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Functional analysis of potato genes involved in quantitative resistance to *Phytophthora infestans*

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Abstract The most significant threat to potato production worldwide is the late blight disease, which is caused by the oomycete pathogen *Phytophthora infestans*. Based on previous cDNA microarrays and cDNA-amplified fragment length polymorphism analysis, 63 candidate genes that are expected to contribute to developing a durable resistance to late blight were selected for further functional analysis. We performed virus-induced gene silencing (VIGS) to these candidate genes on both *Nicotiana benthamiana* and potato, subsequently inoculated detached leaves and assessed the resistance level. Ten genes decreased the resistance to *P. infestans* after VIGS treatment. Among those, a *lipoxygenase* (*LOX*; EC 1.13.11.12) and a *suberization-associated anionic peroxidase* affected the resistance in both *N. benthamiana* and potato. Our results identify genes that may play a role in quantitative resistance mechanisms to late blight.

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Abbreviations

AFLP	Amplified fragment length polymorphism
VIGS	Virus-induced gene silencing
TRV	*Tobacco rattle virus*
PVX	*Potato virus X*
EST	Expressed sequence tag
TDF	Transcript derived fragments
BABA	DL-β-amino-butyric acid
ORF	Open reading frame
ANOVA	Analysis of variance
REML	Restricted maximum likelihood
SSH	Suppression subtractive hybridization
LGR	Lesion growth rate
PTGS	Post-transcriptional gene silencing
PCR	Polymerase chain reaction
NCBI	National Center for Biotechnology Information
LOX	Lipoxygenase
PGSC	Potato Genome Sequencing Consortium

Introduction

The potato (*Solanum tuberosum* L.) ranks as the world's fourth most important food crop, after maize, wheat and rice. Compared to other staple crops, a greater proportion of the potato crop is edible and a higher yield per hectare is achieved. This combined with the high nutritional value of the potato underlines its importance and explains on-going increases in global potato production. The most significant threat to potato production worldwide is the late blight

disease, caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary, which led to Irish famines between 1,845 and 1,852. Late blight is mainly controlled by application of enormous amounts of chemicals, which has raised issues on environmental, healthy and economic impacts. Therefore, there is an urgent need for resistant potato cultivars. Host resistance is a more environmentally benign means of restricting late blight infection but the success of this and other management practices hinge on effective and durable host resistance [1].

The most sustainable strategy to manage late blight is to breed broad-spectrum disease resistance into potato. However, traditional disease resistance breeding that exploits resistance genes (*R* genes) has only been short-life successful. *P. infestans* has a remarkable capacity to rapidly adapt to resistant host plants [1–3], and introduced *R* genes were quickly defeated. Indeed, this feature has led authors to describe *P. infestans* as a pathogen with a "high evolutionary potential" and an "*R* gene destroyer" [1, 3].

In addition to *R* gene-based resistance, another type of resistance occurs in partially resistant potatoes [4, 5]. In order to reveal molecular events that are associated with such quantitative resistance to late blight, many technologies have been used [6]. In our lab, a cDNA library highly enriched for quantitative-resistance-related genes was constructed by suppression subtractive hybridization (SSH) from the quantitatively-resistant potato 386209.10 in previous studies [7]. The same quantitatively-resistant potato 386209.10 was also subjected to cDNA-AFLP analysis to compare gene expression profiles in response to *P. infestans* and to DL-beta-amino-butyric acid (BABA) [8], which can protect potato plants against late blight [9–12]. In this situation, a large amount of gene fragments that may be associated with quantitative-resistance were gathered.

Virus-induced gene silencing (VIGS) provides a powerful tool to enable large-scale genes functional analysis. VIGS describes a technique employing recombinant viruses to specifically reduce endogenous gene activity and is based on post-transcriptional gene silencing (PTGS) [13]. Until now, several RNA and DNA viruses have been modified to develop VIGS vectors [14]. For example, *Potato virus X* (PVX)-based VIGS was successfully applied in *Nicotiana benthamiana* to identify a potato gene, *plastidic carbonic anhydrase*, which was involved in resistance to *P. infestans* [15]. *Tobacco rattle virus* (TRV) is able to spread more vigorously throughout the entire plant, including meristem tissue, yet the overall symptoms of infection are mild compared with other viruses [16, 17]. *N. benthamiana* is a good model plant for VIGS studies [18]. VIGS studies have also been described for potato [19, 20], but are less routinely used.

In this study, we selected candidate genes based on both cDNA microarrays and cDNA-AFLP analysis and cloned them from the quantitatively-resistant potato 386209.10 [8, 21]. These candidate genes were subjected to TRV-based VIGS in both *N. benthamiana* and potato, and following inoculation with *P. infestans*, we monitored the late blight disease symptoms. By these means, we aim to identify target genes that play a role in quantitative resistance to *P. infestans.*

Materials and methods

Plant materials and growth conditions

The potato (*Solanum tuberosum* L.) genotype 386209.10 with quantitative resistance to late blight, but not containing *R*1–*R*11 genes, was kindly provided by the International Potato Centre. This genotype 386209.10 was used for cloning the candidate gene fragments for VIGS. Another potato clone GT12297-4 and *N. benthamiana* were used for functional VIGS studies. In vitro potato plantlets were propagated in sterile culture boxes containing MS medium supplemented with 4 % sucrose and 0.8 % agar and raised in a climate room under controlled conditions (16 h light/8 h dark cycle at 20 °C). Four-week-old plantlets were transplanted to a greenhouse under proper conditions, i.e. with temperature between 20 and 26 °C and the humidity above 80 %.

Database searches

For the ESTs or TDFs, the Potato Genome Sequencing Consortium (PGSC) provides a promising new tool for identifying the corresponding genes [22]. Database searches were carried out using the Blast and Genome Browser Network and the results are shown in Table 1. The similarities of constructed sequences to *N. benthamiana* or *N. tabacum* (Table 1) were determined by Blastn searches in the Sol Genomics Network database (http://solgenomics.net/tools/blast/index.pl?db_id=194) and The Gene Index Project database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/Blast/index.cgi), respectively.

Plasmid constructs and transformation of *Agrobacterium tumefaciens* for VIGS

The purified total RNA prepared from leaves of potato clone 386209.10 were used to synthesize single-stranded cDNA by the ReverTra Ace reverse transcriptase (TOYOBO, Japan) as template to amplify EST and TDF fragments. We used specific primers with restriction enzyme sites of *Eco*RI, *Bam*HI, *Sac*I or *Xba*I (Appendix S1). After purification by the DNA kit (Takara, Japan), the PCR fragments were cloned into accordingly digested pTRV2 (Km, provided by Dinesh-Kumar, Yale), the binary TRV RNA2 vector that facilitates

Table 1 Characteristics of candidate genes

No	Clone ID	GenBank accession no[a]	PGSC accession no[b]	E value	Description	Significant up-expression time	Reference	Identity to *N. benthamiana*[c] (%)	Effect on resistance to *P. infestans*
1	03-F01	CO267885	PGSC0003DMT400042400	3.2e−58	WRKY protein	2 h	[21]	94	
2	02-H08	CO267880	PGSC0003DMT400011430	1.3e−46	Alpha/beta fold hydrolase family protein	2 h	[21]	93	
3	06-G09	CO267903	PGSC0003DMT400050770	1.6e−52	Amine oxidase	2 h	[21]	88	
4	01-G03	DR751726	PGSC0003DMT400008518	3.6e−77	Negative cofactor 2 transcriptional co-repressor	2 h	[21]	87	
5	10-B01	DR751971	PGSC0003DMT400015248	6.8e−42	Ubiquitin-associated/TS-N domain-containing protein	2 h	[21]	86	
6	03-B01	DR751765	PGSC0003DMT400072408	4.4e−67	Cytochrome P450 76A2	2 h	[21]	86	
7	02-E10	DR751747	PGSC0003DMT400007150	1.4e−73	Nucleolar protein nop56	2 h	[21]	95	
8	06-C02	DR751850	PGSC0003DMT400002738	8.7e−98	Brassinosteroid hydroxylase	48 h	[21]	93	
9	01-A10	CO267855	PGSC0003DMT400039723	2.1e−43	Serine-threonine protein kinase, plant-type	8 h	[21]	90	
10	02-E01	CO267874	PGSC0003DMT400047418	2.7e−35	Bacterial spot disease resistance protein 4	12 h	[21]	87	
11	05-E07	DR751823	PGSC0003DMT400041073	2.6e−41	Ammonium transporter AMT2	8 h	[21]	88	
12	05-F08	DR751829	PGSC0003DMT400034555	2.3e−32	Sodium-dicarboxylate cotransporter	8 h	[21]	88	Yes
13	04-E08	DR751798	PGSC0003DMT400004181	1.3e−131	Arginine decarboxylase	6 h	[21]	85	
14	02-G09	DR751757	PGSC0003DMT400037207	1.7e−111	Acyl-CoA synthetase	6 h	[21]	89	
15	02-D09	DR751743	PGSC0003DMT400076175	4.4e−111	3-ketoacyl CoA thiolase 2	2 h	[21]	98	
16	09-C08	DR751937	PGSC0003DMT400059159	2.1e−40	Inactive purple acid phosphatase 27	8 h	[21]	93	
17	09-G07	DR751955	PGSC0003DMT400041159	2.7e−65	UDP-arabinose 4-epimerase	8 h	[21]	90	Yes
18	02-D08	DR751742	PGSC0003DMT400020128	4.9e−82	Citrate synthase	2 h	[21]	84	
19	06-B11	DR751848	PGSC0003DMT400078735	2.7e−36	Pyruvate decarboxylase	8 h	[21]	93	
20	05-G08	DR751833	PGSC0003DMT400029432	9.6e−65	Eukaryotic translation initiation factor 2 gamma subunit	8 h	[21]	93	
21	01-C09	DR751721	PGSC0003DMT400060466	1.9e−46	2-oxoglutarate dehydrogenase	2 h	[21]	89	Yes
22	04-H08	DR751808	PGSC0003DMT400058934	4.2e−63	Lipoxygenase	24 h	[21]	87	Yes
23	02-G03	CO267878	PGSC0003DMT400039670	4.1e−101	Jasmonic acid 2	8 h	[21]	84	
24	02-C05	DR751739	PGSC0003DMT400031825	2.2e−77	2-deoxyglucose-6-phosphate phosphatase	4 h	[21]	91	Yes
25	06-H08	DR751876	PGSC0003DMT400070574	4.2e−52	DUF810 domain containing protein	8 h	[21]	89	
26	10-F01	DR751984	PGSC0003DMT400007542	2.0e−60	Ubiquitin system component Cue protein	8 h	[21]	89	
27	11-A04	DR752001	PGSC0003DMT400013160	9.8e−69	GYF domain-containing protein	12 h	[21]	92	
28	08-B01	DR751905	PGSC0003DMT400079936	6.2e−55	Lactose permease	4 h	[21]	87	
29	10-A02	DR751965	PGSC0003DMT400073575	2.4e−42	RING/c3HC4/PHD zinc finger	8 h	[21]	85	
30	06-F10	DR751870	PGSC0003DMT400080581	3.9e−46	Heat shock 70 kDa protein	2 h	[21]	83	
31	09-B03	DR751934	PGSC0003DMT400071951	6.9e−47	SnRK1-interacting protein 1	2 h	[21]	90	
32	02-H11	CO267881	PGSC0003DMT400004340	4.5e−62	Eukaryotic translation initiation factor 4G	2 h	[21]	85	
33	11-C06	DR752011	PGSC0003DMT400014995	3.6e−64	2, 4-dihydroxyhept-2-ene-1, 7-dioic acid aldolase	2 h	[21]	72	
34	02-F09	DR751752	PGSC0003DMT400032400	1.2e−59	Proton pump interactor 1	2 h	[21]	87	
35	06-B10	DR751847	PGSC0003DMT400007904	5.4e−15	Ethylene-responsive proteinase inhibitor 1	8 h	[21]	90	

Table 1 continued

No	Clone ID	GenBank accession no[a]	PGSC accession no[b]	E value	Description	Significant up-expression time	Reference	Identity to *N. benthamiana*[c] (%)	Effect on resistance to *P. infestans*
36	TDF 6-1	EL732330	PGSC0003DMT400073267	1.5e−23	WRKY transcription factor 5	4 h	[8]	83	
37	TDF 3-13-2	EL732262	PGSC0003DMS000000130	6.1e−16	None	None	[8]	68	
38	TDF 7-13-1	EL732276	PGSC0003DMT400061256	1.0e−09	Fatty acid desaturase	4 h	[8]	88	
39	TDF 1-7-1	EL732277	PGSC0003DMT400028593	2.4e−38	Oxysterol-binding protein	6 h	[8]	88	
40	TDF 5-7-3	EL732318	PGSC0003DMT400040295	5.4e−48	UDP-glucose: glucosyltransferase	4 h	[8]	84	
41	TDF 13-10	EL732349	PGSC0003DMT400045066	3.2e−101	Serine/threonine-protein kinase	36 h	[8]	93	
42	TDF 4-16-1	EL732321	PGSC0003DMS000000837	3.0e−23	None	16 h	[8]	83	
43	TDF 9-6	EL732320	PGSC0003DMT400035343	1.2e−08	46 kDa ketoavyl-ACP synthase	6 h	[8]	76	
44	TDF 16-1-1	EL732311	PGSC0003DMT400043204	1.5e−16	Double WRKY type transcription factor	36 h	[8]	88	Yes
45	TDF 1-5-2	EL732295	PGSC0003DMT400047088	1.6e−40	Aspartic protease	12 h	[8]	94	
46	TDF 5-7-1	EL732306	PGSC0003DMT400020915	2.8e−81	Kiwellin	16 h	[8]	81	
47	TDF 2-14-2	EL732300	PGSC0003DMT400031197	2.4e−22	Non-specific lipid transfer protein	4 h	[8]	83	
48	TDF 15-14	EL732297	PGSC0003DMT400057522	1.8e−18	Suberization-associated anionic peroxidase	4 h	[8]	82	Yes
49	TDF 10-14-1	EL732310	PGSC0003DMT400046538	4.4e−49	Sesquiterpene synthase 2	2 h	[8]	94	
50	TDF 1-2	EL732317	PGSC0003DMT400032975	3.5e−16	Ribulose bisphosphate carboxylase small chain 2C, chloroplastic	4 h	[8]	90	
51	TDF 4-14-1	EL732304	PGSC0003DMT400028158	2.0e−64	linoleate 9S-lipoxygenase 5	4 h	[8]	84	
52	TDF 4-5-2	EL732338	PGSC0003DMT400065130	6.6e−29	ATP binding protein	8 h	[8]	89	Yes
53	TDF 1-4	EL732292	PGSC0003DMT400015740	4.8e−22	Chlorophyll a/b-binding protein 4, chloroplastic	12 h	[8]	86	
54	TDF 3-16-2	EL732323	PGSC0003DMT400011741	1.4e−39	Dihydrolipoamide dehydrogenase	2 h	[8]	88	
55	TDF 7-3-2	EL732335	PGSC0003DMT400006600	6.0e−42	TATA binding protein associated factor	12 h	[8]	90	Yes
56	TDF 6-11-1	EL732334	PGSC0003DMT400050791	7.0e−42	Microsomal glutathione S-transferase	4 h	[8]	93	
57	TDF 4-6-4	EL732339	PGSC0003DMS000001571	3.5e−28	None	6 h	[8]	91	
58	TDF 7-4	EL732336	PGSC0003DMT400037282	6.8e−36	AT1G24350 protein	6 h	[8]	90	
59	TDF 3-16-1	EL732298	PGSC0003DMT400002284	2.9e−48	NAD(P)-linked oxidoreductase	4 h	[8]	86	
60	TDF 4-6-2	EL732294	PGSC0003DMS000001495	5.2e−41	None	12 h	[8]	88	
61	TDF 11-1	EL732315	PGSC0003DMT400004340	3.0e−26	Eukaryotic translation initiation factor 4G	12 h	[8]	92	
62	TDF 1-1	EL732280	PGSC0003DMT400052945	2.7e−23	LRR repeats and ubiquitin-like domain-containing protein	20 h	[8]	92	
63	STMCD18	BQ111806	PGSC0003DMT400034819	1.7e−112	ATP binding protein	2 h	[38]	85	Yes

[a] Accession number of candidate genes in GenBank database

[b] Accession number of candidate genes in PGSC database

[c] The similarity of constructed gene fragments to *Nicotiana benthamiana* homologs

VIGS in *N. benthamiana* [21]. As some ESTs contained internal cutting sites, the digested fragments of the corresponding genes were smaller than PCR products (Appendix S1). TRV: ESTs or TRV: TDFs binary constructs were then transformed into *Agrobacterium* sp. strain GV3101 by electroporation. The construct TRV: *StPDS* was constructed in the same way. Primers with *Kpn*I and *Mlu*I sites (5′-AAA*GGTACC*TTGAATGAGGATGGGAGTGT-3′ and 5′-AA*ACGCGT*AATGGCCGACAAGGTTCACA-3′) for amplifying the *PDS* fragment were derived from the potato *PDS* gene (AY484445). Both primers derived from the TRV2 vector (5′-*GATTCTGTGAGTAAGGTTACC*-3′ and 5′-*TAATGTCTTCGGGACATGCC*-3′) and specific genes were used to check the single clone of

recombinant plasmids. Then the inserts of the positive clones were sequenced at BGI (Shenzhen, China).

VIGS using TRV: ESTs or TRV: TDFs recombinants and inoculation with *P. infestans*

For *N. benthamiana*, four-week-old plants were treated by coinfiltration of *Agrobacterium tumefaciens* strain GV3101 carrying pTRV1 and the various pTRV2 recombinants, in a 1:1 ratio [23]. For each of the recombinant clones, three plants were used. Three to four weeks after plants treated with TRV: *StPDS* showing initial photobleaching, the newly grown developed leaves compared to negative control plant were detached. They were incubated on the surface of wet filtrate paper in closed boxes and inoculated with 10 µl droplets of freshly produced zoospore-suspension of *P. infestans* isolates at 5×10^4 zoospores ml^{-1}. The mixed isolates (race 1.3.4.7, 3.4.7.10.11 and 1.3.6.7.8.9.10.11) used for the inoculation were collected from west Hubei of China where late blight epidemics occur each growing season. The maintenance and inoculum preparation of *P. infestans* isolates followed the same method described by Vleeshouwers [24]. Inoculated leaves were incubated in a climate room at 18 °C, 90 % relative humidity and a 16 h light/8 h dark photoperiod provided by fluorescent lamps. For potato, two-week-old plants after transplanted in the greenhouse were inoculated in the same way as for *N. benthamiana*, except that agroinfiltration was performed in the stem instead of the leaf.

Estimation and analysis of lesion growth rate (LGR) and lesion size (LS)

Disease lesions were quantitatively assessed. For *N. benthamiana*, the disease lesion dimensions were measured at 3, 4 and 5 days post inoculation (dpi) using a caliper. The experiment was repeated three times and for each experiment eight leaflets were set up for each candidate gene. For potato, the disease lesion dimensions were measured just at 5 dpi, and three replicates with six leaflets for each candidate gene were used. The lesion size (LS) was calculated based on the following formula: $LS = 1/4 \times \pi \times \text{length} \times \text{width}$. The lesion radius calculated from square root transformation of the area was converted into lesion growth rate (LGR, mm/day) [24]. LGR and LS were estimated and analyzed with REML and ANOVA respectively, using GenStat 13.2.

Trypan blue staining of infected leaves

The trypan blue staining was employed to visualize the colonization with sporulating mycelium of *P. infestance*. Trypan blue stock solution was prepared by mixing 10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml water and 0.02 g of trypan blue together. A working solution was prepared by diluting the stock solution with 75 % ethanol (1:2 v/v). Infected leaves were transferred into a plastic jar with diluted trypan blue solution. The jar (lid slightly unscrewed) was placed in a heated water bath and the staining solution was boiled for one minute. After that, the leaves were left overnight in the staining solution. The next day, leaves were distained by replacing the staining solution with 75 % ethanol and the distaining solution was replaced several times.

Results

Candidate genes were selected for functional screening

In total, 63 candidate genes highly expressed during the early biotrophic infection were selected for the functional screening, which include 35 ESTs from the cDNA microarrays, 27 TDFs from the cDNA-AFLP profile and an extra gene STMCD18 from the publication (Table 1). We compared the similarities of the constructed parts of the candidate genes with *N. benthamiana* and most candidate genes showed high identities (above 80 %) to the orthologues of *N. benthamiana* (Table 1).

VIGS is effective on *N. benthamiana* as well as potato

To test whether VIGS studies are suitable for our purpose, we tested *N. benthamiana* as well as potato with control treatments. The photobleaching phenotype was used by suppressing the expression of the endogenous *phytoene desaturase* gene (*PDS*). A fragment about 524 bp of the potato *StPDS* (91 % identity to *N. benthamiana*) was cloned into pTRV2 and transformed into *A. tumefaciens* strain GV3101. For *N. benthamiana*, we used four-week-old plants and agroinfiltrated *StPDS* control construct into the lower-side of leaves. Photobleaching was observed after approximately one week and the top leaves became totally white at 12 dpi (Fig. 1a). This indicates that VIGS with the heterogenous *PDS* construct worked well on *N. benthamiana*. For potato, *StPDS* control fragment was agroinfiltrated into two-week-old stems of potato clone GT12297-4. The top leaves were almost white at 25 dpi in a proper greenhouse condition, with temperature between 20 and 26 °C and the humidity above 80 % (Fig. 1b). During initial VIGS test, we found that VIGS developed slowly on potato, which normally took three weeks or longer to show photobleaching. Besides, the VIGS treatment on potato often resulted in less uniform and weaker silencing of the gene throughout an infected plant compared to *N. benthamiana*. After we consistently observed photobleaching symptoms with *StPDS* on potato in

repeated experiments, we conclude, therefore, that potato could be used for VIGS to screen our candidate genes.

Three candidate genes cause morphological changes in *N. benthamiana*

The phenotypic effects of the candidate genes on plant morphology were also investigated by VIGS. We cloned all 63 candidate gene fragments into pTRV2 and treated four-week-old *N. benthamiana* together with pTRV1 in a 1:1 ratio by agro-coinfiltration. Then we monitored the plants for altered morphology over time. Over all, VIGS-treated *N. benthamiana* plants grew slower compared to non-treated plants, but generally, most constructs did not cause morphological changes compared to the healthy plants. In contrast, three different constructs, i.e. TRV: 7, TRV: 35 and TRV: 50 caused morphologically different phenotypes

Fig. 1 Photobleaching phenomenon on *Nicotiana benthamiana* and potato after VIGS treatment of *StPDS*. **a** VIGS effect of *StPDS* on *N. benthamiana* at 12 days post inoculation; b. VIGS effect of *StPDS* on potato at 25 days post inoculation

(Fig. 2). The candidate genes 7, 35 and 50 revealed similarity to a *nucleolar protein nop*56, an *ethylene-responsive proteinase inhibitor* 1 and a *ribulose bisphosphate carboxylase small chain* 2*C*, respectively (Table 1) by Blastn searches against *S. tuberosum* cv *phurjea* genome.

Two candidate genes cause altered resistance to *P. infestans* in both potato and *N. benthamiana*

To test whether the 63 candidate genes have an effect on resistance to *P. infestans*, we subjected them to VIGS on four-week-old *N. benthamiana.* Plants treated with TRV: *StPDS* were used as negative control. Three to four weeks after the negative control plants showed initial photobleaching, newly grown developed leaves were detached and inoculated with *P. infestans* zoospore suspension. We rated the resistance by measuring disease lesions on 3 dpi, 4 dpi and 5 dpi and estimated LGR. Statistical analysis showed that resistance to *P. infestans* was significantly reduced after VIGS treatment with TRV vectors containing candidate gene fragments 22, 44, 48, 52, 55 and 63 (Table 2). According to Blastn searches against *S. tuberosum* cv *phurjea* genome database, these genes are hinted as a *lipoxygenase*, a *double WRKY type transcription factor*, a *suberization-associated anionic peroxidase*, an *ATP binding protein*, a *TATA binding protein associated factor* and another *ATP binding protein*, respectively (Table 1).

After scoring lesions, we treated infected leaves with trypan blue. The infected areas of plants that were treated with TRV: 22, TRV: 44, TRV: 48, TRV: 52, TRV: 55 and TRV: 63 were much larger than those of healthy and negative control plants. Representative pictures of *P. infestans* infections on *N. benthamiana* are shown in Fig. 3 (TRV: 22, TRV: 63 not shown). From the microscopic observation, most of the blue-stained tissue is colonized with sporulating mycelium (Fig. 4). These data confirm that VIGS treatment with TRV: 22, TRV: 44, TRV: 48, TRV: 52, TRV: 55, and TRV: 63 resulted in enhanced susceptibility to *P. infestans*.

Functional screening of candidate genes was also performed on potato. In this case, LS was investigated at 5 dpi. Statistical analysis showed that VIGS treatments with several candidate genes enhanced susceptibility, i.e. TRV: 12, TRV: 17, TRV: 21, TRV: 22, TRV: 24 and TRV: 48 (Table 2). From our results, we concluded that two candidate genes 22 and 48, identified as *lipoxygenase* and *suberization-associated anionic peroxidase* were found to have a significant effect on disease resistance in *N. benthamiana* and potato (Table 1). The other genes 12, 17, 21, 24 are hinted as a *Tonoplast dicarboxylate transporter*, a *UDP-arabinose* 4-*epimerase* (EC 5.1.3.5), a 2-*oxoglutarate dehydrogenase* and a 2-*deoxyglucose*-6-*phosphate phosphatase*, respectively (Table 1).

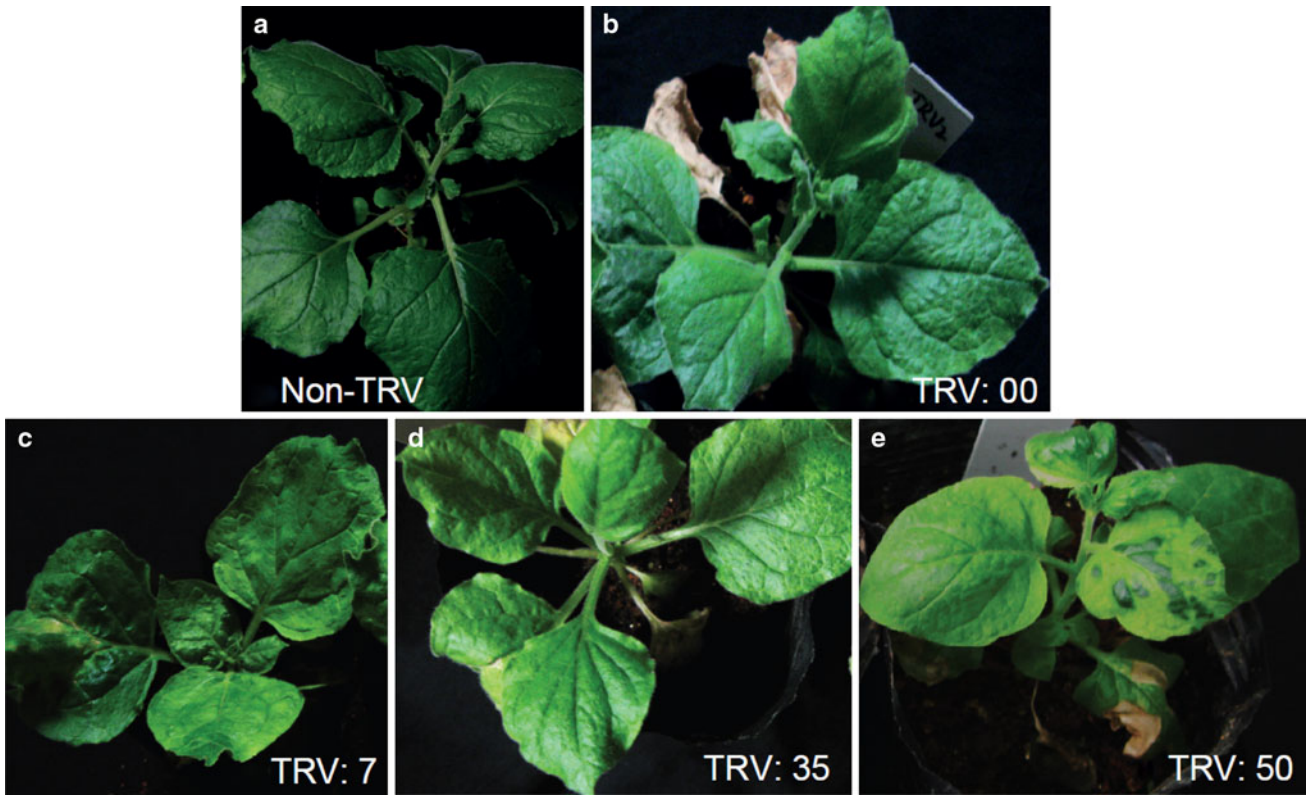

Fig. 2 Morphological changes of *Nicotiana benthamiana* after VIGS treatment at 16 days post inoculation. **a** A healthy plant. **b** A negative control plant treated with TRV: 00. **c** After treatment with TRV: 7, the stem had stopped growing and leaves became thick and small. **d** After treatment with TRV: 35, plants had stopped differentiating the terminal buds. **e** After treatment with TRV: 50, plants grew slowly and leaves became thin, small and chlorosis

Table 2 Decreasing resistance to *Phytophthora infestans* in potato and *Nicotiana benthamiana* after VIGS treatment of some genes

Construct no.	*N. benthamiana* Lesion growth rate ± standard error	Potato LS (mm^2)
TRV: 00[a]	1.8 ± 0.4	12.0 ± 1.3
Non-TRV[b]	1.7 ± 0.4	12.5 ± 0.9
TRV: 12	1.8 ± 0.5	34.7 ± 7.7*
TRV: 17	2.6 ± 0.6	26.8 ± 5.8*
TRV: 21	nd	20.4 ± 4.9*
TRV: 22	3.0 ± 0.6*	28.9 ± 6.1*
TRV: 24	1.9 ± 0.5	20.3 ± 7.0*
TRV: 44	3.2 ± 0.6*	8.3 ± 0.9
TRV: 48	3.5 ± 0.8*	35.2 ± 5.5*
TRV: 52	3.0 ± 0.5*	13.8 ± 0.5
TRV: 55	3.1 ± 0.5*	9.4 ± 0.6
TRV: 63	2.7 ± 0.7*	nd

nd no data, *LS* lesion size

[a] Negative control plants inoculated with pTRV2

[b] Healthy plants

*Significantly different at $P < 0.05$ probability level compared to TRV: 00 by REML analysis

Discussion

To identify genes involved in quantitative resistance to potato late blight, 63 candidate genes were selected based on their high expression levels during the early infection stages with *P. infestans* and the genes were subjected to VIGS treatment and detached leaf assay in both *N. benthamiana* and potato. Candidate genes were first subjected to VIGS treatment and after photobleaching caused by TRV: *StPDS* occurred, the leaves were detached and inoculated with *P. infestans.* We found 10 candidate genes caused enhanced susceptibility after VIGS treatment in *N. benthamiana* and potato (Tables 1, 2). This suggests that enhanced expression of these genes may cause enhanced resistance to late blight.

A *lipoxygenase* (*LOX*) and a *suberization-associated anionic peroxidase*, represented by candidates 22 and 48, respectively, were identified to affect the late blight resistance in both potato and *N. benthamiana* background. In addition, we found that candidate genes 44, 52, 55 and 63 decreased the resistance to late blight in *N. benthamiana* and candidate genes 12, 17, 21 and 24 in potato. These candidates share similarities with various enzymes, e.g.

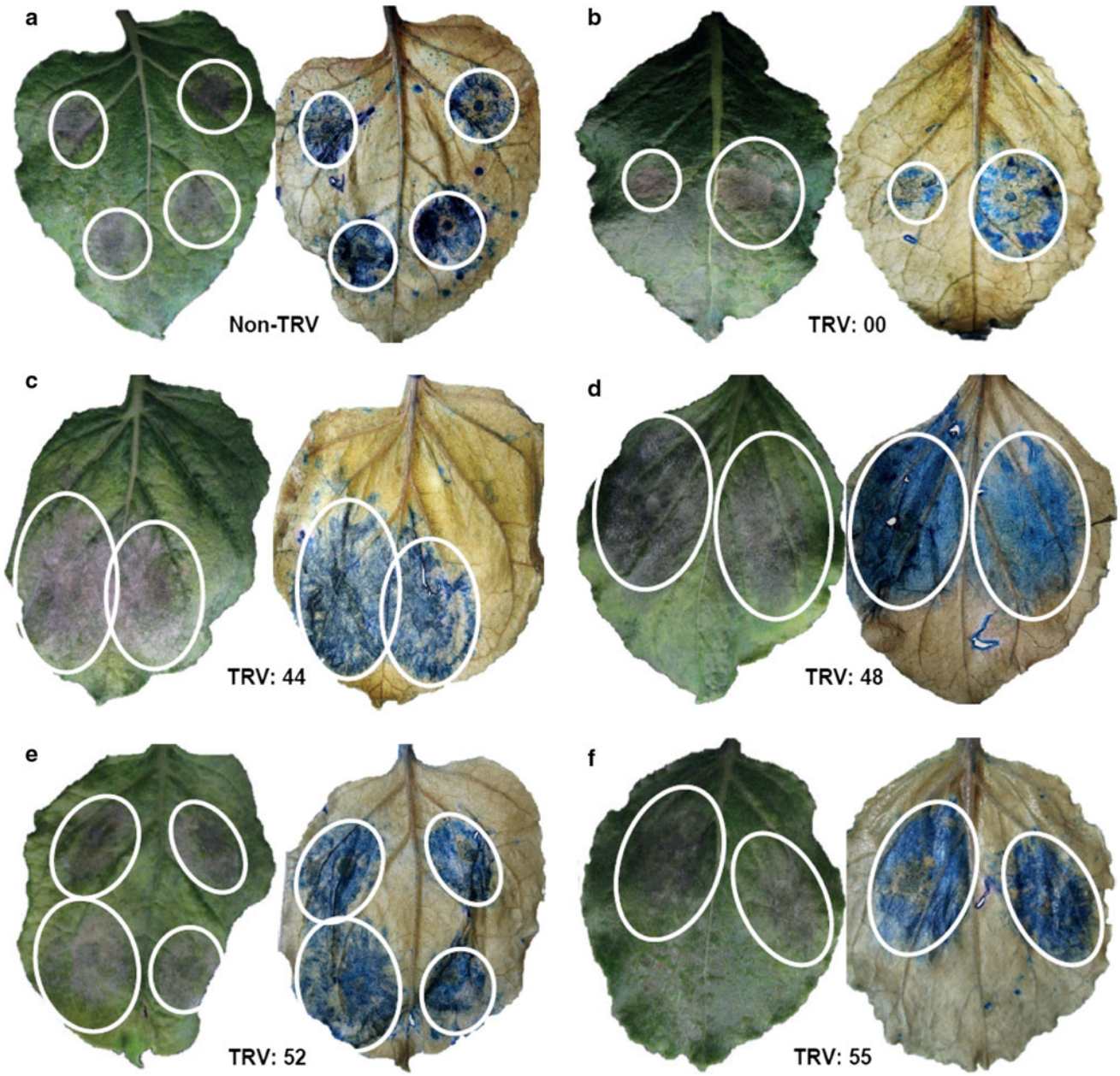

Fig. 3 Susceptibility to *Phytophthora infestans* after VIGS treatment of some genes in *Nicotiana benthamiana*. **a**–**f** Comparisons of the lesions infected by *P. infestans* at 6 days post inoculation on *N. benthamiana* before and after trypan blue staining

UDP-arabinose 4-*epimerase*, 2-*oxoglutarate dehydrogenase* and 2-*deoxyglucose*-6-*phosphate phosphatase*, two different *ATP binding proteins*, a *Sodium-dicarboxylate cotransporter*, a *double type WRKY transcription factor*, and a *binding protein associated factor* (Table 1). We hypothesize that overexpression of those genes in potato may contribute to quantitative resistance to late blight.

LOXes are dioxygenases that catalyze the hydroperoxidation of polyunsaturated fatty acids or their esters that contain a cis, cis-1, 4-pentadiene moiety. Candidate gene 22 was identified as a *LOX*. In plants, *LOXes* have been implicated in the biosynthesis of stress-responsive signaling molecules, such as traumatin [25] and jasmonic acid [26]. Another *LOX* gene from potato, *POTLX*-3 (GenBank/EMBL accession no. U60202), was also reported to be involved in defense to *P. infestans* [27]. This is agreement with our finding that decreased expression of the putative *LOX* candidate gene 22 decreased resistance to *P. infestans* in both potato and *N. benthamiana*.

Suberization-associated anionic peroxidases were reported to play an important role of specific cell wall suberization in plant defense [28–30]. Cell wall suberization can result in strengthening the cell wall, and thereby inhibit pathogen invasion. We identified the candidate gene

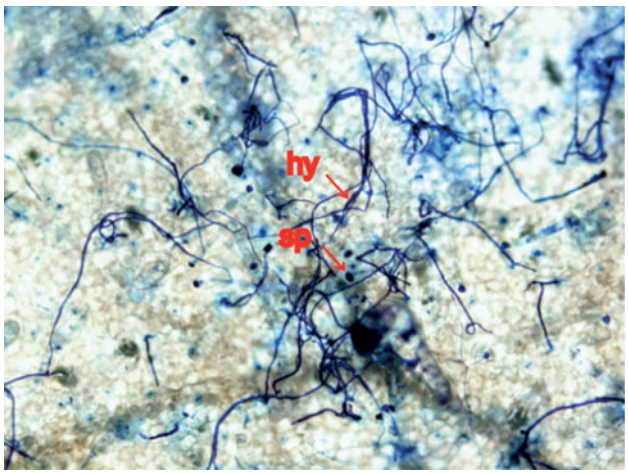

Fig. 4 Microscopic observation of an infected leaf after trypan blue staining. *sp* sporangium, *hy* hypha

48 as a putative *Suberization-associated anionic peroxidase*. This candidate gene was also found to decrease resistance to late blight after VIGS treament in potato and *N. benthamiana*, and we consider it as a good candidate to contribute to quantitative resistance.

With respect to cell wall strengthening, a *UDP-arabinose* 4-*epimerase* (EC 5.1.3.5), alternately named *UDP-D-xylose* 4-*epimerase*, was reported to play a rate-limiting role in the control of cell wall biosynthesis [31]. In our study, a putative *UDP-arabinose* 4-*epimerase* was identified, which shared high similarity with candidate gene 17. VIGS treatment of this gene resulted in decreased resistance to late blight in potato, which supports the hypothesis that modifying the cell wall may play a prominent role in quantitative resistance to late blight [32].

WRKYs are a large family of plant-specific transcription factors that bind to the W-box of promoter regions of many pathogenesis-related (PR) genes [33, 34]. PR genes are thought to be associated with regulating defense responses to both abiotic and biotic stresses [35, 36]. Phosphorylation of the NbWRKY8 (GenBank/EMBL accession no. AB445392.1), a *double WRKY transcription factor* from *N. benthamiana*, was recently reported to play a role in the defense response [37]. Candidate gene 44 showed high identity to *NbWRKY*8, and VIGS treatment of it resulted in a rapid development of the disease.

Candidate gene 63 showed similarity to an *ATP binding protein*, which is co-localizing with a QTL for late blight resistance on chromosome XI of potato population PCC1 (developed at International Potato Center) [38]. Most plant disease resistance (*R*) genes identified today encode proteins with a central nucleotide binding site (NBS) and a C-terminal Leu-rich repeat (LRR) domain. The NBS contains three ATP/GTP binding motifs known as the kinase-1a or P-loop, kinase-2, and kinase-3a motifs. Perhaps this candidate gene is involved in both a quantitative resistance response and *R*-gene related defense responses [39, 40].

We also identified the other candidate genes 12, 21, 24 and 55, representing a *Sodium-dicarboxylate cotransporter*, a *2-oxoglutarate dehydrogenase*, a *putative 2-deoxy-glucose-6-phosphate phosphatase* and a *putative TATA binding protein*, respectively, in our study. To our knowledge, there are no reports on functions in plant disease resistance for these genes and they might represent novel genes associated with quantitative resistance to late blight.

The fact that silencing a same gene in potato and *N. benthamiana* showed different levels of susceptibility to *P. infestans* might for example be explained by the fact TRV-based silencing in potato is weaker and less uniform, as shown from the photobleaching control experiments.

In conclusion, the combination of VIGS and detached leaf assays on both *N. benthamiana* and potato has proven to be a fast and powerful tool for identifying genes that may play a role in quantitative resistance to *P. infestans* in potato. Further studies of these identified target genes functions will help us clarify the resistance mechanisms involved in the quantitative resistance to *P. infestans.*

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