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TECHNICAL ADVANCE

Virus-induced gene silencing in *Solanum* species

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Summary

Virus-induced gene silencing (VIGS) has been used routinely in *Nicotiana benthamiana* to assess functions of candidate genes and as a way to discover new genes required for diverse pathways, especially disease resistance signalling. VIGS has recently been shown to work in *Arabidopsis thaliana* and in tomato. Here, we report that VIGS using the tobacco rattle virus (TRV) viral vector can be used in several *Solanum* species, although the choice of vector and experimental conditions vary depending on the species under study. We have successfully silenced the phytoene desaturase (*PDS*) gene in the diploid wild species *Solanum bulbocastanum* and *S. okadae*, in the cultivated tetraploid *S. tuberosum* and in the distant hexaploid relative *S. nigrum* (commonly known as deadly nightshade). To test whether the system could be utilised as a rapid way to assess gene function of candidate resistance (*R*) genes in potato and its wild relatives, we silenced *R1* and *Rx* in *S. tuberosum* and *RB* in *S. bulbocastanum*. Silencing of *R1*, *Rx* and *RB* successfully attenuated *R*-gene-mediated disease resistance and resulted in susceptible phenotypes in detached leaf assays. Thus, the VIGS system is an effective method of rapidly assessing gene function in potato.

Keywords: virus-induced gene silencing, VIGS, *Solanum*, TRV vector, resistance.

Introduction

Potato is one of the most economically important crops following wheat, maize and rice (Hawkes, 1994). However, its use as a model species in plant genetics is hampered by its ploidy (potato is a tetraploid with tetrasomic inheritance) and the development of severe inbreeding depression after just a few rounds of self fertilisation (Ortiz and Peloquin, 1994). Thus, gene discovery in potato is a lengthy process.

Although T-DNA insertional mutagenesis and transposon tagging have been reported in dihaploid potato (ElKharbotly *et al*., 1996; Pereira *et al*., 1992; Van Enckevort *et al*., 2000, 2001), this approach is time consuming because interesting material (usually tetraploid) has to be taken first to the diploid level. When tetraploid *Solanum tuberosum* is diploidised, through either anther culture or the use of certain dihaploid inducer *S. phureja* clones (Ortiz and Peloquin, 1994), a powerful gametophytic self incompatibility system (Clarke *et al*., 1989; Hermsen, 1978; Pushkarnath, 1942) complicates further genetic analyses. Inbreeding depression is also problematic (Ortiz and Peloquin, 1994). Furthermore, unlike tomato or *Arabidopsis*, diploid mutagenised collections are not readily available.

Map-based gene cloning and confirmation of function in potato is slow. Most tests of candidate genes use a transgenic approach, and confirmation of gene function is usually performed by complementation through stable transformation of a suitable genetic background. Impairment of gene function in potato has been achieved using transformation with antisense constructs (Veramendi *et al*., 2002; Visser *et al*., 1991). However, generation of stable

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transformants in potato is complicated by somaclonal variation (Dale and McPartlan, 1992; Heeres *et al.*, 2002; Rietveld *et al.*, 1993) and by the need to optimise tissue culture techniques for any given cultivar. Furthermore, the time required to regenerate transgenic potatoes is usually 6 months under favourable conditions.

An attractive approach of providing a rapid assessment of gene function prior to stable transformation is virus-induced gene silencing (VIGS; for recent reviews on the mechanisms and applications of gene silencing, see Lu *et al.*, 2003; Pickford and Cogoni, 2003; Zamore, 2002). VIGS has been successfully applied to *Arabidopsis* (Dalmay *et al.*, 2000; Lu *et al.*, 2003; Turnage *et al.*, 2002) and Solanaceae, including *Nicotiana* species (Baulcombe, 1999; Jin *et al.*, 2002; Liu *et al.*, 2002a, 2003; Peele *et al.*, 2001) and, recently, tomato (Liu *et al.*, 2002b). VIGS offers an easy way to test the function of several genes in a short time, as it only requires a fragment of the candidate gene cloned into a suitable viral vector. Infection of the host plant with this recombinant viral vector will induce mRNA degradation of the expressed gene (or gene family) homologous to the cloned sequence. An additional advantage of VIGS over antisense transformation and mutagenesis is that it allows the study of genes whose functions are essential for plant viability.

Attempts to silence genes in potato using VIGS had been unsuccessful in the past, and there are no reports in the literature of VIGS employed in potato for gene function studies. However, this could potentially be a fast and efficient way to assess candidate gene phenotypes in this important crop species.

Several viral vectors for VIGS have been described based on viruses that infect Solanaceae. One of the first vectors described is the potato virus X (PVX) vector (Chapman *et al.*, 1992). Although PVX is very efficient in *Nicotiana* species, the tobacco rattle virus (TRV)-based vector has advantages, including uniformity of silencing, invasion of meristems (Ratcliff *et al.*, 2001) and stability of the recombinant virus (Ana M. Martín-Hernández, unpublished results). Currently, there are two TRV-based vectors. The vector developed by Ratcliff *et al.* (2001) has been used successfully in several *Nicotiana* species and in *Arabidopsis* (Dalmay *et al.*, 2000; Lu *et al.*, 2003). The second vector developed by Liu *et al.* (2002a) incorporates several modifications aimed at improving viral expression and has been used in *N. benthamiana*, as well as in tomato (Liu *et al.*, 2002a,b). We have tested both vector systems in different *Solanum* species using the phytoene desaturase (*PDS*) gene as a visual marker of silencing. We have also been successful in silencing three different pathogen resistance (*R*) genes: *R1* that confers race-specific resistance to the oomycete *Phytophthora infestans*, *Rx* that confers resistance to PVX (both in a *S. tuberosum* background) and *RB* that confers broad-spectrum resistance to *P. infestans* in *S. bulbocastanum*. Our results show that the TRV vector system is useful in providing an early indication of gene function in *Solanum* species, including cultivated potato.

Results and discussion

Choice of plant material

In all other successful VIGS systems, the starting material is usually young (seedlings) and grown from seed. VIGS using old *N. benthamiana* plants (more than 6 weeks after germination) is very inefficient, as infection is more difficult to establish in adult plants (unpublished results). In potato, this problem is exacerbated by the phenomenon of adult plant resistance, which makes potato more resistant to some pathogens, especially viruses, as the plant reaches maturity (Beemster, 1987).

Potato is traditionally propagated vegetatively by tubers or cuttings, and previous attempts to establish VIGS did not succeed. Cuttings are not truly young plants, and plants emerging from tubers tend to be very vigorous compared to seedlings. Only with *S. bulbocastanum* were we successful in triggering silencing in plants grown from very small tubers (see sections under 'Silencing of PDS in tuber-grown *S. bulbocastanum*' and 'Silencing of resistance (*R*) genes: *RB*, *R1* and *Rx*') as well as from seed. Therefore, unless indicated, all silencing experiments described in the following sections were carried out using 1.5–2-week-old plants (four-leaf stage) grown from seed.

Silencing of PDS using the TRV vectors: initial fine-tuning

In order to choose the most efficient way of silencing a large number of plants, we tested the following variables: inoculation method, temperature and TRV vector. All variables were assessed visually by taking advantage of the photobleaching that results from PDS silencing. Experiments to test the inoculation method and the optimal temperature were performed with the TRV vector developed by Liu *et al.* (2002b).

Three methods of inoculation were used: spray inoculation of the *Agrobacterium tumefaciens* cell suspension (Liu *et al.*, 2002b), direct agro-infiltration (English *et al.*, 1997) or sap inoculation with extracts of agro-infiltrated *N. benthamiana* leaves (see Experimental procedures). The three methods of inoculation were compared on *S. bulbocastanum* and *S. okadae*. All methods produced silencing of PDS, although the onset of silencing in the spray-inoculated plants was 1 week later than in the agro-infiltrated and sap-inoculated plants (Figure 1). Both agro-infiltration and sap inoculation resulted in a high number of silenced plants, although agro-infiltrated plants were smaller and necrosis was observed frequently when compared to

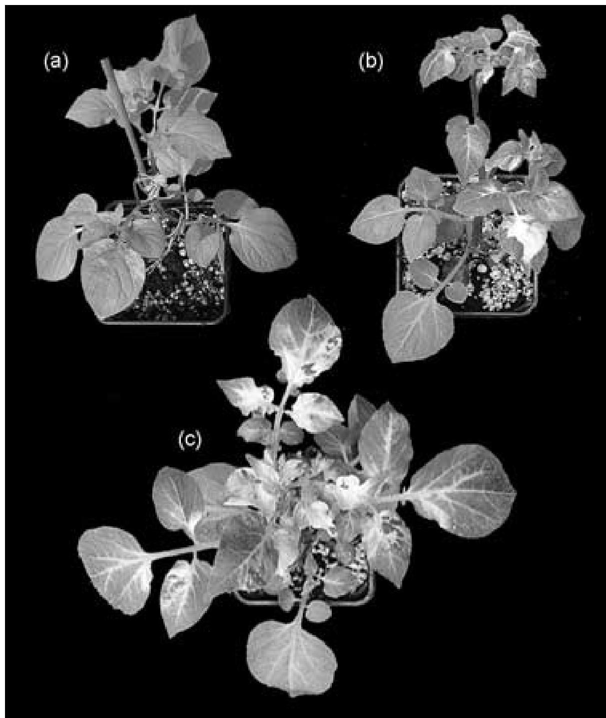


Figure 1. Comparison of inoculation methods.
(a) Spray inoculation.
(b) Agro-infiltration.
(c) Sap inoculation.
S. okadae plants showing PDS silencing. All plants were inoculated at the same time and pictures were taken at 3 weeks post-inoculation. The plant in (a) shows the initial stages of PDS silencing, while plants in (b,c) show a more developed photo-bleaching.

sap-inoculated plants (data not shown). To avoid necrosis and stunting, we decided to use sap inoculation in subsequent experiments. Sap inoculation is also more versatile as it allows inoculation of a large number of plants in a shorter time.

Another variable under study was temperature. We performed experiments using *S. okadae* seedlings under two different glasshouse temperatures, 22–24 and 16–18°C. It has been reported that high temperatures (24°C) favour the accumulation of short interfering RNAs (Szittya *et al.*, 2003), which in our case should correlate with a more extended photo-bleaching in plants grown under higher temperatures. However, our results show comparable establishment of PDS silencing under both conditions. In fact, plants grew more vigorously under the cooler conditions. As 16–18°C is the normal temperature range for potato growth, we decided to perform subsequent silencing experiments at this temperature. The choice of temperature is one variable that will have to be considered depending on the species under study. In addition, if using direct agro-infiltration, expression from the T-DNA will be favoured by an initial lower temperature during the first 24 h following infiltration (16–18°C, Brande Wulff, personal communication).

The efficiency of the two available TRV vectors (Liu *et al.*, 2002a; Ratcliff *et al.*, 2001) was also tested. For the purposes of this paper, we have termed 'A' the vector developed by Ratcliff *et al.* (2001) and 'B' the vector developed by Liu *et al.* (2002a). Therefore, 'A1' and 'A2' refer to RNA1 and RNA2 from vector A, and 'B1' and 'B2' refer to RNA1 and RNA2 from vector B, respectively. The main difference between the two vectors is that B1B2 has a double 35S promoter driving RNA1 and RNA2 transcription and a ribozyme in the 3′ end of the non-translated sequence that should improve processing of the mature transcript. Therefore, this vector should produce a higher level of TRV expression than the A1A2 vector. The same tomato-derived PDS fragment was used in both vectors and has been described previously by Liu *et al.* (2002b). Both TRV vectors were used either as they were originally developed or in all possible combinations of RNA1 and RNA2. We also tested several *Solanum* species that differ in their ploidy level; among them, the diploid *S. okadae*, the tetraploid *S. tuberosum* (F_1 progeny derived from cultivar Cara) and the hexaploid *S. nigrum*.

Our results show that both vectors and their combinations are able to trigger silencing of PDS in *S. okadae* and *S. nigrum* (Figure 2). However, vector B1B2-PDS or the A1B2-PDS combination showed a higher frequency of silenced plants in *S. okadae*. In this species, viral symptoms were also always stronger when vector B1B2-PDS or the A1B2-PDS combination were used. This result suggests that a higher level of viral expression, albeit detrimental for the plant in terms of disease symptoms, yields a higher number of silenced plants.

The highest efficiency of PDS silencing was achieved in the species *S. nigrum* regardless of the combination of vector components used, where all plants inoculated developed PDS silencing (Figure 2a,d). Nevertheless, there was a qualitative difference in terms of silencing uniformity, with vector B1B2-PDS being slightly better than A1A2-PDS in triggering larger sectors of photo-bleached tissue (data not shown). There was no obvious difference in terms of viral symptomatology between vectors in this species.

In the case of the F_1 progeny derived from *S. tuberosum* cv. Cara, silencing efficiency was lower than in the other species tested and only vector A1A2-PDS was capable of triggering PDS silencing (Figure 2a,c). In a separate experiment, we obtained the same result but this time using the F_1 progeny derived from *S. tuberosum* cv. Pentland Ivory (data not shown). We did not observe silencing with vector B1B2-PDS or any of the vector combinations in progeny from either cultivar.

Silencing of PDS in tuber-grown S. bulbocastanum

Solanum bulbocastanum is a wild diploid relative of potato that is native to Mexico. Natural populations of this species

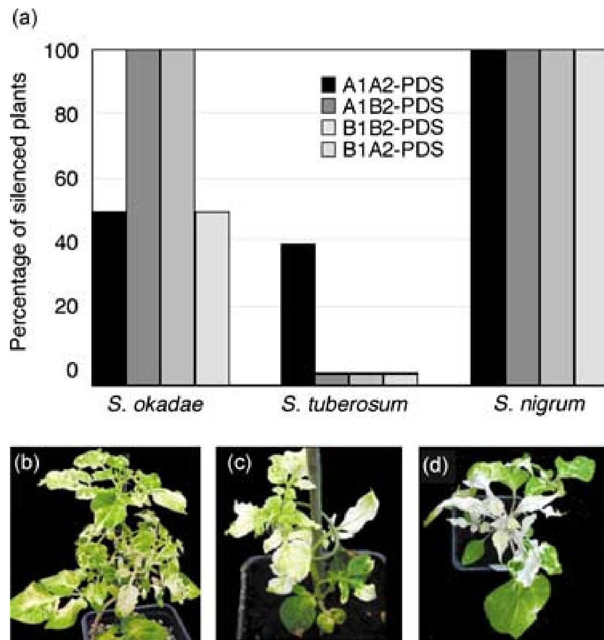


Figure 2. PDS silencing using different vectors and vector combinations in three *Solanum* species.
(a) Percentage of plants showing PDS silencing after inoculation with TRV vectors A1A2-PDS (Ratcliff *et al.*, 2001) and B1B2-PDS (Liu *et al.*, 2002b) or their RNA1 and RNA2 combinations (A1B2-PDS and B1A2-PDS). Forty plants per species, and 10 per vector combination, were used in the experiment. The experiment was performed twice with similar results.
(b) Typical PDS silencing in *S. okadae*. The plant shown was inoculated with the A1B2-PDS construct combination.
(c) PDS silencing in *S. tuberosum* cv. Cara after inoculation with A1A2-PDS.
(d) PDS silencing in *S. nigrum* after inoculation with B1B2-PDS.

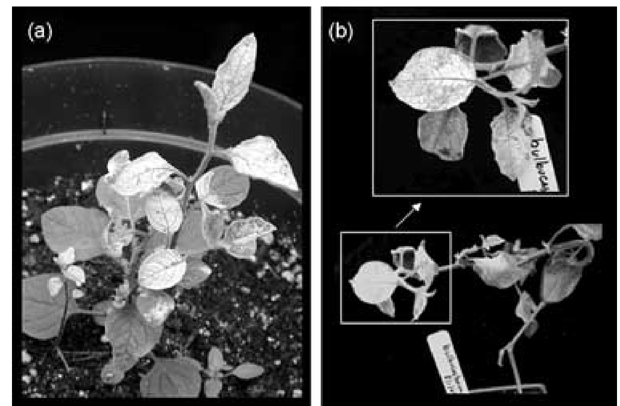


Figure 3. Silencing of PDS in *S. bulbocastanum*.
(a) Seed-germinated *S. bulbocastanum*.
(b) Tuber-generated *S. bulbocastanum* PT-29 showing a close up (framed) of a PDS-silenced sector.
Plants shown were inoculated with A1A2-PDS (Ratcliff *et al.*, 2001) and pictures were taken at 4 weeks post-inoculation.

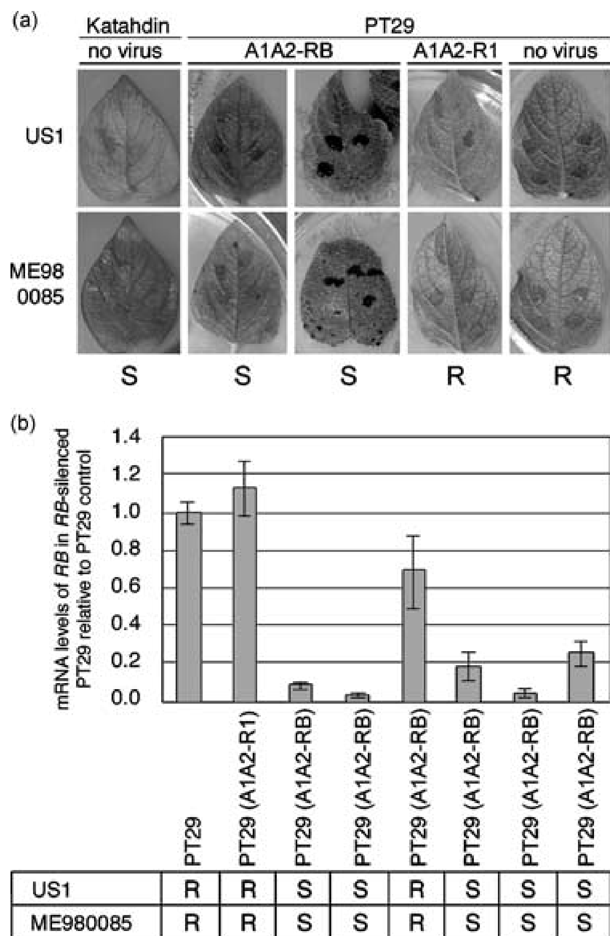
are known to be highly resistant to most races of *P. infestans*, and it is considered to be a good source of resistant germplasm (Niederhauser and Mills, 1953). The gene *RB* from *S. bulbocastanum* Accession PI 243510 (clone PT-29) confers resistance to all races of *P. infestans* and has recently been cloned (Song *et al.*, 2003). As *S. bulbocastanum* is self-incompatible (almost all diploid potato relatives are obligate out-breeders), a specific genotype can only be propagated through tubers or cuttings making it a difficult host for genetic studies. In this species, we also focused our efforts in implementing VIGS on tuber-grown material as our ultimate goal is to study the function of candidate genes responsible for disease resistance-signalling pathways in the resistant hosts.

S. bulbocastanum plants emerging from small tubers or tuber pieces usually grow less vigorously. We particularly chose those small plants, which resemble those grown from seed for VIGS. Two-week-old plants were inoculated with sap extracts from *N. benthamiana* agro-infiltrated with A1A2-PDS. In contrast to *S. tuberosum*, in which no silencing was observed from tuber-grown plants, the percentage of silenced plants in this species was quite high, reaching between 60 and 70%. Similar results were obtained using B1B2-PDS. Thus, it is feasible to conduct VIGS in tuber-grown *S. bulbocastanum*, although the number and size of photo-bleached sectors per silenced plant was higher when the same experiment was performed in plants grown from seed (Figure 3).

Silencing of R *genes:* RB*,* R1 *and* Rx

We wanted to establish VIGS in *Solanum* species to rapidly assess candidate genes for their role in plant disease resistance without the need to use stable transformation on a heterologous system. Several disease *R* genes have been cloned from *Solanum* species. We have successfully applied the VIGS system to silence three of them: *RB*, *R1* and *Rx*.

Silencing of RB. *RB* confers resistance to all races of *P. infestans.* It was cloned recently and belongs to the class of *R* genes defined by coiled coil domains, nucleotide binding sites and leucine rich repeats (CC–NB–LRR) (Song *et al.*, 2003). To silence *RB*, we used young tuber-grown *S. bulbocastanum* PT-29 plants carrying *RB*. A total of 28 plants from four independent experiments were inoculated with sap extracts from *N. benthamiana* agro-infiltrated with A1A2-RB. The plants were challenged with *P. infestans* zoospores 3–4 weeks after virus infection using the detached leaf assay (see Experimental procedures). Two different races of *P. infestans* were used: US1, which carries the avirulence (Avr) gene *AvrR1*; and ME980085, which carries *AvrR3*, *AvrR4* and *AvrR6*–*AvrR9*. Growth of the pathogen was recorded 12 days later. A total of 17 out of 28 PT-29 plants pre-infected with A1A2-RB displayed a susceptible phenotype after inoculation with both races of *P. infestans* (Figure 4a). On average, *RB*



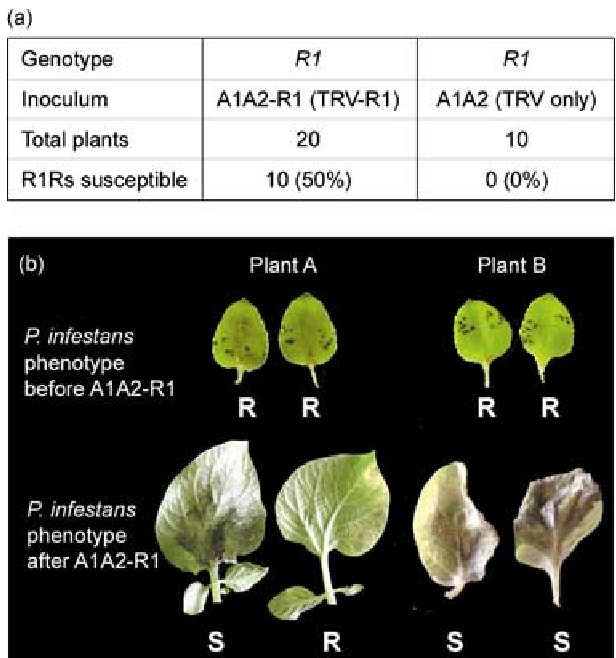
	PT29	PT29 (A1A2-R1)	PT29 (A1A2-RB)	PT29 (A1A2-RB)	PT29 (A1A2-RB)	PT29 (A1A2-RB)	PT29 (A1A2-RB)	PT29 (A1A2-RB)
US1	R	R	S	S	R	S	S	S
ME980085	R	R	S	S	R	S	S	S

Figure 4. Silencing of *RB* in *S. bulbocastanum* PT-29.
(a) Late blight detached leaf assay after *RB* silencing. Reaction to inoculation with *P. infestans* of *S. tuberosum* cv. Katahdin (inoculation control), A1A2-RB-pre-inoculated (*RB*-silenced) PT-29, A1A2-R1-pre-inoculated PT-29 (silencing control) and non-inoculated PT-29 plants (resistance control). Pictures were taken 12 days after *P. infestans* inoculation with race US1 (top panel) and race ME980085 (lower panel). R, resistant; and S, susceptible.
(b) Correlation of *RB* mRNA levels and reaction to inoculation with *P. infestans* 3 weeks after TRV inoculations. The relative level of *RB* gene expression in *RB*-silenced PT-29 was measured by real-time RT-PCR and compared to PT-29 wild-type control. The bottom panel showed the pathogen assay results of A1A2-RB-pre-inoculated PT-29 inoculated with *P. infestans* strain US1 and ME980085. S, susceptible; and R, resistant.

expression as assessed by real-time (RT-)PCR (Figure 4b) was reduced 70–90% in A1A2-RB-inoculated plants, but it varied between 0 and 95%. The susceptibility phenotype correlated with the efficiency of *RB* silencing (Figure 4b). Detached leaves of *S. tuberosum* cv. Katahdin were used as inoculation controls as it is susceptible to all races of *P. infestans.* Leaves from healthy and A1A2-R1 pre-infected PT-29 plants were used as resistance controls. When the same experiments were performed using B1B2-RB vector, no *RB* silencing was observed (data not shown).

Silencing of R1. *R1* was the first gene for resistance to late blight to be cloned from potato and belongs to the CC–NB–LRR class of *R* genes. In contrast to *RB*, *R1* is race-specific and confers resistance to races of *P. infestans* carrying *avrR1* (Ballvora *et al*., 2002). To silence this gene, we used self progeny derived from *S. tuberosum* cv. Pentland Ivory that carries *R1*. This cultivar also has a low degree of resistance to TRV (Stegemann and Schnick, 1985). A total of 40 F_1 seedlings were scored for their reaction to inoculation with *P. infestans* (isolate R1RS: avirulent in plants carrying *R1*) prior to A1A2-R1 inoculation. As expected, three-fourths of the seedlings displayed resistance to the pathogen and therefore carried *R1*. Resistant plants were inoculated with sap extracts from *N. benthamiana* agro-infiltrated with A1A2-R1 or the A1A2 vector (Ratcliff *et al*., 2001) and later challenged with *P. infestans* zoospores using the detached leaf assay (see Experimental procedures). Four independent *P. infestans* inoculation experiments were performed during four consecutive weeks, starting at 3 weeks after virus infection. The A1A2 vector was used as a negative control of silencing. Leaves from susceptible F_1 seedlings were used as *P. infestans* inoculation controls.

After all four rounds of *P. infestans* inoculations, 10 out of 20 plants inoculated with A1A2-R1 and carrying *R1* became susceptible to *P. infestans* (Figure 5), indicating that the


(a)

Genotype	*R1*	*R1*
Inoculum	A1A2-R1 (TRV-R1)	A1A2 (TRV only)
Total plants	20	10
R1Rs susceptible	10 (50%)	0 (0%)

Figure 5. Breakdown of the *R1*-mediated resistance in *S. tuberosum* cv. Pentland Ivory.
(a) Number of plants showing *R1* silencing after inoculation with A1A2-R1. The numbers reflect the totals from four rounds of challenges with *P. infestans* isolate R1RS.
(b) Reaction to *P. infestans* inoculation before and after A1A2-R1 inoculation. Leaves from two different plants are shown. In plant A, only one of the tested leaves showed a shift in phenotype. R, resistant; and S, susceptible. Pictures were taken 8 days after *P. infestans* inoculation.

gene was silenced in 50% of the plants, whereas none of the plants inoculated with the empty vector supported growth of the pathogen and instead displayed a typical hypersensitive response (HR). The number of *R1*-silenced plants increased progressively during the period of sequential inoculation. We conclude from this observation that there is between-plant variation that could be a consequence of the genetic heterogeneity in the population of F_1 seedlings.

When we performed the same experiment using B1B2-R1, none of the plants carrying *R1* became susceptible to the oomycete, confirming our previous PDS silencing results indicating that the A1A2 vector is more efficient for VIGS in *S. tuberosum*.

Silencing of Rx. *Rx* was the first *R* gene to be cloned from potato and confers extreme resistance to most strains of PVX. *Rx* also belongs to the CC–NB–LRR class of *R* genes (Bendahmane *et al*., 1999). To silence this gene, we used self progeny derived from *S. tuberosum* cv. Cara that carries *Rx* in the heterozygous state. This cultivar has a medium to low degree of resistance to TRV (Stegemann and Schnick, 1985). *Rx* was detected with molecular markers in 30 out of 40 F_1 seedlings, as expected (see Experimental procedures). We selected 20 *Rx* plants and inoculated them with A1A2-Rx. The remaining 10 *Rx* plants were inoculated with empty A1A2 vector as negative controls of silencing. Plants without *Rx* (*rx* plants) were used as positive PVX infection controls.

Three weeks after TRV inoculations, six plants developed a generalised HR (indicating the presence of resistance to the vector constructs) and had to be discarded. Three leaves from each of the remaining plants were challenged with a GUS-tagged PVX by agro-infiltration and were collected 8 days later to be tested for GUS expression (see Experimental procedures). HR was used as an indicator of an active *Rx*-mediated resistance response and GUS expression as an indicator of viral replication in the infiltrated leaf patches. Although HR is not associated with the *Rx* response under normal conditions, PVX can trigger this reaction when delivered by agro-infiltration (Bendahmane *et al*., 1999).

We assessed development of HR and GUS expression in 42 leaves collected from 14 *Rx* plants that were pre-inoculated with A1A2-Rx and in 19 leaves collected from 10 *Rx* plants pre-inoculated with empty A1A2 (Figure 6). When PVX-GUS caused a strong HR in the absence of or with limited GUS staining, this indicated *Rx* function. As VIGS is normally patchy, plants were recorded as showing susceptibility to PVX if any of the tested leaves showed absence of HR and presence of GUS expression in the infiltrated patch. A total of 11 out of 14 (78.6%) *Rx* plants pre-inoculated with A1A2-Rx showed susceptibility to PVX (HR− and GUS+). From these results, we concluded that A1A2-Rx had silenced *Rx*. In the control experiment with the A1A2 vector

(a)

Genotype	*Rx*	*Rx*
Inoculum	A1A2-Rx (TRV-Rx)	A1A2 (TRV only)
Total plants	14	10
PVX susceptible	11 (78.6%)	1 (10%)

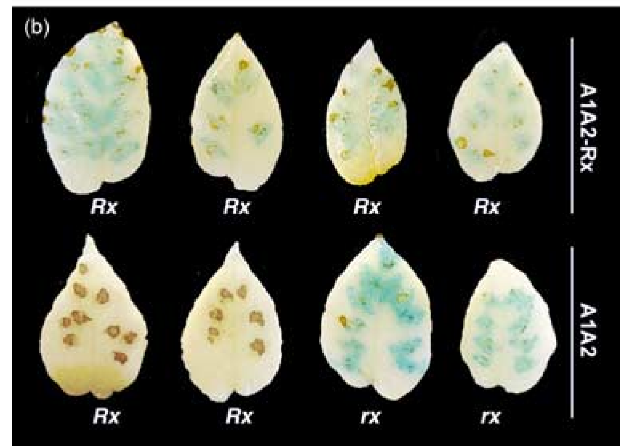


Figure 6. Breakdown of the *Rx*-mediated resistance in *S. tuberosum* cv. Cara.
(a) Number of plants showing *Rx* silencing after inoculation with A1A2-Rx. Typically, three leaves per plant were challenged with PVX-GUS and scored for HR and GUS expression 8 days later. Plants were considered PVX-susceptible when HR failed to develop and GUS staining was observed in at least one of the sampled leaves.
(b) Reaction of *Rx*-silenced leaves to PVX-GUS infiltration. Top panel: four leaves from A1A2-Rx inoculated *Rx* plants showing GUS expression and reduced or no HR. Lower panel: leaves from plants inoculated with empty A1A2. The two leaves on the left are from *Rx* plants and show a fully developed HR and no GUS expression. The two leaves on the right are from *rx* plants and show high GUS expression and no HR. Pictures were taken 8 days after PVX-GUS agro-infiltration.

inoculated into *Rx* seedlings, 9 out of 10 plants did not exhibit any evidence of susceptibility. One plant showed reduced HR but essentially no GUS expression (Figure 6a). Genetic variation for other factors in the segregating plant population could account for this altered *Rx* activity.

Final remarks

The work described here is intended to provide guidelines for the use of VIGS in potato and its wild relatives. We have shown that we can use this approach to test for gene function in two different cultivars of tetraploid potato and in wild diploid and hexaploid *Solanum* species. Although emphasis has been made in plant pathogen resistance, VIGS can prove to be useful in many other aspects of plant molecular biology.

A rapid assay like VIGS can be very useful when there are many candidates for a particular function. In these situations, VIGS can be used to reduce the number of candidate genes (or even single out a unique gene) so that the stable

transformation of candidates that is needed for final confirmation of gene function need only be carried out on a limited scale.

We have tested different inoculation methods, conditions and TRV vectors and have set up the best conditions for VIGS in the *Solanum* species used in this study. We found that it is important to take into account a number of other variables that can affect VIGS in other species or cultivars when designing an experiment.

Choice of viral vector

During our initial experiments using the different vectors and their combinations, we observed different percentages of silenced plants that varied depending on the species under study. One possible explanation for this variation is that sap inoculum contains virions that originate directly from the agro-infiltrated *N. benthamiana* leaves. Each RNA particle is individually encapsidated, and a balance between the amount of RNA1 and RNA2 is important for a successful infection, and therefore silencing establishment. As vector B has been modified to enhance its transcription in relation to vector A, the balance between RNAs might be altered. This could be advantageous or not to the virus depending on the host species. Furthermore, RNA2 carries the gene sequence that will trigger VIGS, and is not necessary for RNA1 multiplication and spread. Therefore, in some species, RNA2 can be lost during infection and this will result in a failure to trigger VIGS, as it has been observed for *Arabidopsis* (A. M. Martín-Hernádez, unpublished results).

Degree of resistance to TRV

In order to interpret correctly silencing results of a target gene in a given *Solanum* species or cultivar, it is advisable to check beforehand the degree of resistance, if any, of the host to TRV. In hosts with a partial or medium resistance to TRV, some degree of HR will develop after TRV infection. This can mask results if silencing a *R* gene, as was the case in some of the *S. tuberosum* cv. Cara progeny in our *Rx*-silencing experiments.

Type of genes amenable to silencing

Virus-induced gene silencing is especially useful when studying genes whose silencing phenotype is easily observable or when there is an assay available to analyse the extent of silencing. In the first case, silencing genes involved in pigment production will generate a colour different from that of control plants. In the second case, silencing of a *R* gene will require the use of a pathogenicity assay to test for susceptibility to the corresponding pathogen. Likewise, VIGS of a gene whose function involves producing a measurable amount of substrate will require a quantitative assay to analyse the extent of silencing.

Timing for establishment of silencing

In *N. benthamiana*, full silencing is established 2 weeks after TRV inoculations. In the case of *Solanum* species, this process appears to be slower and at least 3 weeks are required before testing for the candidate gene phenotype. Therefore, the time required will be dependent on the *Solanum* species under study. It is advisable to design experiments to always include a visual marker, like PDS, that will indicate the onset and spread of silencing throughout the plant's tissues.

Experimental procedures

Plant materials and pathogens

Solanum bulbocastanum (Accession PI243510) was obtained from NRSP-6, the US potato GenBank, and the clone PT-29 was obtained from Jiming Jiang's Laboratory at University of Wisconsin. *S. okadae* seed was generated from a cross between siblings from cross Soka 145 performed in the Sainsbury Laboratory. Parents for Soka 145 came originally from Accessions CGN18279 and CGN18108. *S. okadae* and *S. nigrum* (Accession CGN21367) were obtained from The Center for Genetic Resources, the Netherlands (CGN). *S. tuberosum* cv. Cara and Pentland Ivory were obtained from the Scottish Crop Research Institute (SCRI) Commonwealth Potato Collection. The self crosses used in this study were generated in the Sainsbury laboratory.

P. infestans races US1 and ME980085 were kindly provided by Dr William Fry. The R1RS *P. infestans* isolate was kindly provided by Dr David Shaw and is unable to infect *S. tuberosum* cultivars carrying *R1*. The PVX construct (described below) is based on strain UK3.

Plasmids and primers

Construction of pTRV and pTV derivatives. All the constructs used for VIGS were built into pTRV2 vector (B2; Liu *et al.*, 2002a) and pTV-00 vector (A2; Ratcliff *et al.*, 2001).

PDS constructs. pTRV2-PDS (B1B2-PDS), containing a fragment of PDS gene from tomato, was kindly provided by Dr Dinesh-Kumar (Liu *et al.*, 2002b). The same PDS fragment was PCR amplified from this plasmid using the primers 5′-AAAA-GGGCCCGGCACTCAACTTTATAAACC-3′ and 5′-GTCAGGTACC-CTCAGTTTTCTGTCAAACC-3′ and cloned into pTV-00 (A2-PDS; Ratcliff *et al.*, 2001) using the restriction sites *Apa*I and *Asp*718 (underlined).

R1 *constructs.* A 521-bp fragment of the potato *R1* gene was amplified from *S. tuberosum* cv. Pentland Ivory genomic DNA. Primers 5′-TTAAAAGGGCCCACAAAGCATGCTCACTTG-3′ and 5′-TTGTCAGGTACCAATTCTGCATCCTCAACACG-3′ were used for the PCR amplification. The fragment was cloned into pTV-00 (A2-R1) using *Apa*I and *Asp*718 restriction sites (underlined) and as a blunt-end fragment into pTRV2 (B2-R1) using a *Sma*I restriction site.

Rx *constructs.* pTV Rx (A2-Rx) has been described by Peart *et al.* (2002).

RB *constructs.* A 632-bp fragment of the potato *RB* gene was amplified from *S. bulbocastanum* PT-29 genomic DNA. Primers 5′-AAAAATCGATAGTTACTGAGCATTTCCATTCC-3′ and 5′-AAAAGGATCCGCACAATACGCAAAGCATTGTT-3′ were used for the PCR amplification, and the fragment was cloned into pTV-00 (A2-RB) using the restriction sites *Cla*I and *Bam*HI. The same fragment was re-amplified with primers 5′-AAAACTCGAGAGTTACTGAGCATTTCCATTCC-3′ and 5′-AAAAGGTACCGCACAATACGCAAAGCATTGTT-3′, and the product was cloned into pTRV2 (B2-RB) using the restriction sites *Xho*I and *Kpn*I.

PVX construct. The PVX construct used in the *Rx*-silencing experiments is derived from pGC3 (Chapman *et al.*, 1992). In order to deliver the virus by agro-infiltration, the viral cDNA on pGC3 was excised using *Sph*I and *Sac*I restriction sites, blunt-ended and transferred into *Sma*I-digested pBIN61 (Bendahmane *et al.*, 2002). The new plasmid PSLD2100 (PVX-GUS) was transformed into *A. tumefaciens* strain C58C1 harbouring the pCH32 helper plasmid (Hamilton *et al.*, 1996).

Rx *molecular markers.* Detection of *Rx* in segregating self progeny of *S. tuberosum* cv. Cara was performed by PCR using closely linked flanking markers IPM3–IPM5. Primers and PCR conditions are described by Bendahmane *et al.* (1997).

TRV inoculation

Spray inoculation of *A. tumefaciens* cell suspensions was performed as described by Liu *et al.* (2002b).

Infiltration of *A. tumefaciens* cells (agro-infiltration) was performed to amplify the TRV vector inoculum in *N. benthamiana* or to infiltrate test plants directly. *A. tumefaciens* cells carrying pTRV1 (TRV vector RNA1, B1) and constructs derived from pTRV2 (TRV vector RNA2, B2) were cultured as described by Liu *et al.* (2002b). *A. tumefaciens* cells carrying pBINTRA (TRV vector RNA1, A1) and the constructs derived from pTV-00 (TRV vector RNA2, A2) were cultured as described by Ratcliff *et al.* (2001) before infiltration. In the case of direct agro-infiltration of *Solanum* species, the first two true leaves of four-leaf stage plants were agro-infiltrated to trigger silencing directly.

In the cases in which we amplified the TRV inoculum in *N. benthamiana*, two young but fully extended leaves of a 3–4-week-old *N. benthamiana* were infiltrated with the cell suspension mixture using a 2-ml syringe without needle. Agro-infiltrated plants were left in the glasshouse for 3 days before collection of the infiltrated leaves. Sap inoculation was performed using extracts from these leaves. Agro-infiltrated leaves were ground in 50 mM phosphate buffer, pH 7.2 (3 ml per leaf), and this extract was used to directly rub-inoculate four-leaf stage *Solanum* plants that had been previously dusted with carborundum. After 1–2 min, inoculated leaves were rinsed with distilled water to avoid leaf damage.

Resistance assays

P. infestans *inoculation.* A detached leaf assay was used to test for resistance to *P. infestans* (modified from Vleeshouwers *et al.*, 1999). Two leaves per plant were detached, inserted in a small portion of wet florist sponge and placed on a Petri dish. Leaves were inoculated with droplets of a zoospore suspension (10 ul cm^{-2}; 20 000–50 000 zoospores ml^{-1}), and the inoculum was gently spread over the abaxial leaf surface with an artist's brush. Petri dishes were wrapped in plastic film and incubated 7–12 days under controlled environmental conditions (18°C; light/dark cycle) before scoring phenotypes. Plants with leaves showing sporulating lesions were scored as susceptible; plants with leaves showing no visible symptoms or necrosis in the absence of sporulation were scored as resistant.

PVX infection. PSLDB2100 (PVX-GUS) was used to test for the breakdown of the *Rx*-mediated resistance through agro-infiltration (English *et al.*, 1997). Several leaves of *Rx* plants selected from a self cross of cultivar Cara were agro-infiltrated with the PVX-GUS construct and analysed for HR elicitation and GUS expression 8 days post-infiltration. GUS staining was performed as described by Angell and Baulcombe (1995).

Real-time RT-PCR

An Oligo dT17 adaptor was used for mRNA reverse transcription. Real-time PCR was performed with ABI PRISM 7000 using SYBR Green I (ABI; Applied Biosystems, Foster City, CA, USA). Primers were designed with PRIMER EXPRESSION 2.0 (ABI) to anneal to sequences in regions outside the region targeted for silencing to ensure that only the endogenous gene transcripts would be amplified. The primers used to amplify a 118-bp fragment of the potato ubiquitin gene were 5′-CTGGCAAGACCATAACTCTCGA-3′ and 5′-CTTTCCAGCAAAGATCAGCCTT-3′. The primers used to amplify a 117-bp fragment of the *RB* gene were 5′-CAATTGTGTTGCGCACTAGAGA-3′ and 5′-GCTGCAATCCCTCTGGTAAAC-3′. The comparative threshold cycle (Ct) method was applied according to the manufacturer's recommendation (ABI User Bulletin #2).

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