent in plant resistance to viruses. It was shown that about 50% of the 200 known virus resistance genes identified till 2005 are recessively inherited (Kang et al. 2005). Surprisingly, all natural recessive resistance genes (14 in total) characterized so far from crops belong to translation initiation factors (eIFs),

mainly eIF4E or eIF4G and their isoforms (Wang & Krishnaswamy 2013). Although resistance caused by loss-of-function mutations in eIF(iso)4E or eIF(iso)4G is prominently effective to potyviruses (e.g. potato virus Y), resistance to other plant viruses has been documented, including the carmovirus *Melon necrotic spot virus*, the Waikavirus *Rice tungro spherical virus*, the sobemovirus *Rice yellow mottle virus* (Wang et al. 2013; Truniger & Aranda 2009). Some of the mutations in eIF4E have been used successfully in agriculture for several decades (Wang et al. 2013). Till now, only one case is reported that resistance in the lettuce *mol* mutant has been overcome (Abdul-Razzak et al. 2009).

Gene editing promises an optimistic future for the deployment of plant *S* genes in resistance breeding

In crops (mostly diploid crops like tomato), loss-of-function mutations in *S* genes can be obtained by searching for natural mutations and mutations induced by mutagens, such as EMS (ethyl methanesulfonate) (Pathirana 2011). Several natural recessive mutants in *S* genes have been used in crop cultivation for centuries, such as barley *mlo*-mutants, the rice *xa-5* and *xa-13* mutants for resistance to *X. oryzae* pv. *oryzae,* the mutants in isoforms of eIF4E and eIF4G for resistance to *Potyviridae* in several crops (Pavan et al. 2010). In the past, many recessive resistance loci have been identified in crops for resistance to different pathogens, some of which are mapped in chromosomal regions where *S* genes are nowadays found. For example, cucumber resistance to downy mildew is mostly inherited recessively and the underlying genetic loci often co-localize with orthologs of Arabidopsis *S* genes to either powdery mildew or downy mildew (Schouten et al. 2014), which suggests that mutations in these *S* gene orthologs of cucumber could contribute to powdery/downy mildew resistance. Such a co-localization provides a starting point to isolate mutant alleles of casual *S* genes in crops. In addition to natural mutation of *S* genes, induced mutants can be obtained through mutagenesis, such as EMS treatment. In the CAB review paper of Pathirana (2011), many examples have been given on resistant cultivars that are derived from induced mutations using the treatment with mutagens. Screening for natural and induced mutations has been facilitated by TILLING (targeting-induced local lesion in genome) and HRMC (high resolution melting curve) techniques (Minoia et al. 2010). Today, next–generation sequencing opens up a fast way to detect mutations in target genes.

Evidence suggests that certain *S* genes, besides being involved in plant-pathogen interactions, are required for other important physiological processes. Therefore, complete loss-of-function mutations of such *S* genes reduce fitness. For example, the rice *Xa13* gene is required for both the growth of *X. oryzae* and for pollen development

(Chu et al. 2006) and down regulation of the *DMR1* gene in Arabidopsis and tomato is associated with severely reduced plant growth (Huibers et al. 2013). As to mutations in the MLO protein, pleiotropic effects were observed in barley *mlo*-mutants and pepper plants in which *CaMLO2* was silenced via virus-induced gene silencing (VIGS) (Chu et al. 2006; Lyngkjaer 2000), but not in the natural tomato *mlo*-mutant (Bai et al. 2008). Despite the pleiotropic effects, barley *mlo* mutants have been used in cultivation since 1940s, showing that fitness cost can be compensated in different genetic backgrounds (Jørgensen 1992; Dreiseitl et al. 2013). On the other hand, it has been also demonstrated that loss-of-function mutations of certain *S* genes, such as *PMR4* and *DMR6*, can result in broad-spectrum resistance without fitness costs (Vogel & Somerville 2000; Bai et al. 2008; Van Damme et al. 2005; Huibers et al. 2013). Nowadays, new techniques are available which can be applied to design superior mutant alleles by editing plant *S* genes in such that their other biological functions are not disturbed. These novel techniques include site-directed mutagenesis using Zinc-finger nucleases (Lloyd et al. 2005), TALEN (transcription activator-like effector nuclease)-based gene editing for allele design (Li et al. 2012a) and Crispr-Cas9 (clustered regularly interspaced short palindromic repeats–associated nuclease Cas9) technology (Belhaj et al. 2013; Xie & Yang 2013). For example, the TALEN technology was successfully used to edit the promoter of the rice *S* gene *Os11N3*, which contains an effector-binding element for AvrXa 7 of *X. oryzae* pv. *oryzae* (Li et al. 2012a). Thus, gene editing makes it feasible to alter exclusively the *S* gene function without disturbing other biological functions.

In contrast to diploid plant species, it is a challenge to obtain mutant alleles of *S* genes homozygous in crops with allo-polyploid and autopolyploid genomes, such as wheat and potato, respectively. For those crops, instead of searching for natural or random-induced mutants, silencing *S* genes using RNAi may offer a better chance than the use of mutant *S* gene alleles for breeding disease resistance. Fortunately, gene editing techniques mentioned above can be nowadays applied to engineer targeted modifications of *S* genes in polyploid genomes. For example, Wang et al (2014) have, using TALEN and CRISPR-Cas9 technologies, successfully introduced targeted mutations in the three homoeo-alleles coding the MLO protein in hexaploid bread wheat. The resulted mutations of all three *TaMLO* homoeologs in the same plant confers heritable broad-spectrum resistance to powdery mildew.

Conclusion

In summary, plant resistance can be obtained by introgression of *R* genes (via crosses or GMO approach) or by disrupting *S* genes (via mutations or RNAi) (Fig. 1). The latter was proposed by us in 2010 as a novel breeding strategy for durable and broad-spectrum resistance (Pavan et al. 2010). By then, we summarized about 30 genes that had been experimentally identified or cloned as potential plant *S* genes. In the past few years, more and more plant *S* genes have been found in crops via forward and reverse genetic screens and via identification of host effector targets (Eckardt et al. 2002; Hückelhoven et al. 2013; Lapin et al. 2013). More attention has been given to the concept of deploying altered host *S* genes to control plant disease (Hückelhoven et al. 2013; Gawehns et al. 2013; Dangl et al. 2013; van Schie & Taken 2014).

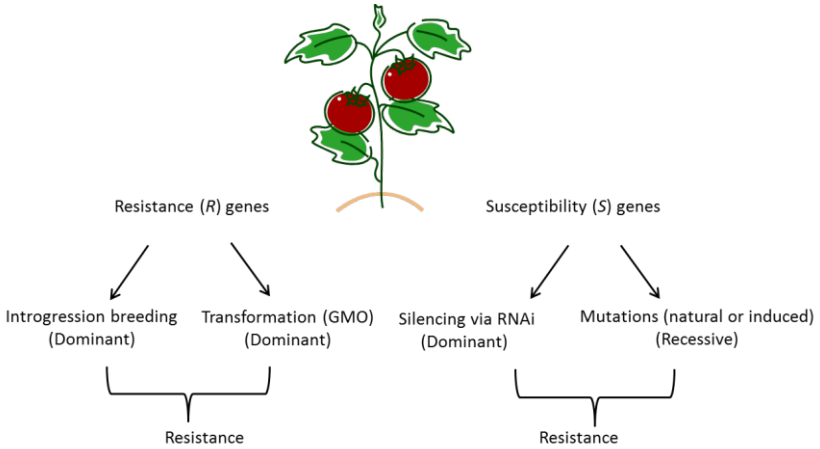

Fig.1. Shelton drawing to show the use of plant resistance (*R*) and susceptibility (*S*) genes in breeding crops with resistance to pathogens and pests. **Left panel:** An *R* gene is genetically defined as a dominantly inherited resistance factor. In crop breeding, an *R* gene is usually identified in wild relatives of a crop. There are at least two ways to transfer such a *R* gene from wild species to cultivated crops, introgression breeding (via interspecific crosses and back-crosses) and transformation (cloning the *R* gene and expressing it in cultivated crops resulting in genetic modified organisms (GMO)). Both ways result in cultivated crops with dominant resistance. **Right panel:** An *S* gene is genetically defined as a gene which loss-of-function results in recessively inherited disease resistance. In plant breeding, using loss-of-function alleles of a *S* gene can be achieved in at least two ways, silencing and identifying loss-of-function mutation of the *S* gene involved. Silencing via RNAi is a GMO approach and the resulting resistance trait will be inherited dominantly. Loss-of-function mutations in an *S* gene lead to recessively inherited resistance and can occur naturally, be induced via non-GMO mutagenesis and be engineered by gene editing approaches.

This review, by illustrating that the *S* genes identified in Arabidopsis are conserved for their function as susceptibility factors in other plant species, promotes further the potential exploitation of orthologs of Arabidopsis *S* genes in resistance breeding of crop species. For many crop species genome sequences are or will soon become available, which will facilitate the identification of orthologs of *S* genes in other plant species. For using mutant alleles of *S* genes in plant breeding, mutations that alter only the *S* gene function are desired and can be obtained by screening natural/random-induced mutant alleles in populations and/or by the targeted mutagenesis approaches mentioned above. Thus, deploying altered host *S* genes opens an alternative way for durable resistance in controlling plant diseases.

CHAPTER 3

Silencing of six susceptibility genes results in potato late blight resistance

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Abstract

Phytophthora infestans, the causal agent of late blight, is a major threat to commercial potato production worldwide. Significant costs are required for crop protection to secure yield. Many dominant genes for resistance (*R* genes) to potato late blight have been identified, and some of these *R* genes have been applied in potato breeding. However, the *P. infestans* population rapidly accumulates new virulent strains that render *R* genes ineffective. Here we introduce a new class of resistance which is based on the loss-of-function of a susceptibility gene (*S* gene) encoding a product exploited by pathogens during infection and colonization. Impaired *S* genes primarily result in recessive resistance traits in contrast to recognition-based resistance that is governed by dominant *R* genes. In *Arabidopsis thaliana*, many *S* genes have been detected in screens of mutant populations. In the present study, we selected 11 *A. thaliana S* genes and silenced orthologous genes in the potato cultivar Desiree, which is highly susceptible to late blight. The silencing of five genes resulted in complete resistance to the *P. infestans* isolate Pic99189, and the silencing of a sixth *S* gene resulted in reduced susceptibility. The application of *S* genes to potato breeding for resistance to late blight is further discussed.

Keywords: late blight; potato; resistance; RNAi; susceptibility gene

Introduction

The plant immune system comprises an intricate network of active and passive mechanisms that successfully prevent the colonization of a host by a pathogen (Jones & Dangl 2006) (Fig. 1). In many cases, defense is actively triggered upon first contact between a plant and pathogen. Plasma membrane receptors perceive pathogen-associated molecular patterns (PAMPs) or apoplastic effectors (Fig. 1). This perception leads to intracellular signal transduction events, culminating in defense responses that, when effective, induce PAMP-triggered immunity (PTI). A known example is the receptor-like protein ELR (elicitin response). ELR was isolated from the wild potato species *Solanum microdontum* and can mediate the broad-spectrum recognition of elicitins (referred to as oomycete PAMPs) from several *Phytophthora* species (Du et al. 2015). The second layer of defense relies on proteins encoded by resistance genes (*R* genes) that recognize intracellular avirulence (Avr) effectors. This recognition results in effector-triggered immunity (ETI).

Potato late blight, caused by *Phytophthora infestans,* is considered to be the most serious potato disease worldwide. An asexual cycle of sporangial proliferation is completed within five days (Fry 2008). Depending on environmental conditions, an unprotected potato field with a susceptible cultivar (cv) can be devastated within 10 days after infection with *P. infestans* (Fry 2008). The control of potato late blight is dependent on fungicide sprays and the use of cultivars carrying dominant *R* genes (Haverkort et al. 2009). All late blight *R* genes identified thus far belong to the coiled-coil nucleotide binding leucine-rich repeat (CC-NB-LRR or NLR) class and reside inside the plant cell where these genes recognize *P. infestans* avirulence effectors (Avr) of the RxLR class.

Because the resistance conferred by *R* genes is, in general, race-specific, ETI can be broken due to the rapid evolution of pathogen effectors in agricultural practice (Vleeshouwers et al. 2011). For example, *Rpi-vnt1*, isolated from *S. venturii*, confers resistance to a broad spectrum of *P. infestans* lineages and races in European potato growing areas (Pel et al. 2009). However, this resistance can be overcome by the EC1 lineage, which is abundant in Ecuador. Thus, in addition to exploiting dominant *R* genes, combinations of resistance traits that are effective against the prevailing *P. infestans* population are needed for durable late blight resistance. Therefore, pyramiding ETI receptors is expected to enhance resistance durability (Kim & Hwang 2012; Rietman et al. 2012; Zhu et al. 2013).

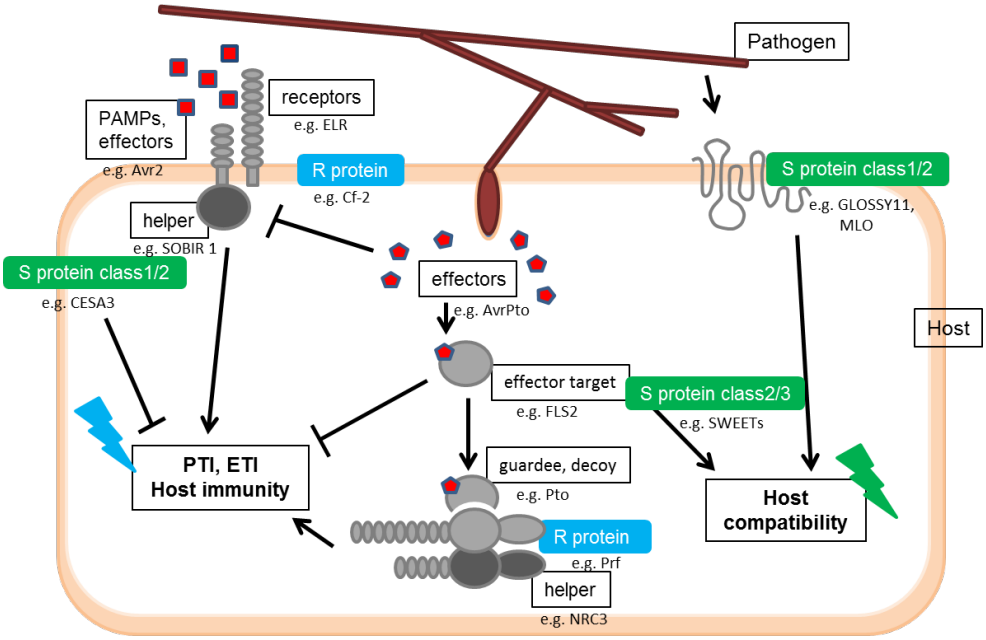

Fig. 1. Plant innate immunity: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). Apoplastic pathogen-associated molecular patterns (PAMPs/apoplastic effectors), intracellular effectors or modified effector targets are actively perceived by receptors in the plasma membrane or resistance (R) proteins in the cytoplasm, resulting in the activation of PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). Helper proteins and guard proteins/decoys are involved in the co-perception of pathogen-derived components (Césari et al. 2014). Pathogens use host proteins (S proteins) encoded by plant susceptibility genes (*S* genes) to facilitate entry and growth, resulting in host compatibility (Doehlemann & Hemetsberger 2013). MLO, CESA3 and SWEETs are examples of S proteins in class 1, 2, or 3 according to van Schie and Takken (2014). Class 1 genes provide features that facilitate the entrance of a pathogen, class 2 genes increase innate immunity when the gene is disabled, and class 3 genes encode substrates essential for the pathogen.

The resistance conferred by *R* genes is based on pathogen recognition followed by the induction of defense responses. Another type of resistance, based on the loss-of-function of a susceptibility gene (*S* gene), has recently been introduced (Eckardt, 2002; Pavan et al. 2011). Plant genes are termed *S* genes when a pathogen takes advantage of them for its own benefit during the colonization of the plant. Pathogens are impeded from colonizing the plant when these genes become dysfunctional as a result of a recessive mutation or non-expression. Thus, when disabled, *S* genes can induce a resistance phenotype resembling that of healthy plants. For example, the loss-of-

function mutant in the *Powdery Mildew Resistance 6* (*PMR6*) gene prevents powdery mildew growth (Vogel et al. 2002). Based on the mechanism, *S* genes have been divided into three classes (van Schie & Takken 2014). The first class comprises the genes required in early pathogen infection steps. One example is the maize (*Zea mays*) wax mutant *glossy 11*, which limits powdery mildew (*Blumeria graminis*) spore germination (Hansjakob et al. 2011; Fig. 1). The second class of *S* genes encodes negative regulators of plant immunity, such as the *CesA3* gene, which is involved in cellulose synthesis (Ellis et al. 2002; Ellis and Turner 2001; Fig. 1). The presence of homozygous recessive mutant alleles of the CesA3 gene can confer resistance to multiple pathogens, as a result of an increase of *in planta* levels of abscisic and jasmonic acid and ethylene. The third class of *S* genes includes genes involved in pathogen sustenance, such as metabolite biosynthesis and sugar transport. For example, the *Downy Mildew Resistant 1* (*DMR1*) gene encodes homoserine kinase (HSK). Arabidopsis *dmr1* mutants are resistant to the downy mildew fungus *Hyaloperonospora parasitica* (Huibers et al. 2013; Van Damme et al. 2008; Van Damme et al. 2009) and the fungi *Fusarium graminearum* and *F. culmorum,* which cause Fusarium Ear Blight (FEB) disease on small grain cereals (Brewer et al. 2014). Other examples include the genes encoding SWEET proteins, identified as factors that are required for susceptibility to *Xanthomonas oryzae*, which provide a carbon source to the pathogen (Chen et al. 2010; Streubel et al. 2013; Yuan et al. 2010; Fig. 1).

The aim of the present study was to impair *S* genes in plants to obtain durable resistance (Pavan et al. 2010). In tomato, we showed that silencing *DMR1* and *PMR4* orthologues resulted in resistance to the powdery mildew fungus *Oidium neolycopersici* (Huibers et al. 2013), suggesting that *S* genes, such as *PMR4* and *DMR1,* are conserved among plant species and that impaired orthologues confer resistance to the associated pathogens in other plant species. This finding prompted us to identify potato orthologues of *S* genes reported in other plant species (particularly *A. thaliana*) and to generate a proof of the *S* gene concept in potato. We silenced 11 *S* genes in potato via RNAi and showed that the silencing of five of these genes conferred complete resistance to late blight in potato, while the silencing of a sixth gene only conferred reduced susceptibility to this disease.

Materials and Methods

Identification of potato orthologues of *S* genes

Previously, tomato orthologues of 11 *A. thaliana S* genes were identified as described in Huibers et al. (2013). Briefly, *A. thaliana* protein sequences were used as a query in

a TBLASTN programme against the SGN Tomato Combined database (http://solgenomics.net/tools/blast/) to search for homologous sequences. The tomato and Arabidopsis amino acid sequences were aligned, and the tomato sequences that showed a high level of homology with the *A. thaliana S* genes were considered orthologues. To identify potato orthologues, *A. thaliana* protein sequences were used in a BLASTP analysis at the Spud DB Potato Genomics Resource website (http://solanaceae.plantbiology.msu.edu/blast.shtml). Subsequently, protein and mRNA sequences with the lowest E-values were downloaded. Next, phylogenetic analyses were performed by aligning *A. thaliana*, tomato and potato protein sequences in MEGA5.1 (Tamura et al. 2011) using standard parameters (Supplementary Fig. 1). The tomato and potato genes showing the highest level of homology (on amino acid level) to *A. thaliana S* genes were considered to be *S* gene orthologues and were indicated with the prefix *Sl-* or *St-* to represent *S. lycopersicum* and *S. tuberosum*, respectively.

Construction of the silencing vectors

The binary vector pHellsgate8 (CSIRO, Australia) has a kanamycin resistance gene as a selectable marker and was used to generate RNAi constructs (Helliwell & Waterhouse 2003; Waterhouse & Helliwell 2003). This vector contains a CaMV 35S promoter which drives the expression of the inverted repeat. For all genes, except *SR4, DND2*, and *PMR4,* primers were designed to amplify fragments of the target genes ranging from 150 to 300 bp from tomato cDNA sequences of the cv Moneymaker (Supplementary Table 1). For *SR4* and *DND2*, primers were designed to amplify potato cDNA sequences of the cv Desiree. The forward primer contained CACC at the 5′ end for directional cloning into the pENTR/D-TOPO vector (Thermo Fisher Scientific). Total RNA was isolated using a RNeasy kit from Qiagen (Germany). RNA was treated with RNAse-free DNase (Qiagen). Subsequently, this RNA was used as a template for cDNA synthesis using an iScript cDNA synthesis kit (Bio-Rad). The PCR products were amplified using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), inserted into the pENTR/D-TOPO cloning vector and transformed into One-Shot TOP10 *E. coli* cells. The plasmid DNA of the clones was sequenced to verify the insert. To generate the *SlPMR4* silencing construct (Huibers et al. 2013), we synthesized a 101-bp DNA fragment that was identical to the first 97 bp of the predicted coding sequence of Solyc07g053980 (the tomato *PMR4* ortholog, Supplementary Fig. 1) and contained CACC at the 5′ end flanked by attL sites in pUC57 (Genscript, USA). For *PMR6*, the silencing fragment was based on the coding sequence of tomato Solyc05g014000, but could potentially cross-silence multiple potato *PMR6*-like homologs (Sotub05g015080, Sotub11g012470, Sotub06g029220 and Sotub03g023350). The primer sequences are provided in Supplementary Table 1.

For expression *in planta*, the RNAi fragments were transferred from the entry clone through an LR clonase reaction to the pHellsgate8 vector.

Growth and development of transgenic potato plants

The tetraploid potato cv Desiree (susceptible to late blight) was used for transformation according to the protocol of Visser et al. (1991). The transformants were transferred from MS medium (Murashige & Skoog 1962) supplemented with vitamins, 30 g l^{-1} sucrose and 100 mg l^{-1} kanamycin to similar fresh MS medium without kanamycin. After three weeks of growth at 24°C and a light intensity of 100 W/m^2, the rooted transformants were transferred to plastic pots containing potting soil in a growth compartment at 21°C and 19°C during 16-h days and 8-h nights, respectively. For each RNAi construct, more than eight independent primary transformants were randomly selected and cultured in a greenhouse and subsequently tested for resistance. Three biological replicates were grown for each transformant.

Pathogen inoculations and detached leaflet assay (DLA)

The *P. infestans* isolate Pic99189 (race 1.2.5.7.10.11) (Flier et al. 2002) was used in the present study. For each experiment, the isolate was grown on rye agar medium supplemented with 2% sucrose for 10-15 days at 15°C in closed Petri dishes to induce sporangia formation (Caten & Jinks 1968). To release zoospores from sporangia, ice-cold tap water was added to the Petri dishes, followed by incubation for three hours at 4°C. The zoospore concentration was assessed by bright field microscopy using a Fuchs-Rosenthal counting chamber and adjusted to 5×10^4 spores/ml. The resistance of potato RNAi transformants to Pic99189 was examined using a 10-µl droplet inoculation in detached leaflet assays (DLA) (Vleeshouwers et al. 1999). The leaves were harvested from plants after five to six weeks of greenhouse growth. The fourth or fifth fully developed leaf (counted from the bottom) was used. The lesion diameters were measured from three to six days post-inoculation using an electronic calliper (Helios DIGI-MET®).

RNA isolation and quantitative real-time (qRT)-PCR

The kanamycin-resistant transformants were confirmed by PCR using Fw-NPTII and Rv-NPTII primers (Supplementary Table 1). The PCR-positive transformants were transferred to the greenhouse. More than eight independent transformants were randomly selected per gene, and the silencing levels of the transformants were evaluated by qRT-PCR using gene-specific primers (Supplementary Table 1, -qPCR), producing products of approximately 200 bp. Plant total RNA was extracted using a

MagMAX-96 total RNA Isolation kit (Ambion). The quantity of the isolated RNA was measured using a Nanodrop Spectrophotometer ND-1000 (Isogen), and the cDNA was produced using an iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed in triplicate using a C1000TM Thermal Cycler PCR system (Bio-Rad) with iQ SYBR Green supermix (Bio-Rad). The potato *EF1a* (Sotub06g010680) transcript was used as an internal control to determine the relative transcript levels. The relative level of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001; Nicot et al. 2005). For the qRT-PCR assay, three technical replicates were included for each experiment, and the expression of each gene was investigated in three biological replicates.

Results

Identification of potential potato *S* gene orthologues

To identify potato orthologues of the 11 *S* genes listed in Table 1, we used the amino acid sequences of *A. thaliana* in a BLAST analysis of the potato sequence database. Potato sequences with an amino acid identity higher than 75% were selected and used in phylogenetic studies (Supplementary Fig. 1). Based on multiple sequence alignments, sequences showing the highest degree of homology with the *S* gene in *A. thaliana* were considered to be potential orthologues in potato (Table 2, column 2). The closest homolog to *AtSR1* in the potato database was Sotub01g012330, and silencing fragments were designed for this gene. However, when a subsequent TBLASTN search was conducted using the NCBI database, the closest homolog of *AtSR1* in the potato RefSeq_RNA database was XM_006355276.1, which corresponds to Sotub04g020530. This gene on chromosome 4 was closer to *AtSR1* in the phylogenetic tree than to Sotub01g012330 (Supplementary Figure 1). Because Sotub01g012330 was closer to *AtSR4* and *SlSR4* (Yang et al. 2012), we referred to Sotub01g012330 as *StSR4* in Table 2.

Table 1. Selected *S* genes identified in Arabidopsis. Resistances conferred by mutated alleles, and phenotypic side effects are indicated per gene.

Gene name	Mutant resistance to pathogens						Mutant Notes	Class[7]	References
	PS[1]	PM[2]	*PP*[3]	*HP*[4]	*BC*[5]	*AB*[6]			
CESA3	nd	+	nd	nd	nd	nd	High level of resistance to the herbicide, gametophytic lethal	2	Ellis et al. (2002); Ellis and Turner (2001)
DMR1	nd	+	nd	+	nd	nd	Chlorosis and reduced growth	3	Huibers et al. (2013); Van Damme et al. (2005); Van Damme et al. (2009)
DMR6	–	+	nd	+	nd	nd		2	Van Damme et al. (2005); Van Damme et al. (2008)
DND1	+	nd	nd	+	+	+	Smaller plant, early senescence, moderate lesion mimic	2	Ahn (2007); Clough et al. (2000); Genger et al. (2008); Govrin & Levine (2000); Jurkowski et al. (2004); Su'udi et al. (2011)
SR1	+	+	nd	nd	+	nd	Sensitive to herbivore attack, forms chlorotic lesions on leaf lamina	2	Doherty et al. (2009); Du et al. (2009); Galon et al. (2008); Kim et al. (2013); Laluk et al. (2012); Nie et al. (2012); Qiu et al. (2012)
PMR4	nd	+	nd	+	+	–	Resistance to the green peach aphid *Myzus persicae*, enhanced susceptibility to the fungal pathogens *Pythium irregulare*	2	Nishimura et al. (2003)
BIK1	+	nd	nd	nd	–	–	Altered root growth	2	Veronese et al. (2006)
CPR5	+	nd	+	nd	nd	nd	Spontaneous development of necrotic lesions; affected trichome development	2	Bowling et al. (1997); Jing et al. (2007); Jing & Dijkwel (2008); Love et al. (2007)
DND2	+	nd	nd	+	+	+	Smaller plant, early senescence, moderate lesion mimic	2	Ahn (2007); Clough et al. (2000); Genger et al. (2008); Govrin & Levine (2000); Jurkowski et al. (2004); Su'udi et al. (2011)
PMR5	–	+	–	nd	nd	nd	Altered cell wall composition; exhibit a strong increase in total uronic acid content in cell walls and a more severe reduction in plant size, relative to the single mutants; slight increase in rosette leaf number	3	Chandran et al. (2013); Vogel et al. (2004)
PMR6	–	+	–	nd	nd	nd	Smaller plants than wt; altered leaf morphology: leaves are shorter, rounder and cupped slightly upward compared to wt; altered cell wall composition containing more pectin than wt, altered hydrogen bonding structure of cellulose	3	Chandran et al. (2013); Vogel et al. (2002)

+, significantly reduced susceptibility; –, no reduced susceptibility; nd, not determined

[1]*Pseudomonas syringae*; [2]PM, powdery mildews: *Erysiphe cichoracearum*; *Erysiohe orontii; Oidium lycopersicum*; *Blumeria graminis*; *Golovinomyces cichoracearum*; *Golovinomyces orontii*; [3]*Peronospora parasitica*; [4]*Hyaloperonospora parasitica*; [5]*Botrytis cinerea*; [6]*Alternaria brassicicola*;

[7]According to van Schie and Takken (2014)

Silencing of six potato *S* genes results in reduced susceptibility to *P. infestans*

To assess the significance of these potential *S* genes for susceptibility to *P. infestans* in potato, RNAi constructs of all of the selected potato orthologues were generated and used to transform the potato cv Desiree, which is susceptible to late blight, using *Agrobacterium tumefaciens*-mediated transformation. From each of the 11 *S* genes, several independent transformants with highly reduced transcript levels (>60%) of the targeted *S* gene were selected (Table 2, column 3, Supplementary Fig. 2). These transformants were further indicated as well-silenced transformants, and those with no or a low reduction in the transcript level (<60%) of the targeted *S* gene were considered to be negative controls in the following experiments (Table 2, column 4, Supplementary Fig. 2).

Table 2. Selected potato orthologs of 11 *S* genes and their effects in transformants obtained after RNAi silencing

Gene name	Potato *S* gene homologs	No. plants							
		Tested by qPCR	Reduced transcription		DLA by Pic99189[1]		Dwarfing	Auto-necrosis	Color loss
			> 60% (+)	< 60% (-)	R	S			
CESA3	*StCESA3*(Sotub01g026250)	8	5	3	5	3	–	–	–
DMR1	*StDMR1* (Sotub04g008400)	12	5	7	5	7	4+/8–	–	5+/7–
DMR6	*StDMR6* (Sotub06g027890)	12	6	6	4	8	–	–	–
DND1	*StDND1* (Sotub02g034320)	16	12	4	12	4	7+/9–	13+/3–	7+/9–
SR1	*StSR4* (Sotub01g012330)*	25	11	14	5	20	–	–	–
PMR4	*StPMR4* (Sotub07g019600)	27	13	14	8	19	–	–	–
BIK1	*StB1K1* (Sotub04g010100)	8	5	3	0	8	–	–	–
CPR5	*StCPR5* (Sotub04g022770)	12	4	8	0	12	4+/8–	–	4+/8–
DND2	*StDND2*(Sotub10g007010)	8	4	4	0	8	–	–	–
PMR5	*StPMR5* (Sotub06g006190)	27	10	17	0	27	–	–	–
PMR6	*StPMR6* (Sotub11g012470)	12	7	5	0	12	–	–	–

[1]DLA, detached leaf assay; R, resistant; S, susceptible

* See explanation in text

To determine whether the silencing of these genes impacted the susceptibility to late blight, the leaves of 4-to-5-week-old silenced plants were inoculated with the *P. infestans* isolate Pic99189. The leaves of cv Desiree and A13-013 (a transformant containing the dominant resistance gene *Rpi-vnt1.1* in the genetic background of cv Desiree and conferring resistance to Pic99189, Zhu et al. 2013) were used as susceptible and resistant controls, respectively. The inoculated leaves were visually

inspected (Fig. 2a-c). Three to six days post inoculation (dpi), the lesion size was measured, and lesion growth over time was plotted (Fig. 2d-f). Large lesions were observed on the inoculated leaves of the cv Desiree and transformants in which *StBIK1*, *StCPR5*, *StDND2, StPMR5* or *StPMR6* were silenced (Fig. 2a). The lesion size showed a steady increase from 3 to 6 dpi (Fig. 2d).

Smaller lesions were observed on inoculated leaves of *StPMR4*-silenced potato plants compared with those of Desiree (Fig. 2b). At 6 dpi, the inoculated parts of Desiree leaves were completely blighted with obvious sporulation, while on the leaves of RNAi::*StPMR4* potato plants, the sporulation was only visible through a binocular. The lesion growth on the leaves of these transformants was much slower than on Desiree leaves (Fig. 2e). Thus, the silencing of *StPMR4* resulted in reduced susceptibility to *P. infestans*.

In contrast, up to 6 dpi, no lesion growth was visible on the leaves of resistant A13-013 control plants and transformants in which the five individual *S* genes (*StCESA3*, *StDMR1*, *StDMR6*, *StDND1* and *StSR4*) were silenced (Fig. 2c & f). For each of these five genes, at least two independent well-silenced transformants (with three plants per transformation event) were tested (Table 2). All inoculated leaves showed similar results.

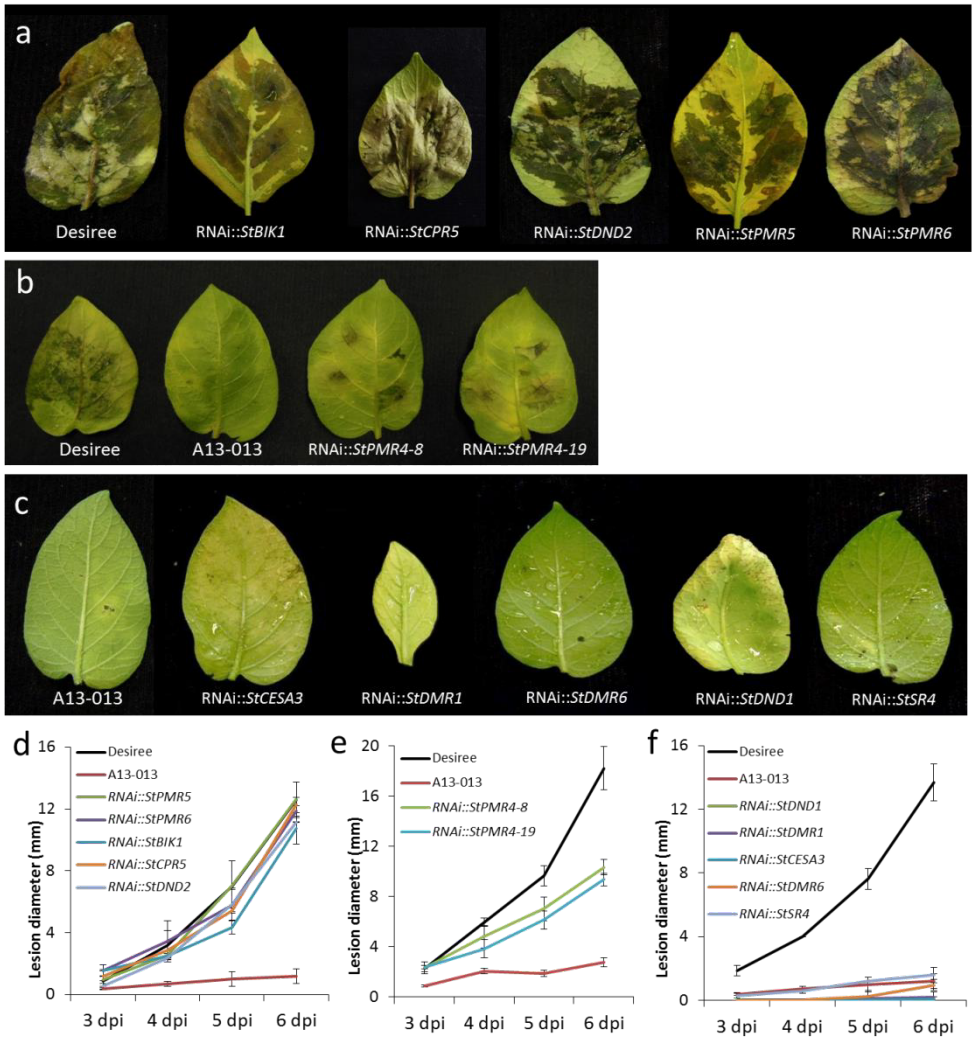

Fig. 2. Detached leaf assay (DLA) of potato RNAi transformants with *Phytophthora infestans* isolate Pic99189. **a** Leaves of the five RNAi transformants (*StBIK1*, *StCPR5*, *StDND2*, *StPMR5*, *StPMR6*) showing a diseased phenotype similar to that of cv Desiree at 7 days post inoculation (dpi). **b** Leaves of two independent RNAi::*StPMR4* transformants compared with the susceptible control Desiree and the resistant control A13-013 at 6 dpi. Both transformants #8 and #19 show strong silencing of *StPMR4* (Table 2). **c** Leaves of the resistant control A13-013 and the five RNAi transformants (*StCESA3*, *StDMR1*, *StDMR6*, *StDND1*, *StSR4*) showing no disease symptoms at 7 dpi. **d-f** Development of lesion size on the inoculated leaves in Fig. 2a, b, and c, respectively. Data were collected at 3, 4,

5, and 6 dpi. Untransformed Desiree plants were included as the susceptible control and A13-013 plants were included as the resistant control in this experiment. For each experiment, more than four well-silenced transformants per investigated gene were tested (three individual plants per transformant, one leaf per plant). Three independent experiments were performed with similar results.

Fitness costs associated with *S* gene silencing

Altered phenotypic characteristics in non-inoculated plants were observed for some RNAi transformants, such as reduced growth (dwarfing), necrosis, and lighter green leaves (colour loss, Table 2). *StDMR1*-silenced plants showed reduced growth and light green leaves compared with cv Desiree (Fig. 3a & b), whereas *StDND1*-silenced plants displayed auto-necrotic spots only in the leaves of older plants (Fig. 3c). Plants of a few well-silenced *StDND1*-tranformants also showed dwarfing, but not as severe as that of *StDMR1*-silenced transformants. The light green leaf colour and dwarfing were observed for plants of all of the well-silenced RNAi::*StCPR5* transformants (Fig. 3d), and these plants were susceptible to late blight (Fig. 2a)

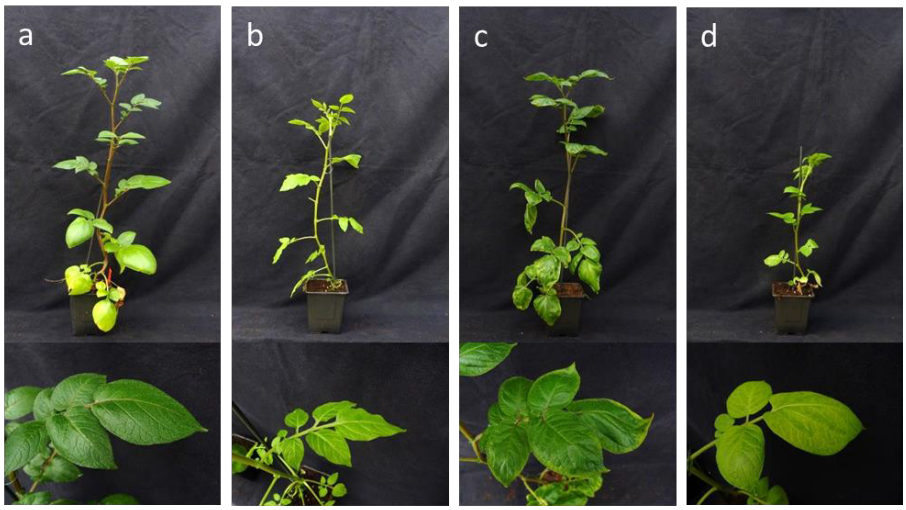

Fig. 3. Plants and leaflets of three potato RNAi transformants compared with cv Desiree. **a** Potato cv Desiree. **b** *StDMR1*-silenced transformant, showing a dwarf phenotype and light green leaves. **c** *StDND1*-silenced transformant showing auto-necrotic spots on older leaves. **d** *StCPR5*-silenced transformant showing dwarfing and light green leaves.

Discussion

Impaired potato *S* genes: a new source of durable resistance against *P. infestans*?

Potato is the third largest food crop in the world, with the efficient usage of resources and high nutritional quality. For over 180 years, the most devastating disease in potato is late blight disease, caused by *P. infestans*, which costs billions of Euros annually for crop protection to prevent crop losses. Many potato *R* genes have been cloned and characterized, and some *R* genes have been used in breeding. Still, durable resistant potato varieties have not been generated, reflecting new virulent *P. infestans* races that render resistance genes ineffective. Only a few potato varieties carrying multiple *R* genes show a sufficient level of durable resistance, such as the cultivar Sarpo Mira, which possesses multiple major *R* genes (Rietman et al. 2012; Tomczyńska et al. 2014). Thus, pyramiding *R* genes show the potential to achieve durable resistance.

The results of the present study showed that silencing the potato orthologues of six *A. thaliana S* genes resulted in complete or partial resistance to late blight in potato. These six *S* genes were not originally identified as susceptibility factors towards late blight. Thus, these results showed that orthologues of the *S* genes in *A. thaliana* might be functionally conserved across plant species. Furthermore, the impairment of orthologues of the same *S* gene in different plant species could potentially result in resistance to different pathogens.

Because the *S* gene concept is relatively new, it is not clear whether the resistance resulting from impaired *S* genes is durable. Several natural loss-of-function alleles of plant *S* genes have been identified in agriculture as providing durable disease resistance (Pavan et al. 2010). A well-known example is the barley *mlo* mutant for non-race specific resistance to the powdery mildew *Blumeria graminis*, which has been successfully used in European agriculture for more than 35 years (Büschges et al. 1997; Lyngkjaer et al., 2000). Natural mutant alleles of the *MLO* gene have been identified in several plant species, such as tomato (Bai et al. 2008), pea (Pavan et al. 2011) and cucumber (Berg et al. 2015), which confer resistance against different powdery mildew species. Whether impaired potato *S* genes are a new source of durable resistance to late blight needs further study. We are currently testing the RNAi transformants with different races of *P. infestans* to verify whether resistance is broad spectrum. Moreover, an examination of the resistance

mechanisms associated with impaired *S* genes might shed light on the resistance spectrum and durability (van Schie & Takken, 2014).

Fitness costs associated with impaired potato *S* genes

Plant *S* genes exploited by pathogens during infection might have other intrinsic functions that are important for the plant. Therefore, impairing the function of particular *S* genes might have adverse effects. Prior to using impaired *S* gene alleles in crop breeding, potential negative side effects should be identified and reduced or even prevented. Mutant alleles of the 11 *S* genes, except *DMR6* and *PMR4,* were associated with phenotypic changes in *A. thaliana* (Table 1). Remarkably, the silencing of only three of the 11 tested *S* gene orthologues generated negative pleiotropic effects in potato (Table 2), suggesting that fitness costs could be plant species-dependent (Bai et al. 2008). For the RNAi constructs in the present study, we used the cauliflower mosaic virus 35S promoter, which is a strong and constitutively active promoter in most tissues. It is likely that the use of native *S* gene promoters could reduce or prevent the negative side effects on plant performance. In the present study, we identified six *S* genes whose impairment by RNAi reduced susceptibility to late blight, of which four *S* genes were not associated with negative side effects under the conditions tested (Fig. 2, Table 2). Further studies are needed to determine whether pleiotropic effects are also absent under conditions relevant to agricultural practices.

Strategies for exploiting impaired *S* genes in potato breeding

As shown in the present study, silenced *S* genes are a new source of resistance to late blight. Loss-of-function mutations in *S* genes lead to recessively inherited resistance, which is difficult to exploit in an autotetraploid crop, such as potato. In the heterozygous (wild) potato gene pool, only dominant *R* genes have been identified. In contrast, the resistance conferred by recessive alleles of *S* genes is not readily selected in both the evolution and breeding of heterozygous species, such as potato. As shown in the present study, the RNAi approach can be applied to knockdown the expression of *S* genes, which leads to resistance. RNAi has previously been applied in potato to silence the Granule-Bound Starch Synthase I (GBSSI) gene to produce potatoes with amylose-free starch (Visser et al. 1991). Currently, advanced gene-editing technologies are available for the targeted

modification of all alleles of a gene. For example, TALEN-induced mutation and CRISPR-Cas9 technology have been used to generate mutant alleles of the *MLO* gene in all three genomes of allohexaploid wheat (Wang et al. 2014). In addition to these advanced techniques, F_1 hybrid potato breeding (Lindhout et al. 2011) will be advantageous for the use of mutated *S* genes. With a homozygous diploid line, knockout mutations can be obtained by mutagenesis, such as ethyl methanesulphonate (EMS) treatment. Thus, a realistic outlook towards late blight resistance is within reach by using *S* gene mutants with advanced gene-editing technology or EMS in hybrid potato breeding.

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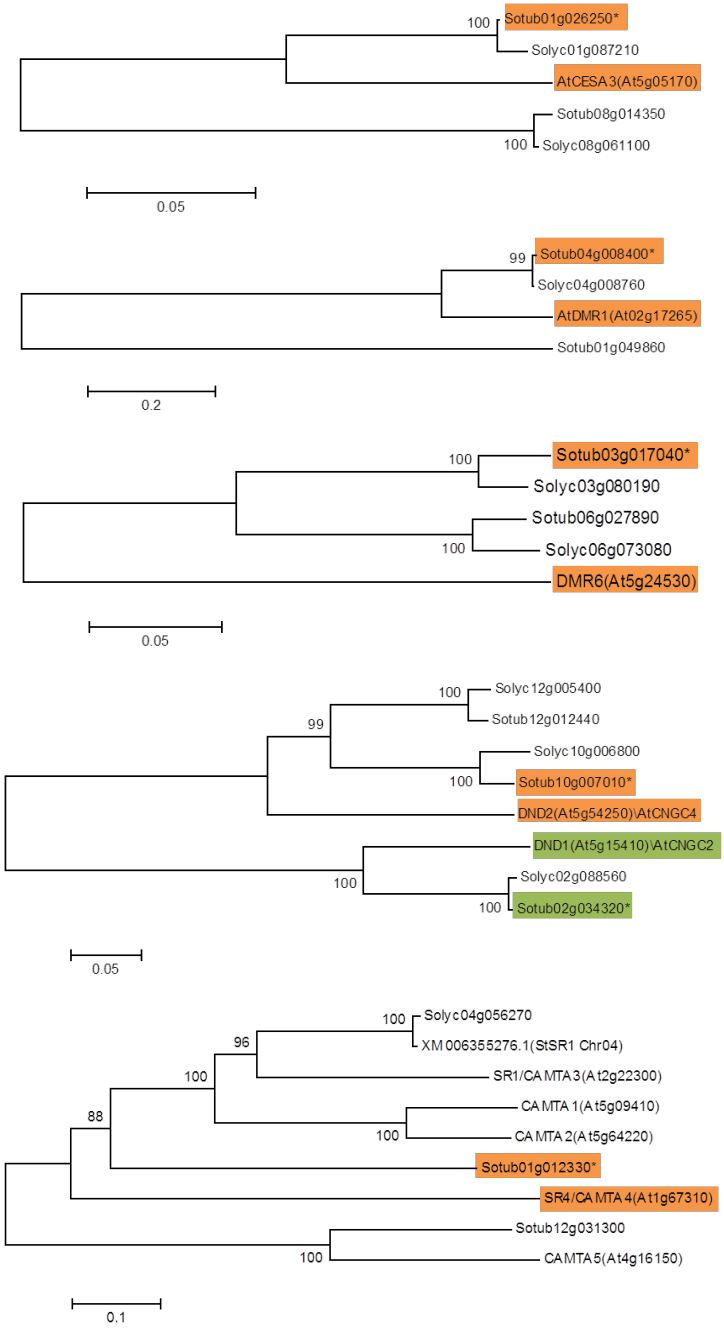
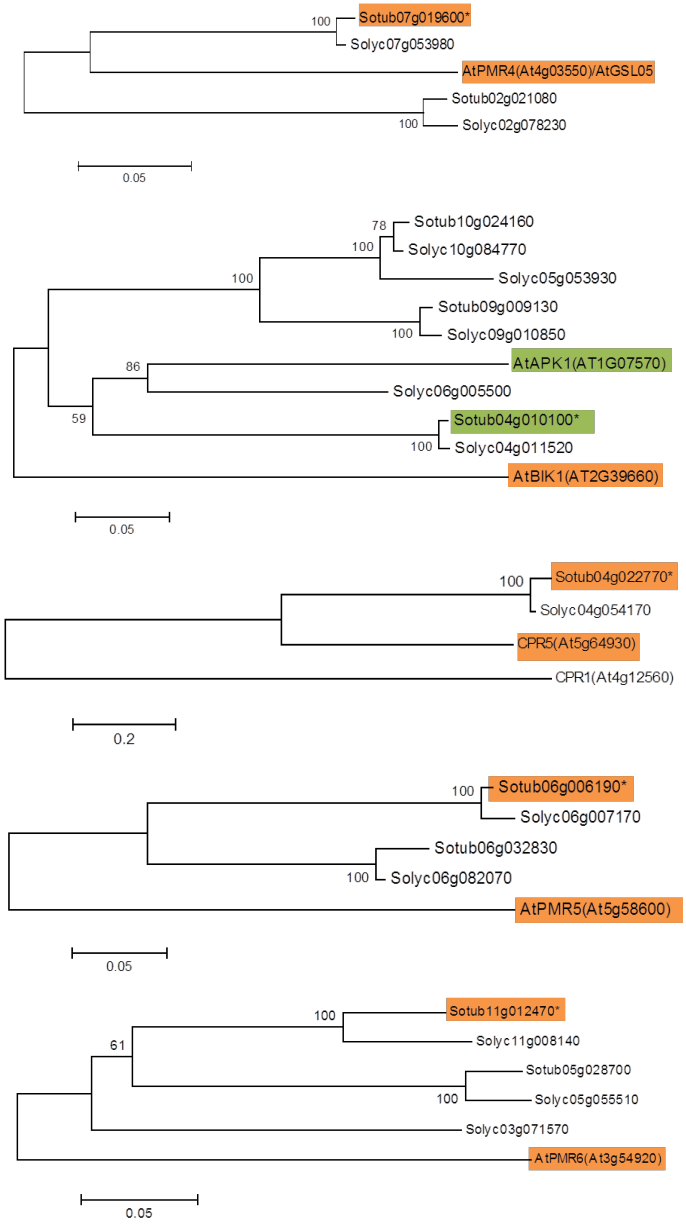
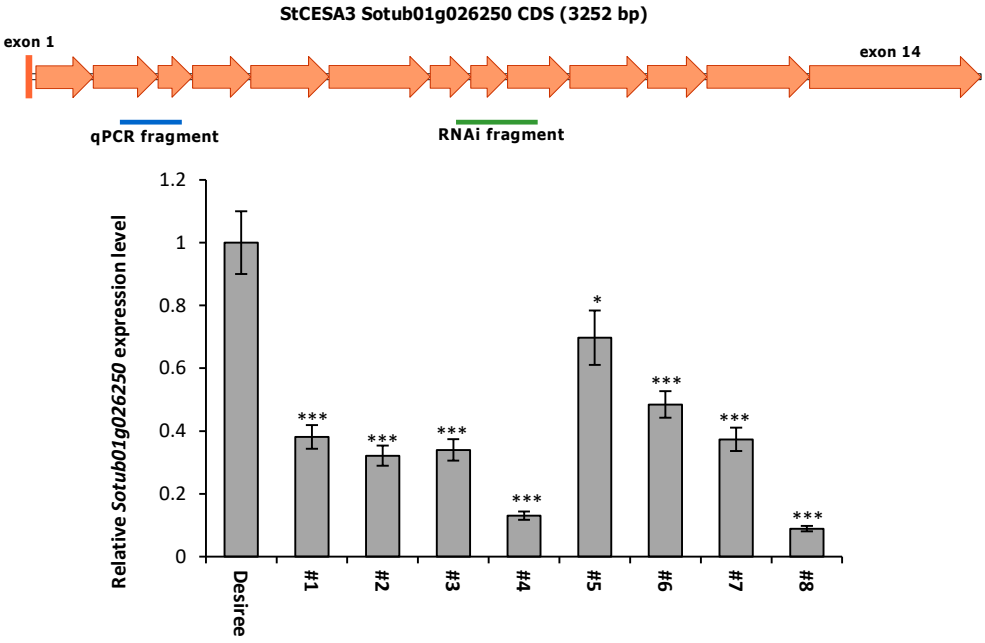
Supplementary Material





Supplementary Fig. 1. Individual phylogenetic trees of all 11 candidate susceptibility genes (*S* genes) including homologs from tomato and potato. Sequences with an asterisk (*) which showed the highest degree of homology with the Arabidopsis *S* gene were considered as the orthologs in potato. These are highlighted in orange, or in green in the case of multiple Arabidopsis *S* genes in the same tree. The scale bar indicates amino acid substitutions per position. In the trees, the numbers above nodes indicate bootstrap support values based on 10,000 replicates.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qPCR fragment
RNAi fragment
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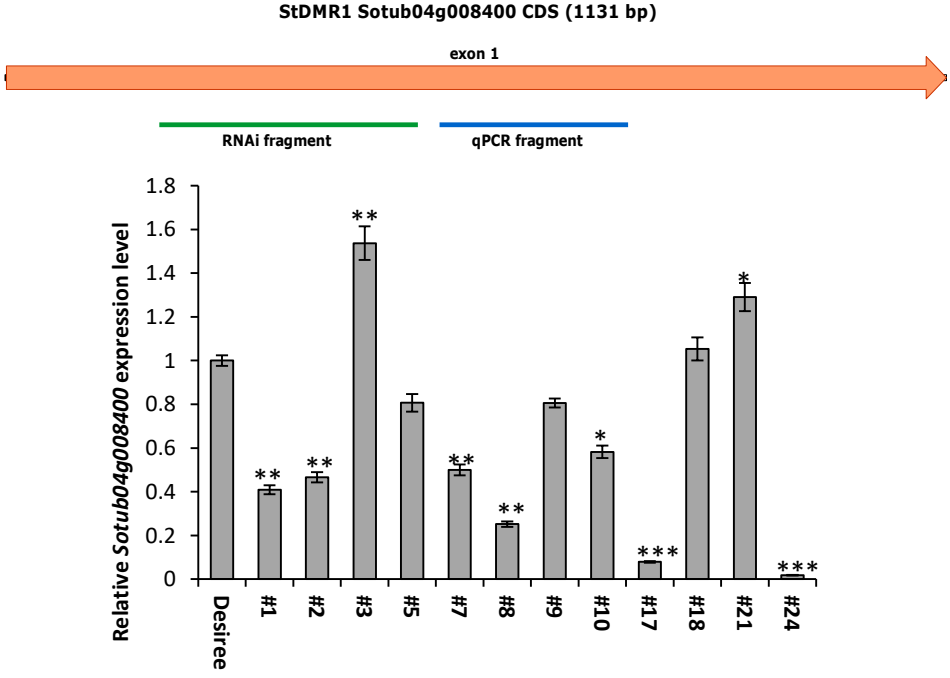
0.4
0.2
0
Desiree
#1
#2
#3
#4
#5
#6
#7
#8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RNAi fragment
qPCR fragment
Relative Sotub04g008400 expression level
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1.4
1.2
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0.8
0.6
0.4
0.2
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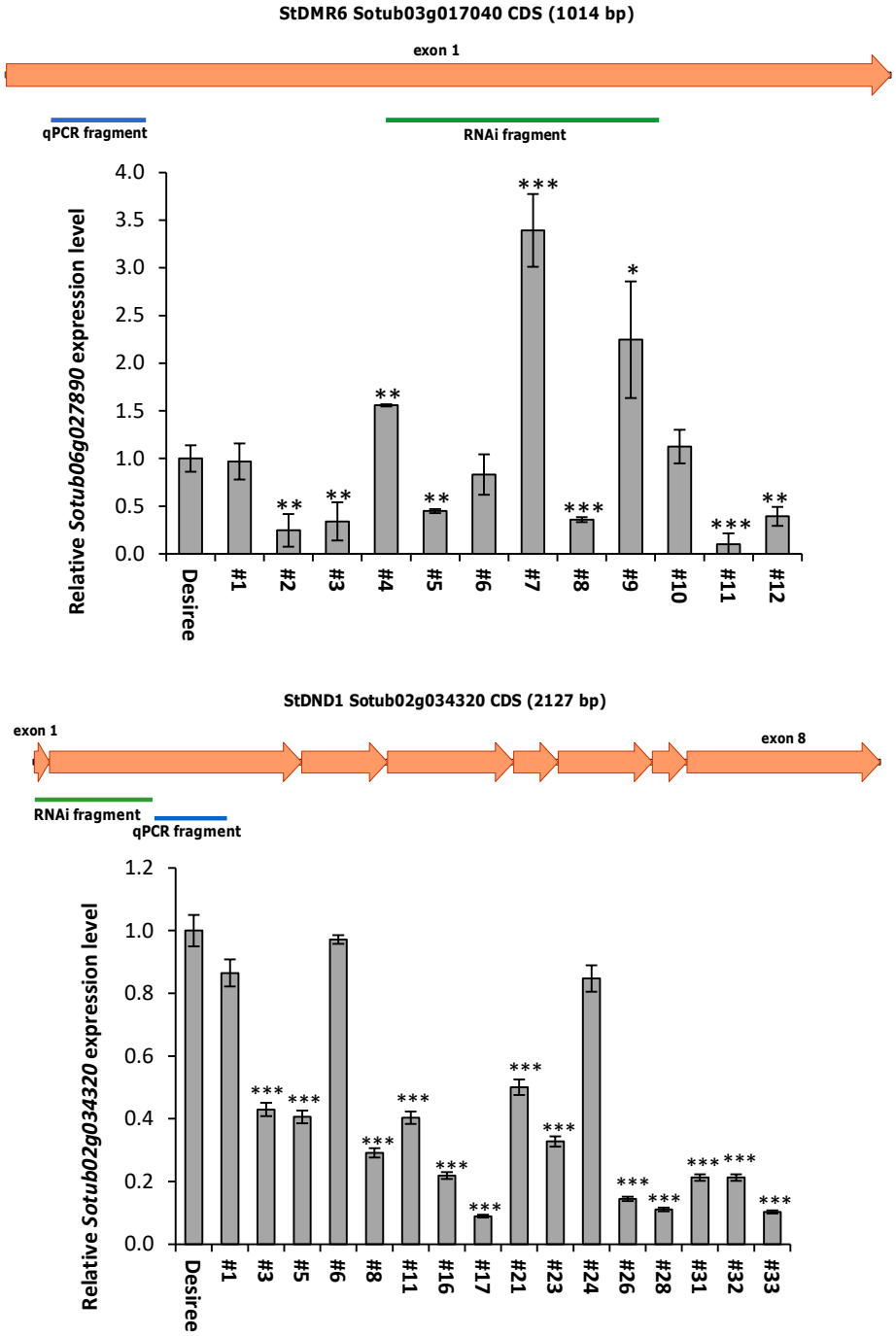
Desiree
#1
#2
#3
#5
#7
#8
#9
#10
#17
#18
#21
#24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qPCR fragment
RNAi fragment
Relative Sotub06g027890 expression level
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3.0
2.5
2.0
1.5
1.0
0.5
0.0
Desiree
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
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exon 1
exon 8
RNAi fragment
qPCR fragment
Relative Sotub02g034320 expression level
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0.6
0.4
0.2
0.0
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#1
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#8
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#23
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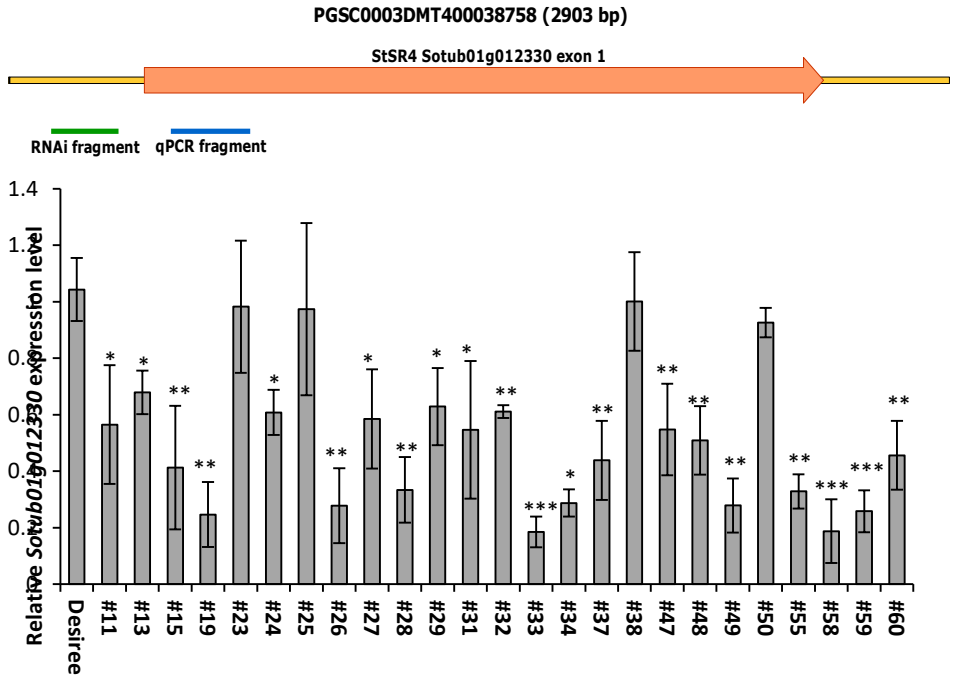
#28
#31
#32
#33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RNAi fragment
qPCR fragment
Relative Sotub01g012330 expression level
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#13
#15
#19
#23
#24
#25
#26
#27
#28
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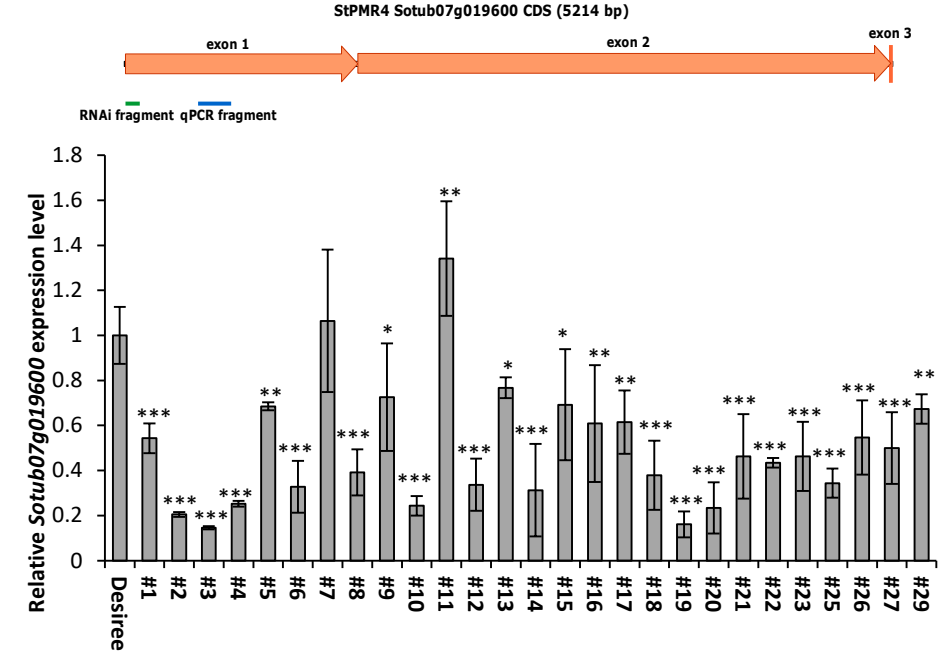
#58
#59
#60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exon 1
exon 2
exon 3
RNAi fragment
qPCR fragment
Relative Sotub07g019600 expression level
Desiree
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
#13
#14
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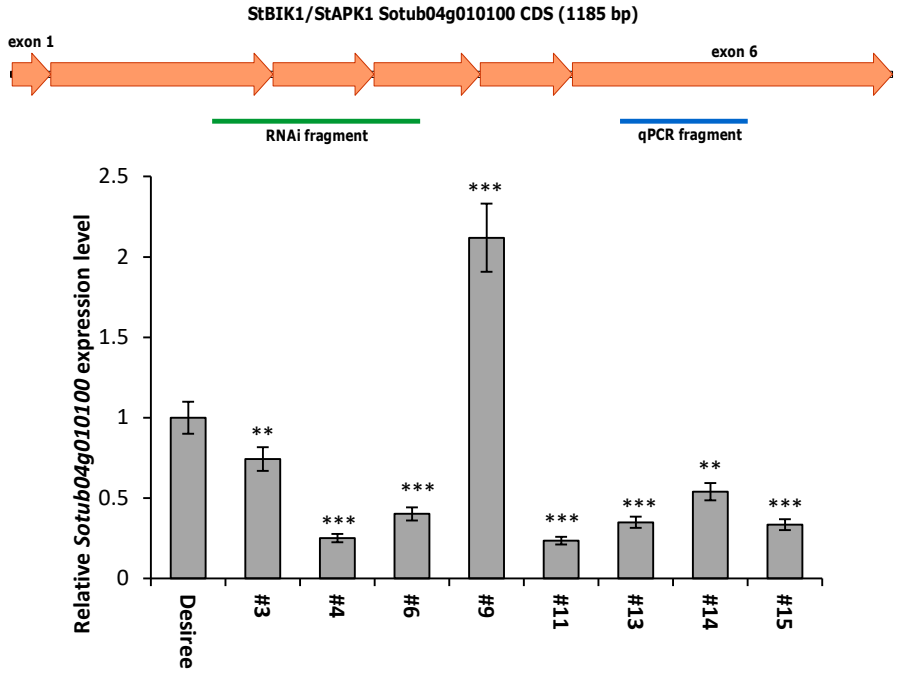
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RNAi fragment
qPCR fragment
Relative Sotub04g010100 expression level
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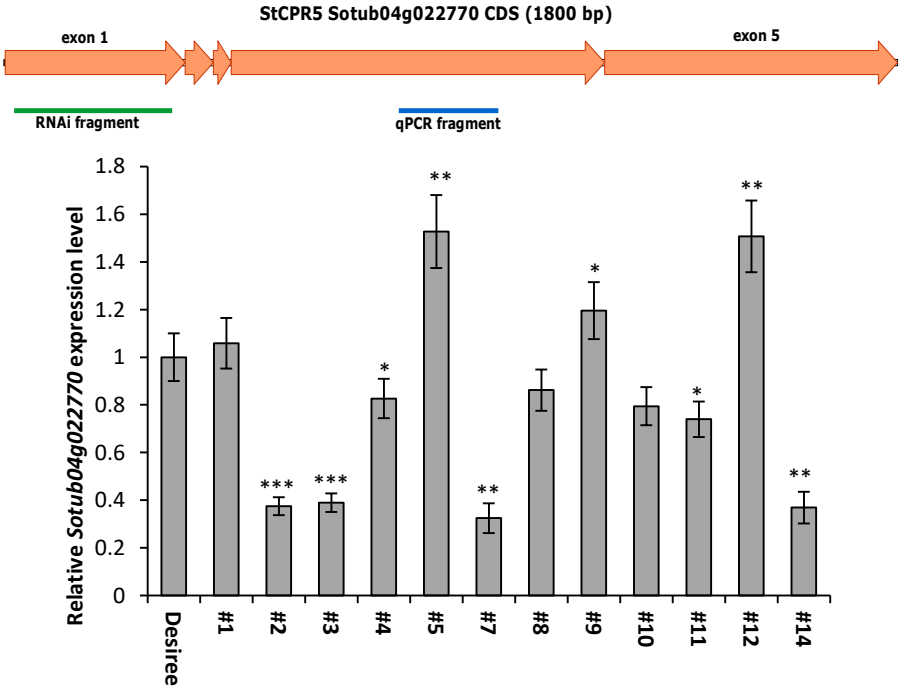
#3
#4
#6
#9
#11
#13
#14
#15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qPCR fragment
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0.6
0.4
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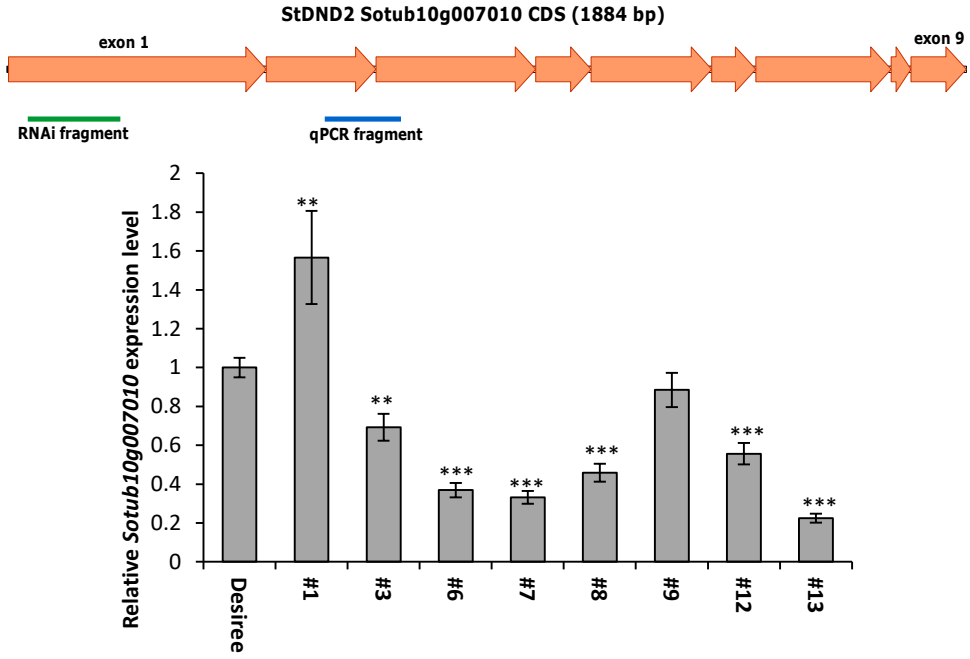
#5
#7
#8
#9
#10
#11
#12
#14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qPCR fragment
Relative Sotub10g007010 expression level
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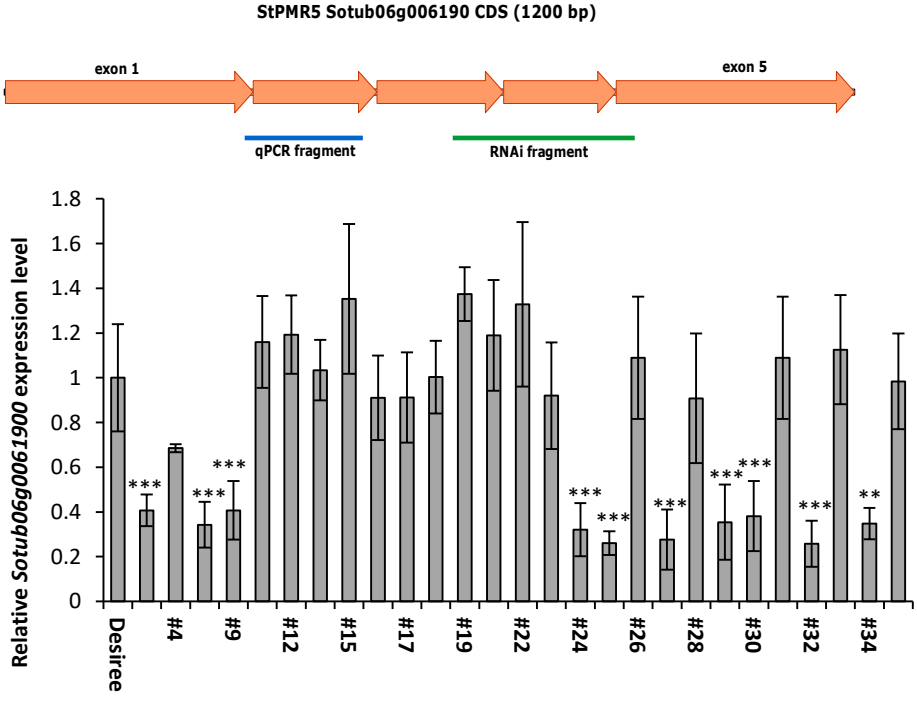
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
***
***
***
***
***
Desiree
#1
#3
#6
#7
#8
#9
#12
#13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qPCR fragment
RNAi fragment
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1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
***
***
***
***
***
***
**
Desiree
#4
#9
#12
#15
#17
#19
#22
#24
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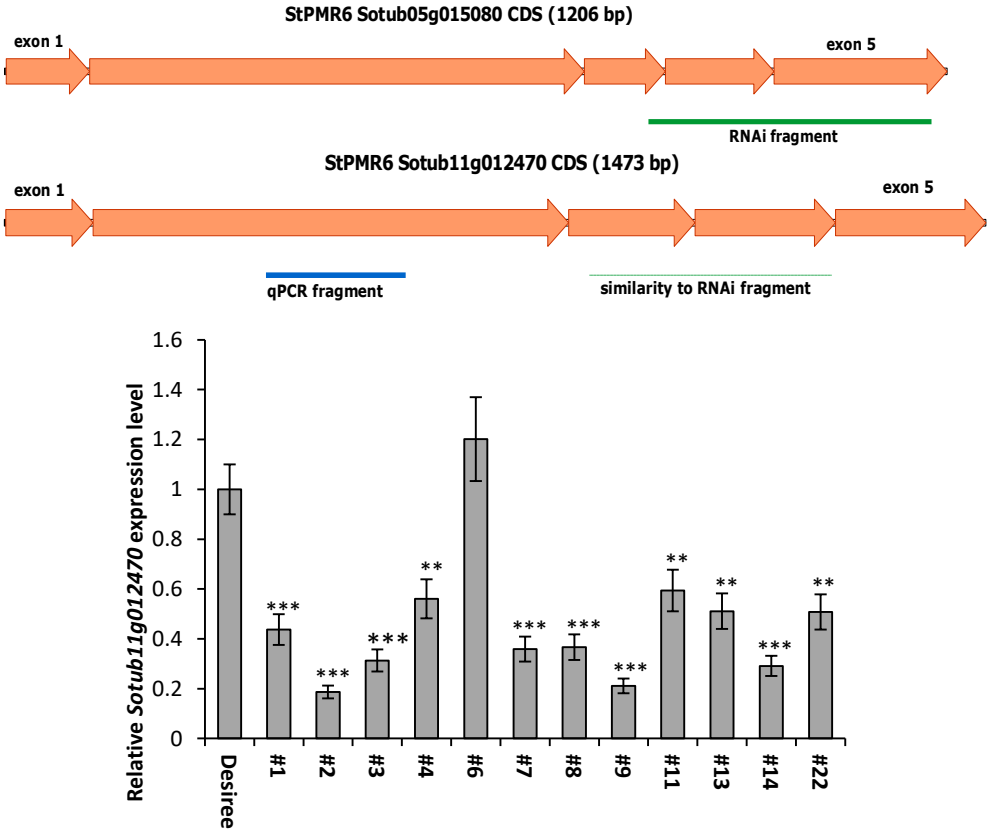
#28
#30
#32
#34



Supplementary Fig. 2. Information of RNAi silencing constructs and silencing effects in RNAi potato transformants. Location of the targeted regions of the RNAi constructs and qPCR fragments in 11 susceptibility genes (*S* genes). Relative expression level of 11 potential *S* genes in leaves of Desiree and independent potato RNAi transformants. Asterisks indicate degree of significant difference compared to Desiree plants (*p<0.05, **p<0.01, ***p<0.001)

Supplementary Table 1. Primers used in this study

Primer name	Sequence (5' to 3')	Used for	Gene
Fw-SlCESA3	caccGCACACCATGGCCTGGAAATAACACC	RNAi::*SlCESA3* cloning	Solyc01g087210
Rv-SlCESA3	AAAAGCACATTGCTTCTCTCAAAGCC		
Fw-StCESA3-qPCR	GAAGCAAAAAGTGGCTGACC	Determining relative transcript levels	Sotub01g026250
Rv-StCESA3-qPCR	GATGTGTTTTGCACCACCAG		
Fw-SlDMR1	caccAGTCGTTTGCTCCGGCCACTG	RNAi::*SlDMR1* cloning	Solyc04g008760
Rv-SlDMR1	CGCCGCAGCACTAGCAGCAC		
Fw-StDMR1-qPCR	TGGACGGAAGTTGAGTGTTG	Determining relative transcript levels	Sotub01g026250
Rv-StDMR1-qPCR	CCCTCATCTTCTTGGTTGGA		
Fw-SlDND1	caccTGTCTTCTCACCAAGACGTCCGC	RNAi::*SlDND1* cloning	Solyc02g088560
Rv-SlDND1	CGATGTGTGCCGGCTGCGAC		
Fw-StDND1-qPCR	GTTCGGGCGTGTATTAGACC	Determining relative transcript levels	Sotub02g034320
Rv-StDND1-qPCR	GAATCACCGTGACGATAGCC		
Fw-SlDMR6	caccTCATCTTTCAGGGAAATCGTGAGCAG	RNAi::*SlDMR6* cloning	Solyc03g080190
Rv-SlDMR6	TGACAAAGGCGTCAGGTTGAGG		
Fw-StDMR6-qPCR	AGTTACATCCGACCCGAATC	Determining relative transcript levels	Sotub06g027890
Rv-StDMR6-qPCR	GACGGATTATTTGAGCTTGGTC		
Fw-StSR4	caccAGAATAGGTGCCTCCCGTCT	RNAi::*StSR4* cloning	PGSC0003DMT400038758/ Sotub01g012330
Rv-StSR4	GCGACCTCGAGAACTCCATA		
Fw-StSR4-qPCR	CTGGTAAAGCTCTGGCGAAC	Determining relative transcript levels	Sotub01g012330
Rv-StSR4-qPCR	GCAGTCTCCACCGATCTCTC		
	ATGAGCCTCCGGCAACGTTCAACGCCGGCGGCGAGACAAGTTTCTATAGATGAAGAACCATATAACATCATTCCGATTCATAATCTTCTAGCTGACC*	RNAi::*SlPMR4* cloning	Solyc07g053980
Fw-StPMR4-qPCR	GGGGTGAGTCAGCGAATCTA	Determining relative transcript levels	Sotub07g019600
Rv-StPMR4-qPCR	GGGCAGTACCATTCCGACTA		
Fw-SlBIK1	caccAGCTGCAAGGCCAGGGACTG	RNAi::*SlBIK1* cloning	Solyc04g011520
Rv-SlBIK1	AGCTGCCTCGAGAGCAACCTTC		
Fw-StBIK1-qPCR	TCGTCGAGTGATGGACAAAA	Determining relative transcript levels	Sotub04g010100
Rv-StBIK1-qPCR	CAGGCATTTGACAGCAAGAA		
Fw-SlCPR5	caccTCTCAACCCGTCGATCCGCC	RNAi::*SlCPR5* cloning	Solyc04g054170
Rv-SlCPR5	TGCCGAGAGGAAGTGCAAGTG		
Fw-StCPR5-qPCR	GCAATTGGCTTCTGCTTCTT	Determining relative transcript levels	Sotub04g022770
Rv-StCPR5-qPCR	CTCTCCAAAAGGTTCGCATC		

*Artificial sequence

Supplementary Table 1 (continued)

Primer name	Sequence (5' to 3')	Used for	Gene
Fw-StDND2	caccTGACGATGAAGATGAAGATGATG	RNAi::*StDND2* cloning	Sotub10g007010
Rv-StDND2	CCCATTGTGCTTTAGGGTCT		
Fw-StDND2-qPCR	TTTGCCTCTTGAGAAGAATGC	Determining relative transcript levels	Sotub10g007010
Rv-StDND2-qPCR	ATTTGGCTGCCCTTTGAATA		
Fw-SlPMR5	caccTGCTTGGAGAGGTGTTGATGTCTTG	RNAi::*SlPMR5* cloning	Solyc06g007170
Rv-SlPMR5	ACGTGCCGGTGGTCCAATCG		
Fw-StPMR5-qPCR	TGAAATTCCAAGGTTCAATGG	Determining relative transcript levels	Sotub06g006190
Rv-StPMR5-qPCR	GAGGGTCACCCGTGATGTAT		
Fw-SlPMR6	caccGGCTTGTGCAAAGGATGCCAAGATG	RNAi::SlPMR6 cloning	Solyc05g014000
Rv-SlPMR6	TGCAACCAAGTGCACCAGCATTC		
Fw-StPMR6-qPCR	CCGTAATCCAGGAAGAACCA	Determining relative transcript levels	Sotub11g012470
Rv-StPMR6-qPCR	TATTCCCCGACGGTAAACAG		
Fw-NPT II	ACTGGGCACAACAGACAATC	Verification of transgenic plants	
Rv-NPT II	TCGTCCTGCAGTTCATTCAG		
Fw-StEF1a	ATTGGAAATGGATATGCTCCA	Normalisation in qRT-PCR assays	Sotub06g010680
Rv-StEF1a	TCCTTACCTGAACGCCTGTCA		

Supplementary Table 2. Details of susceptibility genes identified in Arabidopsis

Gene name	Subcellular location	Function	Susceptibility mechanism	References
CESA3	Golgi apparatus, endosome, integral component of membrane, plasma membrane, plasmodesma, trans-Golgi network	Cellulose synthase	Probably DAMP induced defense suppression (JA, ET)	Ellis et al. (2002); Ellis and Turner (2001)
DMR1	Chloroplast, chloroplast stroma	Homoserine kinase	Amino acid metabolism	Huibers et al. (2013); Van Damme et al. (2005); Van Damme et al. (2009)
DMR6	Cytoplasm	2-oxoglutarate-Fe oxygenase	Defense suppression (SA?)	Van Damme et al. (2005); Van Damme et al. (2008)
DND1	Plasma membrane	Cyclic nucleotide-gated ion channel	Defense suppression (SA, JA), regulator NO synthesis	Ahn (2007); Clough et al. (2000); Genger et al. (2008); Govrin & Levine (2000); Jurkowski et al. (2004); Su'udi et al. (2011)
SR1	Nucleus	Ca^{2+}/calmoduline-binding transcription factor	Defense suppression (SA, ET)	Doherty et al. (2009); Du et al. (2009); Galon et al. (2008); Kim et al. (2013); Laluk et al. (2012); Nie et al. (2012); Qiu et al. (2012)
PMR4	1,3-beta-D-glucan synthase complex, Golgi apparatus, integral component of membrane, plasma membrane, plasmodesma	Callose synthase	Probably DAMP induced defense suppression (SA, JA)	Nishimura et al. (2003)
BIK1	Chloroplast, cytoplasm, nucleolus, nucleus, plasma membrane	Membrane-anchored protein kinase	Defense suppression PTI (SA?, ET)	Veronese et al. (2006)
CPR5	Chloroplast, integral component of membrane, membrane, nucleus	Transmembrane protein	Defense suppression (SA, JA, ET)	Bowling et al. (1997); Jing et al. (2007); Jing & Dijkwel (2008); Love et al. (2007)
DND2	Integral component of plasma membrane, membrane, plasma membrane	Cyclic nucleotide-gated ion channel	Defense suppression (SA, JA), regulator NO synthesis	Ahn (2007); Clough et al. (2000); Genger et al. (2008); Govrin & Levine (2000); Jurkowski et al. (2004); Su'udi et al. (2011)
PMR5	Endoplasmic reticulum, integral component of membrane	Unknown	Altered pectin composition of cell wall affecting either nutrition or endoreduplication	Chandran et al. (2013); Vogel et al. (2004)
PMR6	Anchored component of membrane, anchored component of plasma membrane, plasma membrane	Pectate lyase-like protein	Pectin accumulation in the extrahaustorial matrix possibly resulting in decreased nutrient availability to the pathogen	Chandran et al. (2013); Vogel et al. (2002)

CHAPTER 4

Down-regulation of *Arabidopsis DND1* orthologs in potato and tomato leads to broad-spectrum resistance to late blight and powdery mildew

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Abstract

Multiple Susceptibility genes (*S*), identified in *Arabidopsis*, have been shown to be functionally conserved in crop plants. Mutations in these *S* genes result in resistance to different pathogens, opening a new way to achieve plant disease resistance. The aim of this study was to investigate the role of *DND1* (*Defense No Death*) in susceptibility of tomato and potato to late blight (*Phytophthora infestans*). In *Arabidopsis*, the *dnd1* mutant has broad-spectrum resistance against several fungal, bacterial, and viral pathogens. However this mutation is also associated with a dwarfed phenotype. Using an RNAi approach, we silenced *AtDND1* orthologs in potato and tomato. Our results showed that silencing of the *DND1* ortholog in both crops resulted in resistance to the pathogenic oomycete *P. infestans* and to two powdery mildew species, *Oidium neolycopersici* and *Golovinomyces orontii.* The resistance to *P. infestans* in potato was effective to four different isolates although the level of resistance (complete or partial) was dependent on the aggressiveness of the isolate. In tomato, *DND1*-silenced plants showed a severe dwarf phenotype and autonecrosis, whereas *DND1*-silenced potato plants were not dwarfed and showed a less pronounced autonecrosis. Our results indicate that *S* gene function of *DND1* is conserved in tomato and potato. We discuss the possibilities of using RNAi silencing or loss-of-function mutations of *DND1* orthologs, as well as additional *S* gene orthologs from *Arabidopsis,* to breed for resistance to pathogens in crop plants.

Keywords: late blight; powdery mildew; resistance; RNAi; susceptibility (*S*) gene; *DND1*

Introduction

Potato and tomato are important food crops that are grown worldwide. Diseases such as powdery mildew and late blight, cause substantial economic losses in these crops. Potato late blight, which is caused by the oomycete *Phytophthora infestans*, has been the most devastating potato disease over 150 years (Fry & Goodwin 1997; Haverkort et al. 2008). Currently, the most effective way of controlling the damage by *P. infestans* is by regular application of fungicide to protect both the infected foliage and tubers. The frequent fungicide applications not only raises environmental concerns, but also gives rise to fungicide-resistant strains of *P. infestans* (Altamiranda et al. 2008; Eschen-Lippold et al. 2010; Bengtsson et al. 2013).

The most common method of resistance breeding to late blight is the introgression of major resistance genes (*R*). After the massive outbreak of late blight in the 1850's in potato, efforts to detect *R* genes have been made in the 20th century. This resulted in identification and use of a number of *R* genes (NBS-LRR genes e.g. *R1, R2, R3,* and *R10*) from the Mexican wild species *Solanum demissum* (Malcolmson 1969; Wastie 1991). The introgression of *R* genes into cultivated potato species through interspecific crosses and backcrosses can be time consuming and associated with linkage drag (Schouten et al. 2006). Resistance mediated by *R* genes is frequently associated with a hypersensitive reaction (HR), as a result of the interaction between the *R* gene and a specific avirulence gene of the pathogen (Lokossou et al. 2010). Due to new virulent races of *P. infestans,* introduced *R* genes were rapidly overcome by the pathogen after only a few years in the field (Vleeshouwers et al. 2011). An option to achieve a broad spectrum, and more durable resistance, is to stack *R* genes against many isolates (Kim & Hwang 2012; Zhu et al. 2012). In recent years, additional late blight *R* genes have been identified and cloned from other *Solanum* species, including *S. bulbocastanum*, *S. stoloniferum*, *S. venturii*, *S. mochiquense*, and *S. chacoense* (Vossen et al. 2011). The cloning and pyramiding of these *R* genes is facilitated by the availability of genome sequences for potato and tomato as well as information on *P. infestans* effectors (Jo et al. 2011; Vleeshouwers et al. 2011; Gebhardt 2013). Also, rotation of potato cultivars carrying different *R* genes is a good option to extend the effectivity of resistance conferred by *R* genes in the field.

Supplementary to the use of *R* genes, in 2010 we proposed a novel breeding strategy based on "loss-of-function of susceptibility genes" for durable and broad-spectrum resistance (Pavan et al. 2010). Plant genes coding for susceptibility factors are named susceptibility genes (*S* genes), which can be functionally divided into immunity-related and immunity-unrelated groups (Hückelhoven et al. 2013). Immunity-related *S* genes

play a role in plant defense responses, while immunity-unrelated genes provide entrance, accommodation and nutrients for pathogens. In a recent review (van Schie & Takken 2014), *S* genes were divided into three groups based on the time of their action. The first group (*S* gene allowing basic compatibility) includes those that provide early pathogen establishment, such as the *MLO* (*Mildew resistance Locus O*) gene, encoding a membrane-anchored protein (Büschges et al. 1997) that is required for susceptibility to powdery mildews. The second group (*S* genes encoding immune suppressors) contains those that interfere negatively with host defense responses such as the *Cellulose synthase 3* (*CESA3*) gene. The *CESA3* (*cev1*) mutant shows constitutively activated JA and ethylene defense signalling pathways. The third group (*S* genes allowing sustained compatibility) covers those involved in feeding of the pathogen, such as the rice *Xa13* and *Xa 25* genes. These two genes encode sugar transporters providing nutrients to the pathogen, *Xanthomonas oryzae* (Chen et al. 2010).

From a genetic point of view, we consider a gene as an *S* gene, when impairment of its function can lead to recessively inherited resistance (Pavan et al. 2010). In *Arabidopsis*, many loss-of-function mutations are known to cause pathogen resistance. Examples are *pmr* (powdery mildew resistance; Vogel & Somerville 2000; Vogel et al. 2002) and *dmr* mutants (downy mildew resistance to *Hyaloperonospora parasitica*; McDowell et al. 2005). However, only few *S* genes have been tested for their conserved function in crops. The best-studied example is the *Mlo* gene. Mutations in certain *Mlo* homologs in plant species (e.g. barley *Mlo*, *Arabidopsis AtMlo2*, pea *PsMlo1* and tomato *SlMlo1)* result in disease resistance by preventing the penetration of the adapted powdery mildew species (e.g. Bai et al. 2008; Pavan et al. 2011; Zheng et al. 2013). The first identified barley *mlo* mutant has been successfully used in barley cultivars in European agriculture for more than 35 years, illustrating the durability of *mlo*-based resistance (Lyngkjaer et al. 2000; Humphry et al. 2006).

Our research is aimed at identifying *S* genes for susceptibility to different pathogens in tomato and potato. We have tested the susceptibility function of several *S* genes identified in *Arabidopsis* and demonstrated that in tomato, silencing *PMR* and *DMR* orthologs gives rise to resistance against the mildew fungus *Oidium neolycopersici* (Huibers et al. 2013). Our results suggest that *S* genes such as *MLO*, *PMR* and *DMR* genes are functionally conserved among plant species and that silencing orthologs in other plant species can give resistance to fungi. In this study, we focused on the *Arabidopsis AtDND1* (*Defense No Death 1*) gene (Yu et al. 1998) encoding a cyclic nucleotide-gated cation channel (CNGC; also known as *AtCNGC2*, Clough et al. 2000). In *Arabidopsis*, *dnd1* mutants were reported to show resistance to *Pseudomonas syringae* and other fungal and viral pathogens (Yu et al. 2000; Jurkowski

et al. 2004; Genger et al. 2008). Here, we show that silencing tomato and potato *DND1* orthologs resulted in resistance to both late blight and powdery mildew.

Materials and Methods

Plant growth and cultivation

Tomato (*S. lycopersicum* cultivar Moneymaker, MM), tetraploid potato *S. tuberosum* cultivar Desiree, diploid potato genotype SH83-92-488 (SH) (van de Voort et al. 1997) and potato transformant A13-013 carrying the *Rpi-vnt1.1* resistance gene in Desiree background (Foster et al. 2009; Pel et al. 2009) were used. Plants were grown in greenhouses at 21°C and 19°C during the 16h day and 8h night periods respectively. Relative humidity was 70% and light intensity was supplemented with 100 W/m^2 when light intensity dropped below 150 W/m^2. Potato transgenic plants were also grown in large 5-liter pots in an insect-free screen cage on a concrete floor under outdoor summer conditions. All transgenic plants were grown with a GMO permit according to Dutch GMO regulations.

Identification of tomato and potato *DND1* orthologs

The *Arabidopsis thaliana* DND1 (GenBank accession number BAE99132) amino acid sequence was used as a query in a BLASTP program against the Sol Genomics Network (SGN) Tomato Genome protein sequences (ITAG release 2.40) or Potato PGSC DM v3.4 protein sequences database (http://solgenomics.net/tools/blast/) or against an *Arabidopsis* protein database (http://www.arabidopsis.org/Blast/index.jsp) to search for homologous sequences in tomato, potato and *Arabidopsis*, respectively. The alignment of the protein sequences was done by DNASTAR Lasergene 8 and phylogenetic trees were constructed by using Mega4.0 (Tamura et al. 2007). The evolutionary history was inferred using the neighbour-joining method (Saitou & Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown next to the branches.

Generation of RNAi transgenic plants

The binary vector pHellsgate 8 (CSIRO, Australia) was used to generate RNAi constructs (Waterhouse and Helliwell 2003; Helliwell and Waterhouse 2003) with a CaMV35S promoter and containing a kanamycin resistance gene as selectable marker. Primers were designed to amplify fragments of the target genes ranging from 150bp to 300bp (Supplementary Table 1). The forward primer contained CACC at the 5′ end for TOPO cloning. Tomato MM cDNA was used as PCR template. The PCR products

were cloned into the pENTR/D-TOPO cloning vector (Invitrogen, USA) and were sequenced. The final RNAi vector was produced by an LR clonase reaction between the entry clone and pHellsgate8 vectors. Transformation of MM seeds was carried out as described by Huibers et al. (2013). For potato transformation the protocol described by Visser (1991) was used.

Nucleic acid extraction and RNA expression analyses.

Nucleic acid extraction was carried out as described by Huibers et al. (2013). Quantitive real-time PCR (qRT-PCR) was performed to determine the expression levels of potato and tomato *DND1* in different tissues without pathogen stress conditions. Tomato RNA samples were isolated from 12-week-old MM plants grown in the greenhouse. Potato samples were isolated from 8-week-old Desiree plants from the field. The qPCR was performed in triplicate with a C1000 light cycler system (Bio-Rad) using SYBR Green mix (Bio-Rad) with gene-specific primers for *DND1* (Supplementary Table 1). qRT-PCR data for *DND1* were compared with RNAseq data from potato and tomato. Potato FPKM values for *Sotub02g034320* were obtained from the Spud DB website (http://solanaceae.plantbiology.msu.edu/index.shtml). Tomato RPKM values for *Solyc02g088560* were obtained from http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi using library D004: Transcriptome analysis of various tissues in tomato cultivar Heinz and the wild relative *Solanum pimpinellifolium*.

The expression level of *StPR1* (GenBank AJ250136) in Desiree and transgenic potato plants was analyzed by qRT-PCR with RNA samples isolated from six-week-old plants grown in the greenhouse. In addition, *StPR1* expression was analyzed in selected transgenic plants compared with wild type Desiree after mock inoculation or inoculation of six-week-old plants grown in the greenhouse with *P. infestans* isolate Pic99189. Gene-specific primers are shown in Supplementary Table 1. Relative quantification was performed by qRT-PCR using the ΔΔCt method (Livak & Schmittgen, 2001). The potato and tomato elongation factor 1-a (EF1a, *Sotub06g010680* and *Solyc06g005060*) transcripts were used as the internal control to calculate the relative transcript levels. The gene-specific primers used for development of RNAi constructs, qPCR, and *O. neolycopersici* quantification are shown in Supplementary Table 1. For the qRT-PCR assays, three technical replicates were performed for each experiment and the expression of each gene was investigated in three biological replicates.

Disease assays

For detached leaf assays (DLA, Vleeshouwers et al. 1999) with *P. infestans*, several isolates were used. Information on virulence factors, mating type and data concerning the collection of *P. infestans* isolates is presented in Supplementary Table 2. These isolates were cultured on rye sucrose medium (Caten & Jinks 1968) in the dark at 15°C for 10-14 days. Sporulating mycelium was flooded with cold water and the sporangiospore suspension was incubated at 4°C for 1-2 hours. The inoculum was adjusted to a concentration of 5×10^4 zoospores/ml. Potato leaves were taken from plants grown in the greenhouse. The lesion size on leaflets was measured using a calliper with digital display (DIGI-MET®, Helios Preisser, Germany) at 3-6 days after inoculation. For potato tuber assays, the protocol described in Zhu et al. (2012) was applied. Tubers were taken from both greenhouse and screen cage-grown plants. Two assays with greenhouse tubers, and one assay with screen cage tubers were performed. For each transformant, four plants were tested as biological replicates.

For the test with tomato powdery mildew, the Wageningen isolate of *Oidium neolycopersici,* On-Ne (Bai et al. 2005) was used which has been maintained on plants of MM. Spore suspensions were obtained by washing heavily infected MM leaves in water. Tomato plants of about 4 weeks old were sprayed with an inoculum of 2.5x 10^4 spores per ml. Fungal quantification by qPCR was performed on leaf samples collected between 8-14 days post inoculation (dpi). *Oidium* Internal Transcribed Spacer (ITS)-specific primers (Huibers et al. 2013) are shown in Supplementary Table 1.

For the nematode test, *Globodera rostochiensis* line 19 (the Ro1 pathotype) was used. Potato plants were obtained from sprouting tubers in sterile clay pots covered with sterile sandy soil. Four to five weeks after tuber planting the rooting plants were inoculated with the nematodes. To collect the eggs from the cysts, the cysts were soaked overnight in water. Then the cysts were crushed with a special glass pestle. After that, the suspension was filtered over a 100µm sieve. The final number of eggs for inoculation was around 10.000 eggs per pot. The cysts were extracted from roots 6 weeks post inoculation and counted by following the protocol described in Van Bezooijen (2006).

For disease test with potato virus Y (PVY), the PVY^{NTN} strain (isolate PRI-757) was used which was maintained in tobacco plants, *Nicotiana tabacum* cultivar Samsun. Potato transformants were regenerated from *in vitro* plantlets and grown in the greenhouse in soil under controlled environmental conditions (16/8 light/dark cycle, 24 °C) for 4 weeks before *PVY* inoculation. Three newly developed upper leaves were dusted with carborundum powder and rubbed with cheesecloth dipped in a sap

prepared from the leaves of the *PVY*-infected tobacco plants. After 10 min, the leaves were washed liberally with tap water. In mock inoculations, water was used instead of the sap. The presence of PVY^{NTN} in plants was monitored using a polyclonal rabbit antiserum, raised against purified virus, in a double antibody sandwich (DAS) enzyme-linked immuno-absorbent assay (ELISA) (Van den Heuvel & Peters 1989). All plants, both the inoculated plants and the mock non-inoculated plants were checked for the presence or absence of virus 14 dpi by DAS-ELISA. Each treatment had four plants as biological replicates.

Pathogen identification

To verify the identity of powdery mildew species affecting potato in the greenhouse, DNA was extracted from powdery mildew infected leaves using DNeasy® Plant Mini Kit (QIAGEN, Germany). Fungal ITS primers, ITS5A (5'-TTGGAAGTAAAAGTCGTAAC-3', derived from ITS5) and ITS4 (5'-TCCTCCGCTTATTGATAGC-3') were used (White et al. 1990). The PCR products were sequenced and blasted against the NCBI database (http://www.nibi.nlm.nih.gov/BLAST/).

Time lapse video

A time lapse video was produced to visualize *P. infestans* development on leaves of wild type potato Desiree and transgenic RNAi::DND1A plants. A 12-cm square Petri dish was filled with autoclaved demineralized water containing 1% agar to a thickness of 5 mm. Detached potato leaflets were transferred into the Petri dish and kept until the medium was solidified. Leaflets were taken from the same plants as those used in the DLA (see above). They were inoculated with *P. infestans* isolate Pic99189 zoospores using the same inoculum concentration as in the DLA (5×10^4 zoospores/ml). Then, the Petri dish was sealed with Parafilm and placed in a closed box containing a Nikon D90 DSLR camera with a Nikon 60 mm lens. To achieve a 16 h light / 8 h dark cycle, the Petri dish was illuminated with Philips LED modules (4x white, 2x blue and 2x deep red modules; light intensity 37.9µE m-2 s-1) connected to a microprocessor (Arduino Uno) controlling the light. With the help of Nikon Camera Control Pro 2 software, photos were automatically taken once every fifteen minutes. The video is played back at 10 frames per second, and edited using Adobe Photoshop Lightroom 5 software. Depending on the growth conditions such as light and composition of the medium in this experiment, plants can be observed in the Time lapse system for up to five days.

Results

Identification of tomato and potato *DND1* orthologs

Using BLAST analysis we identified a single tomato gene (*Solyc02g088560*) encoding a protein with a high level of amino acid identity (75%) with AtDND1 (*At5g15410*), and hereafter referred to as *SlDND1*. In potato, a single gene was also identified (*Sotub02g034320*) encoding a protein having 75% and 99% identity with *Arabidopsis* AtDND1 or tomato SlDND1, respectively (Supplementary Fig. 1a), hereafter referred to as *StDND1*. In *Arabidopsis* there are 20 CNGC genes (Chin et al. 2013). In a protein-based phylogenetic tree SlDND1 and StDND1 showed higher homology to AtDND1 (CNGC2) than to any other *Arabidopsis* CNGC family member (Supplementary Fig. 1b). Therefore, *SlDND1* and *StDND1* are the most likely candidates for being *AtDND1* orthologs.

Transcript levels of *DND1* in various tissues of tomato and potato

Expression levels of *DND1* were examined in leaf, root, flower, fruit (tomato only), and tuber (potato only) samples by quantitative real-time PCR. As shown in Supplementary Fig. 2, *DND1* mRNA was detected in all tissues, but at different levels. The *DND1* expression level was highest in flowers, more than 15 fold as high as that of the leaves. In contrast, *DND1* transcripts were low in roots, tomato fruit or potato tubers (Supplementary Fig. 2a & b). These measured expression levels of *DND1* correlated well with RNAseq data (RPKM or FPKM values) for tomato cultivar Heinz and potato genotype RH (Supplementary Figure 2c,d respectively).

StDND1-silenced potato plants are resistant to late blight

Two RNAi constructs (RNAi::DND1A and RNAi::DND1B*)* were made based on the tomato *SlDND1* sequence (Supplementary Fig. 3a). These two constructs were designed to silence both tomato and potato *DND1* orthologs (Supplementary Fig. 3b), with RNAi::DND1A targeting the 5' part of the coding sequence and RNAi::DND1B targeting the 3' end.

Both RNAi constructs were used to transform potato cultivar Desiree, and in total 187 transgenic plants were obtained. For each RNAi construct, eight plants were randomly selected and the expression level of *StDND1* was measured (Supplementary Fig. 3c). Compared with Desiree, the expression level of *StDND1* in the transformants was reduced 10-90%. Three transformants of each construct, DND1A-5, -8 -17 and DND1B-6, -8, -11, which had a high silencing level of *StDND1* (well-silenced and indicated as +) and a plant morphology and growth similar to wild type Desiree, were

selected for further experiments. As negative controls, transformants with a low degree of silencing (indicated as –), DND1A-6 and DND1B-4, were selected and included in further analyses. The selected plants were multiplied by *in vitro* cuttings and tubers were harvested after growth of these cuttings in the greenhouse.

To assess the response of the selected plants to *P. infestans* infection, they were inoculated with the isolate Pic99189 in a DLA assay. The expression level of *StDND1* was verified (Fig. 1a). Infection lesion diameter on infected leaves was determined daily from 3 to 6 dpi (Fig. 1b). The resistant control A13-013 carrying the *Rpi-vnt1.1* gene showed no lesions during the whole period. In contrast, leaves of the susceptible control Desiree were almost fully covered by late-blight mycelia at 7 dpi. Three DND1A (+) transformants and two DND1B (+) transformants were as resistant as A13-013. Only DND1B-8(+) showed restricted lesion growth and a more variable level of *StDND1* expression compared to the other (+) transformants (Fig. 1a). Plants of the two *DND1* weak-silenced (–) transformants were as susceptible as Desiree (Fig. 1c). Development of symptoms on detached leaves of Desiree and transformants DND1A-6(–), DND1A-5(+) and DND1A-17(+) is also shown in the time lapse movie Supplementary Video 1. The transformants in the DLA assay were also subjected to a tuber slice assay with *P. infestans*. Similar to the DLA results, tubers of the well-silenced transformants showed significantly lower levels of *StDND1* expression (Fig. 2a) and almost no late blight sporulation compared to Desiree and the weak-silenced transformants (Fig. 2b).

For both DLA and tuber assays, three independent experiments were performed with similar results, demonstrating that the RNAi plants in which *StDND1* is well silenced could lead to complete resistance to *P. infestans* isolate Pic99189 in both leaves and tubers. To analyse whether the resistance observed under greenhouse conditions was stable in other environmental conditions, a tuber slice assay was performed using tubers that developed on plants grown in a screen cage under outdoor conditions. Results obtained with these tubers (Supplementary Fig. 4) were similar to those from greenhouse-grown tubers (Fig. 2b).

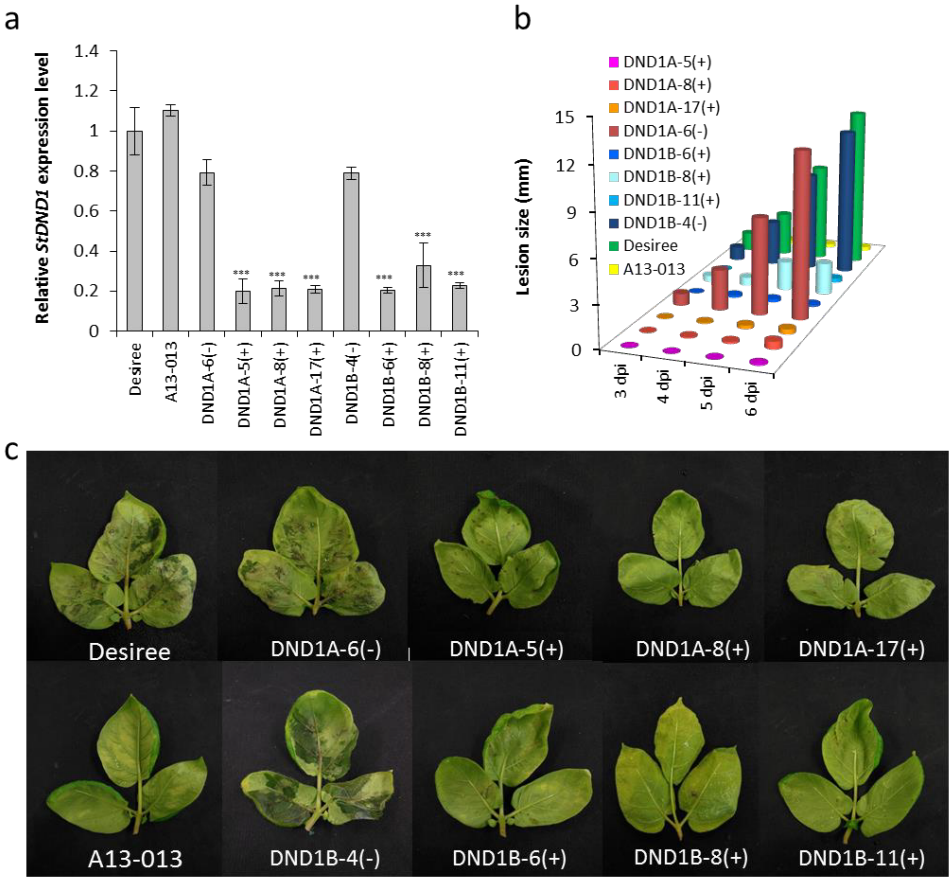

Fig. 1 Late blight resistance in potato leaves by silencing *StDND1*. **a** Relative expression level of *StDND1* in leaves of Desiree, A13-013 and eight independent potato RNAi::*DND1* plants (DND1A-5, 6, 8 and 17, DND1B-4, 6, 8 and 11). **b** Development of lesion size on plants infected with *Phytophthora infestans* isolate Pic99189. Data were collected at 3, 4, 5 and 6 days post inoculation (dpi). Well-silenced (+) plants showed significantly lower growth rate compared to untransformed Desiree and weak-silenced (-) plants. **c** Pictures of potato leaves taken at 7dpi. Note: the same plants were used for all the measurements. For each transformant, four plants were tested (one leaf per plant). Three independent experiments were performed with similar results.

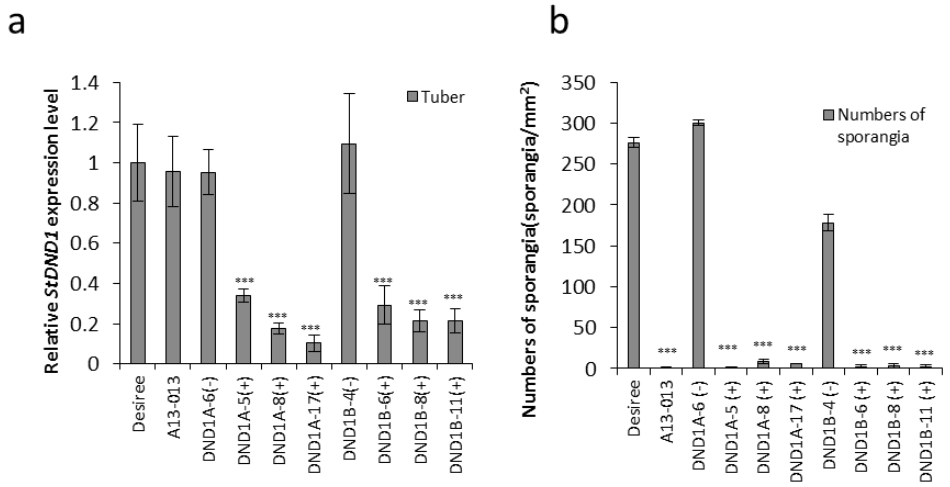

Fig. 2 Late blight resistance in potato tubers by silencing *StDND1*. **a** Relative expression level of *StDND1* in tubers of Desiree, A13-013 and eight independent potato RNAi::*StDND1* plants (DND1A-5, 6, 8 and 17, DND1B-4, 6, 8 and 11). **b** Numbers of sporangia on tubers infected with *Phytophthora infestans* isolate Pic99189. For each transformant, four plants were tested (one tuber per plant). Asterisks indicate degree of significance compared to Desiree plants (***p<0.001).

4

***StDND1*-silenced potato plants show resistance to multiple isolates of late blight**

In order to investigate whether *StDND1*-silenced potato plants show race non-specific resistance to *P. infestans*, the above tested RNAi transformants were challenged in a DLA assay with three additional *P. infestans* isolates; Pic99177, USA618 and EC#1. Desiree was considered as the susceptible control and A13-013 as the resistant control for isolates Pic99177 and USA618. Both Desiree and A13-013 are susceptible to the aggressive isolate EC#1. Development of lesion sizes on the infected leaves was determined from 3 to 7 dpi (Fig. 3). The experiment with the aggressive isolate EC#1 was, however, terminated at 6 dpi because of serious disease symptoms. Similar to the results obtained with the isolate Pic99189, RNAi plants in which *StDND1* is well-silenced, showed resistance to isolates Pic99177 and USA618 (Fig. 3). With the aggressive isolate EC#1 significantly smaller lesion sizes were observed for *StDND1* well-silenced (+) transformants compared to Desiree and A13-013 (Fig. 3). In general, lesion growth on the susceptible plants (Desiree, A13-013 and non-silenced transformants) started from 3 dpi, while DND1A (+) and DND1B (+) transformants showed a delayed and restricted lesion growth starting from 4 dpi. These experiments were performed twice with similar results, showing that the acquired resistance by

silencing of *DND1* in potato is not specific to the tested isolates, though the resistance level may partially depend on the aggressiveness of the *P. infestans* isolates.

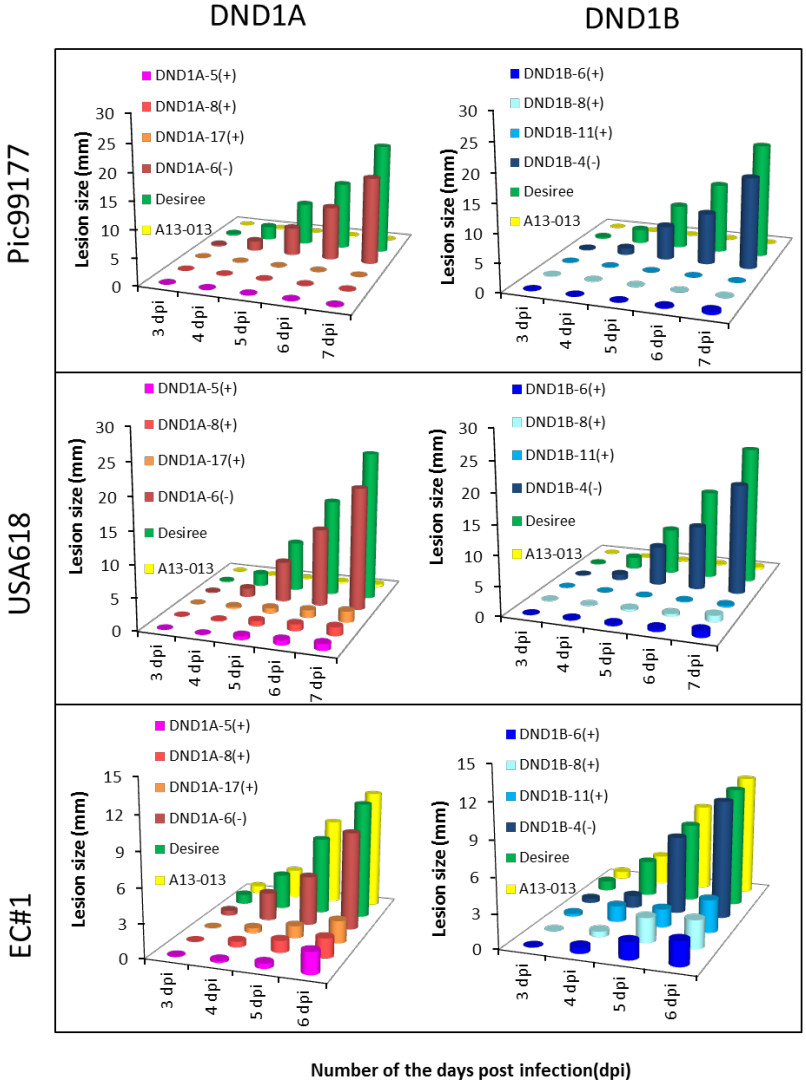

Fig. 3 Broad spectrum resistance to late blight in potato by silencing *StDND1*. Graphs show the development of lesion size of infected leaves of different genotypes after inoculation with three *Phytophthora infestans* isolates Pic99177, USA618 and EC#1. Cuttings from the same transformants as in Fig. 1 and 2 were used. For each transformant, four plants were used. From each plant, three leaves were detached and each was inoculated with one isolate. The experiment was repeated twice with similar results.

StDND1-silenced potato plants are resistant to powdery mildew

When potato plants are grown in the greenhouse they can be naturally infected by powdery mildew (Supplementary Fig. 5a). To verify the identity of the powdery mildew species infecting potato plants, DNA was isolated from infected potato leaves and used as a template for PCR amplification with fungal ITS (Internal Transcribed Spacer) primers (Supplementary Fig. 5b). The sequence amplified in this ITS region was identical to that of *Golovinomyces orontii* MUMH 2003 (GenBank AB427188, Uchida et al. 2009). Sporulation (characterised with a Disease Index (DI) score of about 2.5, Supplementary Fig. 5c) was observed on the upper side of older leaves of Desiree as well as on old leaves of the two weak-silenced transformants, DND1A-6(–) and DND1B-4(–). In contrast, no fungal sporulation (DI score of 0, Supplementary Fig. 5c) was observed on leaves of well-silenced (+) transformants. Natural infection was uniform and plants were randomized. Fungal biomass was quantified (Supplementary Fig. 5d). All *StDND1*-silenced (+) plants showed significantly lower fungal biomass compared to the controls (Desiree and A13-013) and *StDND1*(–) plants.

Resistance by silencing of *DND1* is specific to certain pathogens

To test whether *DND1*-silenced plants show resistance to a broad range of pathogens, *StDND1*-silenced potato plants were tested with nematode *Globodera rostochiensis* and potato virus Y (*PVY*). Compared with the susceptible control Desiree plants, all *StDND1*-silenced potato plants were susceptible to *PVY* (Supplementary Fig. 6). In the nematode test, diploid potato genotype SH carrying the *H1* resistance gene was the resistant control. SH showed almost no cysts growing on the roots, while Desiree showed a high number of cysts (on average 250 per gram dry root weight (Supplementary Fig. 7). A lower number of cysts was observed on the well-silenced *DND1* transformants. This reduction was however in general not significantly different from cyst number in Desiree.

Silencing of tomato *SlDND1* results in powdery mildew and late blight resistance

Tomato plants of cultivar Moneymaker (MM) were transformed with RNAi::DND1A and the expression level of *SlDND1* was measured in leaves of 5-week-old T1 plants. Among 10 transformants, three T1 plants (T1#2, #5 and #6) showed about 80% reduction of *SlDND1* expression, compared to MM (Fig. 4a). These three independent *SlDND1* silenced T1 transformants and untransformed MM were multiplied by cuttings. Ten cuttings of each T1 plant were inoculated with *O. neolycopersici*. To assess the disease severity, fungal biomass was measured. Compared with MM,

significant lower amounts of fungal biomass were detected in these three T1 plants (Fig. 4b).

These T1 plants were maintained for seed production. Unfortunately, due to low male fertility of the T1 transformants, selfed progeny could only be obtained from one T1 plant; T1#2. The resulting T2 family (T2#2) was inoculated with *O. neolycopersici*, and Disease Index (DI) was scored at 10 dpi. The T2#2 plants harbouring a silencing construct (+) showed significantly lower DI score compared to untransformed MM plants and T2#2 plants not harbouring a silencing construct (–) (Fig. 4c). The same T2#2 plants were tested by DLA with spores of *P. infestans* isolate Pic99177. A significant difference in DI scores at 6 dpi was observed between T2#2 plants harbouring (+) and not carrying (–) the silencing construct (Fig. 4d). Thus, silencing of *SlDND1* in tomato resulted in resistance to powdery mildew (*O. neolycopersici*) and *P. infestans* showing agreement with the results obtained in potato.

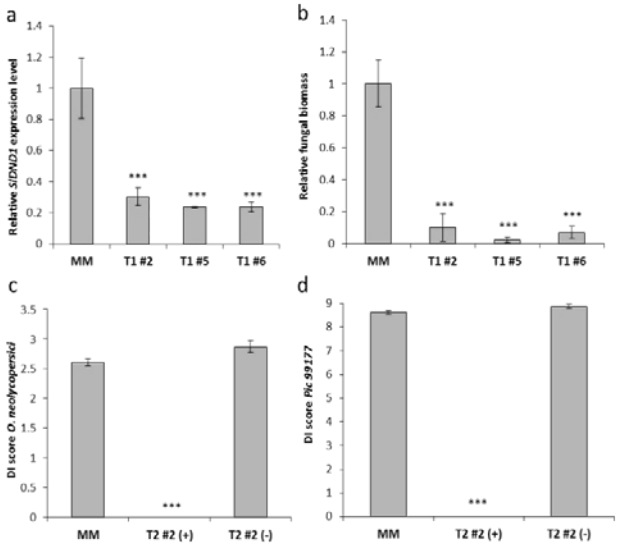

Fig. 4 Powdery mildew and late blight resistance in tomato by impairment of *SlDND1*. **a** Relative *SlDND1* transcript levels in plants of untransformed cultivar Moneymaker (MM) and 3 independent T1 plants (T1 #2, #5 and #6) transformed with a silencing construct specifically targeting *SlDND1*. **b** Relative fungal biomass of *Oidium neolycopersici* on MM and the 3 T1 Plants in (**a**) measured 9 days post inoculation (dpi). **c** Disease index (DI) scores of T2 progeny (T2#2 family derived from T1#2) harbouring a *SlDND1* silencing construct (+, n=13) or not (-, n=1) at 10 dpi with *O. neolycopersici*. DI scores range from 0 (resistant) to 3 (susceptible). **d** DI scores of the same plants as in (**c**) but inoculated with *Phytophthora infestans* isolate Pic99177. DI scores range from 0 (resistant) to 9 (susceptible) and represent a mean of 5 leaflets of the 3rd leaf (from the bottom) per plant. After harvesting the 3rd leaf for testing with *P. infestans*, the same plant was then inoculated with *O. neolycopersici.* For both powdery mildew and late blight, a significant reduction in DI scores was observed on the T2 plants harbouring a silencing

construct (+) compared to plants of MM and T2 plants not harbouring a silencing construct (-). Asterisks indicate degree of significance compared to MM (***p<0.001).

Silencing of *DND1* leads to side effects

Strong RNAi silencing of *SlDND1* in tomato, resulted in two phenotypic side effects, dwarfing and necrosis (Fig. 5a). Autonecrosis (spontaneous cell death without pathogen infection) was observed on non-inoculated leaves of tomato transformants. Autonecrosis was also found on leaves of *StDND1*-silenced potato plants (Fig. 5b) However, it was much less severe than that in the tomato *SlDND1*-silenced transformants. In tomato, necrotic spots were present on both old and young leaves, while in potato necrosis occurred only on older leaves at greenhouse conditions. When the potato *StDND1*-silenced transformants were grown in a screen cage under outdoor conditions the plants looked healthy. Only after three months, very small autonecrotic spots developed on the older leaves (Supplementary Fig. 8).

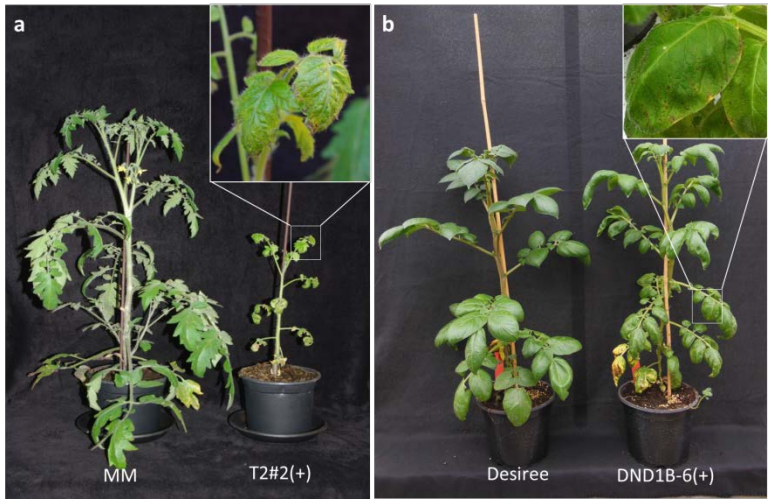

Fig. 5 Morphological alterations observed on tomato and potato by impairment of *DND1* orthologs. **a** Picture of tomato cultivar Moneymaker (MM) compared to *SlDND1*-silenced MM transformant T2#2(+). Both plants were five weeks old. The T2#2(+) plant shows a dwarf phenotype and autonecrotic spots on all leaves. **b** Photo of a potato cultivar Desiree plant compared to *StDND1*-silenced Desiree transformant DND1B-6(+). The DND1B-6(+) plant shows similar growth vigour as Desiree. Autonecrotic spots are visible on older leaves only, not on younger leaves.

Expression of defense-related gene *StPR1* in *StDND1*-silenced potato

In the *Arabidopsis dnd1* mutant an elevated level of salicylic acid (SA) and a constitutively high expression level of *PR1* (pathogenesis related protein 1) have been reported (Clough et al. 2000; Ahn 2007). To gain some insight in the mechanism of

resistance in *DND1*-silenced potato transformants transcript levels of *StPR1* were monitored in Desiree and the *DND1* transformants by qRT-PCR. First, the expression level was measured in all eight potato transformants studied without pathogen infection. All six well-silenced transformants (+) showed a high constitutive expression level compared with Desiree, while the weak-silenced transformants (–) showed the same low level of expression as Desiree (Supplementary Fig. 9a). Next, the influence of inoculation with *P. infestans* on *StPR1* expression level was measured in three selected transformants; two well-silenced (+) and one weak-silenced (–) transformant compared with Desiree. Expression was measured in both mock-inoculated and *P. infestans*-inoculated leaves. As is shown in Supplementary Fig. 9b the expression of *StPR1* was highly up-regulated 6 days after *P. infestans* inoculaton in both the transformants and Desiree. However, the level of expression was significantly higher in *DND1* well-silenced (+) transformants compared with Desiree and weak-silenced (–) transformants. Thus, both the constitutive and the pathogen-induced level of expression of *StPR1* was increased after silencing *StDND1*.

Discussion

Loss of susceptibility factor as an alternative disease resistance type

To combat *P. infestans*, a highly flexible pathogenic oomycete, it is important to identify new types of resistance, in addition to the classical *R* gene-based resistance (Vleeshouwers et al. 2008). Recessive resistance, not associated with HR reaction, is widely used in crops to provide resistance against viruses, such as resistance to *PVY* in pepper or to *Bymoviruses BaYMV and BaMMV* in barley (Ruffel et al. 2002; Stein et al. 2005). Recently, this kind of resistance was also reported against other pathogens, such as the recessive resistance alleles *rk1* and *rhg1* against root-knot nematodes in cowpea and cyst nematodes in soybean, respectively (Das et al. 2008; Afzal et al. 2009). The observation in *Arabidopsis* that loss-of-function mutations in many more *S* genes can lead to resistance to pathogens is remarkable. Evidence is accumulating that the susceptibility function of these *Arabidopsis S* genes is conserved across plant species. For example, down regulation of orthologs of *PMR4* and *MLO* leads to powdery mildew resistance in tomato (Bai et al. 2008; Huibers et al. 2013). More surprisingly, resistance resulting after silencing of orthologs of these *Arabidopsis S* genes in most cases appears to be effective against a wide range of pathogens. For example, the *Arabidopsis dmr1* gene was originally identified in a screen for resistance to downy mildew caused by *H. parasitica*, and silencing the tomato ortholog resulted in powdery mildew resistance (Huibers et al. 2013). The *Arabidopsis dnd1* mutant was previously reported to be resistant to bacterial, fungal and viral diseases (Yu et al. 1998). In this

study, we show for the first time that the *DND1* gene can be considered as an *S* gene for late blight and powdery mildew diseases. It is likely that a susceptibility factor encoded by *S* genes is used by multiple pathogens to promote disease development. Therefore, the identified *Arabidopsis S* genes can be tested in crops to achieve broad-spectrum resistance via loss-of-function mutations and/or RNAi technique. Both approaches are possible in self-fertilizing diploid crops like tomato, while, in tetraploid potato, RNAi is the most realistic possibility. The advantage of the RNAi approach is that resistance obtained via RNAi silencing behaves as a dominant resistance trait, which is preferable for practical breeding programs especially of polyploid, vegetatively propagated crops. Very recently, it was shown that reduced expression of the putative *S* gene *SYNTAXIN-RELATED1* (*StSYR1*), an ortholog of *AtSYP121/AtPEN1* of *Arabidopsis*, decreased susceptibility to *P. infestans* in potato via the RNAi approach (Eschen-Lippold et al. 2012a & b).

Fitness costs associated with loss of susceptibility factors can be plant-species dependent

S genes are expected to have important biological functions in plants besides being a susceptibility factor (Pavan et al. 2010). For example, the rice *Xa13* gene is required for both the growth of *X. oryzae* and for pollen development (Chu et al. 2006). Thus, resistance to pathogens achieved by silencing of *S* genes is potentially associated with negative side effects on other agronomic traits, as shown by the severe dwarfing and autonecrosis in *DND1*-silenced tomato plants (Fig. 5a). Therefore, more phenotypic studies are needed to determine whether loss-of-function of *DND1* can lead to plants which are acceptable for agriculture/horticulture. Such plants can then also be used as a new source of resistance in breeding. This resistance is inherited recessively after mutation induction or dominantly after RNAi transformation. Among the *StDND1*-silenced potato transformants we could already select plants with a good level of resistance combined with an almost normal phenotype in the screen cage (Supplementary Fig. 8a). These transformants were obtained with an RNAi construct containing the strong 35S promoter. We will try using a native promoter for the expression of the RNAi construct to investigate whether it is possible to obtain plants with a high level of late blight resistance but without fitness costs.

The phenotypic effects of dwarfing and autonecrosis were also described for the original *dnd1* mutant in *Arabidopsis* (Clough et al. 2000). In *DND1*-silenced tomato plants, it was shown that male fertility was decreased and thus offspring after selfing could not be obtained from all transformants. In *Arabidopsis*, DND1/CNGC2 has been reported to play a role in flowering timing and fertility (Chaiwongsar et al. 2009; Chin

et al. 2013). Fortuna et al. (2015) reported that the role of DND1 in flowering timing is independent from SA accumulation, and independent from its role in pathogen defense. Further investigations are necessary to determine whether disease resistance can be achieved in tomato without reduced fertility by decreased expression level or altered functionality of *SlDND1*.

Interestingly, a much lower level of autonecrosis was found in *DND1*-silenced potato plants compared to tomato, although they were obtained using the same silencing constrcuts. Surprisingly, dwarfing was not observed at all in *DND1*-silenced potato plants under the tested growth conditions, indicating that the fitness costs associated with loss-of-function of *S* genes seems to depend on the plant species. One explanation for this apparent difference between tomato and potato could be the higher ploidy level combined with a high level of heterozygosity (multi-allelism) of potato that could provide a better genetic buffer against pleiotropic, multi-genic and multi-allelic effects at expression level than in diploid homozygous tomato (Gebhardt 2013). Alternatively, the fitness costs after knocking-out plant *S* genes may depend on the crop species, as was shown for the *mlo* mutation. In both *Arabidopsis* and barley, *mlo* mutants showed early senescence, which is however not observed in the natural *ol2* mutant of tomato (Bai et al. 2008). Since yield is the final measure of fitness costs, it will be necessary to test *StDND1*-silenced potato plants in the field under natural environmental conditions.

Mechanisms of *dnd1*-mediated resistance

The *DND1* gene was identified in *Arabidopsis* with a screen designed to discover additional components of the *avrRpt2-RPS2* disease resistance pathway (Yu et al. 1998, 2000). Compared to the wild type Col-0, the loss-of-function mutant *dnd1* displayed an inability to produce a HR in response to avirulent isolates of *Pseudomonas syringae* pv. *glycinea*. In addition, the *dnd1* mutant showed reduced growth, a constitutively elevated level of expression of the *PR1* gene, HR-like spontaneous lesions without pathogen infection, and resistance to unrelated pathogens including *H. parasitica, Botrytis cinerea* (Clough et al. 2000) and *Pectobacterium carotovorum* (the bacterial agent causing soft rot disease on Korean cabbage, Ahn 2007). It may be argued that *dnd1*-associated autoimmune responses (such as autonecrosis and dwarfing) result in stressed plants, which in turn may enhance resistance to pathogens. However, we have shown that the *DND1*-silenced potato transformants show resistance to late blight and powdery mildew, a constitutively elevated level of expression of the *StPR1* gene and small autonecrotic lesions, but no reduced growth. Furthermore, the severity of autonecrosis in potato was dependent on environmental conditions. This has also been reported for the *dnd1* mutant in *Arabidopsis* (Clough et al. 2000; Moeder et al. 2011).

However, we observed that the level of pathogen resistance was not influenced by environmental conditions, at least in the tuber slice assay. In addition, there is evidence showing that defense-related signalling pathways are required for resistance in the *dnd1* mutants, but not for autoimmune phenotypes (Clough et al. 2000; Jirage et al. 2001; Jurkowski et al. 2004). Thus, the resistance resulting from loss-of-function of *DND1* is not necessarily associated with severe autoimmune phenotypes.

The *DND1* gene encodes a cyclic nucleotide-gated cation channel (CNGC) belonging to a large family consisting of 20 members in *Arabidopsis* (Chin et al. 2013). The CNGCs have a role in conducting Ca^{2+} into plant cells and are involved in various physiological processes (Sherman & Fromm 2009). The precise role of *AtCNGC2 (DND1)* in plant defense responses remains elusive, although evidence has been obtained that activation of multiple defense pathways are required for the broad-spectrum resistance in *dnd1* mutants (Genger et al. 2008; Moeder et al. 2011). For example, the resistance to *P. syringae* and *H. parasitica* in *dnd1* mutants is dependent on SA accumulation (Genger et al. 2008). Further, the PR1 protein has been shown to have inhibitory activity against *P. infestans* (Niderman et al. 1995). Thus, the highly elevated *PR1* expression in DND1-silenced plants may lead to increased PR1 protein content, which can play an important role in resistance against *P. infestans*. Resistance to *B. cinerea* could be abolished by disrupting ethylene signalling (Genger et al. 2008). Further experiments are needed to study the resistance mechanisms associated with the resistance to late blight and powdery mildew in *DND1*-silenced potato and tomato plants.

Perspectives of *S* genes for resistance breeding

Deployment of plant *S* genes in breeding crops with resistance to pathogens and pests is a relatively new approach as proposed by us in 2010 (Pavan et al. 2010). In the past few years, more and more examples have arisen, where resistance can be achieved by altering plant genes that are required by pathogens to establish a compatible interaction with hosts (reviewed in Van Schie & Takken 2014). Here, we show that *dnd1*, which plays a pivotal role in disease resistance, may be an interesting gene in resistance breeding of crops. Although we observed negative side effects upon gene silencing via RNAi, especially in tomato, it may be possible to minimise these fitness costs by investigating sufficient numbers of RNAi transformation events, by transformation of different genotypes and/or by sexual crossing followed by selection for offspring plants with normal phenotypes in the presence of pathogen resistance. The high level of heterozygosity and multi-allelism in potato could open up the possibility to select for a more favourable genetic background when using this approach. As shown in this study,

silencing *DND1* resulted in less fitness costs in potato than in tomato (Fig. 5). In diploid crops like tomato, variation in loss-of-function mutations can be obtained by assessing natural mutations and mutations induced by chemical mutagenesis, such as EMS (Emmanuel & Levy 2002; Minoia et al. 2010). In potato, the approach using mutation induction is possible, as earlier shown for the recessive *amf* mutant with amylose-free starch (Hovenkamp-Hermelink et al. 1987). However, breeding at the tetraploid level with recessive traits is complicated, although it has recently become more realistic by using allele-specific markers. This approach would be much more appropriate after realisation of the method of hybrid seed propagated potato varieties which are based on a cross between self-compatible diploid homozygous parental lines (Lindhout et al. 2011). Nowadays, new techniques are available to enable the discovery and design of superior allele variants, which combine favourable disease resistance levels with no or less detrimental side effects. Such advanced technologies include TILLING (targeting induced local lesions in genomes); site-directed mutagenesis using Zinc-finger nucleases (Colbert et al. 2001), TALEN (Transcription activator-like effector nuclease)-based gene editing for allele design (Lloyd et al. 2005; Li et al. 2012) and CRISPR-/Cas9 (clustered regularly interspaced short palindromic repeats–associated nuclease Cas9) (Belhaj et al. 2013; Xie & Yang. 2013). For example, the TALEN technology was successfully used to edit the rice gene *Os11N3* for bacterial blight susceptibility resulting in a specific allele altering this *S* gene function (Li et al. 2012). Combining TALEN and CRISPR-/Cas9 techniques, Wang et al. (2014) simultaneously edited three homeoalleles of the *MLO* gene in hexaploid wheat providing resistance to powdery mildew. Taken together, these approaches hold substantial promise in defining and deploying altered host *S* genes for durable resistance in controlling plant diseases (Dangl et al. 2013).

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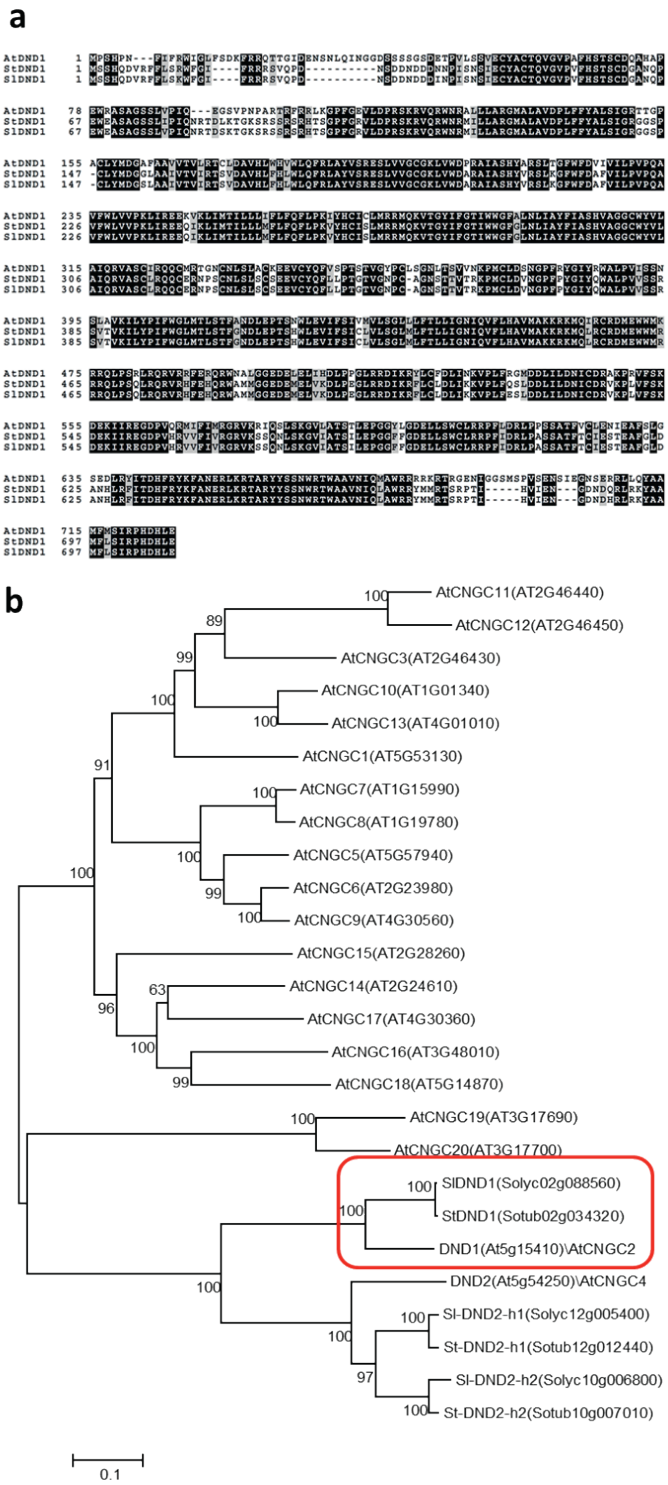
Supplementary Material

Supplementary Table 1. Primers used in this study

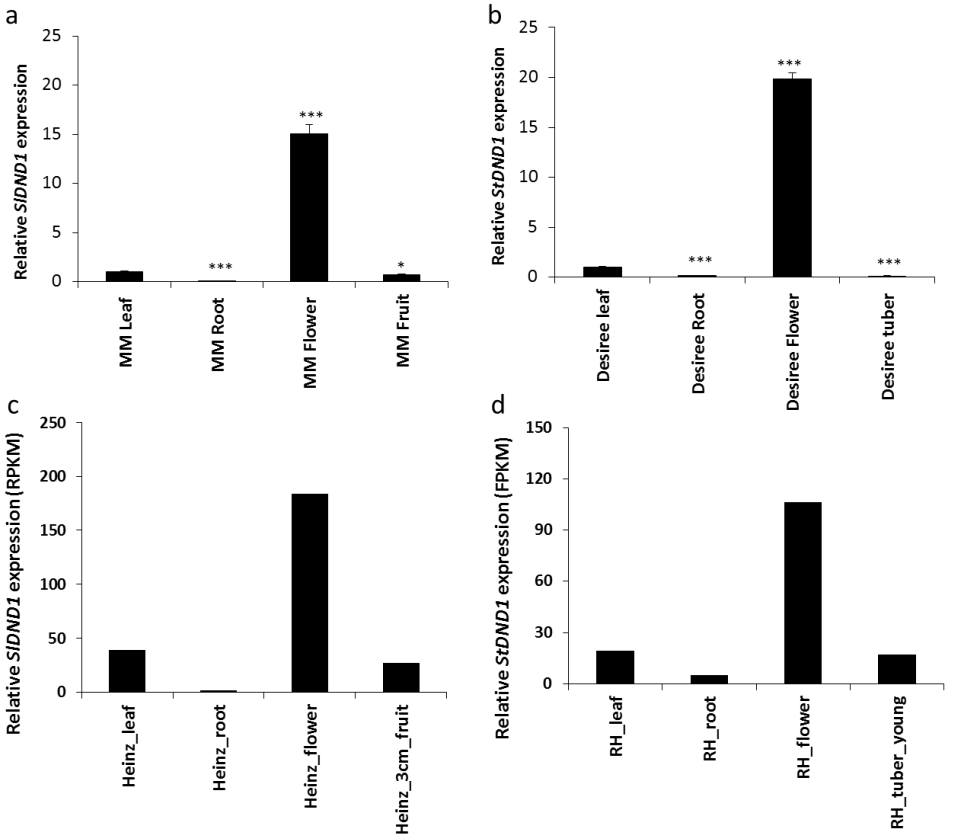
Primer name	Sequence (5' to 3')	Gene/Accession	Used for
Fw-DND1A	caccTGTCTTCTCACCAAGACGTCCGC	Solyc02g088560	RNAi:: DND1A cloning
Rv-DND1A	CGATGTGTGCCGGCTGCGAC		
Fw-DND1B	caccTGATGAGGACTAGCCGTCCCAC	Solyc02g088560	RNAi:: DND1B cloning
Rv-DND1B	AGCAAATAATTCCCGGGCCCTTC		
Fw-SlDND1-qPCR	GGGCCGTTCGGGCGTGTATT	Solyc02g088560	Determining relative transcript levels
Rv-SlDND1-qPCR	CCACCGCGGCCGATGGATAA		
Fw-StDND1-qPCR	GTTCGGGCGTGTATTAGACC	Sotub02g034320	Determining relative transcript levels
Rv-StDND1-qPCR	GAATCACCGTGACGATAGCC		
Fw-SlEF1a	ATTGGAAACGGATATGCCCCT	Solyc06g005060	Normalisation in qRT-PCR assays
Rv-SlEF1a	TCCTTACCTGAACGCCTGTCA		
Fw-StEF1a	ATTGGAAATGGATATGCTCCA	Sotub06g010680	Normalisation in qRT-PCR assays
Rv-StEF1a	TCCTTACCTGAACGCCTGTCA		
Fw-On	CGCCAAAGACCTAACCAAAA	EU047564	Quantification of *Oidium neolycopersici*
Rv-On	AGCCAAGAGATCCGTTGTTG		
Fw-StPR1a	TGGTGATTTCACGGGGAGGG	AJ250136	
Rv-StPR1a	CGAACTGAGTTGCGCCAGAC		

Supplementary Table 2. Background information of Phytophthora infestans isolates used in this study

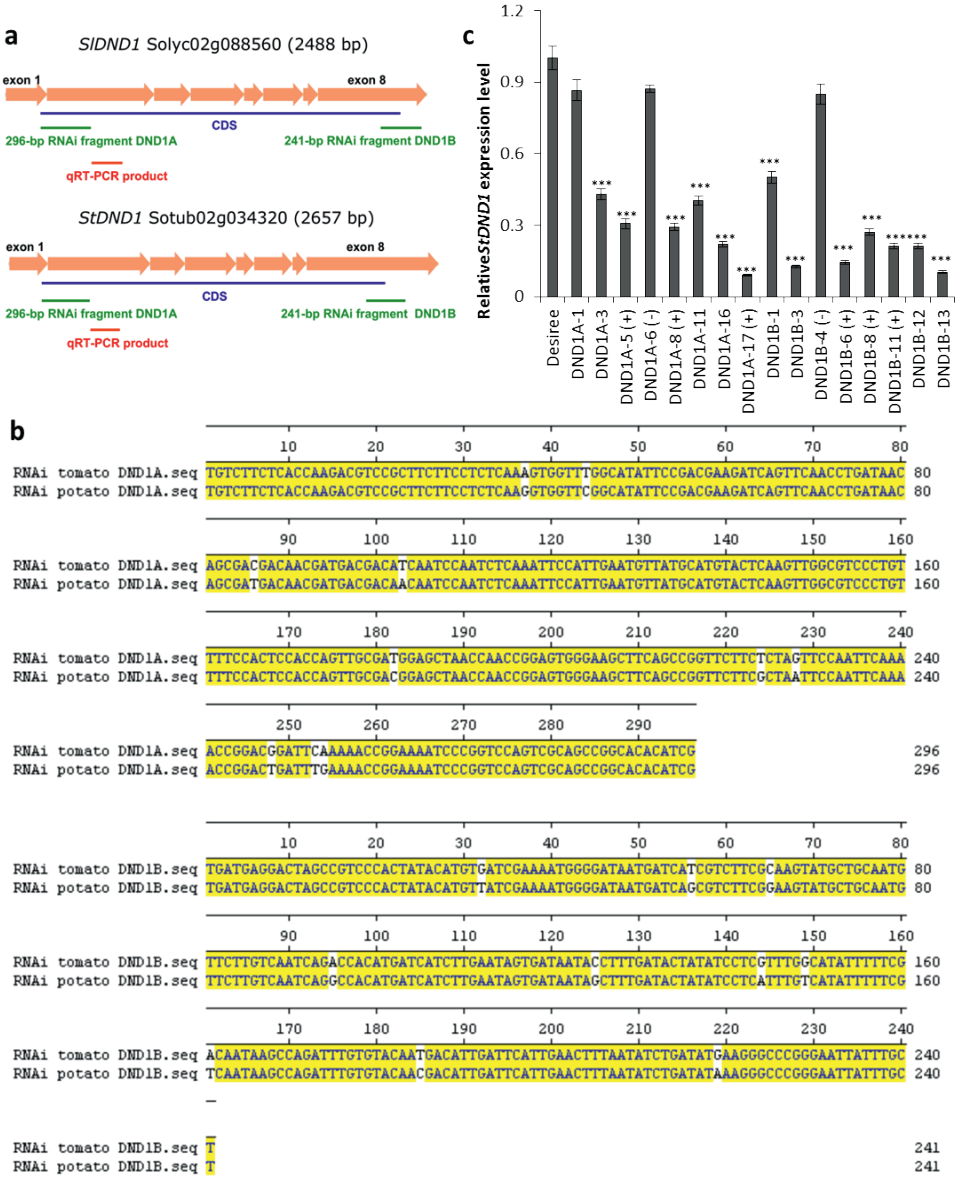
Isolate	Race	Mating type	Origin			Provider of isolate	Reference
			Year of collection	Country	Source		
Pic99177	1.2.3.4.7.9.11	A2	1999	Mexico	*Solanum stoloniferum*	Kessel, WUR, Netherlands	Flier et al.2002
Pic99189	1.2.5.7.10.11	unknown	1999	Mexico	*Solanum stoloniferum*	Kessel, WUR, Netherlands	Flier et al.2002
EC#1	1.3.4.7.10.11	unknown	unknown	Ecuador	unknown	Birch, SCRI, Scotland	Armstrong et al.2005
USA618	1.2.3.6.7.10.11	A2	unknown	Mexico	Potato crop	Fry, Cornell, USA	Goodwin et al. 1994



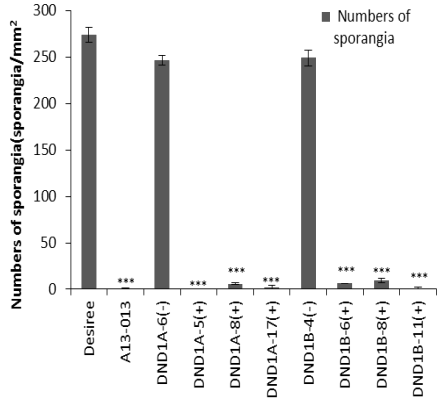
Supplementary Fig. 1. Identification of *DND1* orthologs in tomato and potato. **a** Sequence alignment of *Arabidopsis* AtDND1, tomato SlDND1 and potato StDND1 protein sequences. The numbers on the left indicate the amino acid position. Identical residues in all these proteins are shown in a dark blue background. Dots indicate gaps introduced for optimal alignment. The *SlDND1* (*Solyc02g088560)* and *StDND1* (*Sotub02g034320)* genes are regarded as *DND1* orthologs in tomato and potato, respectively. **b** Phylogenetic tree of *Arabidopsis* DND1 and other *Arabidopsis* AtCNGC members, together with tomato SlDND1 (*Solyc02g088560)* and potato StDND1 (*Sotub02g034320)*.



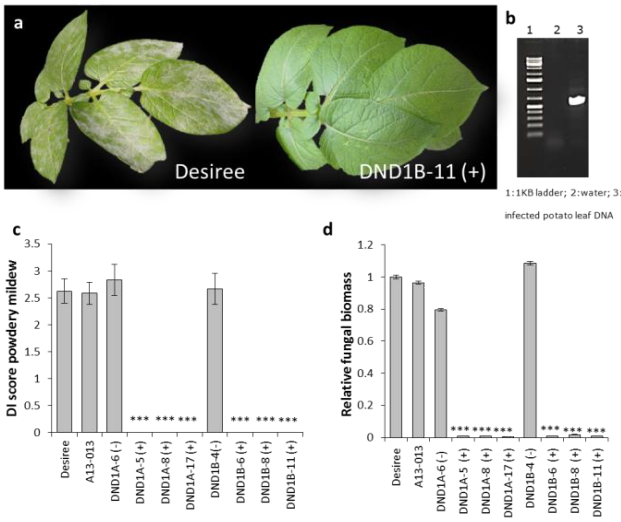
Supplementary Fig. 2. Transcript profiles of *DND1* in major tissues of tomato (**a**, *SlDND1*) and potato (**b**, *StDND1*). The relative expression level of *DND1* in leaf, root, flower, fruit (tomato only), and tuber (potato only) samples were evaluated by qPCR. For each sample, the transcript level of tomato or potato gene *EF1a* was used as reference. For both tomato and potato, the relative expression level in leaves was defined as 1. Data indicate the mean of 3 biological replicates with error bars representing the standard error. Asterisks indicate degree of significance compared to MM or Desiree leaves, respectively. (*$p<0.05$, ***$p<0.001$). **c** and **d** RNAseq data of *SlDND1* (*Solyc02g088560*) and *StDND1* (*Sotub02g034320*), respectively.



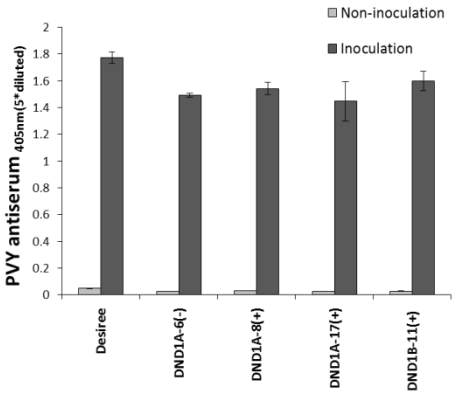
Supplementary Fig. 3. Information of RNAi silencing constructs and silencing effects on RNAi potato transformants. **a** Location of the targeting regions of the two RNAi constructs in *SlDND1* (*Solyc02g088560*) and *StDND1* (*Sotub02g034320*). **b** Alignment of RNAi fragments for the *DND1A* and *DND1B* genes in tomato and potato. **c** Relative expression level of *StDND1* in leaves of Desiree and 16 independent potato RNAi::*DND1* transformants (DND1A-1, 3, 5, 6, 8, 11, 16 , 17 and DND1B-1, 3, 4, 6, 8, 11, 12, 13). Plants marked + (well-silenced) or – (weak-silenced) were used in the following experiments.



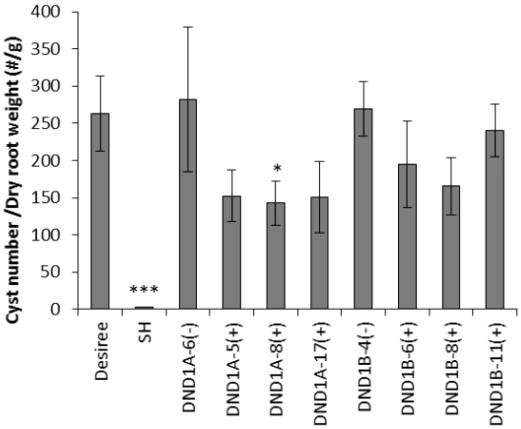
Supplementary Fig. 4 Late blight resistance in potato tubers from the screen cage by silencing *StDND1*. Tubers were harvested from Desiree, A13-013 and eight independent potato RNAi::*DND1* plants (DND1A-5, 6, 8 and 17, DND1B-4, 6, 8 and 11). Numbers of sporangia on tubers infected with *Phytophthora infestans* isolate Pic99189. For each transformant, four plants were tested (one tuber per plant). Asterisks indicate degree of significance compared to Desiree plants (***p<0.001).



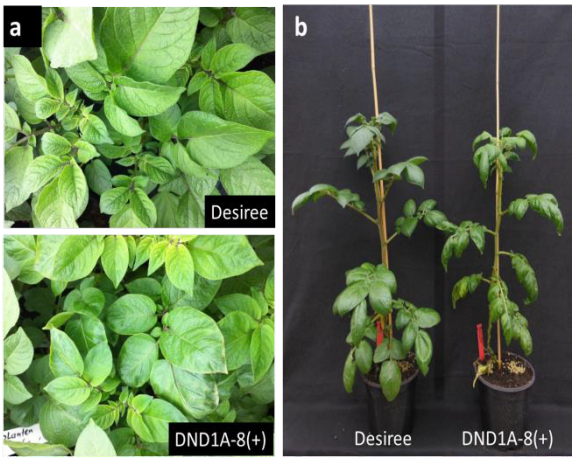
Supplementary Fig. 5. Powdery mildew resistance in potato by impairment of *StDND1*. **a** Photo of a leaf from a Desiree (left) plant compared to a leaf from a *StDND1*-silenced plant (DND1B-11, right). Plants were grown in the greenhouse and naturally infected with powdery mildew. **b** PCR fragment of 621 bp amplified with fungal ITS primers ITS5A and ITS4 using DNA isolated from the powdery mildew-infected leaves. **c** Disease index (DI) scores of the same plants used in Fig. 1 after 6 weeks growth in the greenhouse. DI scores range from 0 (resistant) to 3 (susceptible). All three *StDND1*-silenced (+) plants showed no fungal sporulation (DI=0) compared to the controls Desiree, A13-013 and *StDND1*-silenced (–) plants, which had a DI of about 2.5. **d** Fungal biomass quantification of powdery mildew on the same plants used in Fig. 1 after 8 weeks growth in the greenhouse. Relative fungal biomass was quantified by qRT-PCR with primers ITS5A and ITS4 using *StEF1a* as internal reference. All *StDND1*-silenced (+) plants showed significant lower fungal biomass compared to the controls (Desiree and A13-013) and *StDND1*-silenced (–) plants. Data indicate the mean of 4 biological replicates with error bars representing the standard error. Asterisks indicate degree of significance compared to Desiree plants (***p<0.001).



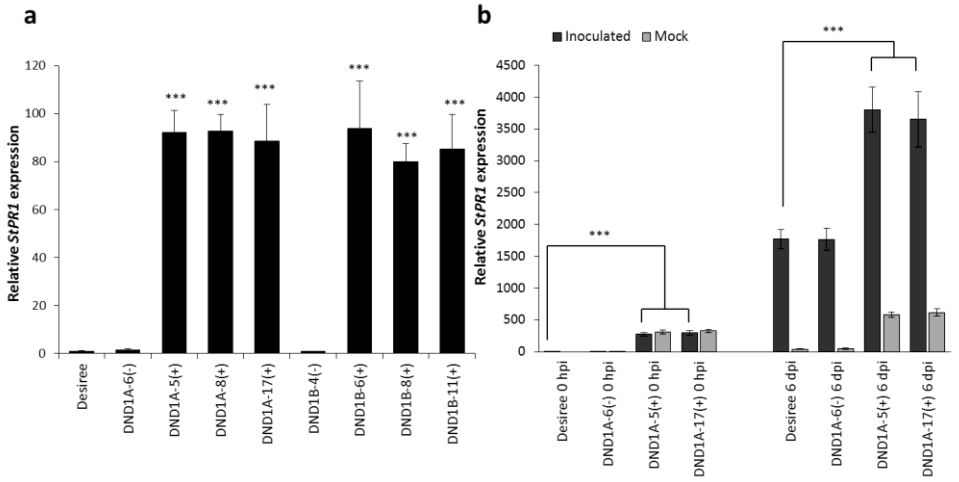
Supplementary Fig. 6. Quantification of PVY anti-serum in Desiree and *StDND1*-silenced plants. Data indicate the mean of four biological replicates with error bars representing the standard error.



Supplementary Fig. 7. Number of nematode cysts formed on plant roots of Desiree, *StDND1*-silenced transformants and the resistant control genotype SH. The cyst nematode *Globodera rostochiensis,* line 19 was used. Data indicate the mean of five biological replicates with error bars representing the standard error.



Supplementary Fig 8. Phenotypes of the wild type Desiree and RNAi::*StDND1* potato plants. **a** Plants grown in a screen cage. Pictures were taken two months after transfer to the screen cage. **b** Plants grown in the greenhouse. Picture was taken five weeks after transfer to the greenhouse. In the screen cage autonecrosis was less severe than in the greenhouse.



Supplementary Fig. 9. Relative *StPR1* (pathogenesis related protein 1) gene expression in Desiree and *StDND1*-silenced plants. **a** Expression level under non-inoculated conditions. **b** Comparison of expression level between mock and *P. infestans* isolate Pic99189-inoculated plants. For each assay, the expression level in non-inoculated or mock-inoculated Desiree was defined as 1. Data indicate the mean of 4 biological replicates with error bars representing the standard error. Asterisks indicate degree of significance compared to Desiree samples, non-inoculated in (A), or inoculated in (B) (***p<0.001).

Supplementary video 1 Time lapse video showing the development of *Phytophthora infestans* isolate Pic99189 on inoculated (I) and mock-inoculated (M) halves of detached leaves of wild type potato Desiree and transformants DND1A-6(-), DND1A-5(+) and DND1A-17(+). Times after inoculation are indicated.
25-06-2015 06:02, clear lesions can be observed on all Desiree and DND1A-6(-) I halves, but not on DND1A-5(+) and DND1A-17(+) I halves.
26-06-2015 10:25, late blight mycelium showing on the Desiree and DND1A-6(-) I halves, DND1A-5(+) and DND1A-17(+) leaflets are still clean and no infection.
27-06-2015 12:25, late blight mycelium covers the (I) halves of Desiree and DND1A-6(-) plants, no lesions and no mycelium growth on DND1A-5(+) and DND1A-17(+) leaflets.
http://www.edge-cdn.net/video_958954?playerskin=37016

CHAPTER 5

Phytophthora infestans infection is hindered at different stages on *StDND1*-, *StDMR1*- or *StDMR6*-silenced potato plants

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Abstract

Many potato varieties are highly susceptible to late blight disease caused by *Phytophthora infestans*. Several dominant genes for resistance (*R*) to potato late blight have been applied in potato breeding, which however can be rapidly overcome by this pathogen. A new source of resistance in plants to many pathogens is needed and found in the loss of function of plant susceptibility (*S*) genes. Using RNA interference, we previously showed that silencing of six potato *S* genes increased resistance to late blight. Three of them, *StDND1, StDMR1* and *StDMR6,* have been further investigated in this study. We monitored microscopically the infection process of *P. infestans* on *StDND1-, StDMR1-* or *StDMR6*-silenced potato plants. Macroscopically, no lesions were found on RNAi silenced plants, in contrast to lesion growth on the susceptible cultivar (cv) Desiree from 3 days post inoculation (dpi). Microscopically at 6 dpi, mycelium was detected on infected cv Desiree plants, in contrast to clean leaf surfaces, small individual or clustered black dots on *StDND1-, StDMR1-* or *StDMR6*-silenced plants, respectively. On *StDND1* silenced plants, attachment of *P. infestans* to the leaf surface was hampered. On *StDMR1* and *StDMR6* silenced plants, growth of *P. infestans* was arrested by a hypersensitive response (HR). Cell death associated with *P. infestans* infection occurred at 24 hours post inoculation (hpi) on cv Desiree plants. Prior to this time point, i. e. between 12 and 16 hpi, epidermal cells underwent HR on *StDMR1-* and *StDMR6*-silenced plants. Single cell HR on *StDMR1*-silenced plants stayed in epidermal cell layer and was effective to stop infection of *P. infestans* at the germinated cyst stage. On *StDMR6*-silenced plants, HR progressed as far as mesophyll cells at 48 hpi onwards. Our results showed that the infection process of *P. infestans* was hindered at different stages on *StDND1-, StDMR1-* or *StDMR6-* silenced potato plants. These results indicate that different mechanisms are possibly involved in the late blight resistance obtained by impairing the three potato *S* genes, *StDND1, StDMR1* and *StDMR6*.

Keywords: Susceptibility (*S*) gene; *DND1*; *DMR6*; *DMR1*; *Phytophthora infestans*, resistance, potato

Introduction

Phytophthora infestans, which belongs to the class of oomycetes, can infect tomato, potato (*Solanum tuberosum*) and some ornamental relatives of these two crops. In potato it can attack leaves, stems, and tubers, and the infected tissues show water soaked symptoms which soon collapse, shrivel and turn brown. Depending on environmental conditions, an unprotected potato field with a susceptible potato cultivar (cv) can be devastated within 10 days after natural infection of *P. infestans* (Fry, 2008). The pathogen reproduces asexually by zoosporangia, which can be spread to healthy plants by wind and rain. After landing on the plant leaf surface, zoospores are encysted and start to germinate (Fig. 1a). Germinated cysts form appressoria that penetrate epidermal cells. Subsequently, intracellular hyphae grow into epidermal cells and haustoria are formed in mesophyll cells (Avrova et al. 2008). The pathogen obtains the nutrients from the host via haustoria, to support intercellular hyphal growth. Later during the infection process (about 72 hpi onwards), new sporangia on sporangiophores will emerge usually through stomata (Pristou & Gallegly 1954; Coffey & Wilson 1983; Pieterse et al. 1992). Few days after infection, the infected areas become necrotic and water soaked lesions are macroscopically visible on the infected plants (Fig. 1b).

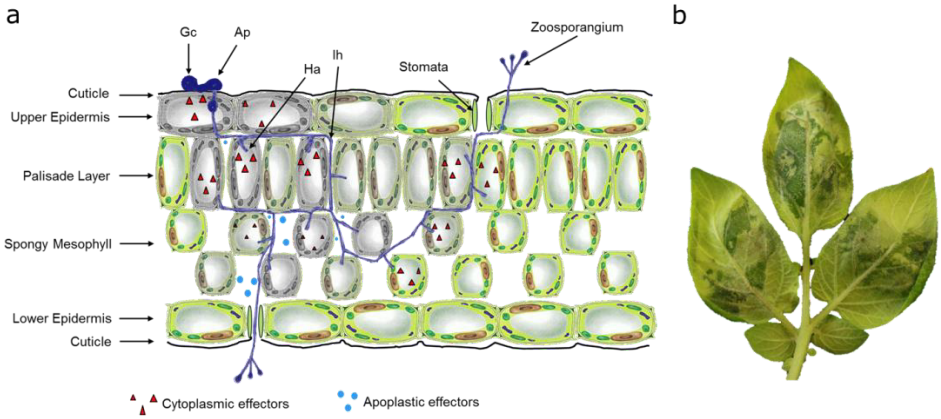


Fig. 1. Infection process of *Phytophthora infestans* in susceptible potato plants. **a** Major events in the infection circle of *P. infestans*. Abbreviations: Gc, germinated cysts; Ap, appressorium; Ha, haustoria; Ih, intercellular hyphae. **b** Abaxial side of a leaf from the susceptible potato cv Desiree, showing lesions caused by *P. infestans* infection at six days post inoculation.

Late blight caused by *P. infestans* is the most important disease worldwide in potato production, which can be controlled effectively by spraying plants on a regular basis with specific fungicides. As a more environmental friendly approach, producing

resistant cultivars has been the target of breeding programs worldwide since the beginning of the 20th century. Many dominant genes conferring resistance to *P. infestans* (*Rpi* genes) have been identified in wild relatives of the cultivated potato, including *S. demissum, S. bulbocastanum*, *S. stoloniferum*, *S. venturii*, *S. mochiquense*, and *S. chacoense* (Haverkort et al. 2016). The introgression of *Rpi* genes into cultivated potatoes through interspecific crosses, followed by backcrosses with cultivated potato, is very time consuming. For example, it took more than 30 years to introgress the *Rpi-blb2* gene from the diploid species *S. bulbocastanum* into potato cultivars Bionica and Toluca (Haverkort et al. 2009). Resistance mediated by these *Rpi* genes is typically race-specific and based on a hypersensitive reaction (HR), resulting from a direct or indirect recognition of proteins encoded by the *Rpi* gene and a specific avirulence gene of *P. infestans* (Lokossou et al. 2010). Mutations occur frequently in *P. infestans* avirulence genes, and this enables the pathogen to evade the recognition by a specific *Rpi* gene. Such adapted isolates are able to overcome *Rpi*-mediated resistance within only a few years after application in the field (Vleeshouwers et al. 2011). To achieve durable resistance, stacking of *Rpi* genes may be an option (Zhu et al. 2012). The best known example for durable resistance is the potato variety Sarpo Mira. This variety is the result of introgression of at least 5 *Rpi* genes (*R3a*, *R3b*, *R4*, *Rpi-Smira1*, and *Rpi-Smira2*) from different sources (Rietman et al. 2012). The risk that such resistant varieties with more than one *R* gene will be overcome by the pathogen is still there. Main reason is that these *R* genes are used individually in varieties which is stimulating breakage of the *R* gene involved. Therefore, it is important also to search for alternative sources of more durable resistance.

Recently, increased research on plant susceptibility genes (*S* genes) has led to a novel breeding strategy of deploying impaired host *S* genes to control diseases (Pavan et al. 2011; Pessina et al. 2014; Sun et al. 2016b). In Arabidopsis, more than 150 *S* genes have been described (van Schie & Takken 2014). There is more and more evidence that orthologs of the Arabidopsis *S* genes are present in crop species (Bai et al. 2008; Sun et al. 2016a). In potato it was shown that reduced expression via RNAi of the putative *S* gene *SYNTAXIN-RELATED1* (*StSYR1*), an ortholog of *AtSYP121/AtPEN1* of Arabidopsis, decreased the susceptibility to *P. infestans* (Eschen-Lippold et al. 2012a & b). In our previous studies (Sun et al. 2016a & b), we selected eleven *A. thaliana S* genes and silenced their orthologs in Desiree, a potato cultivar which is highly susceptible to late blight. RNAi mediated silencing of six of these genes, among which *StDND1, StDMR1* and *StDMR6,* resulted in increased resistance to *P. infestans*. This showed that loss-of-function of a putative *S* gene can be exploited to hamper infection and/or leaf colonisation by *P. infestans* and hence to generate late blight resistance in potato based on mechanisms differing from *R*-gene mediated mechanisms.

StDND1, StDMR1 and *StDMR6* are the orthologs of Arabidopsis *DND1, DMR1* and *DMR6,* respectively. The Arabidopsis *DND1* (*defense no death 1*) gene encodes a cyclic nucleotide-gated cation channel (CNGC; also known as AtCNGC2, Clough *et al.* 2000) and the *dnd1* mutant showed resistance to the bacterium *Pseudomonas syringae* pv. *glycinea* , the oomycete *Hyaloperonospora arabidopsidis,* as well as the fungi *Botrytis cinerea*, and *Alternaria brassicicola* (Ahn 2007; Clough et al. 2000; Genger et al. 2008; Govrin & Levine 2000; Jurkowski et al. 2004; Su'udi et al. 2011). As indicated by the name of "*Defense No Death",* the resistance to *P. syringae* observed in the *dnd1* mutant was not associated with HR (Yu et al. 1998) but with a constitutively elevated expression level of the pathogenesis-related (PR) gene *PR1* (Genger et al. 2008; Yu et al. 1998). Arabidopsis *DMR1* (*Downy Mildew Resistance 1*) encodes a homoserine kinase that catalyses the phosphorylation of homoserine to O-phospho-homoserine (Stuttmann et al. 2011; Van Damme et al. 2009). The Arabidopsis *dmr1* mutants are resistant to the downy mildew *H. arabidopsidis*, the powdery mildew fungus *Oidium neolycopersici*, and two other fungal pathogens *Fusarium culmorum* and *F. graminearum*. The resistance of *dmr1* mutants was associated with accumulation of homoserine (Brewer et al. 2014; Huibers et al. 2013; Van Damme et al. 2005; Van Damme et al. 2009). In *dmr1* mutants, hyphal growth of *H. arabidopsidis* was arrested and underdeveloped haustoria were often surrounded by cell wall appositions containing callose (Van Damme et al. 2005). Arabidopsis *DMR6* (*Downy Mildew Resistance 6*) encodes a 2-oxoglutarate Fe (II) dependent oxygenase (Zeilmaker 2012 & 2015). The *dmr6* mutants showed reduced susceptibility to *H. arabidopsidis, P. syringae* pv. *tomato* (only at adult stage) and *Phytophthora capsici* (Van Damme et al. 2005 & 2008; Zeilmaker 2012). In the *dmr6* mutant, hyphal growth and haustoria of *H. arabidopsidis* were observed but the haustoria often had aberrant shapes and stayed immature (Van Damme et al. 2005). Enhanced expression of a subset of defense-associated genes such as *PR-1* was shown in *dmr6* mutants, which was suggested to contribute to the observed *dmr6*-mediated resistance (Van Damme et al. 2008; Zeilmaker 2012).

In this study, the infection process of *P. infestans* on respectively *StDND1-, StDMR1-* or *StDMR6*-silenced (via RNAi) potato plants was monitored microscopically. The objective was to detect when and how the growth of *P. infestans* was arrested in these RNAi plants.

Materials and methods

Plant and pathogen materials

RNAi transformants designated as RNAi:: *StDND1*-#5, #17*;* RNAi:: *StDMR6*-#5, #6; RNAi:: *StDMR1*-#24, #47 and described in Sun et al (2016b) were grown from *in vitro* plants. The RNAi tranformants are derivatives of the tetraploid potato cv Desiree, which is susceptible to late blight and used as susceptible control. Potato transformant A13-013, that carries the *Rpi-vnt1.1* resistance gene in cv Desiree background (Foster et al. 2009; Pel et al. 2009), was included as a resistant control.

Two *P. infestans* strains were used, one expressing a GFP gene and the other expressing a GUS gene. 14-3-GFP is a derivative of *P. infestans* isolate H30P02 (Bouwmeester et al. 2014) and EY6 from isolate 88069 (van West et al. 1998). For further information see Supplementary Table 1.

Inoculation and histochemical investigation

Three-weeks old and rooted *in vitro* plants were transferred to soil and grown in a greenhouse with 75% relative humidity in 16-h light/ 8-h dark conditions. After 4-6 weeks, when the plants have developed nine or ten leaves, the 5^{th} or 6^{th} leaves above the first expanded leaf were harvested. Full composite leaves with 5 leaflets were arranged in floral foam and placed in plastic boxes with a transparent lid. Detached leaf assays (DLA) were performed as described by Vleeshouwers et al. (1999). *P. infestans* strains were cultured on rye sucrose medium (Caten & Jinks 1968) in the dark at 15°C for 10–14 days. When the plate was covered with mycelium, cold water (4°C) was added. The sporangiospore suspension was pipetted into a closed tube and incubated at 4°C for 1–2 h (Supplementary Fig. 1). Then, zoospores were separated from the sporangiospores by filtration through a 15 μm nylonmesh. The inoculum was adjusted to a concentration of 2.5×10^4 zoospores/ml. *P. infestans* infection assays were performed on detached leaves by drop-inoculation on the abaxial side of the leaf. For each genotype, three plants were tested and three leaves per plant were inoculated. Inoculated leaves were incubated at 18°C (16/8h light/ dark).

For binocular observation, leaves were inoculated on the abaxial side with zoospore suspension of *P. infestans* strains 14-3-GFP and EY6 (6 drops per leaflet, 10 ul per drop). Symptoms were observed with a stereomicroscope (Stemi 508 doc, Zeiss, Germany) at 6 days post inoculation (dpi). Pictures were taken using a BRESSER MikroCam 10.0 microscope camera (Bresser GmbH, Germany) mounted on the stereomicroscope.

To monitor the infection of *P. infestans* with UV light, leaves were drop-inoculated (4 drops per leaflet, 10 ul per drop) with a zoospore suspension of strain 14-3-GFP. After inoculation, leaflets were placed in a 12-cm square Petri dish which was filled with autoclaved demineralized water containing 0.8 % agar to a thickness of 5 mm. Pictures were taken by Nikon S9200 camera with or without UV LED illumination at 6 dpi.

For the histological studies, leaf segments were sampled by punching inoculated areas out of the leaves using a 1-cm-diameter cork bore at 0, 3, 6, 12, 16, 24, 48, 72, and 96 hours post inoculation (hpi) (Supplementary Fig. 1).

To monitor *P. infestans* growth in water, zoospore suspension of strain 14-3-GFP was kept in a closed container with water. The container was placed in the growth chamber (18°C, 16/8 light/ dark) together with the leaves which were used in the DLA. The germination and growth of 14-3-GFP were checked by fluorescence microscopy (Nikon 90i epifluorescence microscope equipped with a GFP filter) at 6 time points (0, 3, 6, 16, 24, 48, 72, and 96 hpi).

The 14-3-GFP inoculated samples were analysed directly by fluorescence microscopy (Nikon 90i epifluorescence microscope equipped with a GFP filter). For histochemical localization of GUS activity, leaf segments inoculated with strain EY6 were incubated overnight at 37°C in staining solution containing 0.5 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl b-D glucuronide, Biosynth AG) in 50 mM phosphate buffer, pH 7.0, 1 mM KFeCN and 0.05% (v/v) Triton-X100. Staining solution was removed by washing the samples with 96% ethanol until the tissue was clear. The leaf segments were mounted on microscope slides in 50% (v/v) glycerol and observed with a bright field microscope (Zeiss, Germany). Pictures were taken with a Canon powershot A620 camera mounted on the microscope. All infection assays were performed at least two times except the histochemical localization of GUS activity.

Time lapse video

A time lapse video was produced to visualize development of *P. infestans* infection on leaves of cv Desiree and on *StDND1-, StDMR1-* or *StDMR6*-silenced potato transformants. The time lapse protocol described in Sun et al. (2016a) was applied. Leaflets were taken from the same plants as those used in the DLA (see above). They were inoculated with zoospores of isolate 14-3-GFP using the same inoculum concentration as in the DLA (2.5×10^4 zoospores/ml). Depending on the growth conditions such as light and composition of the medium (demi water with 0.8% agar) in this experiment, plants could be observed in the time lapse system for up to 4 days.

Results

StDND1-, StDMR1- and *StDMR6*-silenced plants show enhanced resistance to *P. infestans* strains 14-3-GFP and EY6

In our previous studies, RNAi transformants, RNAi::*StDND1*-#5, -#17*;* RNAi::*StDMR6*-#5, and -#6; RNAi::*StDMR1*-#24, and -#47, have been generated (Sun et al. 2016 a and b). The expression levels of the *StDND1*, *StDMR1* and *StDMR6* genes in corresponding transformants have been evaluated by quantitative real-time (qRT)-PCR using gene-specific primers (Sun et al. 2016b). Using the same methods, qRT-PCR was performed on the RNAi transformants used in this study and the reduced expression levels of the targeted *S* genes in the RNAi transformants were confirmed (Supplementary Fig. 2).

StDND1-silenced plants showed resistance to the four tested isolates, Pic99189, Pic99177, USA618 and EC#1 (Sun et al. 2016a). *StDMR1*- and *StDMR6*-silenced plants were found to be resistant to Pic99189, which was the only isolate tested (Sun et al. 2016b). In this study, we planned to use two transgenic *P. infestans* lines, namely strain 14-3-GFP and strain EY6, that carry the reporter genes GFP and GUS, respectively. Therefore, we first tested the response of *StDND1-, StDMR1-* and *StDMR6*-silenced plants to these two strains in a DLA assay. *P. infestans* infection on leaves of *StDND1-*, *StDMR1-* and *StDMR6*-silenced transformants was monitored using a binocular. On the susceptible cv Desiree, both strains formed lesions. Lesion growth caused by strain 14-3-GFP was recorded in a time lapse movie up to 4 dpi (Supplementary Video 1). At 6 dpi the leaf was covered with mycelium and sporangia were formed (Fig. 2). In contrast, no hyphal proliferation was observed on *StDND1-, StDMR1-* and *StDMR6*-silenced plants. *StDND1*-silenced plants showed no visible response, while, *StDMR1-* and *StDMR6*-silenced plants showed a kind of cell death response, with small, dark isolated or clustered black dots that first appeared around 6 dpi (Fig. 2).

Under UV light, we checked the responses to the 14-3-GFP isolate at 6 dpi (Fig. 3). On the inoculated leaves of cv Desiree, fluorescence was visible in the area site where the leaf was colonized. On leaves of *StDMR6*-silenced plants areas around the inoculation sites showed fluorescence, while, on leaves of *StDND1-* and *StDMR1*-silenced plants there was no fluorescence signal visible.

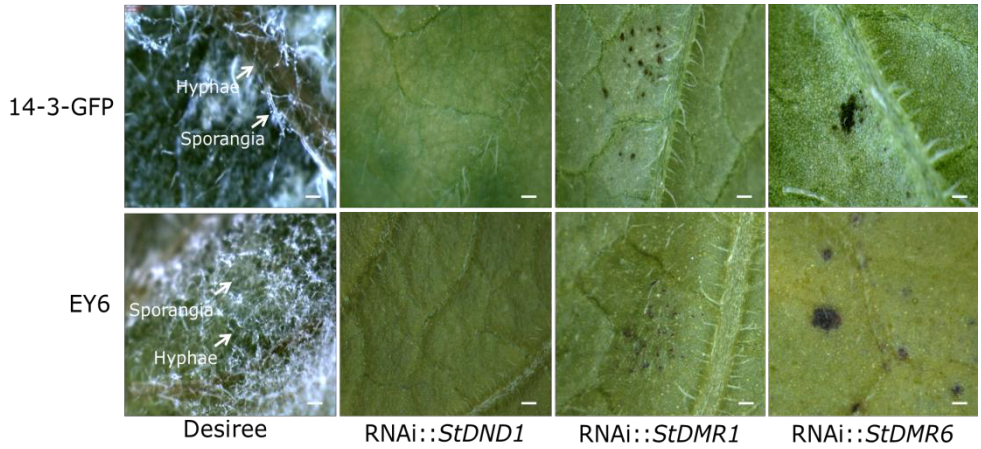


Fig. 2. Late blight resistance on potato leaves after RNAi silencing *StDND1*, *StDMR1* or *StDMR6*, respectively. Disease symptoms on leaves of cv Desiree as well as *StDND1*-, *StDMR1*- and *StDMR6*-silenced potato transformants after inoculation with *Phytophthora infestans* strains 14-3-GFP (upper panel) and EY6 (lower panel). Pictures were taken at 6 days post inoculation and representative for the results obtained in ≥3 inoculated leaves per genotype. Two independent experiments were performed with similar results. Scale bar: 1 mm.

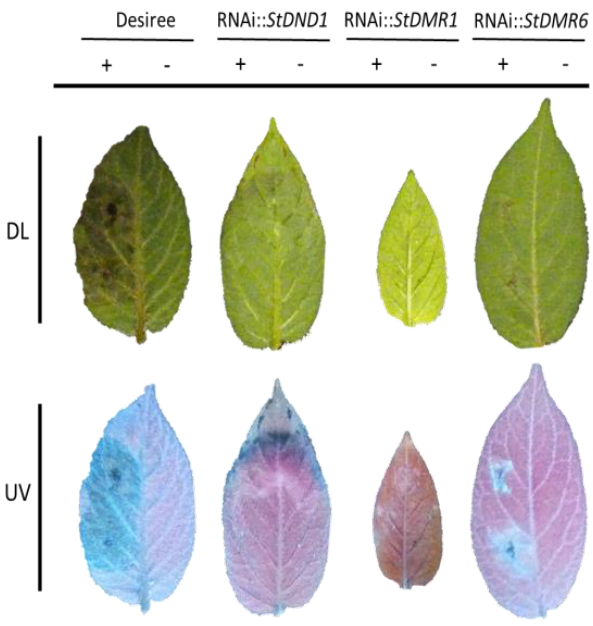


Fig. 3. Disease symptoms on *StDND1*-, *StDMR1*- and *StDMR6*-silenced potato plants after inoculation with *Phytophthora infestans* strain 14-3-GFP. Susceptible control was cv Desiree. Pictures were taken at 6 days post inoculation under daylight (DL, upper panel) and UV light (lower panel) and representative for the results obtained from two inoculated leaves per genotype. +, inoculated with *P. infestans*; -, water-treated. The experiments were repeated three times with similar results.

P. infestans infection is blocked at different stages on *StDND1-*, *StDMR1-* and *StDMR6*-silenced plants

The infection process of strains 14-3-GFP and EY6 was monitored microscopically on inoculated leaves of cv Desiree and *StDND1*, *StDMR1* or *StDMR6* silenced plants at 0, 3, 6, 16, 24, 48, 72, and 96 hpi. With the 14-3-GFP isolate, growth of *P. infestans* could be followed by monitoring the GFP signal. At 0 hpi, cysts were found on all potato genotypes. At 3 hpi, the cysts started to germinate on all plant genotypes, with the exception of *StDND1*-silenced plants where only cysts were observed (Supplementary Fig. 3). At 6 hpi, germ tubes were observed on cv Desiree, and on *StDMR1*- and *StDMR6*-silenced plants, while on *StDND1*-silenced plants cysts just started to germinate. At 16 and 24 hpi, brownish spots, resulting from cell death, appeared on *StDMR1*- and *StDMR6*-silenced plants (Fig. 4, Supplementary Fig. 3). On leaves of *StDND1*-silenced plants, only cysts with a short germ tube were observed from 16 to 96 hpi. At 48 hpi, extensively branched hyphae were detected on cv Desiree, which was associated with cell death (Fig. 4). In contrast, hyphal elongation was arrested on RNAi transformants. On *StDMR1*-silenced plants, the number of single dead cells was increased. On *StDMR6*-silenced plants, multiple cell death occurred covering the inoculation sites. At 72 hpi, collapsed cells were evident at the inoculation areas on cv Desiree (Fig. 4), which led to macroscopic lesions (Supplementary video 1). Images of *StDMR1*- and *StDMR6*-silenced plants were similar to the ones at 48 hpi. At 96 hpi, sporangia were formed on cv Desiree, in contrast to germ tubes found on RNAi transformants. Few spots showed multiple cell death on *StDMR1*-silenced plants.

As a control for HR response, we included the potato transformant A13-013 carrying the *Rpi-vnt1.1* resistance gene in Desiree background. On the inoculation site, single cell HR was observed on A13-013 at 48 hpi and multiple cell HR occurred sometimes at 72 hpi (Supplementary Fig. 4).

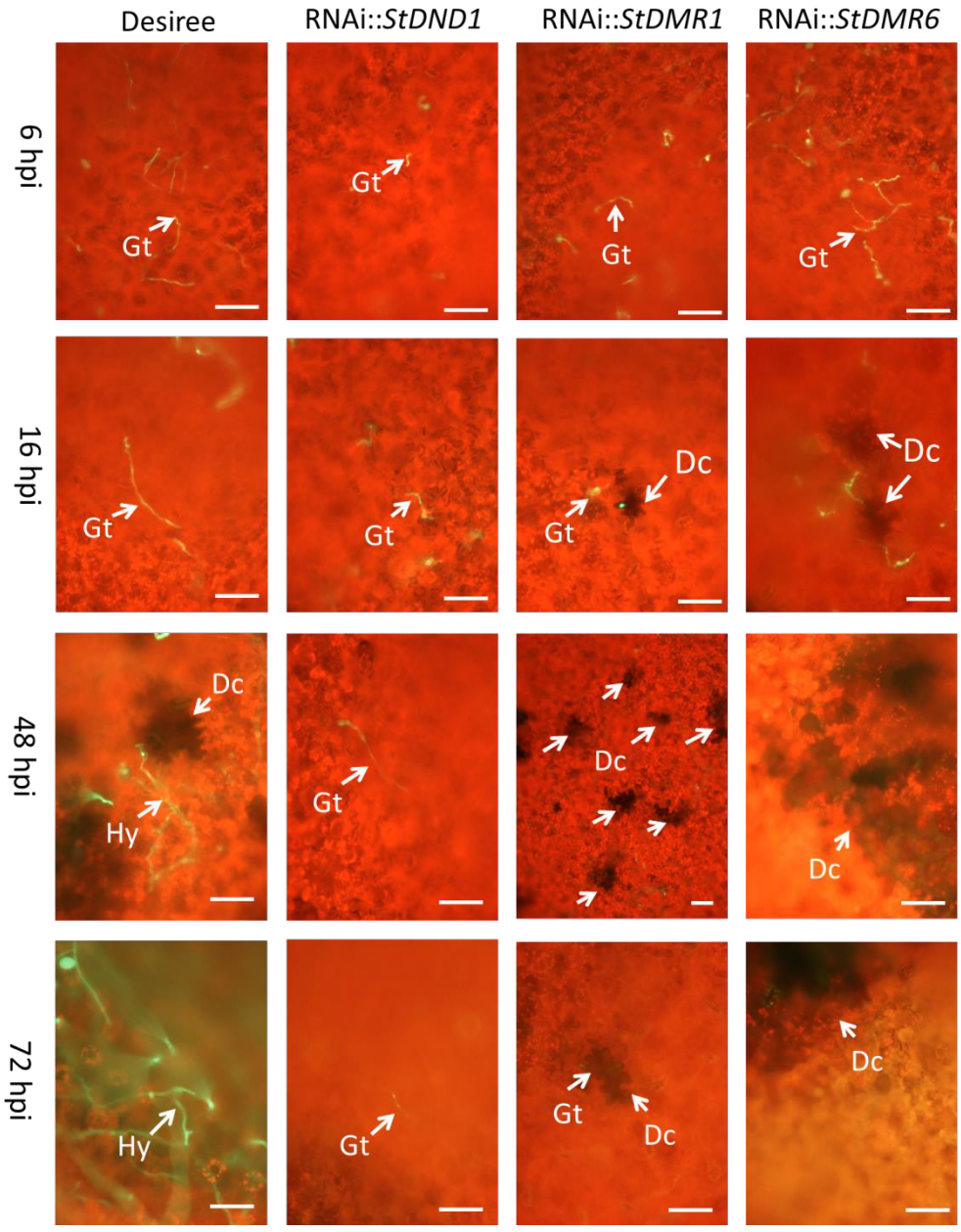


Fig. 4. Infection process of *Phytophthora infestans* (strain 14-3-GFP) on cv Desiree and *StDND1*-, *StDMR1*- or *StDMR6*-silenced potato plants at four time points (6, 16, 48 and 72 hour post inoculation (hpi) (Scale bar = 100 µm). From each genotype, two plants were tested (one leaf with 5 leaflets per plant) and four slides per time point per plant were observed. Pictures are representative for the results observed in six preparations per genotype. Abbreviations: Gt, germ tube; Dc, dead cell(s); Hy, hyphae.

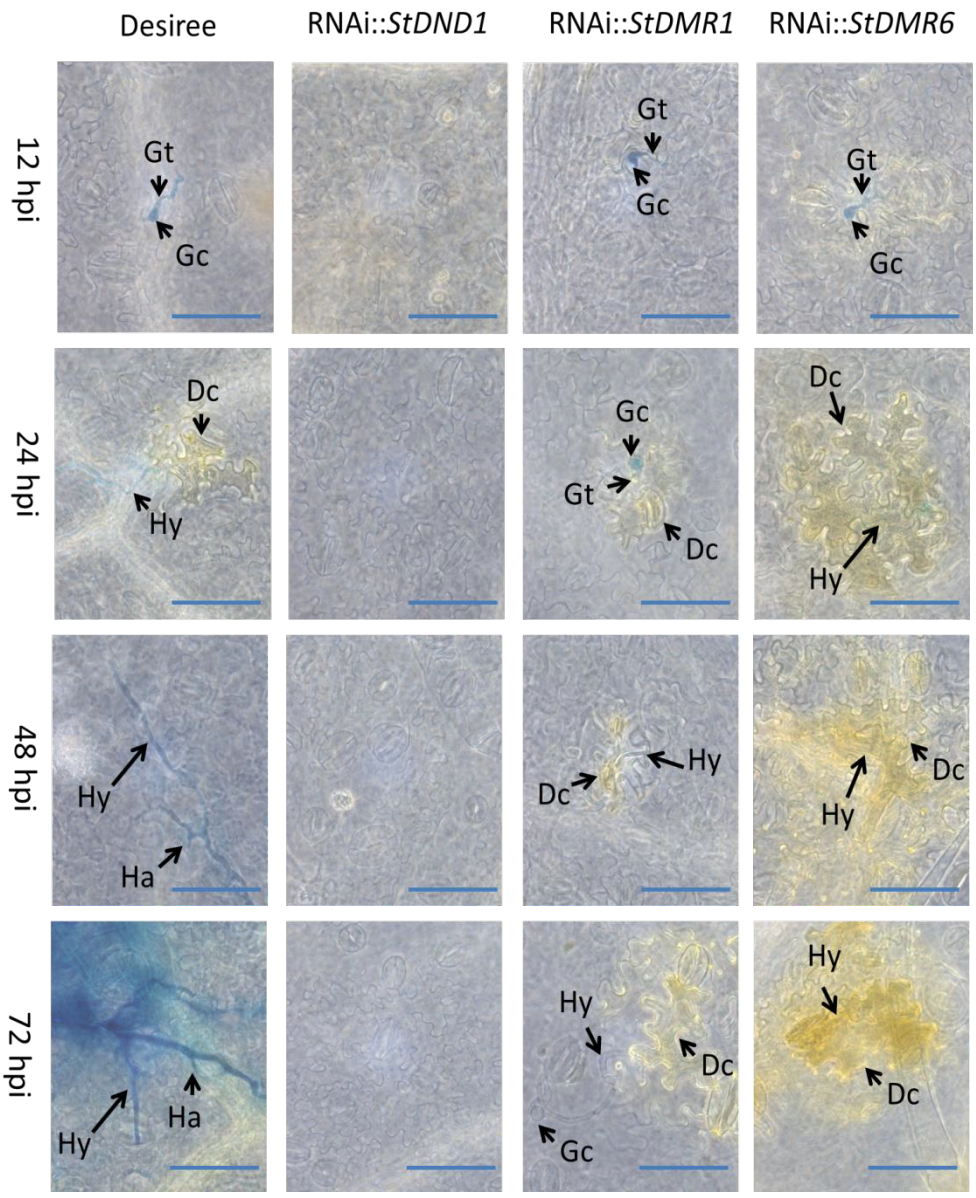


Fig. 5. Infection process of *Phytophthora infestans* (strain EY6) on cv Desiree and *StDND1*-, *StDMR1*- or *StDMR6*-silenced potato plants at four time points (12, 24, 48 and 72 hour post inoculation (hpi) (Scale bar = 100 μm). Cuttings from the same transformants as in Fig. 2 were used. From each genotype, two plants were tested (one leaf with 5 leaflets per plant) and four slides per time point per plant were observed. Pictures are representative for the results observed in six preparations per genotype. Abbreviations: Ap, appressorium; Dc, dead cell(s); Gc, germinated cyst; Gt, germ tube; Ha, haustorium; Hy, hyphae.

With the GUS-expressing strain EY6, the development of *P. infestans* followed similar modes from 12 hpi as observed with the GFP-expressing strain 14-3-GFP. At 12 hpi, cysts germinated on all genotypes except for *StDND1*-silenced transformants. At this time point, primary appressoria were formed on leaves of cv Desiree, and areas underneath the germinated spores became yellowish on leaves of *StDMR6* silenced plants. At 24 hpi on leaves of *StDMR1*- and *StDMR6*-silenced plants, epidermal cells underneath the germinated spores turned brownish (Fig. 5; Supplementary Fig. 5). At 48 and 72 hpi, blue-stained hyphae were found intracellularly on leaves of cv Desiree. While on *StDMR1*- and *StDMR6*-silenced plants, brownish cells occurred in more areas where cysts with a short germinated tube and intercellular hyphae were found on *StDMR1*- and *StDMR6*-silenced plants, respectively (Fig. 5; Supplementary Fig. 5). It is important to note that, at earlier time points, 0 to 6 hpi, no zoospores were found on all genotypes, indicating that they were not attached to the leaf surface and thus were likely washed off during slide preparation (Supplementary Fig. 5). Interestingly, through all observed time points, no spores were observed on leaves of *StDND1*-silenced plants (Fig. 5).

Discussion

Using RNA interference, we previously showed that silencing of six *S* genes in potato resulted in resistance to *P. infestans* (Sun et al. 2016a & b). In this study, three of them, *StDND1, StDMR1* and *StDMR6* have been further investigated. The results showed that the infection process of *P. infestans* was hindered at different stages on *StDND1-, StDMR1-* or *StDMR6*-silenced potato plants (Fig. 6). Attachment of *P. infestans* to the leaf surface was hampered on *StDND1* silenced plants at all investigated time points. Hyphal growth of *P. infestans* was arrested by cell death on *StDMR1*- and *StDMR6*-silenced plants starting around 16 hpi, prior to the cell death associated with *P. infestans* infection happened at 24 hpi on the susceptible cv Desiree. The cell death on *StDMR1*- and *StDMR6*-silenced plants was considered as HR, since it was before the cell death on susceptible cv Desiree and effective in stopping *P. infestans* infection. On *StDMR1* silenced plants single cell HR occurred at the epidermal cell layer and was effective to stop *P. infestans* growth at the germinated cyst stage. On *StDMR6* silenced plants, HR occurred in both epidermal and mesophyll cells.

The *dnd1* mutant of Arabidopsis was selected in a genotype which carries a *R* gene conferring HR-based resistance to *Pseudomonas syringea* (Yu et al. 1998). The *dnd1* mutant showed resistance to *P. syringea* without HR reaction, suggesting that the resistance reaction occurs prior to the HR reaction induced by the *R* gene. In this study, it was that zoospore encystment on *StDND1*-silenced plants, was not hampered but cyst

germination was delayed for 3 hours comparing with cysts on cv Desiree (strain 14-3-GFP, Fig. 4). Germ tubes did not attach to the leaf surface since all spores of the GUS-expressing EY6 strain were washed off during slide preparation (Fig. 5). Obviously, the attachment of spores to the plant surface of *StDND1*-silenced plants appeared to be blocked. This observation may indicate that leaf surface of *StDND1*-silenced plants are chemically and/or physically altered. The *DND1* gene encodes a protein, member of the CNGC family (Clough et al. 2000) which plays a role in conducting Ca^{2+} into plant cells and is involved in various physiological processes (Sherman & Fromm 2009). The Arabidopsis *dnd1* mutant displays elevated SA levels, which are likely required for its resistance to a broad range of pathogens, such as *P. syringae* and *B. cinerea* (Genger et al. 2008). In the *StDND1*-silenced potato plants, constitutively elevated *PR1* expression was also observed (Sun et al. 2016a). The highly elevated *PR1* expression in these *DND1*-silenced plants may lead to increased PR1 protein content, which has been shown to have inhibitory activity against *P. infestans* (Niderman et al. 1995). Alternatively, the leaf surface on *StDND1*-silenced potato plants may be physically altered although there is no report on direct involvement of the CNGC family members in the cell wall biogenesis.

In Arabidopsis *dmr1* mutants, resistance to *H. arabidopsidis*, *O. neolycopersici*, *F. culmorum* and *F. graminearum* was associated with accumulation of homoserine (Van Damme et al. 2005; Huibers et al. 2013; Brewer et al. 2014). Microscopic observation was performed on *dmr1* mutants to *H. arabidopsidis* and it was shown that hyphal growth was inhibited without visible HR after the first underdeveloped haustorium is formed (Van Damme et al. 2005). In this study, our results showed clearly that single epidermal cells underwent HR on *StDMR1*- silenced-potato plants around 16 hpi after *P. infestans* inoculation. The pathogen was arrested at the very early stage of germinated cysts. The length of the germ tubes was comparable to the one observed on a glass plate (Supplementary Fig. 6), indicating no haustorium was formed to support hyphal elongation. *P. infestans*, as a hemibiotrophic oomycete, requires a biotrophic phase in which nutrients from living host cells are acquired via intracellular haustoria-like structures. Thus, rapid host cell death in *StDMR1* silenced plants has hindered the biotrophic phase of this pathogen. In Arabidopsis, the resistance in the *dmr1* mutants was associated with accumulation of homoserine and independent of known defense pathways (Van Damme et al. 2009). Although exogenous application of L-homoserine conferred resistance to *H. arabidopsidis*, *F. culmorum* and *F. graminearum*, it did not inhibit conidiophore germination of *H. arabidopsidis* or mycelium growth of *P. capsici* (Van Damme et al. 2009; Huibers et al. 2013; Brewer et al. 2014). Therefore, it was suggested that a novel homoserine-induced resistance plays an active role in plant defense in the *dmr1* mutants (Van Damme et al. 2009). Further experiments are needed

to verify whether exogenous application of L-homoserine is harmful or leads to resistance to *P. infestans*; and whether any known defense pathway plays a role in the HR-based resistance to *P. infestans* in *StDMR1*-silenced potato plants.

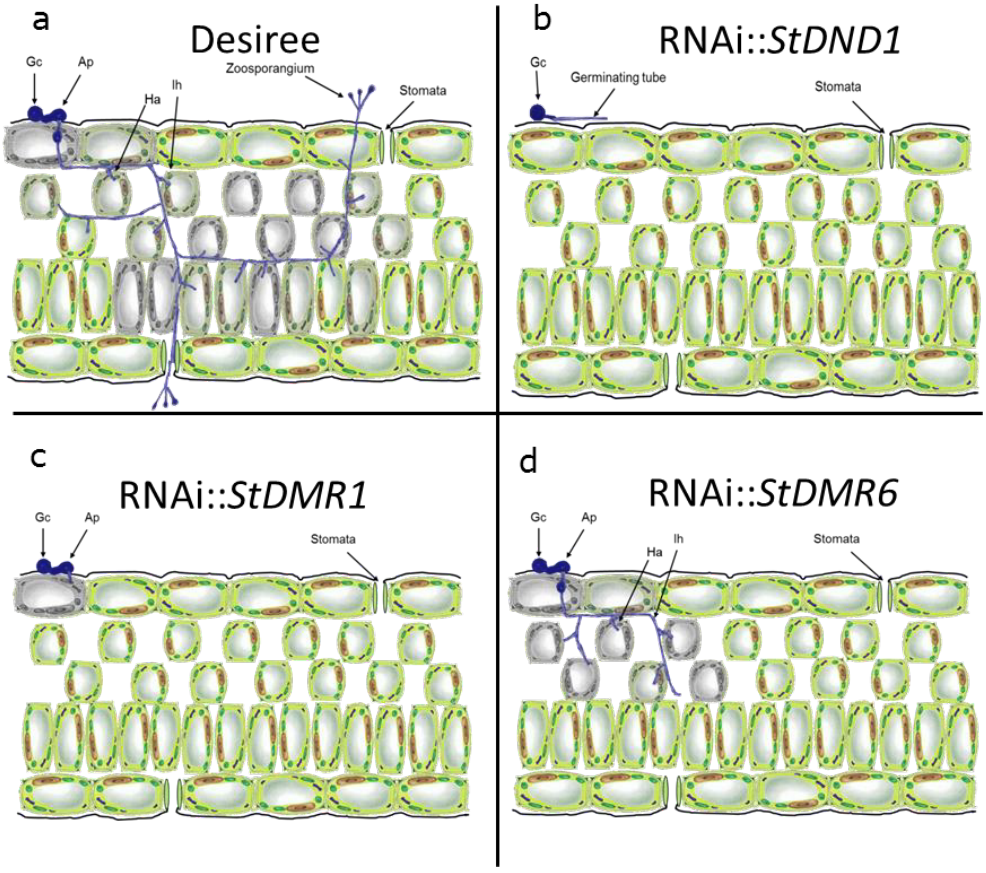


Fig. 6. Schematic representation of the infection process of *Phytophthora infestans* on inoculated leaves of cv Desiree and RNAi transformants silenced for *StDND1, StDMR1* or *StDMR6*. **a** The infection process of *P. infestans* on susceptible cv Desiree; **b** The growth of *P. infestans* is arrested soon after cyst germination on the *StDND1*-silenced transformants; **c** Single epidermal cell death (black) occurs on *StDMR1*-silenced plants, which blocks further hyphal growth; **d** Multiple cell death occurs on *StDMR6*-silenced plants, which arrests hyphal growth at the mesophyll layer likely after forming haustoria. Abbreviations: Gc, germinated cysts; Ap, appressorium; Ha, haustoria; Ih, intercellular hyphae

Arabidopsis *dmr6* mutants were shown to have a reduced susceptibility to *H. arabidopsidis, P. syringae* (only at adult stage) and *P. capsici* (Van Damme et al. 2005,

2008; Zeilmaker et al., 2012). In the *dmr6* mutant, hyphal growth of *H. arabidopsidis* could occur and haustoria were formed although most of the formed haustoria were with aberrant shapes and stayed immature (Van Damme et al. 2005). In this study, our results showed that multiple cells of *StDMR6*- silenced potato plants underwent HR around 16 hpi after *P. infestans* inoculation. Intercellular hyphal growth was found suggesting haustorium formation occurred on *StDMR6*-silenced potato plants (Fig. 5). We hypothesized that the HR occurred in mesophyll cells is due to hyphal growth or haustorium formation. Alternatively, the necrotic plant cells may act as reservoirs for providing secondary elicitors to stimulate HR in surrounding plant cells (Keen 1990, Seifi et al. 2012). *DMR6* encodes a 2-oxoglutarate Fe (II) dependent oxygenase. The resistance to *H. arabidopsidis* could be caused by the accumulation of a toxic DMR6 substrate or the absence of a DMR6 metabolic product required for *H. arabidopsidis* growth (Van Damme et al. 2008). Later, it was shown that resistance to *H. arabidopsidis, P. syringae* and *P. capsici* was accompanied with elevated salicylic acid (SA) levels, and that DMR6 oxygenase has a suppressive effect on defense regulation system to tightly control the SA level (Zeilmaker et al., 2012). Future work is planned to investigate whether the *P. infestans* resistance achieved by silencing *StDMR6* in potato is associated with enhanced defense gene expressions or elevated SA level.

The three *S* genes studied here were not originally identified as susceptibility factors towards late blight disease. Our results showed that orthologues of Arabidopsis *S* genes can be functionally conserved across plant species. For example, down regulation of the *DND1* orthologue in potato and tomato led to resistance to powdery mildew and late blight (Sun et al. 2016a). Down regulation of *DMR1* in tomato and pepper to enhanced resistance to powdery mildew and *P. capsici*, respectively (Huibers et al. 2013; Rehrig et al. 2014). The tomato *dmr6* mutants generated by CRISPR/Cas9 were resistant to *P. syringae* pv. *tomato* and *P. capsici* (de Toledo Thomazella et al. 2016). These results show that the impairment of orthologues of the same *S* gene in different plant species could potentially result in resistance to different pathogens. Further, genome editing technologies, such as CRISPR/Cas9, definitely makes *S* gene editing a very promising strategy for resistance breeding in crops.

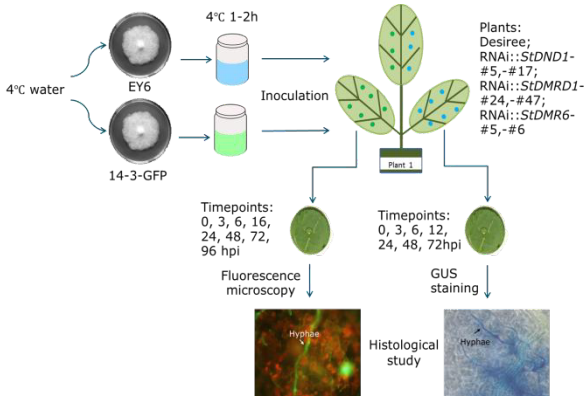
Acknowledgements

This research was made possible by a financial grant from TKI Uitgangsmaterialen (project number EZ-2012-07) and a grant from University Fund Wageningen. We thank Dirk Jan Huigen and Gerda van Engelenhoven for maintenance of the potato plant material. We thank Ageeth van Tuinen and Marjon Arens for assisting in DLA experiments and Johan Bucher for excellent help with the Time Lapse Video system.

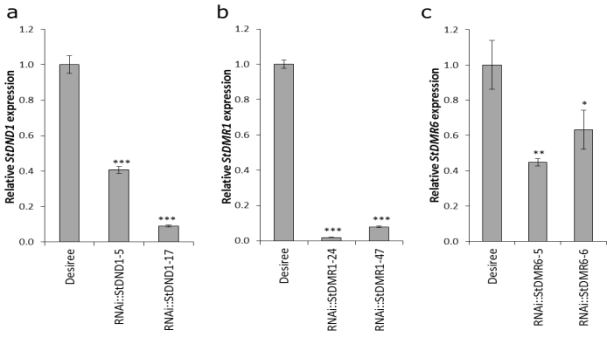
Supplementary Material

Supplementary Table 1. *Phytophthora infestans* transgenic strains used in this study

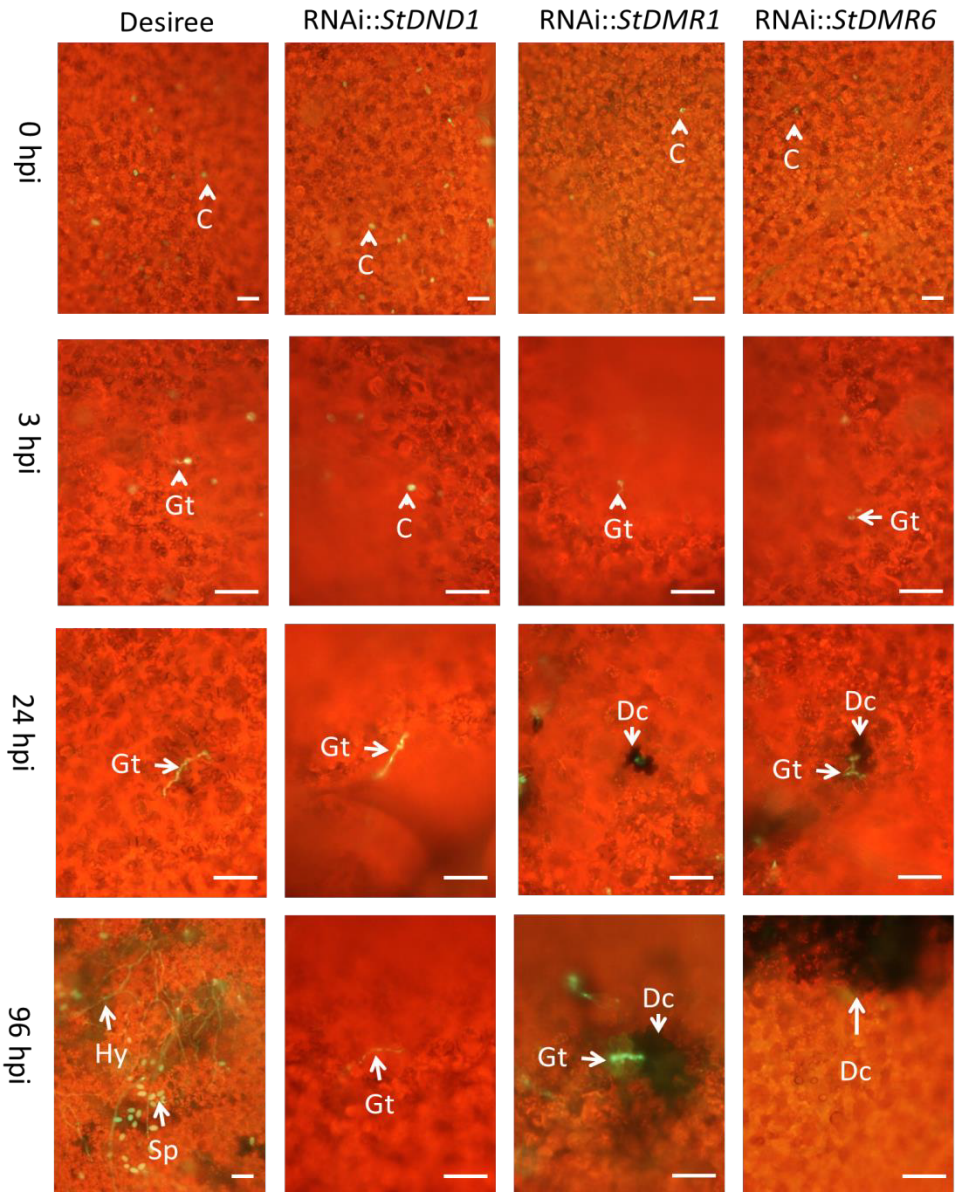
Isolate	Background	Race	Mating type	Provider of isolate	Reference
14-3-GFP	H30P02	3a.7.10	unknown	Prof. Govers, WUR, Netherlands	Bouwmeester et al. 2014
EY6	88069	1.3.4.7	A1	Prof. Govers, WUR, Netherlands	van West et al 1998; Kamoun et al. 1998



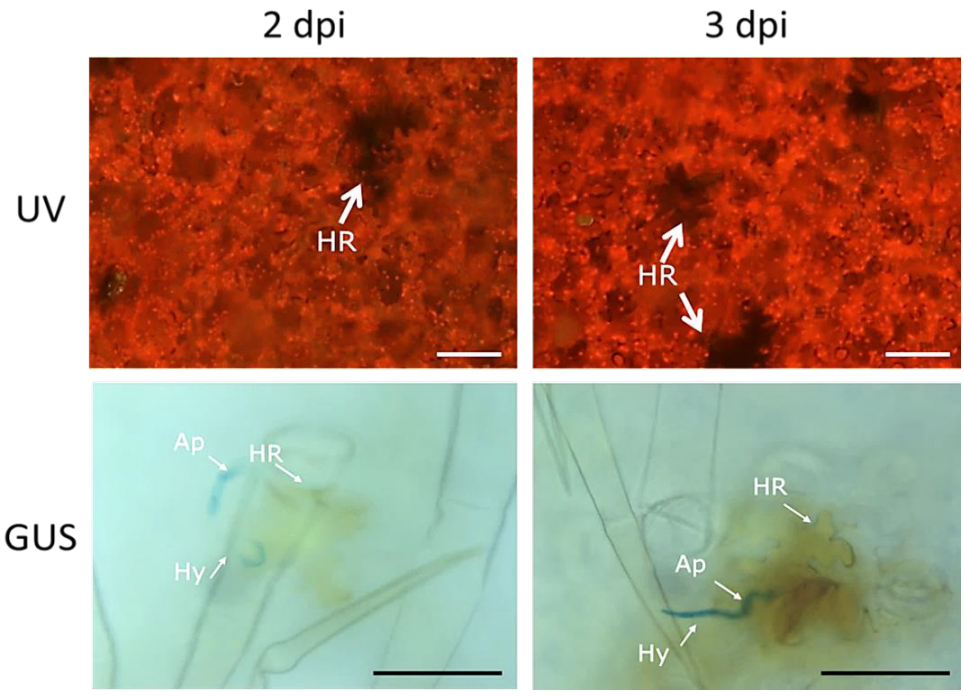
Supplementary Fig. 1. Flowchart of the histological study on cv Desiree and *StDND1*-, *StDMR1*- or *StDMR6*-silenced potato plants using two transgenic *Phytophthora infestans* strains 14-3-GFP and EY6. From each genotype (cv Desiree and *StDND1*-, *StDMR1*- or *StDMR6*-silenced transformants), two plants were tested in a detached leaf assay. Per plant, one leaf with 5 leaflets was drop inoculated. Leaf segments were sampled at different hours post inoculation (hpi) for histological study. Six leaf segments were collected per time point per plant and four per plant were used in microscopic observations.



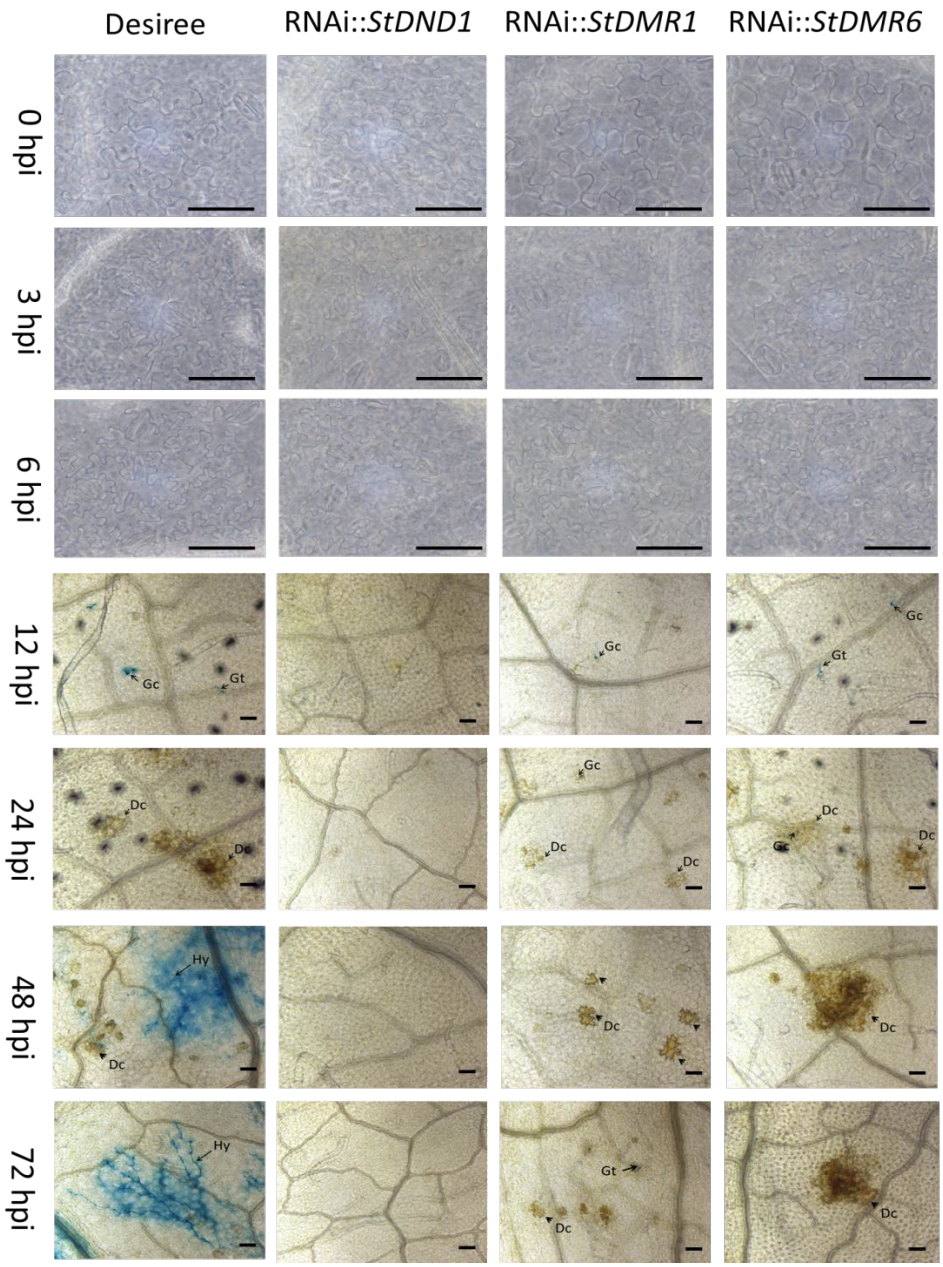
Supplementary Fig. 2. Relative expression level of *StDND1*, *StDMR1* and *StDMR6* in leaves of cv Desiree and multiple independent potato RNAi transformants. Asterisks indicate the degree of significant difference compared to cv Desiree plants (*p<0.05, **p<0.01, ***p<0.001). Three plants were used for cv Desiree and each transformant. Methods for RNA isolation and qRT-PCR using gene-specific primers were presented in the study of Sun et al. (2016b).



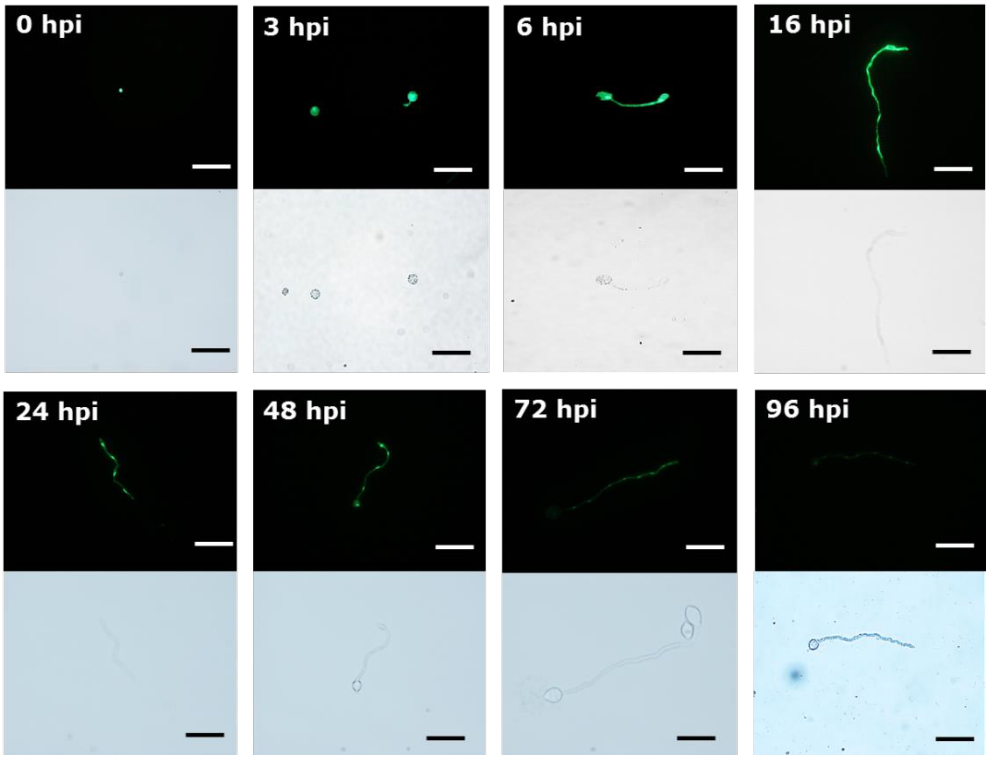
Supplementary Fig. 3. Infection process of *Phytophthora infestans* (isolate 14-3-GFP) on cv Desiree and *StDND1*-, *StDMR1*- or *StDMR6*-silenced potato plants at four time points (0, 3, 24 and 96 hour post inoculation (hpi) (Scale bar = 100 µm). Cuttings from the same transformants as in Fig. 2 were used. From each genotype, two plants were tested (one leaf with 5 leaflets per plant) and four slides per time point per plant were observed. Pictures are representative for the results observed in six preparations per genotype. Abbreviations: C, Cyst; Gt, germ tube; Dc, dead cell(s); Hy, hyphae; Sp, sporangium /zoosporangium.



Supplementary Fig. 4. Hypersensitive response (HR) in potato transformant A13-013 carrying the *Rpi-vnt1.1* resistance gene in cv Desiree background. Pictures were taken at 2 and 3 days post inoculation with the *P. infestans* strains 14-3-GFP (UV, upper panel) and EY6 (GUS, lower panel). (Scale bar=100 µm). Abbreviations: Ap, Appressorium; Hy, hyphae.



Supplementary Fig. 5. Infection process of *Phytophthora infestans* (strains 14-3-GFP and EY6) on cv Desiree and *StDND1*-, *StDMR1*- or *StDMR6*-silenced potato plants at seven time points (0, 3, 6, 12, 24, 48 and 72 hour post inoculation (hpi) (Scale bar = 100 µm). Cuttings from the same transformants as in Fig. 2 were used. Pictures are representative for the results observed in six preparations per genotype. Abbreviations: Dc, dead cell(s); Gc, germinated cyst; Gt, germ tube; Hy, hyphae.



Supplementary Fig. 6. Microscopy images of the *P. infestans* strain 14-3-GFP at 6 time points. Photos show the germination and growth of 14-3-GFP between 6 time points (0, 3, 6, 16, 24, 48, 72, and 96 hpi) in a closed container with water. For each time point, the upper panels of the images show the green fluorescent signal and the lower panels show the bright fluorescent signal. Scale bar:100 µm.

Supplementary video 1 Time lapse video showing the development of *Phytophthora infestans* strain 14-3-GFP on inoculated (+) and mock-inoculated (-) halves of detached leaves of cv Desiree and *StDND1*-, *StDMR1*- or *StDMR6*-silenced potato plants. Times after inoculation are indicated.
https://www.dropbox.com/s/iezyleqnux8il8i/Supplymental%20video.mp4?dl=0

CHAPTER 6

Silencing of *DND1* in potato and tomato decreases susceptibility to *Botrytis cinerea*

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Abstract

Botrytis cinerea, a necrotrophic pathogenic fungus, attacks many crops including potato and tomato. Major resistance genes to *B. cinerea* are not known in plants, but a few quantitative trait loci have been described in tomato. Loss of function of particular susceptibility (*S*) genes appears to be a new source of resistance to *B. cinerea* in Arabidopsis. In this study, orthologs of Arabidopsis *S* genes (*DND1, DMR6*, *DMR1* and *PMR4*) were silenced by RNAi in potato and tomato (only for *DND1*). After inoculation with *B. cinerea DND1* well-silenced potato and tomato plants showed, in comparison with susceptible cv Desiree, a significantly reduced lesion diameter at 1, 2 and 3 days post inoculation (dpi). Reduced lesion diameter was also observed on leaves of *DMR6* silenced potato plants at 3 dpi. The *DMR1* and *PMR4* silenced transformants were as susceptible as the control cv Desiree. Microscopic analysis was performed to study the mechanism that confers reduced susceptibility to *B. cinerea* to *DND1* well-silenced potato and tomato leaves. A significantly lower number of *B. cinerea* conidia were attached to the leaf surface of *DND1* well-silenced potato and tomato plants and hyphal growth was hampered. The potential mechanisms associated with the reduced susceptibility to *B. cinerea* in *StDND1* well-silenced potato plants as well as breeding aspects are further discussed.

Keywords: Susceptibility gene; *DND1*; *Botrytis cinerea*

Introduction

Botrytis cinerea, a necrotrophic pathogen which causes the grey mold disease, can infect a broad range of plant species including potato and tomato. Every year, global expenses of *Botrytis* control (cultural measures, botryticides, broad-spectrum fungicides, biocontrol) easily surmount €1 billion (Dean et al. 2012). Hence *B. cinerea* has become the most extensively studied necrotrophic fungal pathogen. Necrotrophic pathogens damage plant cells by secreting toxic compounds, lytic enzymes and an array of pathogenicity factors which can subdue host defenses (van Kan 2006). In order to penetrate the plant cuticle and epidermal cells *B. cinerea* produces cutin esterases, pectinases and hemicellulases during the early infection stages between 8 and 16 hours post inoculation (hpi) (Brito et al. 2006; Kars & van Kan 2007; McKeen 1974; Noda et al. 2010; van Kan et al., 1997). At later stages, between 16 and 40 hpi, multiple phytotoxic metabolites such as botrydial, botcinic acid and oxalic acid, as well as several phytotoxic proteins are produced and secreted into epidermal and mesophyll tissue to kill the host cells (Frías et al. 2011; Noda et al. 2010; van Kan 2006; Staats et al. 2007).

No major genes (*R* genes) for complete resistance to *B. cinerea* have been identified in plants. However, quantitative trait loci (QTLs) reducing the outgrowth of *B. cinerea* have been reported in several plant species, starting with Arabidopsis (Denby et al. 2004). In the wild tomato accession LYC4 from *Solanum habrochaites*, ten major QTLs have been described for reduced lesion growth and for reduced disease incidence (Finkers et al. 2007a, Finkers et al. 2007b). In chickpea and *Brassica rapa*, several QTLs have been identified for resistance to *B. cinerea* (Anuradha et al. 2011; Zhang et al. 2016).

Recently, plant genes (susceptibility or *S* genes) that are recruited by pathogens to promote diseases have become the focal point of research attention (van Schie & Takken 2014). In Arabidopsis and tomato, 16 plant genes have been reported that are involved in the compatible interaction of plants with *B. cinerea* (Supplementary Table 1). Examples of these are the tomato genes, *LePG* and *LeEXP1*, which contribute to fruit cell wall softening during ripening and thereby facilitate penetration and colonization by *B. cinerea* (Cantu et al. 2008), as well as the Arabidopsis *CESA4, CESA7* and *CESA8* genes, encoding subunits of the cellulose synthase complex (Hernández-Blanco et al. 2007).

Evidence is accumulating that loss-of-function mutations of certain plant *S* genes can confer resistance to pathogens (Bai et al. 2008; Pavan et al. 2010; Sun et al. 2016a; Van Damme et al. 2005; Vogel & Somerville 2000). In tomato, the ABA deficient mutant

sitiens is reported to be highly resistant to *B. cinerea* (Audenaert et al. 2002; Curvers et al. 2010). In Arabidopsis, the invasion of *B. cinerea* was arrested in the *bre1* mutant (Asselbergh et al. 2007). The *BRE1* gene, also known as the *long-chain acyl-CoA synthetase2* (*LACS2*) gene, is essential for cutin biosynthesis. Furthermore, the Arabidopsis *dnd1* (*defense no death*) mutant showed enhanced resistance against a variety of fungal, bacterial and viral pathogens, including *B. cinerea* (Genger et al. 2008; Jurkowski et al. 2004; Yu et al. 2000). In our previous studies, we reported that silencing of potato *DND1*, *DMR6*, *DMR1*, and *PMR4* orthologs resulted in resistance to late blight (Sun et al. 2016b). These results demonstrated that plant *S* genes are rather conserved across plant species with respect to their function as a susceptibility factor to certain types of pathogens.

In this study, we aimed to obtain resistance to *B. cinerea* in potato by impairing the functionality of plant *S* genes. Potato RNAi transformants, in which orthologs of *DND1, DMR6*, *DMR1* and *PMR4* were silenced, were examined for reduced susceptibility to *B. cinerea*. The infection process of *B. cinerea* on *StDND1* well-silenced potato plants was much slower than in control plants and was, therefore, examined in greater detail. The results that were obtained in *StDND1* well-*silenced* potato plants were verified in tomato. Our results showed that the silencing of *DND1* in potato and tomato plants affects the surface attachment of conidia and the growth of *B. cinerea* germlings, resulting in significantly reduced disease development.

Materials and methods

Plant materials

pHellsgate plasmids with the *S* gene silencing fragments of *StDMR1*, *StDMR6*, *StPMR4* and *StDND1* (Sun et al. 2016b) were transferred to *A. tumefaciens* strain AGL1+virG through electroporation. RNAi transformants of potato cv Desiree for those four *S* genes were previously described (Sun et al. 2016b). Three-week old rooted *in vitro* plants were transplanted into soil and grown in the greenhouse at 75% relative humidity under 16-h light/ 8-h dark conditions. After 4-6 weeks, when the plants were at 9-10th leaf stage, the fourth or fifth fully developed leaf (counted from the bottom) was selected for inoculation. One composite leaf with five leaflets was kept in floral foam in a plastic box covered with a transparent lid.

For *StDMR1*, *StDMR6* and *StPMR4,* two well-silenced transformants for each gene were used in a detached leaf assay (DLA). For *StDND1*, three *StDND1* well-silenced transformants (DND1A-5, DND1A-8 and DND1A-17) and one weakly-silenced transformant (DND1A-6) and wild type cv Desiree were used for DLA. Transformants

DND1A-6, DND1A-5, DND1A-17 and cv Desiree were used for staining of fungal hyphae and analysis of defense responses. Four cuttings from each genotype were used in the analysis. Tomato *SlDND1*-silenced T2 plants which were obtained by selfing primary transformants have been described previously (Sun et al. 2016a). Tomato cultivar Moneymaker (MM) and wild relative *Solanum habrochaites* accession LYC4, showing reduced susceptibility to *B. cinerea* (ten Have et al. 2007), were grown with *SlDND1*-silenced T2 plants in the greenhouse. The 4-6th leaves were harvested from 4-5 week-old tomato plants and used for analysis.

Inoculation and sample collection

The leaflets were inoculated on the adaxial side with 6-8 droplets of 2 µl of the wild type *B. cinera* strain B05.10 or a GMO reporter strain of *B. cinerea* (strain SAS56 with a pCutGUS; van Kan et al. 1997) spore suspension in PDB (Potato dextrose broth, 12 g/L) at a density of 3×10^5 spores/ml. At the same time, extra plants from each genotype were inoculated with only PDB medium as a control. After inoculation, leaves were incubated in closed plastic trays to obtain a humidity of 100% at 20 °C (16 h light / 8 h dark). In the DLA, the lesion diameter on leaflets was measured using a calliper with digital display (DIGI-MET®, Helios Preisser, Germany) at 1–3 days post inoculation (dpi). For GUS (beta-glucuronidase) and DAB (3,3'-diaminobenzidine) staining, leaf discs (including the inoculation sites) were punched out with a 1-cm-diameter cork borer at six time points (0.5, 3, 6, 10, 24, and 48 hpi), from five leaves (three inoculated biological replicates plus two controls) per time point. Leaf samples were collected for RNA extraction at each time point.

Histological study

For histochemical localization of GUS activity, leaf discs of potato and tomato were incubated with 0.5 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl b-D glucuronide, Biosynth AG) in 50 mM phosphate buffer (pH 7.0, 1 mM KFeCN and 0.05% (v/v) Triton-X100) overnight at 37°C. The staining solution was removed and leaf discs were washed with 96% ethanol until tissue cleared. The tissue was mounted on microscope slides in 50% (v/v) glycerol.

In order to detect H_2O_2 generation in detached plant leaves, DAB staining was performed. Leaf discs were placed in a solution of 1 mg/mL 3,3'-diaminobenzidine dissolved in 0.2 M PBS (phosphate buffer) and HCl was used to adjust the pH to 3.8. The leaf samples were placed overnight in light to optimize the staining reaction. The samples were cleared by boiling with 96% ethanol until the leaves were decolorized and transferred to fresh 70% ethanol for storage until microscopic examination.

Twelve preparations were observed for each genotype per time point with a bright field microscope (Zeiss, Germany). Pictures were taken with a Canon PowerShot A620 camera mounted on the microscope. The presence of H_2O_2 is visualized as a brown coloration.

Gene expression analysis

Leaf samples were collected at 6 time points (0.5, 3, 6, 10, 24, and 48 hpi) and stored at -80°C. Leaf material, when taken from the -80°C freezer, was kept in liquid nitrogen until grinding by using mortar and pestle. Total RNA was extracted by using the MagMAX-96 total RNA Isolation kit (Ambion). RNA was treated with RNAse-free DNAse (Qiagen). The concentration of isolated RNA was measured by using the Isogen Nanodrop Spectrophotometer ND-1000. cDNA was made by using the iScript cDNA synthesis kit (Bio-Rad). For the determination of relative transcript levels, the iQ SYBR Green supermix (Bio-Rad) and the C1000^{TM} Thermal Cycler PCR system (Bio-Rad) was used. Housekeeping genes *EF1α* (plant) and actin *Bcact*A (*Botrytis*) were used for normalization. Relative transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Gene-specific primer pairs were designed with Primer 3.0 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/, Supplementary Table 2). Three technical replicates were used.

Results

StDND1* well-silenced potato plants show reduced susceptibility to *Botrytis cinerea

In potato, silencing of the genes *DND1*, *DMR1*, *DMR6* and *PMR4* resulted in reduced susceptibility to late blight. In order to assess whether silencing these *S* gene candidates leads to reduced susceptibility to *B. cinerea,* RNAi well-silenced transformants of *StDMR1*, *StDMR6, StDND1* and *StPMR4* were tested with the wild type strain B05.10 in a DLA. In all these transformants, except transformant DND1A-6, the expression level of the corresponding target gene is significantly down-regulated (> 60% reduced *S* gene expression; Sun et al. 2016b). The leaves of cv Desiree were used as susceptible control. Lesion diameters on infected leaves were measured daily from 1 to 3 dpi (Fig. 1). Compared to cv Desiree, significantly smaller *B. cinerea* lesions were observed on *StDMR6* RNAi well-silenced transformants at 3 dpi and on *StDND1* RNAi well-silenced transformants at all three time points (Fig. 1, Supplementary Fig. 1 & 2).

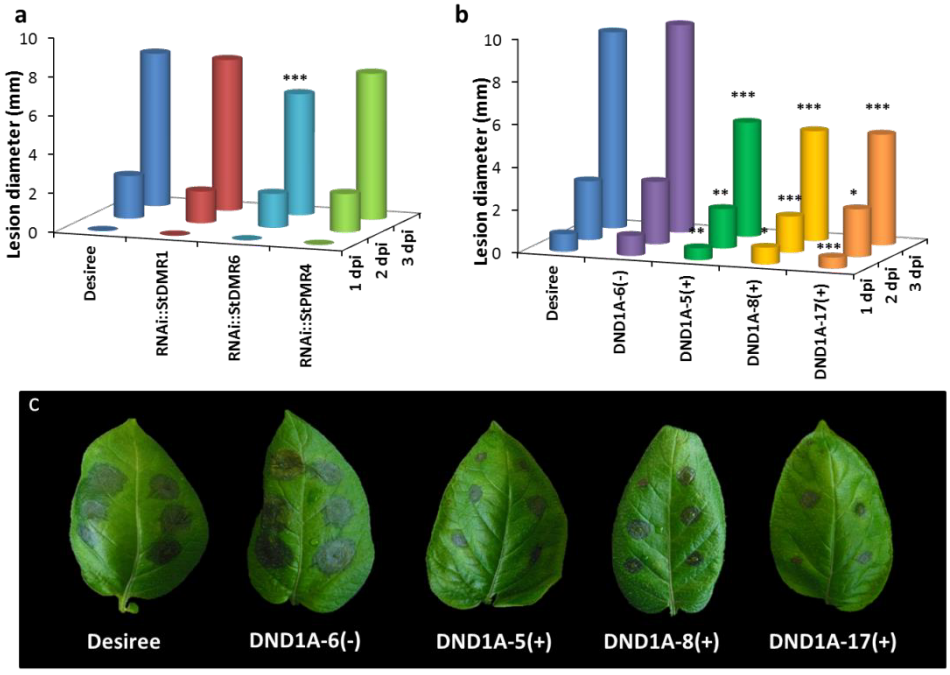

Fig. 1. Detached leaf assay (DLA) of potato RNAi transformants with *Botrytis cinerea* (strain B05.10). **a & b** Lesion diameter on the inoculated leaves of the RNAi transformants. **a** Results from one of the two indepndent experiments performed for *StDMR1*, *StDMR6* and *StPMR4* (two independent well-silenced transformants per gene were used); **b** Results represent one of the three independent experiments for *StDND1*. Four independent transformants were used, one weakly-silenced transformant (-), and three well-silenced transformants (+). Susceptible control was cv Desiree (three plants). An average of all lesion diameters per transformant per time point was calculated. **c** *B. cinerea* disease symptoms on cv Desiree and *StDND1*-silenced transformants. Photos were taken at 3 dpi.

B. cinerea conidial surface attachment and germling growth are reduced on leaves of *StDND1* well-silenced plants

In order to get insight into the mechanism of reduced susceptibility, a histological study was performed on the fungal spore germination and outgrowth of germlings in *B. cinerea*-infected leaves of *StDND1*-silenced potato plants and of cv Desiree. A *B. cinerea* reporter strain was used that contains a GUS gene under control of the promoter of the cutinase gene *Bccut*A, which is inactive in spores but induced from the onset of germination onwards. GUS activity and accumulation of H_2O_2 were monitored

microscopically following histochemical staining and the number of spores per inoculation droplet was counted (Fig. 2). At 0.5 hpi, only few blue stained conidia could be observed on the leaf surfaces of all genotypes, probably because the *BccutA* promoter was at that moment inactive and/or because conidia were washed from the leaf surface in the staining procedure due to their weak attachment to the cuticle. At 3 hpi, on cv Desiree the majority of conidia had germinated and the germ tubes increased in length at subsequent time points, 6 and 10 hpi. Both conidia and germ tubes stained blue due to the induction of the *Bccut*A promoter during infection (Fig. 3). The total number of blue-stained conidia on the leaf surfaces at the three time points tested (3, 6, and 10 hpi) did not significantly differ between cv Desiree and the weakly-silenced plant DND1A-6 (Fig. 2). On the leaves of two *StDND1* well-silenced plants, however, the number of blue-stained conidia was two- to three-fold lower than on cv Desiree at all time points analysed (Fig. 2). Fig. 3 shows that the blue GUS stain of fungal hyphae decreased to some extent at 10 hpi on cv Desiree, but vanished almost entirely in the *StDND1* well-silenced potato plants (see also Supplementary Fig. 3). DAB staining showed that H_2O_2 accumulated on the surface of or inside fungal conidia and germ tubes (Fig. 3). In most cases, there was a correspondence between the GUS staining and DAB staining in or on fungal tissue, with one exception. Notably, at 10 hpi on *StDND1* well-silenced potato plants, DAB staining detected clear accumulation of H_2O_2 inside or on the fungal germ tube surfaces, even though fungal structures were barely detected following the GUS staining (Fig. 3). This observation suggested that fungal hyphae were severely damaged, possibly to such an extent that cytoplasmic content leaked out and GUS activity vanished from the hyphae.

At 24 hpi, clear lesions could be observed on the inoculation sites of cv Desiree leaves (Fig. 4a) which had the size of the initial inoculation droplet. Hyphae of *B. cinerea* could be observed that were extending into the mesophyll layers of leaves (Fig. 4b). On the leaves of *StDND1* well-silenced plants, necrotic lesions were not visible (Fig. 4d) and only a small number of *B. cinerea* hyphae were observed that appeared to be growing on the leaf surface and displayed very weak GUS staining (Fig. 4e). Accumulation of H_2O_2, as shown by dark DAB staining, was observed beyond the inoculation sites on cv Desiree plants (Fig. 4c). In contrast, DAB staining was limited to the inoculation sites on the *StDND1* well-silenced plants and less pronounced (Fig. 4f).

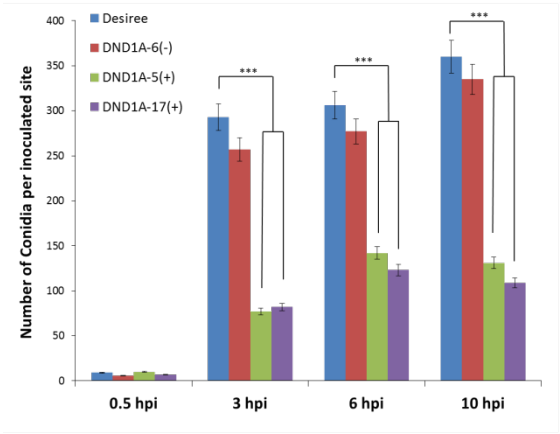

Fig. 2. Number of *Botrytis cinerea* (strain SAS56 containing pCutGUS) conidia on infected leaves of cv Desiree and *StDND1*-silenced potato plants. From cv Desiree and each transformant, three plants were tested (one leaf with 5 leaflets per plant). Bars indicate means ± standard deviation. Asterisks indicate degree of significance compared to cv Desiree plants (***p< 0.001)

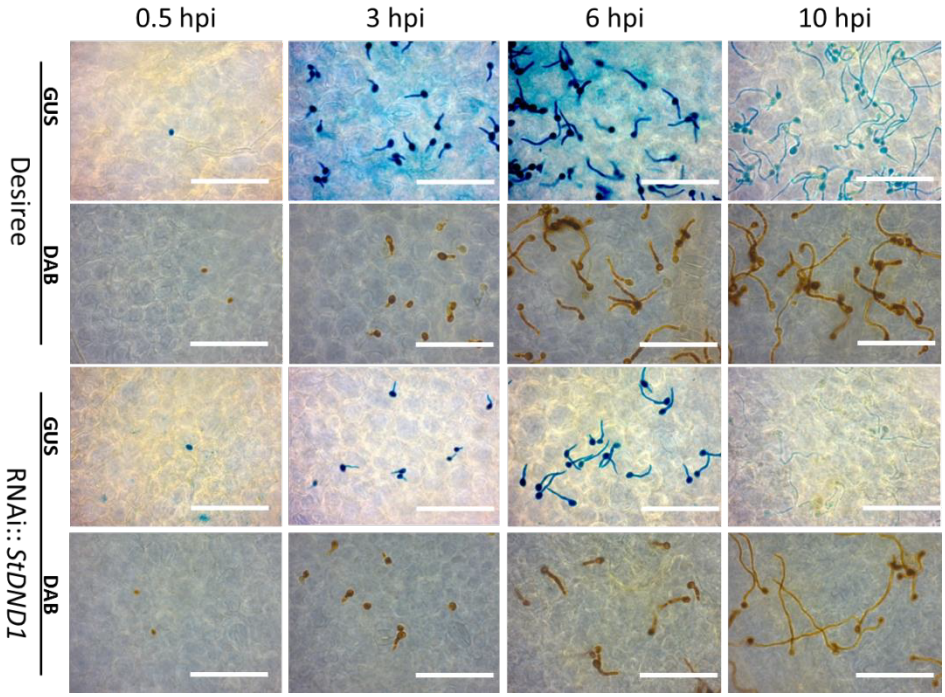

Fig. 3. Microscopic observations after GUS and DAB staining of *Botrytis cinerea* inoculated cv Desiree and *DND1* well-silenced potato plants (Scale bar = 100 µm). The same plants were used in the conidia counting in Fig. 2. Samples were taken for GUS and DAB staining at 0.5, 3, 6, and 10 hour post inoculation (hpi), respectively. From each genotype, three plants were tested (5 leaflets per plant) and four slides per time point per plant were observed. The photos are representative for the results observed in twelve preparations per genotype.

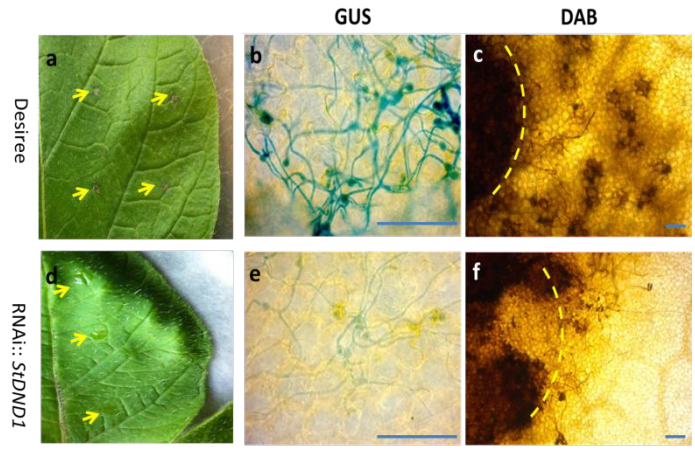

Fig. 4. Disease symptoms of *Botrytis cinerea* (strain SAS56 containing pCutGUS) on inoculated leaves of cv Desiree and *StDND1* well-silenced potato plants at 24 hours post inoculation (Scale bar = 100 µm). **a & d** photos of *B. cinerea* infected leaves of cv Desiree and *StDND1* well-silenced potato plants. Yellow arrows point to the inoculation sites, where lesions can be seen on the cv Desiree leaf but not on the *StDND1* well-silenced leaf. **b & e** and **c & f** GUS and DAB staining of leaf samples from *B. cinerea* infected cv Desiree and *StDND1* well-silenced potato plants.

At 48 hpi, lesion development of *B. cinerea* was observed by naked eye on both cv Desiree and *StDND1* well-silenced potato plants (Fig. 5, a & b). Necrotic spots, surrounded by a water-soaked lesion, developed on inoculated leaves of cv Desiree. Much smaller necrotic spots were observed on the *StDND1* well-silenced potato plants and they were surrounded by chlorotic tissue rather than a water-soaked area typically observed in the control. Leaf samples of the inoculation sites were taken from both cv Desiree and *StDND1* well-silenced potato plants after inoculation with *B. cinerea* (IN) or after mock-treatment (Potato Dextrose Broth, PDB) and were stained with GUS and DAB (Fig. 5c). GUS staining detected deep blue spots on leaves of cv Desiree, reflecting a dense growth of *B. cinerea* hyphae. Leaves of *StDND1* well-silenced potato plants exhibited much smaller blue spots, surrounded by a yellow/brownish ring, indicating a very restricted extent of fungal growth surrounded by plant tissue undergoing cell death (yellow/brownish colour). No fungal growth was observed on leaves inoculated with only PDB (Fig. 5). DAB staining showed that the brown colour in the wild type control extended beyond the borders of the lesion and the inside of the lesion lost colour. In *StDND1* well-silenced potato plants, however, the brown colour seemed to be restricted to the dead necrotic zone, but the entire brown region was smaller than in the control. Moreover, the central part of the inoculation site in *StDND1* well-silenced plants was deep brown and not discoloured like in the control (Fig.5).

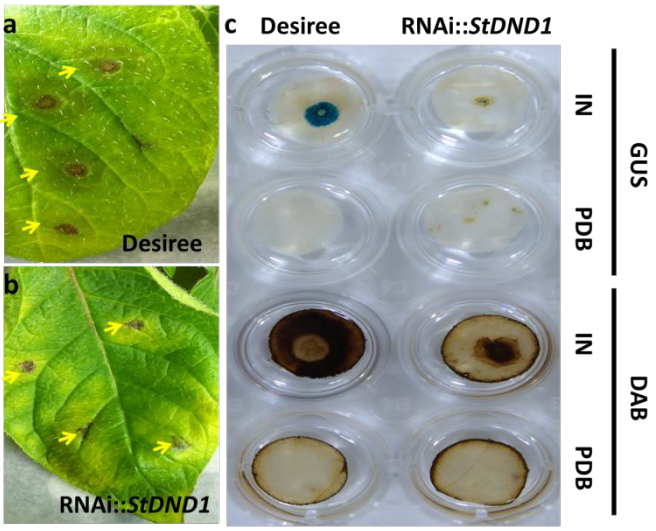

Fig. 5. Disease symptoms of *Botrytis cinerea* (stain SAS56 containing pCutGUS) on inoculated leaves of cv Desiree and *StDND1* well-silenced potato plants at 48 hours post inoculation. **Left panel** (**a** & **b**) photos of *B. cinerea* infected leaves of cv Desiree and *StDND1* well-silenced plants. Yellow arrows point to necrotic spots occurring at the inoculation sites. **Right panel** (**c**) GUS and DAB staining of leaf samples with inoculation sites from cv Desiree and *StDND1* well-silenced potato plants after *B. cinerea* inoculation (IN) or mock treatment (PDB). GUS stains hyphae of *B. cinerea* as blue spots at inoculation sites. DAB stains the H_2O_2 accumulation as brownish areas at inoculation sites. IN, inoculated; PDB, Potato Dextrose Broth medium; GUS, beta-glucuronidase; DAB, 3,3'-diaminobenzidine.

High *PR1* expression is detected in *StDND1* well-silenced potato plants

Previously, a high constitutive expression level of *PR1* (GenBank AJ250136), one of the pathogenesis-related protein encoding genes, was shown in *StDND1* well-silenced potato plants (Sun et al. 2016a). In non-inoculated plants, *StDND1* well-silenced transformants #5 and #17 showed an almost 100x higher expression level of *PR1* than untransformed cv Desiree and the weakly-silenced transformant #6. To investigate whether the salicylic acid (SA) pathway is involved in the reduced susceptibility of the *StDND1* well-silenced potato plants, expression of the *PR1* gene was quantified in a *B. cinerea* infection time course from 0.5 to 48 hpi. In cv Desiree and transformant #6, *PR1* transcripts started to increase from 3 hpi, and kept a high expression level till 48 hpi. In well-silenced transformants #5 and #7, *PR1* expression increased also from 3 hpi, reached a peak at 6 hpi and then decreased steadily. At 48 hpi, the *PR1* expression levels in all four inoculated genotypes were similar to each other (Fig. 6).

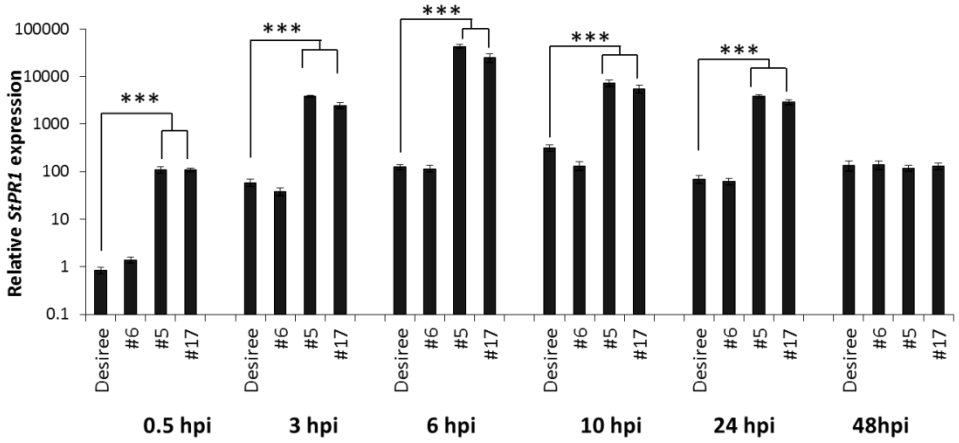

Fig. 6. Relative transcript levels of the *PR1* gene during infection with *Botrytis cinerea*. *PR1* is considered as a marker gene for salicylic acid (SA)-mediated defense pathways. Infected leaves were sampled at 0.5, 3, 6, 10, 24, and 48 hours post inoculation (hpi) for RNA extraction. For each sample, the expression level of the potato *EF1a* gene was used as reference, the relative expression level of *PR1* in cv Desiree leaves at time point 0.5 hpi was defined as 1. Data indicate the mean of three biological replicates with error bars representing the standard error. Three technical replicates of each repeat were analysed, which showed similar results.

Expression of *B. cinerea* endopolygalacturonase gene *Bcpg*1 and cutinase gene *BccutA* are altered in *StDND1* well-silenced potato

For *B. cinerea*, successful penetration of the cell wall requires enzymes capable of degrading pectin like endopolygalacturonases (endo-PGs) and pectin methylesterases (PMEs). In order to assess whether the expression of one of the endo-PG genes was affected in *StDND1* well-silenced potato plants during infection, the transcript levels of *Bcpg1* were investigated from 0.5 to 48 hpi (Fig. 7a). The expression level of *Bcpg1* in leaves of cv Desiree and the *DND1* weak-silenced potato transformant #6 increased 5-10 fold at 3 hpi and continued to rise until the last time point at 48 hpi. The transcript levels of *Bcpg1* in the two *StDND1* well-silenced potato transformants #5 and #17 only started to increase somewhat at 10 hpi, while at 24 hpi they reached values that were comparable to the 6 hpi sample on the control plants. Only at 48 hpi the *Bcpg*1 transcript levels in *StDND1* well-silenced potato plants did approach the level in both cv Desiree and potato transformant #6, but the difference in transcript level was still significant.

To assess whether the expression of the *B. cinerea* cutinase gene was altered in *DND1* well-silenced potato plants, the transcript levels of *BccutA* were also investigated in cv Desiree and *StDND1*-silenced potato transformants from 0.5 to 48 hpi (Fig. 7b). Similar to the transcript pattern of *Bcpg1*, significantly lower transcript levels of *BccutA* were found in *StDND1* well-silenced potato transformants. These results are in agreement with GUS staining (Fig. 3) indicating that the growth of *B. cinerea* is reduced in *StDND1* well-silenced potato plants.

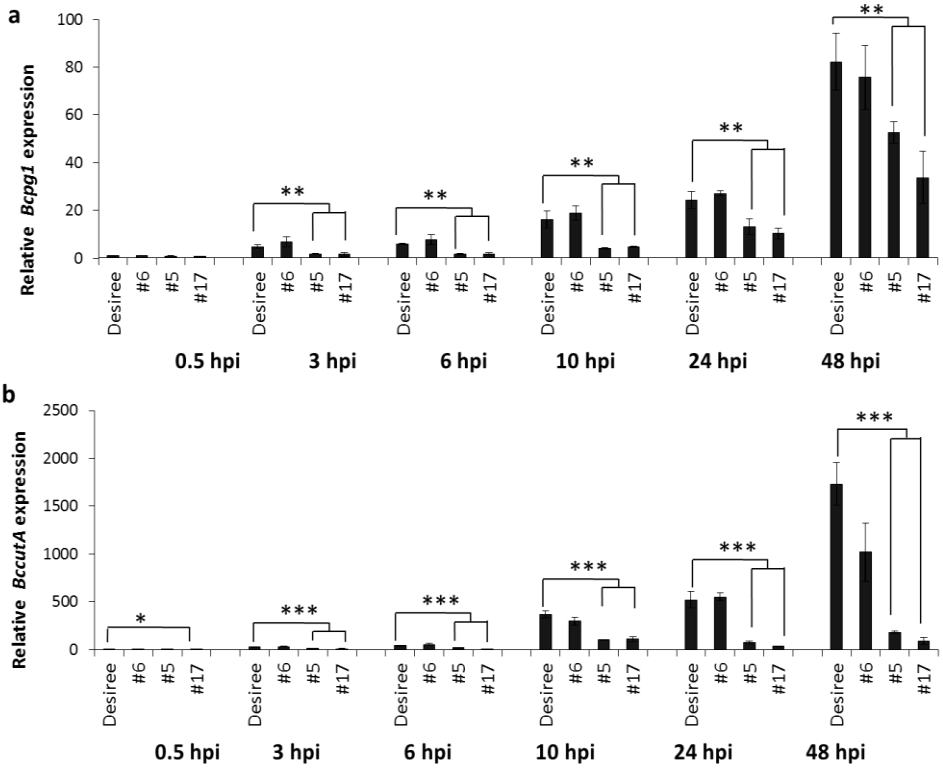

Fig. 7. Relative transcript levels of the *Botrytis* polygalacturonase gene *Bcpg1* and the cutinase gene *BccutA* in cv Desiree and *StDND1*-silenced potato transformants during infection with *B. cinerea* (strain SAS56 with a pCutGUS). Infected leaves were sampled at 0.5, 3, 6, 10, 24, and 48 hours post-inoculation (hpi) for RNA extraction. For each sample, the transcript level of Botrytis gene *Actin* was used as reference, the relative expression level of the target gene in cv Desiree leaves at time point 0.5 hpi was defined as 1. Data represent the mean of three biological replicates with error bars displaying the standard error. Three technical replicates of each repeat were analysed, which showed similar results.

SlDND1 well-silenced tomato plants show reduced susceptibility to *Botrytis cinerea*

In order to investigate whether silencing *DND1* can reduce *B. cinerea* infection also in tomato, the second generation (T2) of RNAi::*SlDND1* tomato plants obtained after transformation of cv Moneymaker (MM) (Sun et al. 2016a) were used in a DLA with *B. cinerea* strain B05.10. T2 plants were screened for the presence or absence of the RNAi construct. Compared with the susceptible control MM, smaller necrotic spots at the inoculation sites were observed on the *SlDND1* well-silenced tomato plants and the partial resistant *Solanum habrochaites* accession LYC4 (Fig. 8a). From 1 to 3 dpi, MM and *DND1* non-silenced tomato plants showed a similar pattern of lesion development (Fig. 8b). The lesion development on *DND1* well-silenced tomato plants was slower than that observed on the partial resistant *S. habrochaites* accession LYC4. Moreover, we performed a histological study with the *B. cinerea* reporter strain containing a GUS gene under control of the promoter of *Bccut*A. Compared to the susceptible MM plants, a lower number of conidia, delayed germination and reduced germ tube growth of *B. cinerea* were observed at 12 hpi on *SlDND1* well-silenced tomato plants (Fig. 8c). At 16 hpi, *B. cinerea* hyphae were branching and forming multiple penetration sites on MM plants. At the same time point, on inoculated leaves of *SlDND1* well-silenced tomato plants, the growth of hyphae was severely delayed and they were just in the stage of producing appressoria, visible as swellings at the tips of germ tubes. At 22 hpi, *B. cinerea* further developed colonies on MM plants, but not on *SlDND1* well-silenced tomato plants.

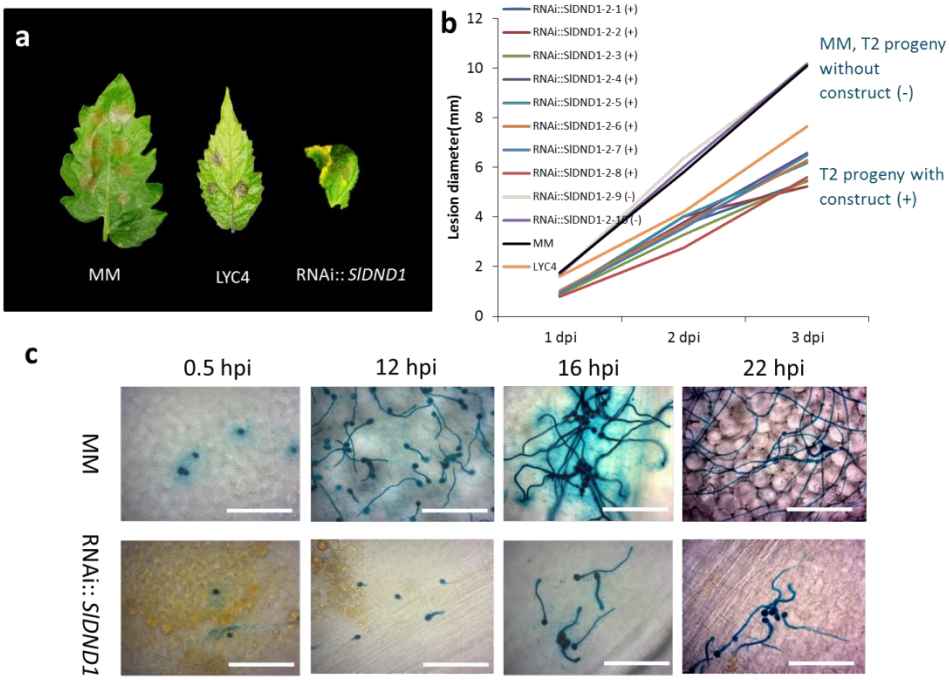

Fig. 8. Infection progress of *Botrytis cinerea* on *SlDND1*-silenced tomato transformants at different time points after inoculation. **a** Disease symptoms of *B. cinerea* on leaves of cv Moneymaker (MM), *S. habrochaites* genotype LYC4 and *SlDND1*-silenced tomato plants. **b** Lesion development in T2 progeny (T2#2 family derived from T1#2) harbouring a *SlDND1* silencing construct (+, n = 8) or their non-transgenic siblings (–, n = 2). Data were collected at 1, 2, and 3 days post inoculation (dpi). For each genotype, five leaflets per plant were inoculated with *B. cinerea* (strain B05.10). **c** Infection progress of *B. cinerea* (strain SAS56 containing pCutGUS) on MM and *SlDND1* well-silenced tomato plants at four time points after inoculation (Scale bar = 100 µm). The photos were taken at 0.5, 12, 16, and 22 hours post inoculation (hpi), respectively. The photos are representative for the results observed in six preparations per genotype.

Discussion

In this study four candidate *S* genes, *DND1*, *PMR4*, *DMR6* and *DMR1*, of which the silencing was previously shown to provide resistance to *P. infestans* (Sun et al. 2016b), were investigated via RNAi in potato for their role in susceptibility to *B. cinerea*. Of these four genes, only *DND1* was tested earlier in relation to *B. cinerea,* and the *dnd1* mutant of Arabidopsis showed increased resistance (Genger et al. 2008; Jurkowski et al. 2004; Yu et al. 2000). Here, it turned out that silencing the *DND1* ortholog in potato

provided a high level of resistance to *B. cinerea*. In addition, silencing the *DMR6* ortholog in potato showed a significantly reduced susceptibility only at 3 days after inoculation. Further microscopic study is needed to see how the growth of *B. cinerea* is affected in *StDMR6*-silenced potato plants. Silencing the *DMR1* or *PMR4* orthologs in potato did not reduce the susceptibility to *B. cinerea*. Further, the tomato *DND1* ortholog was silenced in cv Moneymaker, which also resulted in reduced susceptibility of tomato to *B. cinerea.* Based on the microscopic observations of the infection process of *B. cinerea* on *DND1* well-silenced tomato and potato plants, we discuss here the potential mechanisms by which the silencing of the *DND1* gene confers reduced susceptibility to *B. cinerea*.

The *DND1* gene encodes a protein that belongs to the family of cyclic nucleotide-gated ion channel proteins (CNGCs; Clough et al. 2000). CNGCs have a role in conducting Ca^{2+} into plant cells and are involved in various physiological processes (Sherman & Fromm 2009). In the Arabidopsis *dnd1* mutant AtCNGC2 is disrupted. This mutant exhibits a broad-spectrum resistance in absence of hypersensitive response (HR) to several biotrophic and necrotrophic pathogens including *B. cinerea* (Govrin & Levine 2000; Yu et al. 1998). HR is one of the most effective ways to impede growth of biotrophic pathogens, however it is considered to facilitate the growth of necrotrophic pathogens like *B. cinerea* (Govrin & Levine 2000). Thus, the resistance of the *dnd1* mutant to *B. cinerea* may be logically explained by the cell death suppression. However, in spite of a deficient HR, a *dnd1ein2* double mutant exhibited susceptibility to *B. cinerea* (Genger et al. 2008), showing that the ethylene pathway also plays an important role in the elevated resistance to *B. cinerea*.

Our results showed that silencing the *DND1* ortholog in both potato and tomato led to reduced susceptibility to *B. cinerea*. The attachment of *B. cinerea* spores to the plant surface appeared to be less efficient on *DND1* well-silenced potato and tomato plants than on wild type plants. This observation may indicate that cuticles of *DND1* well-silenced potato and tomato plants are chemically or physically altered. The cuticle consists of a complex polymeric network of esterified hydroxylated fatty acids covered with a wax layer, and it serves, amongst others, as a structural barrier that protects epidermal cells against pathogens (Nawrath 2006). However, in Arabidopsis, several cutin-defective mutants have been identified showing resistance to *B. cinerea*, including *sma4* (also known as *bre1* or *lacs2*), *bodyguard* (*bdg*) and *lacerate (lcr)* (Supplementary Table 1). Spore germination and hyphal elongation of *B. cinerea* were inhibited on the leaves of the *sma4* mutant (Tang et al. 2007). All these mutants are defective in cuticle integrity and have an increased cuticular permeability. The latter may allow the diffusion of toxic compounds with growth-inhibiting activities, as

shown in the *bdg* and *lacs2* mutants (Bessire et al. 2007; Chassot et al. 2007). In our study, GUS staining of *B. cinerea* indicated that at 10 hpi, the hyphae appeared to be dead on the *StDND1* well-silenced potato plants (Fig. 3). This would be in favour of the hypothesis of diffusion of fungitoxic compounds through a defective cuticle.

There is evidence that cuticular permeability can be uncoupled from resistance to *B. cinerea*. For example, the Arabidopsis *Resurrection 1* (*rst1*) mutant exhibits normal cuticular permeability although it has an altered cuticle composition (Mang et al. 2009). This mutant shows resistance to *B. cinerea*, which is associated with attenuated SA-dependent responses and enhanced JA-dependent defense. This resistance was compromised by defective JA and ethylene signalling pathways.

In addition, plant cell walls have a surveillance system. Alteration of cell wall integrity can lead to specific activation of novel defense pathways as in the Arabidopsis *myb46* mutant (Ramírez et al. 2011a). The transcription factor MYB46, a key player in regulating secondary cell wall biosynthesis, functions as a susceptibility gene to *B. cinerea* by downregulation of *CesA* genes (Ramírez et al. 2011b).

We cannot rule out the possibility that the SA signalling pathway plays a role in the weakened *B. cinerea* infection on leaves of *DND1* well-silenced potato and tomato plants. In *StDND1* well-silenced potato plants, constitutively elevated *PR1* expression was observed (Fig. 6 & Sun et al. 2016a). Although *B. cinerea* infection induced *PR1* expression in the susceptible control plants, a significantly higher level of induction was seen from 3 to 24 hpi in *StDND1* well-silenced potato plants (Fig. 6). It is, therefore, worthwhile to investigate further whether SA and SA-mediated defense responses play a role in resistance to *B. cinerea* on leaves of *DND1* well-silenced potato and tomato plants. In Arabidopsis, it has been shown that both JA- and SA-mediated signalling are required for local resistance to *B. cinerea* (Ferrari et al. 2003). The Arabidopsis *dnd1* mutant displays elevated SA levels, which could be required for its resistance to a broad range of pathogens including *B. cinerea*. This resistance to *B. cinerea* in the *dnd1* mutant could be abolished by disrupting ethylene signalling (Genger et al. 2008).

In tomato, resistance to *B. cinerea* is observed in the ABA-deficient *sitiens* mutant by a timely hyper induction of H_2O_2-dependent defenses in the epidermal cell wall (Asselbergh et al. 2007). Further, it is suggested that ABA determines susceptibility to *B. cinerea* by negatively regulating SA signalling (Audenaert et al. 2002). In the tomato *sitiens* mutant, H_2O_2 accumulation was observed from 4 hpi in epidermal cell walls in close contact to the fungal germ tubes (Asselbergh et al. 2007). In our study,

H_2O_2 accumulation was associated with the lesion growth in the susceptible control cv Desiree, while in *StDND1* well-silenced potato plants, lesions were surrounded by a ring of chlorotic tissue where H_2O_2 accumulation was also present (Fig. 5). It is unclear whether H_2O_2 accumulated in the yellow ring contributes to the resistance mechanism or reflects an early indicator of a pathway to cell death.

Further studies need to be performed to examine whether structural changes of epidermal cells and/or hormone signalling are involved in the reduced susceptibility of *DND1* well-silenced potato and tomato plants.

Although the precise mechanisms for resistance to *B. cinerea* by silencing *DND1* in potato and tomato remain elusive, our results demonstrate that knocking-down or knocking-out plant susceptibility genes may open a new way for breeding crops with resistance to all kind of pathogens including *B. cinerea*. The reported Arabidopsis genes (Supplementary Table 1) that facilitate *B. cinerea* infection offer a quick start for a targeted homology-based approach in finding and modifying their orthologs in crops, as performed in this study. Most of these Arabidopsis genes are involved in the cell wall biogenesis and altered plant morphology is detected in their mutants (Supplementary Table 1), which can hamper their application in crops due to fitness costs. However, fitness costs are not always associated with mutants in cell wall architecture. For example, the Arabidopsis *rwa2* mutant displayed both a normal plant morphology and resistance to *B. cinerea* (Manabe et al. 2011). Moreover, new techniques, such as CRISPR/Cas9 (Belhaj et al. 2013; Xie & Yang 2013) are available to create allelic loss-of-function variants, which combine favourable disease resistance levels with less detrimental side effects. With this technology it is even possible to create tetra-allelic gene disruption in tetraploid potato in one step (Andersson et al. 2017).

Acknowledgements

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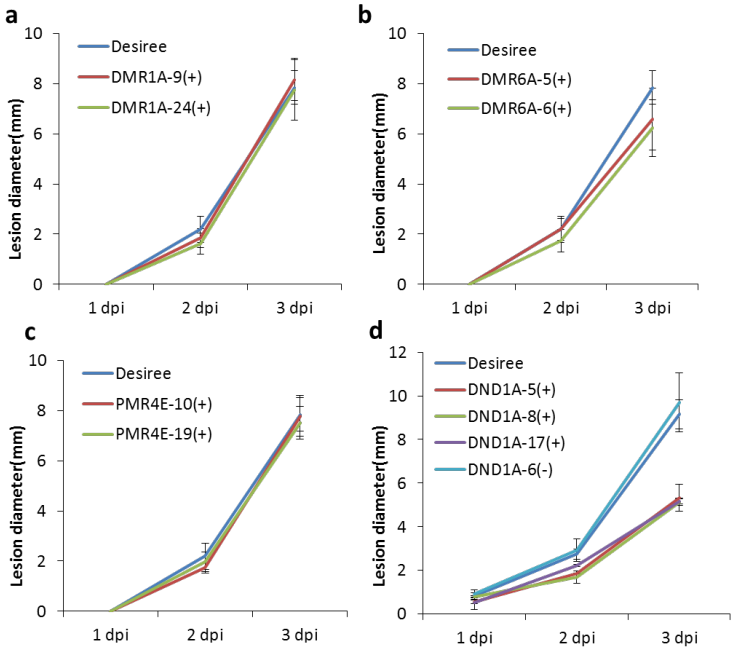
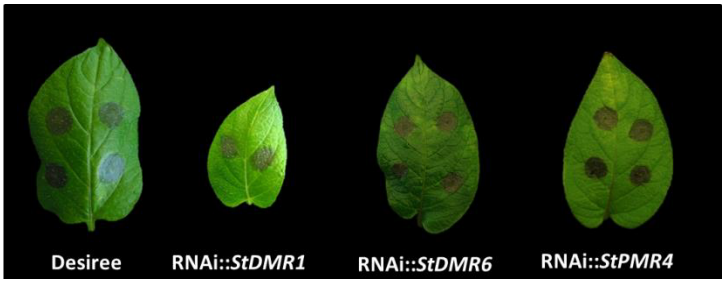
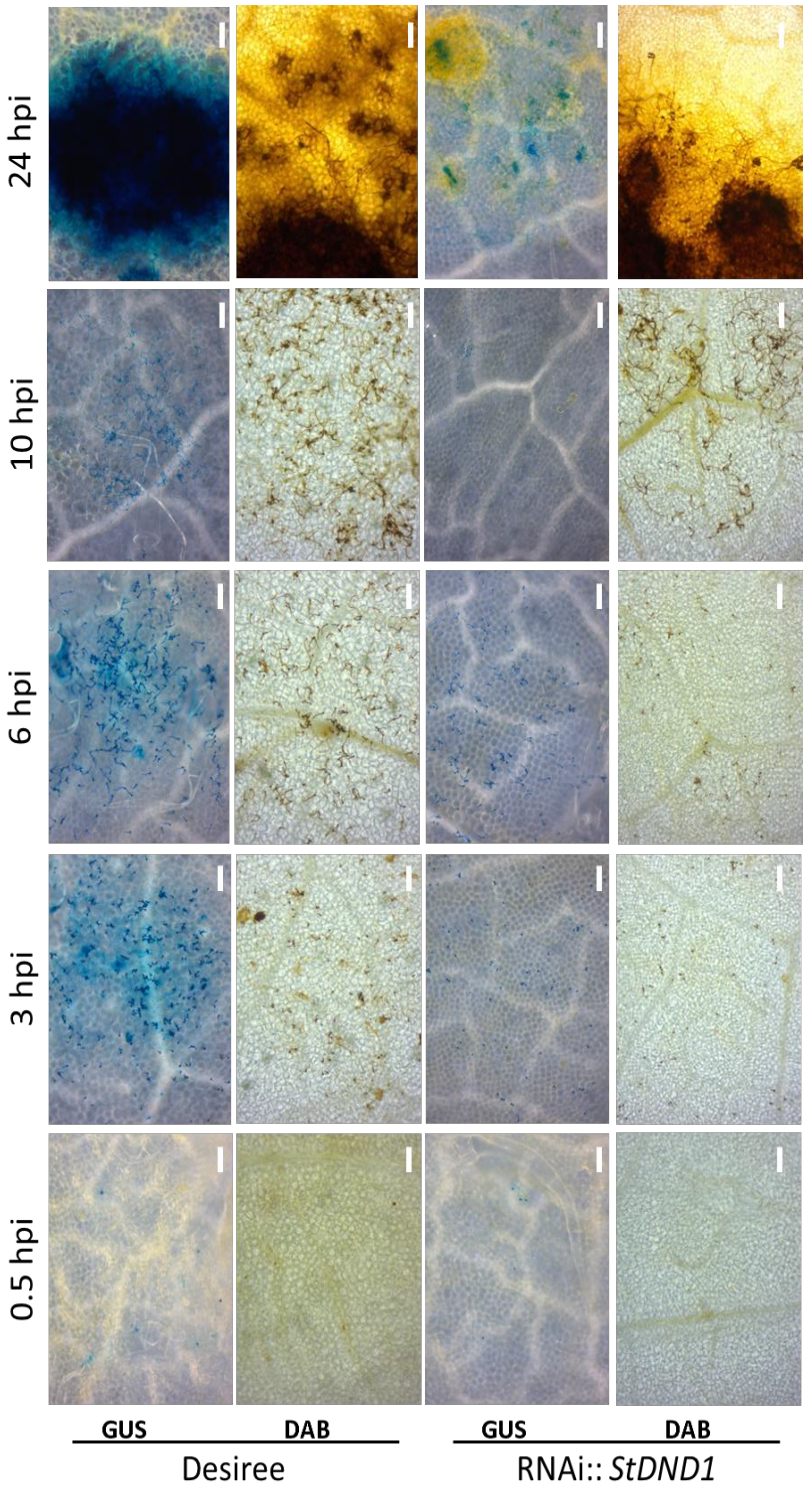
Supplementary material



Supplementary Fig. 1. Detached leaf assay (DLA) of potato RNAi transformants with *Botrytis cinerea* (strain B05.10). **a-d** Lesion diameter on the inoculated leaves of the *StDMR1*, *StDMR6*, *StPMR4*, or *StDND1* well-silenced potato RNAi transformants. Two to four independent transformants per gene were used. For *StDND1*, four independent transformants were used, one weakly-silenced transformant (-), and three well-silenced transformants (+). Susceptible control was cv Desiree. One leaf (the 5th or 6th leaf) with 5 leaflets per plant was drop inoculated (6 to 8 drops per leaf). Data were collected at three time points: 1, 2, and 3 days post inoculation (dpi). An average of all lesion diameters per transformant and per time point was calculated.



Supplementary Fig. 2. Detached leaf assay (DLA) of potato RNAi transformants with *Botrytis cinerea* (strain B05.10). Infection symptoms on cv Desiree, *StDMR1*, *StDMR6*, and *StPMR4* well-silenced transformants. One leaf (the 5th or 6th leaf) with 5 leaflets per plant was drop inoculated (2 to 4 drops per leaf). Photos were taken at 3 dpi.



Supplementary Fig. 3. Differences in interaction of *B. cinerea* with cv Desiree or with *StDND1*-silenced potato plants at different time points after inoculation (Scale bar = 100 µm). The photos were taken at 0.5 hours post inoculation (hpi), 3 hpi, 6 hpi, 10 hpi and 24 hpi, respectively.

Supplementary Table 1. Possible *Botrytis cinerea S* genes identified in Arabidopsis (according to van Schie & Takken 2014)

Gene	Plant species	Susceptibility mechanism	Reported pleiotropic phenotype	References
LePG& *LeEXP1*	Tomato	Cell wall architecture of fruit affects penetration and colonization of *Botrytis*	Reduced fruit softening	Cantu et al. 2008
LACS2/ SMA4/ BRE1	Arabidopsis	Cuticle permeability might affect diffusion of elicitors, ROS and defense compounds	Permeable cuticle, subtle leaf deformation and organ fusion, more susceptible to *Pseudomonas*, increased sensitivity to salt and drought	Bessire et al. 2007; Tang et al. 2007
RWA2	Arabidopsis	Pathogen entry? Unknown, Cuticle/wall permeability affects diffusion of elicitors, ROS and defense compounds?	None reported	Manabe et al. 2011
FDH	Arabidopsis	Cuticle permeability might affect diffusion of elicitors, ROS and defense compounds	Some organ fusion	Voisin et al. 2009
LCR	Arabidopsis	Cuticle permeability might affect diffusion of elicitors, ROS and defense compounds	Some organ fusion	Voisin et al. 2009; Wellesen et al. 2001
ATT1	Arabidopsis	Cuticle permeability might affect diffusion of elicitors, ROS and defense compounds	Permeable cuticle, increased susceptibility to *Pseudomonas*	Tang et al. 2007; Xiao et al. 2004

Supplementary Table 1 (continued)

Gene	Plant species	Susceptibility mechanism	Reported pleiotropic phenotype	References
BDG	Arabidopsis	Cuticle permeability might affect diffusion of elicitors, ROS and defense compounds	Permeable cuticle, more sensitive to osmotic stress, narrow elongated leaves, smaller plants	Asselbergh et al. 2008; Kurdyukov et al. 2006; Voisin et al. 2009; Wang & Dong 2011
Sit	Tomato	ABA affects cuticle composition and defense. Mutant has decreased ABA and increased cuticle permeability (and DAMP induced defense?)	Increased sensitivity to drought, wilting (open stomata), impaired interaction of (beneficial) arbuscular mycorrhizal fungi, early germination (e.g. viviparous)	Asselbergh et al. 2008; Audenaert et al. 2002; Curvers et al. 2010; Groot & Karssen 1992; Harrison et al. 2011; Herrera-Medina et al. 2007; Tal 1966
CESA4/7	Arabidopsis	Mutant has increased ABA/JA/Eth	None reported	Hernández-Blanco et al. 2007
CESA8	Arabidopsis	Mutant has increased ABA/JA/Eth	Increased ABA, enhanced drought tolerance	Chen et al. 2005; Hernández-Blanco et al. 2007
MYB46	Arabidopsis	Regulates CESA4/7/8, CSLA9; probable DAMP induced defense suppression	None reported	Kim et al. 2013; Ramírez et al. 2011a; Ramírez et al. 2011

Supplementary Table 1 (continued)

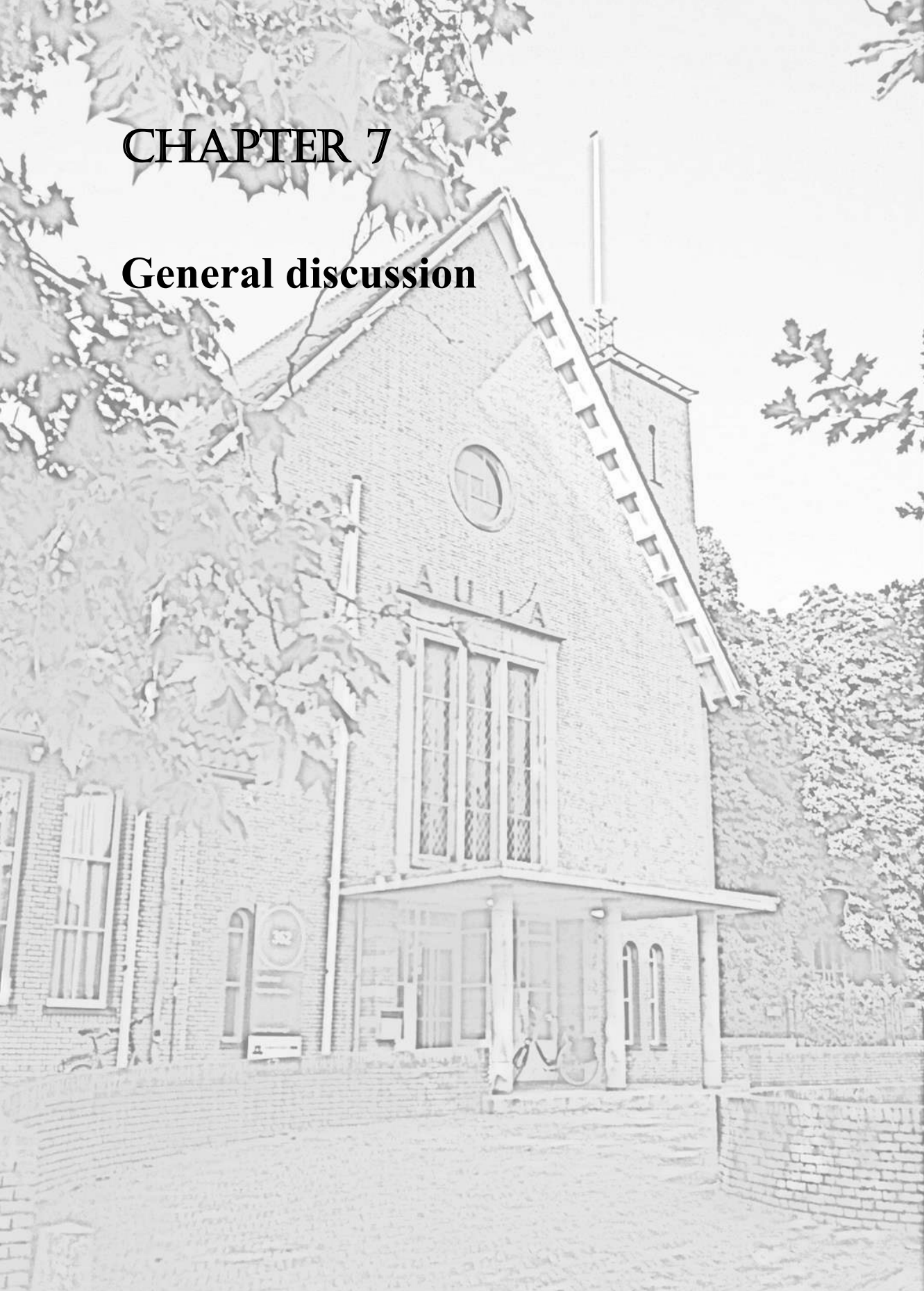
Gene	Plant species	Susceptibility mechanism	Reported pleiotropic phenotype	References
DND1/2	Arabidopsis	Defense suppression, HR and positive regulator of NO synthesis	Smaller plant, early senescence, moderate lesion mimic	Ahn 2007, Clough et al. 2000, Genger et al. 2008, Govrin & Levine 2000, Jurkowski et al. 2004, Su'udi et al. 2011
SR1/ CAMTA3	Arabidopsis	Defense suppression SA	Smaller plants [abolished at higher temp], increased susceptibility to insect feeding (*Trichoplusia, Bradysia*), possible reduced cold tolerance	Doherty et al. 2009, Du et al. 2009, Galon et al. 2008, Kim et al. 2013, Laluk et al. 2012, Nie et al. 2012, Qiu et al. 2012
RST1	Arabidopsis	Defense suppression JA; mutant has increased free (cuticular) lipids and increased JA	Increased sensitivity to biotroph powdery mildew (*E. cichoracearum*), increased embryo abortion, reduced storage lipids in seed	Chen et al. 2005, Mang et al. 2009
PLP2	Arabidopsis	Defense suppression, cell death (oxylipin mediated)	Increased sensitivity to virus (*Cucumber mosaic virus*), no developmental phenotype reported	Camera et al. 2009, La Camera et al. 2005

Supplementary Table 2. Primers used in this study

Primer name	Sequence (5'-3')	Used for
Fw-Botrytis Actin	TCTGTCTTGGGTCTTGAGAG	*Botrytis cinerea* normalisation
Rv-Botrytis Actin	GGTGCAAGAGCAGTGATTTC	
Fw-Bcpg1	AACGTGGTACCGCCTGTACC	determining relative transcript levels
Rv-Bcpg1	AGCCTTGGACTT GGAAGC G	
Fw-BccutA	CCTCCTTCCTCTCTCCGTCT	
Rv-BccutA	GCTGGGTAGTCGACACCATT	
Fw-StEF1a	ATTGGAAACGGATATGCTCCA	normalisation
Rv-StEF1a	TCCTTACCTGAACGCCTGTCA	
Fw-StPR1	TGGTGATTTCACGGGGAGGG	determining relative transcript levels
Rv-StPR1	CGAACTGAGTTGCGCCAGAC	

CHAPTER 7

General discussion



Potato, the world-wide important crop, is affected by several diseases. The most serious problem is potato late blight which is caused by the oomycete *Phytophthora infestans*. Although, resistance can be obtained by introgression of major resistance genes (*R* genes) from wild species, mostly conferred by single *R* genes, this has rarely been durable. Durability could be improved by stacking more *R* genes, as for instance in cultivar (cv) Sarpo Mira is showing (Rietman et al. 2012). Hence, other sources of resistance are highly needed. New research with a focus on loss of function mutations has led to the identification of disease susceptibility (*S*) genes in plants. In Arabidopsis many *S* genes were identified and several of them have been shown to be functionally conserved in other crop plants (Huibers et al. 2013; van Schie & Takken. 2014). Loss-of-function mutations in these *S* genes, which are recessively inherited, resulted in resistance to different diseases caused by pathogenic fungi, bacteria, viruses and oomycetes (Pavan et al. 2010; van Schie & Takken. 2014).

The research in this thesis was aimed at the identification and characterization of potato *S* genes involved in the interaction with *P. infestans* and *Botrytis cinerea*. We selected 11 *A. thaliana S* genes and silenced their potato orthologs by RNAi in the cv Desiree, which is highly susceptible to late blight (chapter 3). The silencing of five genes (*StCESA3*, *StDMR1*, *StDMR6*, *StDND1*, *StSR4*) resulted in complete resistance to the *P. infestans* isolate Pic99189, and the silencing of a sixth *S* gene (*StPMR4*) resulted in reduced susceptibility. Moreover, silencing *StDND1* provided resistance to three additional *P. infestans* isolates Pic99177, USA618, and EC1 (chapter 4). Furthermore, silencing of *StDND1* led to complete resistance to powdery mildew caused by *Golovinomyces. orontii* and reduced the infection of *B. cinerea*. Microscopic analysis showed that spore attachment and/or germination of *P. infestans* and *B. cinerea* was hampered on the leaf surface of *StDND1*-silenced potato plants (chapter 5 and 6). On *StDMR1*- and *StDMR6*-silenced potato plants, hyphal growth of *P. infestans* was arrested by the hypersensitive response, (HR)-like cell death, which is defined as HR in chapter 5. Although the precise mechanisms for resistance to *P. infestans* and/or *B. cinerea* after silencing of the selected *S* genes (*StCESA3*, *StDMR1*, *StDMR6*, *StDND1*, or *StSR4*) in potato remain elusive, our results demonstrate that impairment of plant *S* genes may open a new way for breeding potatoes with resistance to pathogens like *P. infestans* and *B. cinerea*. Here we discuss our findings presented in this thesis in a broader context and their implications for resistance breeding.

S genes: an additional source of disease resistance for plant breeding

We observed in literature and in our research that the susceptibility function of Arabidopsis *S* genes frequently is conserved across plant species. For example, loss of function or knock-down the expression of the *Mlo* gene led to powdery mildew resistance in different plant species, including Arabidopsis, tomato, pepper, wheat, and pea (Bai et al. 2008; Consonni et al. 2006; Pavan et al. 2011; Pessina et al. 2014; Wang et al. 2014; Zheng et al. 2013). As an added bonus, resistance obtained after silencing of orthologs of certain Arabidopsis *S* genes appears to be effective against a wider range of pathogens than one. For example, the Arabidopsis *dmr1* gene was originally identified in a screen for resistance to downy mildew caused by *Hyaloperonospora*

parasitica, and silencing the tomato ortholog resulted in powdery mildew resistance (Huibers et al. 2013). In chapter 3 and 5, down regulation of the potato ortholog of *DMR1* also exhibited resistance to the oomycete *P. infestans*. The Arabidopsis *dnd1* mutant was previously reported to be resistant to bacterial, (biotrophic, hemibiotrophic and necrotrophic) fungal and viral diseases (Yu et al. 1998). In chapter 3, 4, 5, and 6, we showed that suppression of *DND1* orthologs in potato and tomato can reduce susceptibility to the hemi-biotrophic oomycete *P. infestans* causing late blight, the biotrophs *O. neolycopersici* and *G. orontii* causing powdery mildew, and the necrotroph *B. cinerea* causing grey mould. Furthermore, the silencing of potato orthologs of other five *S* genes (*StCESA3*, *StDMR1*, *StDMR6*, *StSR4* and *StPMR5*) resulted in reduced susceptibility to *P. infestans* (chapter 3). Thus, impairment of plant *S* genes may open a new way for breeding potatoes with resistance to *P. infestans* and more pathogens.

However, we also find that some plant *S* genes are specific to certain pathogens. For example, *StDND1*-silenced potato plants had no reduced susceptibility to potato virus *Y* and the cyst nematode *Globodera rostochiensis* (chapter 4). Moreover, silencing *StBIK1*, *StCPR5*, *StDND2*, *StPMR5* and *StPMR6* did not provide resistance to *P. infestans* (chapter 3). These five genes may not be the proper *S* genes for *P. infestans*. On the other hand, it could also be that we did not find the correct potato ortholog(s) or that more than one homolog is needed for the resistance we are searching for. For *BIK1*, no clear orthologs in potato were found. We have chosen the one with the highest similarity (64% identity) to *AtBIK1* from the potato database. For *DND2, PMR6* and *PMR5*, we found more than one homolog in the potato database. Our RNAi constructs were designed to target the closest related homolog in the phylogenetic tree (chapter 3, Supplementary Fig. 1 &2). It may indicate that resistance would be achieved only when all the homologs are impaired simultaneously, as shown in the *Atmlo2/6/12* triple mutant (Consonni et al. 2006). In Arabidopsis, three *MLO* genes, *MLO2*, *MLO6* and *MLO12* showed functional overlap in mediating susceptibility to powdery mildew. A complete penetration based resistance to powdery mildew could only be achieved in the *Atmlo2/6/12* triple mutant (Consonni et al. 2006). *CPR5* is another interesting *S* gene, it participates in signal transduction pathways involved in plant defense. The Arabidopsis *cpr5* mutant provided resistance to multiple diseases caused by the oomycete *H. arabidopsidis*, the bacterium *P. syringae* and *Cauliflower mosaic virus* (Bowling et al 1997; Jing et al 2007; Jing et al 2008; Love et al 2007). The ortholog of *CPR5* in potato was found and silenced by RNAi. Unfortunately, *StCPR5* is not an *S* gene in potato for the oomycete *P. infestans* since in our study *StCPR5*-silenced potato plants did not provide resistance to the oomycete despite the observed occurrence of morphological changes e.g. dwarfing, yellowish leaves (chapter 3).

It is clear from the above mentioned examples that frequently orthologues of Arabidopsis *S* genes can be found in potato and that the observations on Arabidopsis mutants can predict resistance to certain classes of pathogens, but not always. In comparison with *R* genes, loss of function of *S* genes is more often not restricted to resistance to one pathogen. The results of our study demonstrate that knocking-down the expression of plant *S* genes can bring resistance to crops like potato and tomato (chapter 3 and 4).

Pre-penetration or post-penetration based resistance

In this thesis, we investigated cytologically the infection process of the silenced *StDND1, StDMR1*, or *StDMR6* genes in potato and tomato (*StDND1* only). In these studies the infection process could be stopped or slowed down in two different phases of the interaction: pre-penetration or post-penetration.

Pre-penetration based resistance: Before a pathogen can penetrate the host tissue, a spore must be able to germinate and grow on the surface of the plant after landing. The spore attachment and germination can be influenced by multiple factors, such as plant physical barriers and environmental factors (humidity, temperature, light, pH, etc). Our observations in cytological studies showed that spore attachment and spore germination of *P. infestans* or *B. cinerea* were much less efficient on *StDND1*-silenced potato plants, compared with that on wild type cv Desiree (chapter 5 and 6). We therefore proposed that leaf surface of *StDND1*-silenced plants are chemically and/or physically altered. In Arabidopsis, several mutants have been found showing resistance to *B. cinerea*, which are defective in cuticle integrity and have an increased cuticle permeability (Supplementary table 1 in chapter 6). The necrotrophic pathogen *B. cinerea* possesses multiple tools like enzymes, toxic compounds, phytotoxic metabolites to facilitate penetration (van Kan 2006). The increased cuticle permeability in these mutants (e.g. *bdg* and *lacs2*) may allow the diffusion of toxic compounds that inhibit the growth of *B. cinerea* (Bessire et al. 2007; Chassot et al. 2007). There were indications in literature that the *dnd1* mutant of Arabidopsis could provide resistance to *B. cinerea* (Genger et al. 2008; Jurkowski et al. 2004; Yu et al. 2000), but there was no histological study to link the *dnd1* based resistance to *B. cinerea* with reduced pathogen attachment. Our histological observations in chapter 5 fill this gap and provide another possible mechanism to explain *DND1*-silenced resistance to *B. cinerea* (Table 1). It may bring an interesting new research topic to check whether important physical factors are changed on the epidermis of the *StDND1*-silenced plants or not.

Supposing that altered leaf surface of *StDND1*-silenced plants would prevent penetration of a pathogen, we would expect that it would be effective to all the isolates of the same pathogen. However, when the aggressive EC#1 isolate of *P. infestans* was used, lesion growth was observed on *StDND1*-silenced potato plants although it was delayed and restricted compared with that on the susceptible cv. Desiree plants (chapter 4). Therefore, we argue whether the elevated SA signalling pathway in *StDND1*-silenced plants plays a role in the resistance after penetration of *P. infestans* or not. Further experiments are needed to study the infection progress of the EC#1 isolate in *StDND1*-silenced potato plants.

Evidence has been obtained that activation of multiple defense pathways are required for the broad-spectrum resistance in *dnd1* mutants (Genger et al. 2008; Moeder et al. 2011). For example, the resistance to *P. syringae* and *H. parasitica* in *dnd1* mutants is dependent on SA accumulation (Genger et al. 2008). Further, the former study on Arabidopsis *dnd1* indicated that CNGC2 participates in plant innate immunity, acting as a plasma membrane Ca^{2+} channel (Ali et al. 2007). Therefore, ROS production may be interrupted due to *DND1*-impairment which blocks Ca^{2+} influxes, which could slow

down the infection speed of *B. cinerea* in *StDND1*- silenced plants. Therefore, both the altered leaf surface and elevated signalling pathways in *StDND1*- silenced plants *DND1* could have contributed to the observed resistance to *P. infestans* and *B. cinerea.*

Post-penetration based resistance: It is well-known that plants defend themselves against most pathogens through two layers of an innate immune system (Jones & Dangl 2006; Schwessinger & Ronald 2012). The first one is the detection of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors at the plasma membrane, leading to PAMP-triggered immunity (PTI). Successful pathogens secrete effectors that suppress PTI and thus induce disease, resulting in effector triggered susceptibility (ETS). As a counter defense strategy, plants recognise a given effector either directly or indirectly and activate effector-triggered immunity (ETI) resulting in disease resistance (Chisholm et al. 2006; Jones & Dangl 2006). PTI or ETI activate defense responses by triggering events including calcium ion influx, oxidative burst, activation of mitogen-activated protein kinase cascades, reprogramming of gene expression, reinforcing cell wall at pathogen attempt sites, and programmed cell death (Dodds & Rathjen 2010). ETI is generally regarded as a stronger and more rapid response in the post-penetration stage, and is associated with the hypersensitive response (HR) (Jones & Dangl 2006; Schwessinger & Zipfel 2008).

In chapter 5, predominantly single or multiple cell death were observed on the *StDMR1*- or *StDMR6*-silenced potato plants inoculated with *P. infestans*, respectively. These single or multiple cell death reactions were clearly different with the susceptibility reaction of cv Desiree after inoculation with *P.* infestans, we therefore defined the cell death on *StDMR1*- or *StDMR6*-silenced potato plants as HR in chapter 5. Since there is no *R* gene known in cv Desiree, the HR-like cell death could not be explained by the direct or indirect recognition between proteins encoded by an *R* gene and its corresponding effector. Thus, it may indicate that PTI is activated once the *P. infestans* has penetrated on *StDMR1*- or *StDMR6*-silenced plants and stimulate the formation of HR-like cell death. Cell death is normally linked with oxidative burst (ROS accumulation) which is one of the first events occurring upon pathogen attack (Lamb & Dixon 1997). For certain *S* genes which are a negative regulator of ROS, impairment would elevate ROS level and increase susceptibility to necrotrophic pathogens like *B. cinerea* as accumulation of ROS seems to be essential in successful host penetration. That may explain why *StDMR1*- or *StDMR6*-silenced potato plants were unable to significantly reduce the infection of *B. cinerea* (Table 1).

In conclusion of *S* gene related pre-penetration, or post-penetration based resistance, firstly, resistance based on mutation of *S* genes involved in pre-penetration may confer resistance to more than one pathogen. Secondly, cell death can be considered as the fast defense response to restrict pathogen infection, but also can be used by certain (necrotrophic) pathogens as a nutrient source for further growth.

Table 1. The effect of three different *S* genes (*DND1*, *DMR6*, *DMR1*) silenced in potato, the wildtype cv Desiree and the *Rpi-vnt1.1* resistance gene containing cv Desiree transformant on inoculation with *P. infestans*, powdery mildew and *Botrytis cinerea.*

Gene	Pathogens			Cytology e.g. *P. infestans* inoculation
	Biotrophic e.g. Powdery mildew*	Hemi-biotrophic e.g. *P. infestans*	Necrotrophic e.g. *B. cinerea*	
DND1	+	+	+	reduced attachment, no penetration, no cell death
DMR1	+	+	-	attachment, penetration, quick cell death (single)
DMR6	+#	+	±	attachment, penetration, quick cell death (multiple)
Rpi-vnt1.1	-	+	-	attachment, penetration, cell death (single and multiple)
Desiree	-	-	-	attachment, penetration lesion caused cell death

+, significantly reduced susceptibility; –, no reduced susceptibility

**Oidium neolycopersici* and *Golovinomyces orontii*

unpublished data

Silencing of *StPMR4*, *StSR4* or *StCESA3* to achieve disease resistance

The Arabidopsis *PMR4* gene (also called *AtGSL5*) encodes a callose synthase that is required for papillary callose formation. Papillae are defined as the formation of thick cell wall appositions, which can be used by plants to prevent penetration of a fungus into epidermal cells. The well-known *mlo*-based resistance to powdery mildews is associated with the formation of papillae where callose are deposited, suggesting that callose contributes to the observed resistance (Bai et al. 2005; Consonni et al. 2006). However, the reverse was observed in the *pmr4* mutant of Arabidopsis, in which the absence of callose deposition resulted in reduced susceptibility to different pathogens including *E. cichoracearum*, *E. oriontii*, and *Phytophthora parasitica* (Jacobs et al. 2003; Nishimura et al. 2003). In tomato impairment of *SlPMR4* showed enhanced resistance to the powdery mildew fungus *O. neolycopersici* (Huibers et al. 2013).

These results suggested that absence of callose enhances resistance as well. In the *pmr4* mutant, the interplay between SA and JA signalling pathways could be altered upon pathogen attack and the enhanced resistance against *Pseudomonas syringae* was shown to be correlated with constitutive expression of the *PR-1* gene (Flors et al. 2008). In potato, it is known that callose deposition at the penetration sites contributes to the defense response with *P. infestans* (Halim et al. 2007; Vleeshouwers et al. 2000). In our studies, we observed enhanced foliar resistance to *P. infestans* isolate Pic99189 after silencing the potato *PMR4* ortholog (chapter 3). Further experiments are needed to study whether the absence of callose deposition and/or changes in signalling pathways contribute to the reduced *P. infestans* infection in potato by silencing *StPMR4.*

In chapter 3, we identified the potato ortholog *SR4* (also called calmodulin-binding transcription activator 2, *CAMTA2*), which is the closest gene in homology to *SR1* in the CAMTA family. By using RNAi, the *StSR4* silenced potato plants showed resistance to *P. infestans* isolate Pic99189. The *A. thaliana SR1* gene (also known as *CAMTA3*) belongs to a class of Ca^{2+}/calmodulin (CaM)-binding transcription factors and it is suggested as a negative defense regulator of the plant innate immunity (Galon et al. 2008, Du et al. 2009, van Schie & Takken 2014). Loss of function mutation of *SR1* in Arabidopsis showed enhanced resistance to *G. cichoracearum*, *P. syringae*, *B. cinerea*, and *Xanthomonas oryzae* pv.oryzae (Galon et al. 2008; Nie et al. 2012; Rahman et al. 2016). Linking to the results in chapter 3, it may indicate that the function of *SR1* is not only conserved in different crops, but also conserved in the *CAMTA* gene family. Moreover, *AtSR1* has been indicated to play an important role in plant immunity and ethylene signalling by directly regulating *NDR1* and *EIN3* (Nie et al. 2012). Our results in chapter 3 showed that *SR1* and *StSR4* are functionally conserved for their *S* gene function. Considering that the *SR1* gene is a negative defense regulator of the plant innate immunity, the *StSR4* RNAi silenced potato may provide resistance to a broad range of pathogens including fungal and bacterial diseases. This still needs to be investigated.

The *CesA3* gene from Arabidopsis (also called *cev1*, constitutive expression of VSP1), is involved in cellulose synthesis (Ellis et al. 2002). The presence of homozygous recessive alleles of the *CesA3* gene can enhance resistance to biotrophic pathogens like powdery mildew, including *Erysiphe cichoracearum* UCSC1, *E. orontii* MGH, and *O. lycopersicum*. The resistance mechanism of *cesa3* plants was concluded to be the result of an increase of *in planta* levels of abscisic and jasmonic acid (JA) and ethylene (Ellis & Turner 2001). It is well known that jasmonates are required, alone or in combination with ethylene, for defense against insects (McConn et al. 1997) and necrotrophic pathogens (Thomma et al. 2000; Thomma et al. 1999). So, it is not surprising to see that *StCESA3*-silenced potato plants were resistant to the hemi-biotrophic pathogen *P. infestans*. Based on the resistance mechanism of the *CesA3* gene in Arabidopsis, we assume that *StCESA3*-silenced potato also may provide resistance to necrotrophic pathogens such as *B. cinerea* and *Alternaria solani*. Moreover, a possible mechanism for JA- and ethylene-dependent defense is through their synergistic interaction in the induction of many defense-related genes, including *PR5*, *PDF1.2*, basic chitinase

(*CHI-B*), and of a hevein-like protein (Norman-Setterblad et al. 2000; Penninckx et al. 1998; Xu et al. 1994). The expression level of these defense-related genes in *StCESA3*-silenced potato plants has to be analysed and these plants can be used in testing the predicted resistance to different pathogens, including *B. cinerea* and *Alternaria solani*.

How to avoid fitness costs associated with impaired *S* genes

Plants do not carry *S* genes only for establishing susceptibility to pathogens. *S* genes most probably have other intrinsic functions that are important for the plant. For example, *EDR1* is a negative regulator of ethylene pathway (Frye et al. 2001). Arabidopsis *edr1* mutant is resistant to powdery mildew *Erysiphe cichoracearum, O. neolycopersici* and dark leaf spot *Pseudomonas syringae* pv *tomato* (Frye & Innes 1998; Gao et al 2015). However, the *edr1* mutant shows early senescence of the old leaves and is more sensitive to the necrotroph *Alternaria brassicicola* and the hemibiotroph *Colletotrichum gloeosporioides* (Hiruma et al 2011; Tang et al 2005).

In the review of van Schie and Takken (2014), 182 *S* genes were listed, and loss-of-function mutation in 91 of them showed altered plant phenotypes. These 91 *S* genes represent all three categories of *S* genes, of which 55 belong to the second category of *S* genes functioning as a suppressor of the plant immunity system (van Schie & Takken 2014). Loss of function mutations in these 55 genes resulted in constitutively activated defense, and the mutants consequently suffered from deleterious traits such as dwarfing and spontaneous lesions due to elevated SA level, ROS and cell death (Lorrain *et al.* 2003; Moeder & Yoshioka 2008). For example, lesion mimic phenotypes and dwarfing have been reported in Arabidopsis *dnd1* mutants (Clough et al. 2000). However, the negative pleiotropic effects, introduced by impairment of *S* genes, may be crop dependent. In this thesis, we are working with 11 *S* genes, all 11 mutated genes except *DMR6* showed phenotypic changes in Arabidopsis (chapter 3, Table 1). It is remarkable that silencing of only three of the 11 tested *S* gene orthologues showed pleiotropic side effects in potato (chapter 3, Table 2). Of the six *S* genes whose impairment by RNAi led to resistance to late blight, four were without pleiotropic negative side effects under the circumstances tested (chapter 3 Fig 2, Table 2). In our study, the results indicated that the fitness costs after silencing *S* gene *DND1* may be plant species dependent and related to the test conditions (chapter 4). This is in agreement with the situation in Arabidopsis where lesion mimic phenotypes in the *dnd1* mutant were dependent on the environmental conditions (Clough et al. 2000).

In chapter 2, 3 and 4, strategies to avoid fitness costs have been discussed in detail, including using native promoters of the *S* gene in RNAi constructs; creating different mutant alleles in the same *S* gene and using gene editing techniques (also discussed later in this chapter).

How to achieve durable and broad-spectrum resistance in crop plants?

Resistance and susceptibility have always been considered as two sides of the same coin. However, most studies have for a long time been focused on the resistance side, in search for plant major *R* genes. *R* gene based resistance is called vertical as the

resistance is following the gene-for-gene model of Flor (1971) and it is isolate specific. Only few examples were described that one major *R* gene could confer resistance to multiple diseases in the same crop plant (e.g. *Mi-1.2*.confers resistance to potato aphids and also to nematodes, Vos et al. 1998). A promising approach to improve multiple disease resistance of a susceptible variety could be achieved by stacking engineered *R* genes, providing resistance to multiple isolates of late blight and/or to other pathogens (Piquerez et al. 2014; Zhu et al. 2013). But even if the broad disease resistance has been achieved by stacking *R* genes against different pathogens, one of the pathogens could be able to overcome this *R* gene–based resistance after a simple point mutation in the corresponding avirulence gene of the pathogen..

Meanwhile, recessive resistances have been known for many years and a number of recessive alleles for disease resistances have been reported in Singh and Singh (2005) and Stubbs et al (1984). When characterized at the molecular level, the recessively inherited resistances have been shown to be derived from loss-of-function mutations of genes which are required for susceptibility. Although recessive resistances have been used for decades, the definition of a susceptibility gene was first highlighted in 2002 after the identification of *pmr6* (PM resistance) in Arabidopsis (Eckardt 2002; Vogel et al. 2002). *PMR6* was described as "..*a novel form of disease resistance based on the loss of a gene required during a compatible interaction*.." (Vogel et al. 2002), after which the term susceptibility gene was proposed (Eckardt 2002).

So far, the potential of increased resistance by impairment of *S* genes makes *S* genes highly interesting targets for resistance breeding. However, their potential as a new source for resistance breeding is dependent on their durability and the width of resistance spectrum provided after loss of function of *S* genes. The expected durability of *S* gene based resistance in plants has been indicated by van Schie and Takken (2014) as the following "..*For a pathogen to overcome S gene–based resistance rather than evading R gene–based recognition, it must overcome a dependency on a host factor. This may mean the pathogen needs to acquire a new function, which is more difficult to accomplish than loss-of-function. Therefore, we predict that S gene–based resistance is generally more durable than R gene–based resistance*". Here we would like to discuss *mlo* as an example of durable resistance because it is the most frequently mentioned *S* gene in literature. In barley the *mlo* mutation containing varieties were shown to be able to provide durable powdery mildew resistance. It has been used in resistance breeding for more than 70 years, and resistance-breaking strains have not been found in the field (Jørgensen 1992). Moreover, this resistance is broad spectrum, as impairment of *Mlo* in different crops can restrict different species of powdery mildew (Bai et al. 2008; Jiwan et al. 2013; Várallyay et al. 2012; Zheng et al. 2013). van Schie and Takken (2014) claim the *mlo* resistance as the following "*Plants with the recessive mlo allele seem to have increased penetration defense, but more importantly, the plant seems unable to cooperate with pathogen induced membrane and cytoskeleton reorganizations which are needed for the formation of haustoria. This is a mechanistic/structural requirement that the pathogen in the mlo mutant cannot easily overcome*." This may explain why *mlo* induced resistance is so durable and broad-spectrum for related powdery mildews in other crops. Cytological observations on inoculated *DND1*- silenced potato and tomato plants both with *P. infestans* and with *B*

cinerea showed also a blockade at the very beginning of the plant-pathogen interaction (chapters 5 and 6). This could be a good reason to indicate that *DND1* silencing in potato and tomato provides durable resistance to these pathogens as have been observed in the *mlo* mutant of barley.

The other mentioned example of durable resistance is the *pi21* mutant of rice, giving rise to non-race specific resistance to blast disease (Fukuoka et al., 2009). This mutant showed durable resistance in a few varieties. However, in breeding, this mutant showed negative side effects because of pleiotropy or of linkage drag and could not be used in elite cultivars. Cloning of the gene and transformation experiments showed that the negative side effect was not pleiotropic but very tightly linked to the recessive *pi21* allele. Cytological observations showed that penetration rate of hyphae into host cells was normal, but that hyphal growth was highly reduced. This observation shows that blockade of the interaction in somewhat later phase of the disease development can result in durable resistance. This example indicates that also resistance observed in *DMR1* and *DMR6* silenced potato plants can be durable, despite their blockade observed at later stages of the disturbed interaction with *P. infestans* (chapter 5).

As shown in this thesis, impairment of *S* genes provides a new class for late blight resistance in potato and tomato and six different *S* genes have been found with this effect. It is still not clear how durable this resistance is for the individual *S* genes selected. This has to be tested in future with more isolates and finally in varieties which are grown under different field conditions. To improve durability, two or more of these silenced *S* genes could be combined to prevent loss of resistance or it could be combined with major *R* genes.

Moreover, for resistance breeding, it is important to know how major *R* genes can be combined with silenced *S* genes in a way that both are involved in the resistance. Two ways can be followed: 1. Transformation in one step of both RNAi::*DND1* and one or two major *R* genes. The biological effect of silenced *DND1* can be observed during spore germination of late blight at the cell biological level and of the *R* genes by HR after agro-infiltration of the complementing *Avr* genes one by one; 2. Transformation in 2 steps: a. the first step is to generate resistance in *StDMR1*- or *StDMR6*-silenced plants; b. the second step is re-transformation of a resistant RNAi transformant with one or two *R* genes followed by agro-infiltration of the complementing *Avr* genes. It is possible in this way that *R* gene and *S* gene based resistance to late blight is combined. Such a plant brings in addition resistance to powdery mildew and to *B. cinerea*. At this moment, it is not known whether silencing of *StDND1* is bringing negative side effects with respect of becoming more susceptible to other diseases. Considering the results and ideas mentioned in this thesis, *S* gene silencing may open a potentially more durable disease resistance system in crops.

Technologies available to analyse or edit plant *S* genes

It has been shown in this thesis that silencing of *S* genes is a powerful way to test Arabidopsis *S* gene orthologues of potato and tomato for their effect on the interaction with pathogens like *P. infestans* and *B cinerea*. However RNAi is representing a

knock-down system; meaning that the target genes are not turned off completely. In our case it can be said that RNAi is a fruitful system because 6 of 11 silenced *S* genes showed an altered biological effect with pathogens. It is well known in the world that in Europe RNAi is seen as genetic modification, also when no transgenic marker gene like NPTII, which is coding for kanamycin resistance, is used and the intragenic approach is applied by using plant own promoters (Lusser & Davies 2013). The near future of this *S* gene approach has to be found in knock-out mutations. In addition to random mutation induction by EMS or irradiation, the more selection targeted technology like TILLING (targeting induced local lesions in genomes) (McCallum et al. 2000) or the gene targeted mutation induction technologies like ZFN (Zinc Figure Nuclease) (Urnov et al. 2010), TALENs (transcription activator-like effector nucleases) (Wood et al. 2011) and Crispr-Cas9 (Jiang et al. 2013) are possible to be used. The big difference between knock-down and knock-out systems is that the inheritance is dominant for the first mentioned possibility and recessive for the second one. Cultivated potato is an autotetraploid crop, indicating that classical induction and selection of recessive mutations is not preferred because of the very low chance to induce tetra-allelic mutations in one tetraploid cell.

A promising new approach is the recently developed technology of gene editing by targeted mutations with Crispr/Cas9. The recent publications showed tomato mutant alleles of *DMR6* and *Mlo* generated by gene editing with Crispr/Cas9 conferred resistance to *P. capsici* and *O. neolycopersici*, respectively (de Toledo Thomazella et al. 2016; Nekrasov et al. 2017). Meanwhile, it has been used to generate in one step mutant alleles of the *Mlo* gene in all three genomes of allohexaploid wheat (Wang et al. 2014). Also, Crispr/Cas9 has been shown to work in tetraploid potato where four dominant alleles of the major single *GBSSI* (granule bound starch synthase) gene, were mutated in one step, leading to amylose free starch, after using a transient expression approach (Andersson et al. 2017). This direct approach can also be applied for loss of function of *S* genes. Moreover, mutation breeding in potato can best be applied in breeding for hybrid seed potato varieties which are based on a cross between two diploid, self-compatible, inbred lines. Here classical mutation induction for selecting new potato specific *S* alleles, TILLING and even Crispr/Cas9 using transient expression can be applied. All these approaches end up with GM free diploid lines with bi-allelic loss of function mutations of a particular *S* gene.

Conclusion

As shown in this thesis, impairment of *S* genes provides a new class for late blight resistance in potato and tomato and six different *S* genes have been found with this effect. It is not clear how durable this resistance is for the individual *S* genes selected. This has to be tested in future with more isolates in the field. Cell biological investigation of resistance is a good help to determine in which step(s) the interaction between plant and pathogen is disturbed. A big difference between major *R* genes and silenced *S* genes is that *R* gene based resistance is frequently pathogen and isolate specific and that *S* gene based resistance can be broad to multiple isolates of one pathogen and simultaneously to several pathogens.

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Summary

Phytophthora infestans, the causal agent of late blight, is a major threat to commercial potato production worldwide. Significant costs are required for crop protection to secure yield. Many dominant genes for resistance (*R*) to potato late blight have been identified, and some of these resistance (*R*) genes have been applied in potato breeding. However, the *P. infestans* population rapidly accumulates new virulent strains that render *R* genes ineffective. This problem has been complicating resistance breeding. A new source of resistance in plants to many pathogens is needed to fill this gap. In **chapter 2**, we introduce a new class of resistance which is based on the loss-of-function of the susceptibility (*S*) genes encoding a product exploited by pathogens during infection and colonization. Impaired *S* genes by mutation primarily result in recessive resistance traits in contrast to recognition-based resistance that is governed by dominant *R* genes.

Nowadays, there are more than 180 *S* genes identified, mostly in the model organism *Arabidopsis thaliana* but also in crop plants like barley, pepper, tomato and rice. In **chapter 3**, we selected 11 *A. thaliana S* genes and silenced them by RNAi orthologous genes in the potato cultivar Desiree, which is highly susceptible to late blight. Gene silencing is dominantly inherited and can directly be used in tetraploid potato. The silencing of five genes (*CESA3, DMR1, DMR6, DND1* and *SR4*) resulted in complete resistance to the *P. infestans* isolate Pic99189, and the silencing of a sixth *S* gene (*PMR4*) resulted in reduced susceptibility. The other five *S* genes did not provide resistance to late blight but one of them (*CPR5*) showed other effects like dwarfing and light green leaf colour. Two of the five *S* genes, being the earlier mentioned *DND1* and *DMR1*, providing late blight resistance showed in addition also phenotypic effects. This observation means that at least six *S* genes are available now which, after loss of function, can act as a new source for breeding resistance to late blight.

Loss of function mutation in these *S* genes resulted in resistance to different pathogens, opening a new way to achieve plant disease resistance. In Arabidopsis, mutation of *DND1* resulted in broad-spectrum resistance against several fungal, bacterial, and viral pathogens. However this mutation is also associated with a dwarfed phenotype. Using an RNAi approach, we silenced *AtDND1* orthologs in potato and tomato. Our results (**chapter 4)** showed that silencing of the *DND1* ortholog in both crops resulted in resistance to the pathogenic oomycete *P. infestans* and to two powdery mildew species, *Oidium neolycopersici* and *Golovinomyces orontii*. The resistance to *P. infestans* in potato was effective to four different isolates although the level of resistance (complete or partial) was dependent on the aggressiveness of the isolate. In tomato, *DND1*-

silenced plants showed a severe dwarf phenotype and autonecrosis, whereas *DND1*-silenced potato plants were not dwarfed and did show a less pronounced autonecrosis phenotype. Our results indicate that *S* gene function of *DND1* is conserved in tomato and potato. We discuss the possibilities of using RNAi silencing or loss-of function mutations of *DND1* orthologs, as well as other *S* gene orthologs from Arabidopsis, in breeding for resistance to pathogens in crop plants.

In **chapter 5**, we further investigated *DND1, DMR1* and *DMR6* with the aim to monitor microscopically the infection process of *P. infestans* on *StDND1-, StDMR1-* or *StDMR6*-silenced potato plants. Macroscopically, no lesion was visible on the RNAi silenced plants, in contrast to lesion growth on the susceptible cv Desiree from 3 days post inoculation (dpi). Under a binocular, mycelia were seen covering inoculated areas on cv Desiree plants at 6 dpi, while, a clean leaf surface, small individual or clustered black dots were observed on *StDND1-, StDMR1-* or *StDMR6*-silenced potato plants, respectively. On *StDND1*-silenced potato plants, attachment of *P. infestans* to the leaf surface was hampered. On *StDMR1-* and *StDMR6*-silenced potato plants, growth of *P. infestans* was arrested by hypersensitive response (HR). Cell death associated with *P. infestans* infection happened at 24 hours post inoculation (hpi) on cv Desiree plants. Prior to this time point between 12 and 16 hpi, single and multiple epidermal cells underwent HR on *StDMR1-* and *StDMR6*-silenced potato plants, respectively. The single cell HR on *StDMR1*-silenced plants stayed at the epidermal cell layer and was effective to stop *P. infestans* growth at the stage of spores with only short germinated tubes. On *StDMR6*-silenced potato plants, HR reached mesophyll cells from 48 hpi onwards. Therefore, the infection process of *P. infestans* was hindered at different stages on *StDND1-, StDMR1-* or *StDMR6*-silenced potato plants. These results were compared with the loss of function mutants of Arabidopsis for *dnd1, dmr1* and *dmr6*.

Botrytis cinerea, a necrotrophic pathogenic fungus, attacks many crops including potato and tomato. Major resistance genes to *B. cinerea* are not known in plants, but a few quantitative trait loci have been described in tomato and other plants. Loss of function of particular *S* genes appears to be a new source of resistance to *B. cinerea* in Arabidopsis. In **chapter 6**, orthologues of 4 Arabidopsis *S* genes (*DND1, DMR6, DMR1* and *PMR4*) were silenced by RNAi in potato but in tomato only of *DND1*. *DND1* silenced potato and tomato plants showed, in comparison with susceptible cv Desiree and after inoculation with *B. cinerea*, a significantly reduced lesion diameter at all time points (1, 2 and 3 dpi). Reduced lesion diameter was also observed on leaves of *StDMR6*-silenced potato plants at 3 days post inoculation (dpi). The *StDMR1-* and *StPMR4-* silenced transformants were as susceptible as the control cv Desiree. Microscopic analysis was performed to study the mechanism that confers reduced

susceptibility to *B. cinerea* to *DND1*-silenced potato and tomato plants. A significantly lower number of *B. cinerea* conidia was attached to the leaf surface of *DND1* well-silenced potato and tomato plants and hyphal growth was hampered. The potential mechanisms associated with the reduced susceptibility to *B. cinerea* in *DND1* well-silenced potato plants as well as breeding aspects are further discussed.

Our experimental observations are discussed in the General Discussion (**chapter 7**). Based on the results in this thesis, it is clear that loss of function of *S* genes in potato can provide resistance to late blight. It indicated that impairment of *S* genes will be a new source of resistance breeding. We also discussed the potential resistance mechanisms in different infection processes and the possibilities of *S* gene impairment in resistance breeding. A remarkable phenomenon of several *S* genes like *DND1* is that they provide broad spectrum resistance not only to one pathogen but also to several other pathogens at the same time. For *DND1*, it was remarkable that resistance was obtained both to a biotrophic pathogen like *G. orontii*, a hemi-biotrophic pathogen like *P. infestans* and to a necrotrophic pathogen like *B. cinerea*. Cell biological research showed that this is possible because of the very early disturbance of the interaction on the silenced potato plant with spores of both pathogens so that cell necrosis did not play a role. Negative side effects related to *S* gene silencing, such as dwarfing and/or cell necrosis, are the bottlenecks which may restrict the use of *S* genes in resistance breeding. We discuss the possibilities of avoiding or reducing such negative side effects. Furthermore, available technologies used for loss of *S* gene function are discussed as well as the way how to combine *S* gene based and *R* gene based resistance in one genotype to come to durable resistance to late blight. In conclusion, considering the results and ideas mentioned in this thesis, impairment of S genes may open the way to a more durable disease resistance system in crop plants.

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Chunzhi Zhang (张春芝), Dongli Gao (高冬丽), Yiqian Fu (傅伊倩), and Xiaoxue Sun (孙晓雪), thank you to share the joy of life, and, of course, gossiping with you signifies that you are my confidantes. Shu Zhang (张庶), Suxian Zhu (朱素贤), Wenjie Hou (侯文杰), Lina Lou(娄丽娜)，Juan Du (杜鹃), Xi Chen (陈曦) and Weicong Qi (戚维聪), Jihua Cheng (程计华), Ying Li (李颖), Zhe Yan (闫喆), Xiao Lin (林啸), Yajun Wang (王亚军) and Wen Cui (崔文), Xuexue Shen (申雪雪), Mengjing Sun (孙梦婧), Mengyang Liu (刘梦洋), Peirong Li (李佩荣), Hao Hu (胡昊), Huayi Li (李华一), and Shuhang Wang (王舒航), I appreciate the nice lunch time together with you and it keep me still being able to speak fluent Chinese. Xiaonan Guan(关晓楠), Tian Yu(于阗), Chun Wang(王纯), Ruini Wang(汪芮妮), Wen Fang(方文) my ex-corridor mates, chatting with you helps my brain stay young; Xuan Xu (许萱), as you said, we should continue to practice “liu kou cha 六口茶”; Xu Cheng (程旭), and Yongran Ji(季永然), thanks for training and raising my level of table tennis; Bing Bai (白冰), and Xiaolin Wu (吴晓林), I feel really glad to meet my former classmates in Wageningen. My fellows from laboratory of Phytopathology, Yu Du (杜羽), Yan Wang (王燕), Chunxu Song (宋春旭) and Wei Qin (覃伟) (you are almost the part of Phyto),Yin Song (宋银), Guozhi Bi (毕国志), Jinling Li (李金岭), Jinbin Wu (吴锦斌), Hui Tian (田卉), Xiaoqian Shi (史晓倩), Shuqing Yang (杨淑青), thanks for your kind help when I was working in your department and giving me more opportunities to enjoy the authentic Chinese food in the Netherlands. Lingxue Cao (曹凌雪), Xiaotang Di (邸晓棠), Qian Liu (刘倩), Xiaomei Yang (杨晓梅), Lingtong Gai (盖龄同), Lin Ma (马林), Hao Dong (胡栋), Ya Wang (王亚) and Jiao Long (龙蛟), Junyou Wang (王俊友) and Junli Guo (郭军利), XiangDan Meng (孟祥丹), Chen Zhang (张晨), Fengjiao Bu (卜凤娇), Minggang Wang (王明刚), Tonggao Ren (任通高), Jia Ning (宁佳), Yumeng Zhang (张云梦), Tingting Xiao (肖婷婷), Bo Wang (王波), Yanli Wang (王艳丽), Zhen Wei (魏珍), Guiling Ren (任贵玲), Rufang Wang (王汝芳), Qiqi Lu (鲁奇琦), Zhengyi Lin (林正仪), Tao Zhao (赵涛), Mingtian Yao (要明天), and Shuwen Yang (杨舒文), thank you all for the nice time we have had together. Ruie Liu (刘瑞娥), Baowen Huang (黄葆文), Lidong Mo (莫李东), and Yifan Zhang (张怡帆), thanks so much for being the wonderful guide when I travelled to Bordeaux and Zürich.

I also could not miss this group, the Chinese guest researchers in Wageningen throughout these years. Chaohong Zhang (张朝红), Jinshan Miao (苗锦山), Zhechao Pan (潘哲超); and Xueping Chen (陈雪平), thank you for assisting my experiments;

Mengjuan Liu (刘萌娟), Chanjuan Liang(梁婵娟), Haiquan Huang (黄海泉), Shuxin Xuan (轩淑欣), Baosheng Shi(史宝胜), Zheng Wang (王征), Peng Gao (高鹏), Fei Wang (王飞), Yanling Hao (郝艳玲), Xiaotang Ju (巨晓棠), Weibin Ruan (阮维斌), Hongming Zhang (张宏鸣), Qingqin Cao (曹庆芹), Lianwei Qu (屈连伟), Wei Wu (吴未), Xiaohua Du (杜晓华), Fenghua Wang (王凤华), Haiping Wang (王海平), Tong Hu (胡栋), Hongmei Xiao (肖红梅), Jianfei Xu (徐建飞), Lujun Zhang (章炉军), Shuangchen Chen(陈双臣), and Yanming Deng(邓衍明), thanks all for sharing your experience with me , I am sure it will help me to shift my status earlier.

在这里，我要感谢我的母校，河南农业大学，大学四年的学习让我对生物有了更深入的了解。感谢我就读硕士研究生时的导师，生命科学学院的吴建宇教授和夏宗良教授，是您二位的教导，引我走向了科学研究的道路；是您二位给我提供了平台，让我可以把课本上学到的知识在实验室真正的运用起来；也是您二位的培养，使我的动手能力有了极大的提高。感谢园艺学院，是各位领导和同事们的宽容和鼓励，才使我能够顺利的完成博士的学习，我为我能够加入这个大家庭而感到荣幸。

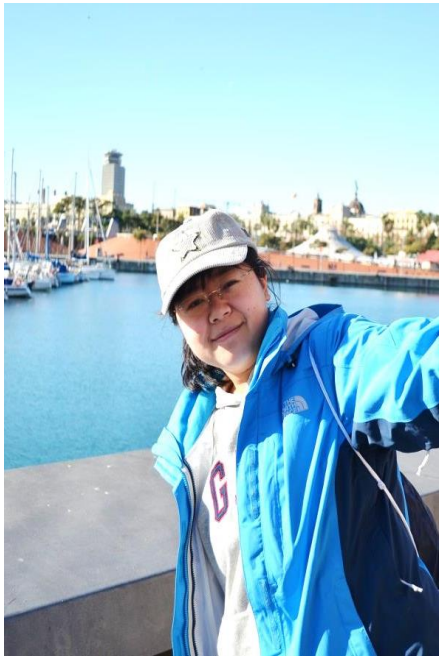
In the end, I just want to tell my big family how much I am grateful to them. 我的姥爷，感谢您每次的鼓励和鞭策，虽然有时候我显得不是很耐烦，但这些鼓励和鞭策却在无形中促进了我的进步；我的姥姥，我还记得在我小时候，您拿出一本厚厚的昆虫图册，带我认里面的大扑棱蛾子，可能那时您已经在我的心中默默种下了我以后学习生物的种子；我的姨夫，虽然我们的研究方向不同，我也可能永远达到不了您的学术水平，但您对科研的那份执着一直会是我前进的动力；我的爸爸，您的脚步走过了省里乃至全国的各个蔬菜基地，教过学生如今也遍布全国，有您这样的父亲我感到骄傲和自豪，我会努力前行不让您失望；妈妈，您总是在为咱们家默默得付出，您对生活的积极乐观，对工作的认真负责，对新知识的耐心学习，都是我学习的榜样，也将会是我以后工作的标准。在这里，我想对我们这个大家庭的每一名成员说：我爱你们，谢谢你们！

Kaile Sun 孙凯乐

20-03-2017, Wageningen

(Please forgive me if your name is missing.)

Curriculum vitae



Kaile Sun was born on March 29, 1986, in Zhengzhou, China. In September 2005, she was enrolled in Henan Agricultural University to study Biotechnology, and obtained her bachelor degree in June 2009. In September 2009, she started her Master education in the same University. There she studied Genetics and worked on functional gene analysis using *Nicotiana tabacum*. When she entered the third year of her Msc phase in 2011, she got an invitation to be a guest researcher in Plant Breeding, Wageningen University & Research (the Netherlands). There she worked on susceptibility genes - a new source of resistance to different diseases in Dr. Yuling Bai's group. In January 2014, she got a fund from the TKI project to start her PhD under the supervision of Dr. Yuling Bai and her two promoters Prof. Dr. Evert Jacobsen and Prof. Dr. Richard Visser. In July 2017, she will start a new job as lecturer in Horticultural College at Henan Agricultural University.

Publication list

Sun K, Wolters AM, Vossen JH, Rouwet ME, Loonen AE, Jacobsen E, Visser RGF, Bai Y(2016). Silencing of six susceptibility genes results in potato late blight resistance. Transgenic research. 25(5): 731-742.

Sun K, Wolters AM, Loonen AE, Huibers RP, van der Vlugt R, Goverse A, Jacobsen E, Visser RGF, Bai Y (2016). Down-regulation of Arabidopsis *DND1* orthologs in potato and tomato leads to broad-spectrum resistance to late blight and powdery mildew. Transgenic research. 25(2): 123-138.

Sun K*, Zheng Z*, Jacobsen E, Visser RGF, Bai Y (2014). Breeding for disease resistance by editing plant susceptibility genes. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources, 9 :5. (* equal contribution)

Xia Z, Wei Y*, **Sun K***, Wu J, Wang Y, Wu K (2013). The maize AAA-type protein SKD1 confers enhanced salt and drought stress tolerance in transgenic tobacco by interacting with Lyst-interacting protein 5. PLoS ONE. 8(7): e69787. (* equal contribution)

Xia Z, **Sun K**, Wang M, Wu K, Zhang H, Wu J (2012). Overexpression of a maize sulfite oxidase gene in tobacco enhances tolerance to sulfite stress via sulfite oxidation and CAT-mediated H_2O_2 scavenging. PLoS ONE 7(5): e37383.

Xia Z, Jia Y, **Sun K**, Wu J (2012). Pathogenic identification of wheat yellow dwarf virus strains in Zhengzhou region and molecular evolution analysis of coat protein genes. Journal of Henan Agricultural University, 46(2), 169-172.

Xia Z, Cao R, **Sun K**, Zhang H (2012).The movement protein of Barley yellow dwarf virus-GAV self-interacts and forms homodimers in vitro and in vivo. Arch Virol, 157(7): 1233-1239

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Kaile Sun**
Date: **16 May 2017**
Group: **Laboratory of Plant Breeding**
University: **Wageningen University & Research**

1) Start-up phase	*date*
► **First presentation of your project**	
Title: Identification, by RNAi silencing, of susceptibility genes in potato involved in *Phytophthora infestans infection*	Jun 16, 2014
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Title: Breeding for disease resistance by editing plant susceptibility genes, CAB Reviews 9 (2014), no.31. doi: 10.1079/PAVSNNR20149031	May 2014
► **MSc courses**	
Breeding for Quality and Resistance (PBR30306)	April 2014
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Get2Gether 2015, Soest, NL	Jan 28-29, 2015
EPS PhD Get2Gether 2016, Soest, NL	Jan 29-30, 2016
► **EPS theme symposia**	
EPS Theme 3 "Metabolism and Adaptation", Utrecht University	Apr 26, 2012
EPS Theme 2 "'Interactions between Plant and Biotic Agents", Utrecht University	Feb 20, 2015
EPS Theme 2 "'Interactions between Plant and Biotic Agents", Leiden University	Jan 22, 2016
EPS Theme 2 "'Interactions between Plant and Biotic Agents", Wageningen University	Jan 23, 2017
► **Lunteren days and other National Platforms**	
Annual Meeting "Experimental Plant Science", Lunteren, NL	Apr 02-03, 2012
Annual Meeting "Experimental Plant Science", Lunteren, NL	Apr 22-23, 2013
Annual Meeting "Experimental Plant Science", Lunteren, NL	Apr 14-15, 2014
Annual Meeting "Experimental Plant Science", Lunteren, NL	Apr 13-14, 2015
Annual Meeting "Experimental Plant Science", Lunteren, NL	Apr 11-12, 2016
► **Seminars (series), workshops and symposia**	
Invited seminar Cornlia Spetea Wiklun: 'lessons from photosynthetic analyses in three widely used Arabidopsis ecotypes'	Feb 21, 2012
PBR research Day 2012: next generation sequencing-what is in it for me	Feb 28, 2012
Invited seminar Inez Hortenze Slamet-Loedin: "Genetic modification for iron biofortification and drought tolerance in rice''	Jun 29, 2012
Invited seminar Hans de Kroon:"Mechanisms and consequences of belowground interactions between grassland species"	Dec 11, 2012
Invited seminar Niels Anten:"Tragedies and cooperation in plant communities: the aboveground perspective"	Dec 11, 2012
Invited seminar Detlef Weigel:"Arabidopsos thaliana as a model system for the study of evolutionary questions"	Feb 27, 2013
Invited seminar Graham Farquhar:"Integrating photosynthetic carbon assimilation from the leaf to the canopy"	Feb 27, 2013
Invited seminar Howard S. Judelson:"Molecular insights into spore biology and metabolism of Phytophthora infestans, the potato blight pathogen"	May 07 , 2013
Invited seminar Rays H.Y. Jiang:"Integrative genomics of destructive pathogens from oomycetes to malaria parasites"	May 07 , 2013
Invited seminar Brian Staskawicz:"Effector-targeted breeding for durable disease control of Xantiomonas diseases in tomato and cassava"	May 21 , 2013
Plant Sciences Seminar Guusje Bonnema and Arnaud Bovy 'The role of Plant Breeding in improving quality of crop plants'	Jun 11, 2013
Invited seminar Hanhui Kuang:"Using the Nicotiana-TMV system to study resistance gene evolution and plant genome stability"	Sep 11, 2013
PBR research Day 2014	Sep 30, 2014

3rd transPLANT user training workshop	Oct 13-14, 2014
Invited seminar Ortrun Mittelsten Scheidr:"Genetics and epigenetics: a complex relationship"	Nov 19, 2014
Mini Symposium Phytopathology 2014	Nov 24, 2014
Invited seminar Yves van de Peer:"The evolutionary significance of gene and genome duplications"	Feb 03, 2015
Invited seminar Monica Höfte:"Towards understanding rice brown spot, a disease induced by physiological stress"	Feb 06, 2015
Invited seminar Michael D. Pirie:"Inferring species trees given coalescence and reticulation"	Mar 18, 2015
Invited seminar Jiming Jiang:"Structure and evolution of centromeres: lessons learned from plants"	Apr 01, 2015
PBR research Day 2015	Sep 29, 2015
► **Seminar plus**	
► **International symposia and congresses**	
Next Generation Plant Breeding-Ede, The Netherlands	Nov 12-14, 2012
The 12th Solanacease Conference-Bordeaux, France	Oct 25-29, 2015
EUCARPIA 20th General Congress-Zurich, Switzerland	Aug 29-Sep 01, 2016
► **Presentations**	
Poster: "Identification, by RNAi silencing, of susceptibility genes in potato involved in P*hytophthora infestans* infection"- EPS Spring school 2014	Jun 02-04, 2014
Poster: "Identification, by RNAi silencing, of susceptibility genes in potato involved in *Phytophthora infestans* infection"- EPS Lunteren 2015	Apr 13-14, 2015
Poster: "Identification, by RNAi silencing, of susceptibility genes in potato involved in *Phytophthora infestans* infection"- The 12th Solanaceae Conference	Oct 25-29, 2015
Talk: "Reduced susceptibility to *Botrytis cinerea* by silencing a potato *S* gene" -PBR research Day 2015	Sep 29, 2015
Talk: "Down-regulation of Arabidopsis *DND1* orthologs in potato and tomato leads to broad-spectrum resistance to late blight and powdery mildew"-EUCARPIA 20th General Congress	Aug 30, 2016
Talk: "Impairment of potato *S* genes reduces susceptibility to late blight and powdery mildew"-Netherlands Society for Plant Biotechnology and Tissue Culture najaarssymposium 2016	Dec 09, 2016
► **IAB interview**	
► **Excursions**	
Company visit: Genetwister and In2Care	Sep 19, 2014
Company visit: Enza Zaden	Jun 12, 2015
Company visit: Tomato world	Oct 14, 2016
Subtotal Scientific Exposure	*15.2 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
EPS Spring School - Host-Microbe Interactomics	Jun 02-04, 2014
The Power of RNA-seq	Feb 10-12, 2016
► **Journal club**	
Participation in literature dicussion group at Plant Breeding, Wageningen UR	2011-2016
► **Individual research training**	
Subtotal In-Depth Studies	*4.7 credits**

4) Personal development	*date*
► **Skill training courses**	
Summer Course for IELTS	Aug 18-29, 2014
EPS introduction course 2015	Jan 20, 2015
Techniques for Writing and Presenting Scientific Papers	Sep 6-9, 2016
► **Organisation of PhD students day, course or conference**	
Assistant organistation of conference "Breeding industry and food security in the Netherlands"	Nov 09, 2013
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**37.9**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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