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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

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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 and Dangl 2006) (Fig. 1). In many cases, defence 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 defence 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
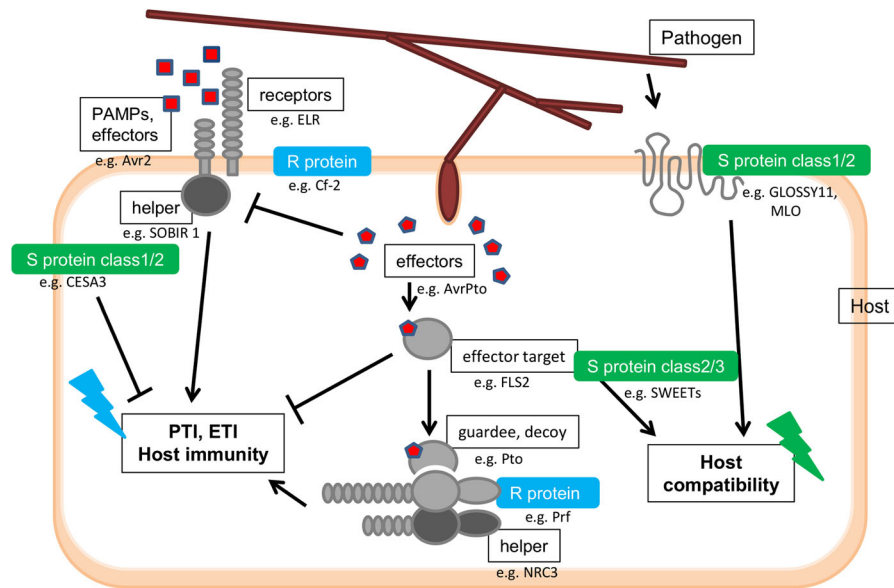
Fig. 1 Plant innate immunity: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). Apoplastic pathogen-associated molecular patterns (PAMPs/apoplastic effectors), intracellular effectors or modified effector targets are actively perceived by receptors in the plasma membrane or resistance (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 and 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

layer of defence 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 5 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 and Hwang 2012; Rietman et al. 2012; Zhu et al. 2013).

The resistance conferred by *R*-genes is based on pathogen recognition followed by the induction of defence 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 and 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 and Waterhouse 2003; Waterhouse and 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 and Skoog 1962) supplemented with vitamins, 30 g l^{-1} sucrose and 100 mg l^{-1} kanamycin to similar fresh MS medium without kanamycin. After 3 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 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 and Jinks 1968). To release zoospores from sporangia, ice-cold tap water was added to the Petri dishes, followed by incubation for 3 h 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 5–6 weeks of greenhouse growth. The fourth or fifth fully developed leaf (counted from the top) was used. The lesion diameters were measured from 3–6 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 $2^{-\Delta\Delta C_t}$ method (Livak and 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 Fig. 1). Because Sotub01g012330 was closer to *AtSR4* and *SlSR4* (Yang et al. 2012), we referred to Sotub01g012330 as *StSR4* in Table 2.

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).

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). 3–6 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.

Fitness costs associated with S-gene silencing

Altered phenotypic characteristics in non-inoculated plants were observed for some RNAi transformants,

Table 1 Selected *S*-genes identified in Arabidopsis

Gene name	Mutant resistance to pathogens						Mutant notes	Class[g]	References
	PS[a]	PM[b]	*PP*[c]	*HP*[d]	*BC*[e]	*AB*[f]			
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, 2008)
DND1	+	nd	nd	+	+	+	Smaller plant, early senescence, moderate lesion mimic	2	Ahn (2007), Clough et al. (2000), Genger et al. (2008), Govrin and 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 and 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 and Levine (2000), Jurkowski et al. (2004), Su'udi et al. (2011)
PMR5	–	+	–	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;	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)

Resistances conferred by mutated alleles, and phenotypic side effects are indicated per gene

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

[a] *Pseudomonas syringae*

[b] PM, powdery mildews: *Erysiphe cichoracearum*; *Erysiohe orontii*; *Oidium lycopersicum*; *Blumeria graminis*; *Golovinomyces cichoracearum*; *Golovinomyves orontii*

[c] *Peronospora parasitica;*

[d] *Hyaloperonospora parasitica*

[e] *Botrytis cinerea*

[f] *Alternaria brassicicola*

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

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 | | | | | Dwarfing | Autonecrosis | Color loss |
| | | Tested by | Reduced transcription | | DLA by Pic99189 | | | | |
		qPCR	>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)[a]	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	–	–	–

DLA detached leaf assay, R resistant, S susceptible

[a] See explanation in text

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).

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 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
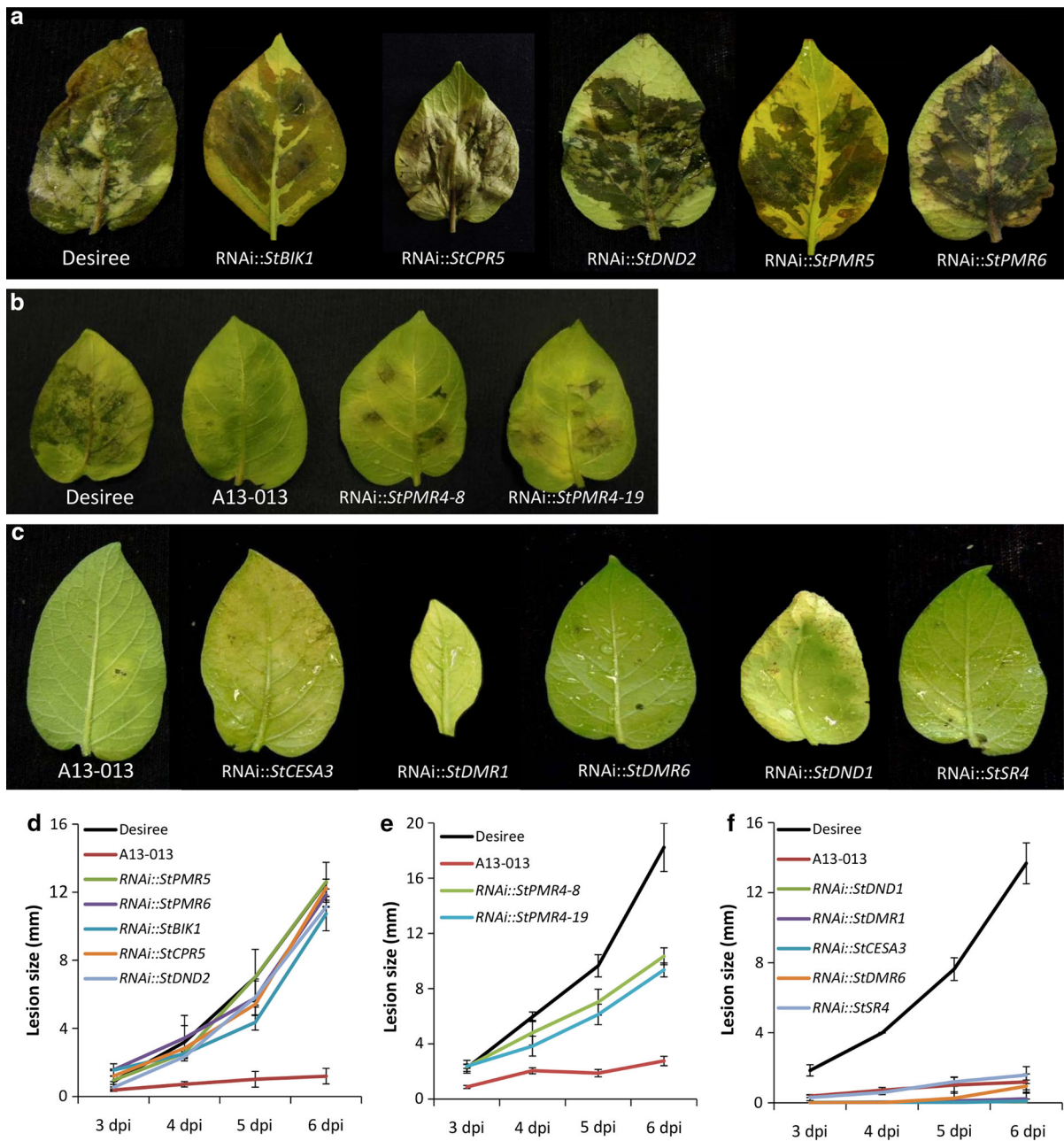
Fig. 2 Detached leaf assay (DLA) of potato RNAi transformants with *Phytophthora infestans* isolate Pic99189. **a** Leaves of the five RNAi transformants (*StBIK1*, *StCPR5*, *StDND2*, *StPMR5*, *StPMR6*) showing a diseased phenotype similar to that of cv. Desiree at 7 days post inoculation (dpi). **b** 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 **a**, **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
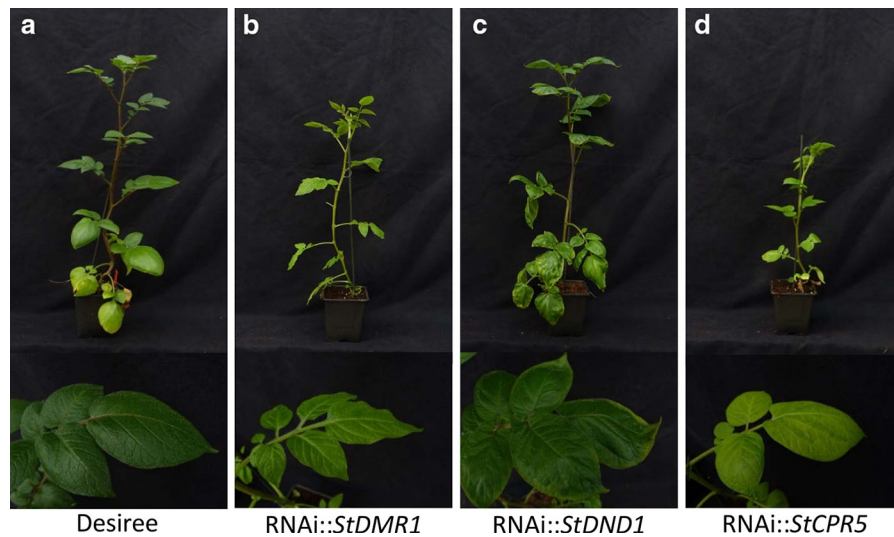
Fig. 3 Plants and leaflets of three potato RNAi transformants compared with cv Desiree. **a** Potato cv. Desiree. **b** *StDMR1*-silenced transformant, showing a dwarf phenotype and *light green* leaves. **c** *StDND1*-silenced transformant showing auto-necrotic spots on older leaves. **d** *StCPR5*-silenced transformant showing dwarfing and *light green* leaves

the powdery mildew *B. graminis*, which has been successfully used in European agriculture for more than 35 years (Büschges et al. 1997; Lyngkjær 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 and 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 (Sun et al. 2016). 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.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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