Potato virus X-induced gene silencing in leaves and tubers of potato

Potato Virus X-Induced Gene Silencing in Leaves and Tubers of Potato[1]

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Virus induced gene silencing (VIGS) is increasingly used to generate transient loss-of-function assays and has potential as a powerful reverse-genetics tool in functional genomic programs as a more rapid alternative to stable transformation. A previously described potato virus X (PVX) VIGS vector has been shown to trigger silencing in the permissive host *Nicotiana benthamiana*. This paper demonstrates that a PVX-based VIGS vector is also effective in triggering a VIGS response in both diploid and cultivated tetraploid Solanum species. We show that systemic silencing of a phytoene desaturase gene is observed and maintained throughout the foliar tissues of potato plants and was also observed in tubers. Here we report that VIGS can be triggered and sustained on in vitro micropropagated tetraploid potato for several cycles and on in vitro generated microtubers. This approach will facilitate large-scale functional analysis of potato expressed sequence tags and provide a noninvasive reverse-genetic approach to study mechanisms involved in tuber and microtuber development.

The recent completion of the Arabidopsis genome (Arabidopsis Genome Initiative, 2000; Yamada et al., 2003) has provided a platform to significantly expedite the rate of knowledge acquisition. To fully exploit this sequence information, however, high throughput screens are required to accurately ascribe gene function. In model species such as Arabidopsis, efficient tools for forward and reverse genetics have been developed (Krysan et al., 1999; Springer, 2000; Tani et al., 2004). Furthermore, insertional mutagenesis programs based on T-DNA or transposon tagging have provided valuable resources for the analysis of gene function (Sessions et al., 2002; Pan et al., 2003).

In Arabidopsis and especially other model plant systems such programs suffer from a number of possible limitations, including: lack of genome-wide coverage, gene target bias, lethality, and functional redundancy (Jeong et al. 2002; Gidoni et al., 2003; Kumar and Fladung, 2003). Additionally, for polyploid crop species such as potato (*Solanum tuberosum*), mutagenesis programs are complicated and resource-intensive. Other approaches, such as dsRNA-mediated suppression of gene expression, have also proved successful in Arabidopsis, although these approaches rely on the generation of large numbers of transgenic lines (Chuang and Meyerowitz, 2000; Smith et al., 2000; Wesley et al., 2001; Harmon and Kay, 2003; Zhao et al., 2003). For many crop species the efficiency of transformation is low and prevents high throughput analysis using this approach.

Virus induced gene silencing (VIGS) is increasingly being used to generate transient loss-of-function assays to assess gene function, as a more rapid alternative to stable transformation (Baulcombe, 1999; Lu et al., 2003a). VIGS triggers an RNA-mediated defense mechanism directly targeting the integrity of the invading viral genome. This sequence-specific phenomenon lowers the titer of the invading virus through an endogenous RNAse-inducible mechanism leading to viral RNA degradation (Baulcombe, 1999; Ratcliff et al., 1999; Goldbach et al., 2003). By introducing host cDNA fragments within the viral genome, it is possible to redirect this mechanism to corresponding endogenous host mRNAs, therefore providing a means to down-regulate host gene expression.

VIGS vectors such as potato virus X (PVX), tobacco rattle virus (TRV) or tomato golden mosaic virus (TGMV) have been optimized in the permissive host *Nicotiana benthamiana* (Kjemtrup et al., 1998; Ratcliff et al., 2001). Recent examples show that VIGS can be extended to model plants such as Arabidopsis using the bipartite geminivirus cabbage leaf curl virus (CbLCV; Turnage et al., 2002), crop species such as tomato using a previously characterized TRV VIGS

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vector (Liu et al., 2002), or developed for monocot species such as barley (*Hordeum vulgare*), using barley stripe mosaic virus (BSMV; Holzberg et al., 2002).

The effectiveness of a VIGS vector relies firstly on the ability of the virus to replicate and accumulate to sufficient levels in the host plant to generate dsRNA molecules that initiate silencing, as recent studies have shown that dsRNA generation is a limiting factor in VIGS (Lacomme et al., 2003). Secondly, the virus must be devoid of strong genome-based, posttranscriptional gene silencing-suppressors such as HC-Pro (potyviruses) or 2b (cucumoviruses; Anandalakshmi et al., 1998; Brigneti et al., 1998) that protect the virus against this RNA-mediated resistance mechanism. Given these conditions, it is likely that a plant virus that is able to trigger a significant VIGS response in the host *N. benthamiana*, has the potential to be used for VIGS in a distinct susceptible host.

As some crop species develop unique organs like tubers or edible fruits, model plants such as Arabidopsis or Nicotiana are often of limited relevance. Potato, which is a member of the Solanaceous family, is the world's fourth largest crop. Furthermore, global production is increasing by 4.5% annually (http://www.cipotato.org/potato/POTstats.htm). The availability of a VIGS vector for potato would significantly accelerate the discovery of genes integral to tuber quality or resistance to potato-specific pathogens, for example.

This paper demonstrates that a PVX-based VIGS vector is effective in triggering a VIGS response in both wild diploid and cultivated tetraploid Solanum species. We show that systemic silencing of a phytoene desaturase (*pds*) gene is observed and maintained throughout the foliar tissues of potato plants and is also extended to tubers and in vitro generated microtubers. This offers the potential for large-scale functional screens to identify genes involved in tuber development, metabolism, and foliar or tuber resistance to commercially important pathogens.

RESULTS AND DISCUSSION

PVX Infects Both Diploid and Tetraploid Solanum Species

A susceptible host is a prerequisite for the development of an efficient VIGS system, as viral replication and in planta accumulation of the virus condition the generation of dsRNA molecules that initiate silencing (Voinnet, 2001). Some plant viruses such as PVX have a relatively broad host-range, including several Solanaceous species (Brunt et al., 1996). In this study, a previously described binary PVX-expression vector (Jones et al., 1999; Lu et al., 2003b) was tested for its capacity to infect both wild diploid and cultivated tetraploid Solanum species. Cultivars were selected due either to their ability to be stably transformed and propagated in vitro (*Solanum tuberosum* L. cv Desiree), or the differential interactions occurring between either susceptible or resistant cultivars (*S. tuberosum* L. cvs Bintje and Stirling, respectively; Birch et al., 1999) to *Phytophthora infestans*, or as a potential source of novel resistance genes to *P. infestans* (*Solanum bulbocastanum*; Song et al., 2003).

Infectivity of PVX in these Solanum hosts was investigated using a PVX construct carrying a GFP insert (PVX.GFP construct, Fig. 1a). Following agroinoculation of young source leaves of potato plantlets, both infiltrated and systemic upper-uninoculated leaves were harvested. Virus accumulation was monitored by immunodetection of viral PVX coat protein (CP) by western blotting (Fig. 1b).

On the basis of semiquantitative western analysis, more PVX CP was detected in both inoculated and upper-uninoculated (systemic) leaves in *S. bulbocastanum* than in *S. tuberosum* L. cvs Desiree, Stirling, or Bintje (Fig. 1b, lower and upper sections). In *S. bulbocastanum* PVX accumulation was comparable to that observed in *N. benthamiana* at the same time postinoculation on both inoculated and systemic leaves (Fig. 1b). In all Solanum species and cultivars that were tested, PVX-CP was also detected in systemic leaves by 14 dpi (Fig. 1b, upper section). Therefore, all plants tested tolerate substantial PVX accumulation.

The PVX Vector Triggers VIGS of Endogenous *pds* in Foliar Tissues in Solanum Species

The silencing effectiveness of the binary PVX vector was assessed by its ability to silence an endogenous *pds* gene in these different Solanum species. Downregulation of endogenous *pds* gene expression leads to a characteristic photobleaching phenotype, therefore providing an indication of gene silencing (Kumagai et al., 1995; Ratcliff et al., 2001). As RNA silencing is homology-dependant, a potato *pds* cDNA fragment was subcloned into PVX. The cDNA fragment selected was a region showing sequence identity of 91% with an *N. benthamiana pds* cDNA (including stretches of 24, 26, 33 and 47 nucleotides of 100% identity between both cDNAs, Fig. 1c). This would allow silencing of the corresponding genes in both species to compare the relative VIGS. The cDNA region was subcloned in antisense orientation into the PVX vector (construct PVX.PDS_{AS}, Fig. 1a). Following challenge with PVX.PDS_{AS}, photobleaching was observed on all *N. benthamiana* plants by 12 to 15 d postinoculation, suggestive of *pds* silencing (Fig. 2l). When the Solanum species and cultivars were infected with PVX.PDS_{AS}, white patches of photobleached tissues were observed by 3 weeks post-inoculation in all infected plants (*S. bulbocastanum,* Fig. 2, d and j; *S. tuberosum* L. cvs Bintje, Fig. 2, a, b, and f; Stirling, Fig. 2g; and Desiree, Fig. 2h) as opposed to plants infected with PVX.GFP where no symptoms of PVX infection were visible (*S. bulbocastanum,* Fig. 2c and *S. tuberosum* L. cv Bintje, Fig. 2e). In silenced plants, the degree of photobleaching varied, however, from covering most of the leaf surface for

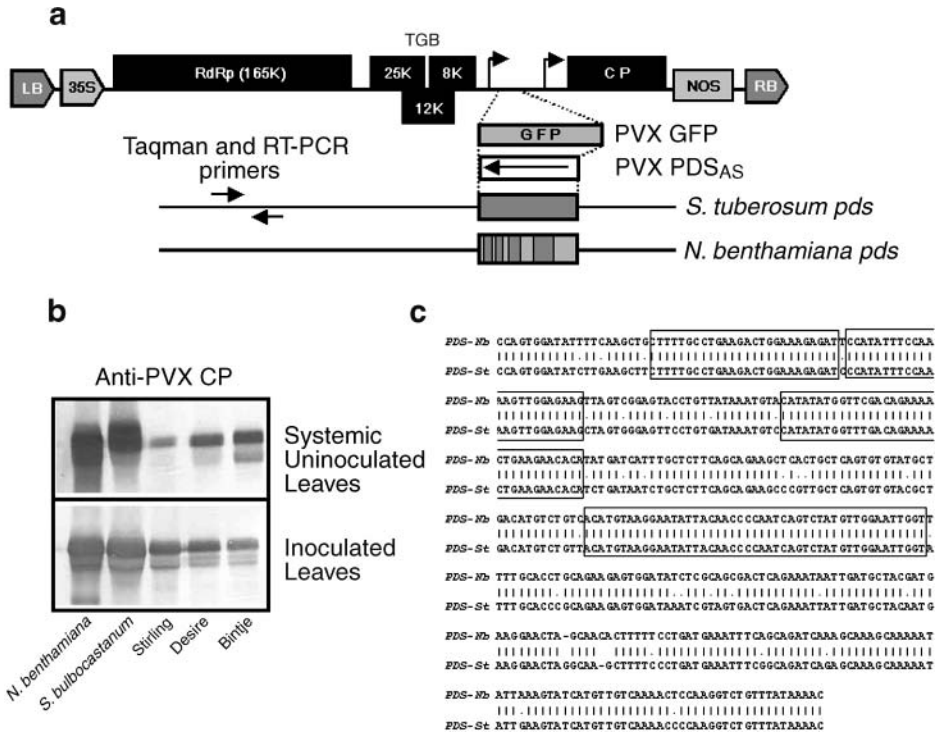

Figure 1. PVX VIGS vector accumulates in a range of Solanum species. a, Schematic representation of a PVX vector in the pGREEN0000 binary plasmid: 35S, 35S promoter of cauliflower mosaic virus; RdRp (165K), PVX 165K RNA-dependent RNA polymerase; TGB 25K, 8K, and 12K, PVX triple gene block movement proteins; CP, viral coat protein gene; NOS, nopaline synthase transcriptional terminator; LB and RB, left and right T-DNA border sequences. Schematic representation of *Solanum tuberosum* and *Nicotiana benthamiana* full-length *pds* cDNAs (black line). The *pds* cDNA region from *S. tuberosum* cloned into PVX is represented as a dark gray box. The corresponding region from *N. benthamiana* is boxed in light gray, sequence stretches of more than 21 nucleotides identical to *S. tuberosum* are represented as dark gray rectangles. Arrows indicate the position of the primers used for RT-PCR and real time RT-PCR. b, Western-blot analysis of PVX CP protein accumulation in *N. benthamiana*, *S. bulbocastanum*, and *S. tuberosum* cvs in inoculated and systemic upper uninoculated leaves at 15 dpi. c, Nucleotide alignment of *pds* cDNA region cloned into PVX from *S. tuberosum* (*PDS-St*) with *N. benthamiana* (*PDS-Nb*). Boxes indicate the identical sequences stretches of more than 21 nucleotides.

S. bulbocastanum (Fig. 2, d and j), to patches uniformly distributed on the leaf surface close to leaf veins for *S. tuberosum* cvs Bintje, Stirling, and Desiree (respectively Fig. 2, f, g, and h). This systemic photobleaching was sustained for the duration of the experiment (up to 3 months postchallenge with PVX.PDS_{AS}) as silenced leaves remained photobleached and newly developing leaves underwent photobleaching as observed in the earlier stages of the VIGS response (Fig. 2, and b; data not shown).

VIGS effectiveness was analyzed at the transcript level by monitoring *pds* mRNA accumulation by reverse transcription (RT)-PCR and real-time RT-PCR. Leaf samples were taken from three to six different plants challenged by either PVX.PDS_{AS} or PVX.GFP (the latter as a control of PVX infection). RT-PCR experiments detected a lower amount of *pds* PCR product in the silenced leaves than in the control samples (Fig. 3a). The levels of control ubiquitin RT-PCR product were similar in all samples tested (Fig. 3a). Real-time RT-PCR was then used to quantify the levels of normalized *pds* mRNA in both silenced and control leaf tissues. A decrease in normalized *pds* mRNA levels ranging from 70% ± 17% to 84% ± 5% was detected in silenced tissues of *S. tuberosum* cv L. Desiree and *S. bulbocastanum* (Fig. 3b) when compared with PVX.GFP-infected control leaves. A comparable decrease in normalized *pds* mRNA was detected between leaves of diploid *S. bulbocastanum* and tetraploid *S. tuberosum* L. cv Bintje, Stirling, and Desiree (respectively 84% ± 5%, 78% ± 17%, 84% ± 15%, and 70% ± 17%). This decrease in *pds* mRNA was similar to that observed during PVX.PDS_{AS} VIGS in *N. benthamiana* (78% ± 10%, Fig. 3b), although the extent of photobleaching appeared greater than that observed with Solanum leaves (Fig. 2l). This corroborates the fact that in these experiments and as previously reported (Ratcliff et al., 2001; Lacomme et al., 2003), the extent of photobleaching does not always correlate with differences in *pds* mRNA levels.

To further characterize the bleached phenotype in potato following *pds* silencing, the levels of phytoene were quantified by reverse-phase HPLC in extracts from both *N. benthamiana* and potato leaves challenged with either PVX.GFP or PVX.PDS_{AS} VIGS constructs. An increase in the level of phytoene, the substrate for

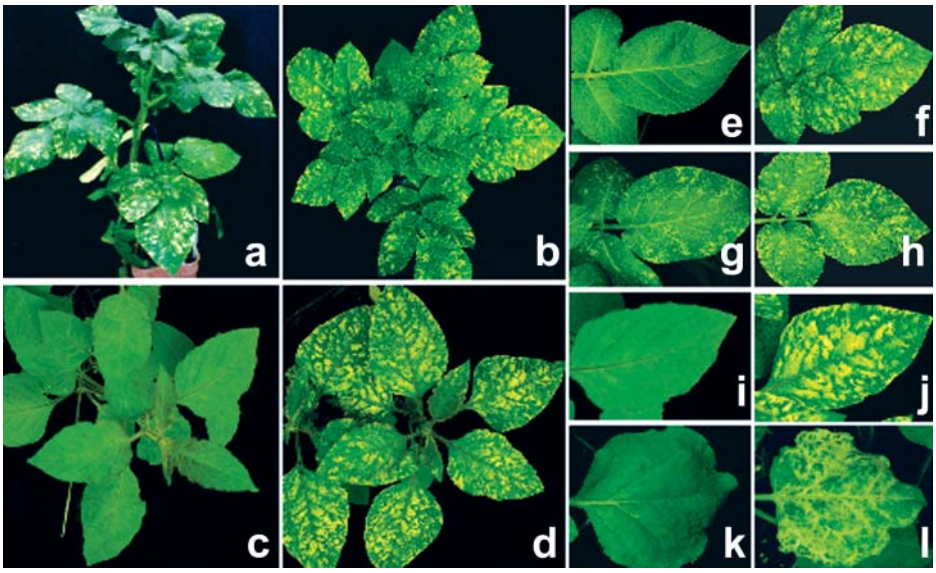

Figure 2. PVX.PDS$_{AS}$ triggers VIGS in diploid and tetraploid Solanum species. Photobleaching phenotypes observed by 21 dpi on tetraploid *S. tuberosum* cv Bintje (a and b) and diploid *S. bulbocastanum* (d) and PVX.GFP control infected plant (c). Close-up on photobleached leaves of *S. tuberosum* cvs Bintje (f), Stirling (g), Desiree (h), *S. bulbocastanum* (j), *N. benthamiana* (l), and on symptomless PVX.GFP infected leaf of cv Bintje (e) and *S. bulbocastanum* (i). Uninfected *N. benthamiana* leaf (k).

PDS, is known to occur during VIGS of the *pds* gene (Kumagai et al., 1995). Phytoene also accumulates to high levels in leaves treated with the herbicide norflurazon, a chemical inhibitor of PDS activity (Kumagai et al., 1995). The peaks in the HPLC chromatogram corresponding to cis- and trans-phytoene were identified by comparison between norflurazon-treated and untreated plants and the characteristic absorption spectra of those peaks (Fraser et al., 2000; data not shown). Increase in phytoene accumulation was quantified as the number of area units under phytoene peaks on the HPLC chromatogram (Holzberg et al., 2002).

Typical HPLC chromatograms are presented in Figure 3c. In both silenced PVX.PDSas infected *N. benthamiana* and *S. tuberosum* plants an increase in 15 cis- and trans-phytoene levels was observed (Fig. 3c, lower left and right sections, respectively) in comparison to PVX.GFP control plants. (Fig. 3c, upper section). In silenced *N. benthamiana* leaves, phytoene levels increased by 5- to 10-fold in comparison to PVX.GFP control plants (Fig. 3d). A comparable result was obtained for all *Solanum* species and cultivars tested (Fig. 3d). Although *N. benthamiana* displayed the strongest photobleaching, the increase in phytoene accumulation was lower than that observed in Solanum species and cultivars. Moreover, although *S. bulbocastanum* displayed the strongest photobleaching phenotype among the *Solanum* genotypes, similar levels of phytoene accumulation were observed in *S. tuberosum* L cv Desiree and Stirling, and these were lower than that observed in cv Bintje (Fig. 3d). This indicates that the increase in phytoene level may vary from 5- to 10-fold in Solanum silenced leaf tissue despite comparable decreases in *pds* mRNA levels. We thus propose that a similar, if not stronger, VIGS of *pds* in Solanum species and cultivars triggers a milder photobleaching phenotype than observed in *N. benthamiana* and this indicates that leaf photobleaching is not quantitatively coupled to the degree of *pds* silencing between different species.

Systemic VIGS of *pds* in Potato Tubers and In Vitro Generated Microtubers

We evaluated the potential of a VIGS-based approach for in vitro grown potato species by down-regulation of *pds* in *S. tuberosum* L cv Desiree micropropagated plants. In vitro material provides a means to substantially reduce glasshouse space requirements for high throughput functional studies. Stab-agroinoculation (Takken et al., 2000; Lu et al., 2003a) of leaves of in vitro grown plants with plated Agrobacteria transformed with either PVX.GFP or PVX.PDS$_{AS}$ constructs was an effective method to generate reproducible PVX infections. By 4 weeks postinoculation, development of systemic photobleached areas on leaves was clearly visible on PVX.PDSas inoculated plants (Fig. 4a, middle and right section) in comparison with PVX.GFP controls (Fig. 4a, left section). Micropropagation of nodal cuttings from silenced plants led to regenerated potato plants displaying a comparable photobleaching phenotype within 3 to 4 weeks (Fig. 4b; data not shown). To determine whether this photobleached phenotype was maintained through several cycles of subculture, micropropagation of the original PVX.PDS$_{AS}$ challenged plant was repeated, and a sustained photobleaching was still observed even after the

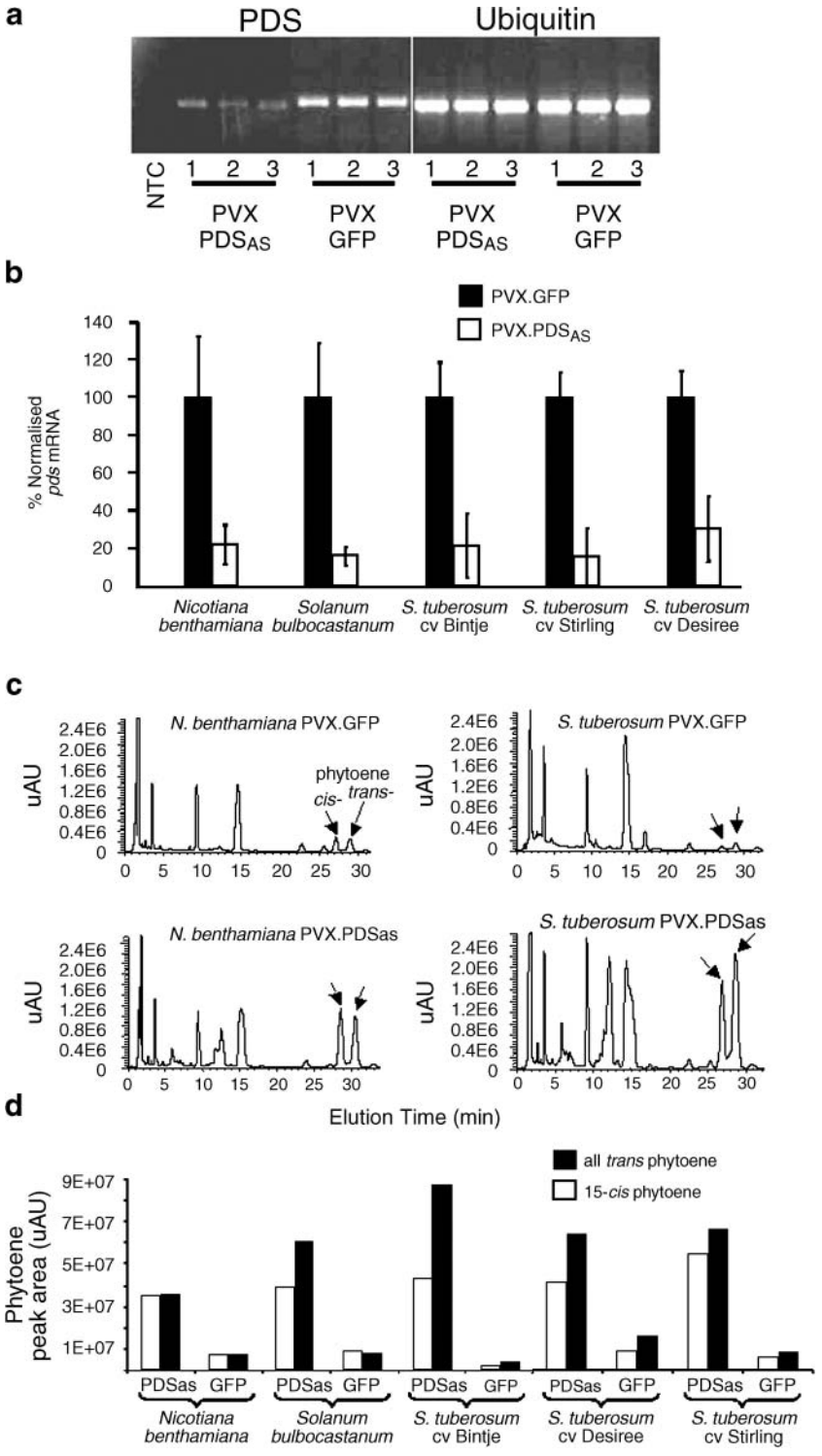

Figure 3. Molecular and biochemical characterization of *pds* VIGS in diploid and tetraploid Solanum species. a, RT-PCR of *S. tuberosum* cv Bintje *pds*-silenced and control plants in response to challenge with PVX constructs. Both RT-PCR products corresponding to endogenous *pds* and *ubiquitin* mRNAs have been assessed. PCR conditions ranging from 20 to 50 amplification cycles were tested in both cases. Presented here are 30 cycles corresponding to the log-linear phase of amplified PCR product in nonsilenced tissues (challenged PVX.GFP construct). NTC, nontemplate control; replicates are leaves from three different challenged plants. b, Real-time RT-PCR determination of normalized relative amounts of *pds* mRNA levels in silenced and control plants challenged with PVX.PDS_{AS} or PVX.GFP constructs (21 dpi). Sampled leaves were cut in half for either RNA extraction (real-time RT-PCR) or phytoene isolation (HPLC analysis). Values are expressed in percentage of normalized *pds* mRNA related to PVX.GFP control. Values represent the means of at least three leaves from different plants per construct per experiment ±SE. For each sample, real-time RT-PCR was carried out in triplicate. c, HPLC analysis of phytoene accumulation induced by PVX.PDS_{AS} VIGS vector. Typical HPLC profile from *S. tuberosum* cv Bintje and *N. benthamiana* is presented here. As before, silenced leaves from plant challenged with PVX.PDS_{AS} and corresponding leaves from PVX.GFP plants were sampled at 21 dpi. Arrows indicate peaks for both 15 cis-phytoene and all trans-phytoene. d, Quantification of phytoene accumulation in silenced and control Solanum species and *N. benthamiana*. Values for 15 –cis-phytoene and trans-phytoene are HPLC area units (arbitrary units) and represent the means of at least three leaves from different plants per construct per experiment (SE < 20%).

fifth consecutive subculture (data not shown). The three first subcultures of in vitro grown plants challenged by either PVX.GFP or PVX.PDS_{AS} (subculture 1 and subculture 3) were analyzed using real time RT-PCR. A significant decrease in *pds* mRNA was observed in both subculture 1 and subculture 3 (Fig. 4c; respectively, 70% ± 7% and 63% ± 11%), the latter representing 12 weeks of in vitro propagation. Phytoene accumulation was measured in these in vitro silenced and control plants. Norflurazon treated in vitro plants developed comparable photobleaching to that observed with glasshouse grown plants, and the chromatogram peak for phytoene was identified as before (data not shown). A similar increase in 15

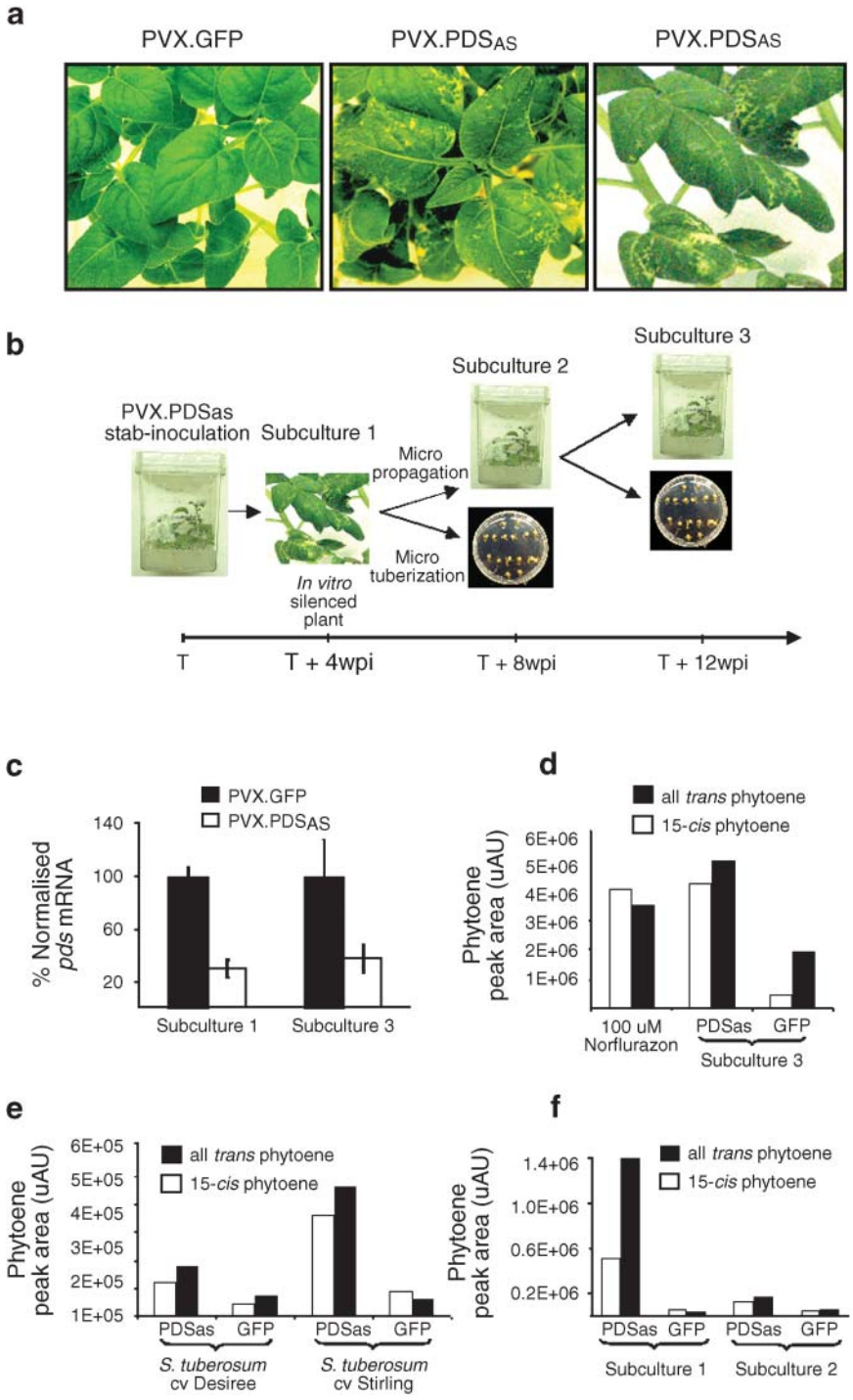

Figure 4. Systemic *pds* silencing in tubers and in vitro propagated plants and microtubers. a, Photobleaching phenotypes observed on in vitro propagated *S. tuberosum* cv Desiree after 3 subcultures (representing 12 weeks of in vitro propagation post-challenge with PVX.PDSas, middle section) or 1 subculture (4 weeks post-challenge with PVX.PDS_{AS}, right section). PVX.GFP control infected plant is shown on the left section. b, Schematic representation of in vitro culture and generation of in vitro grown microtubers. Time scale in week post-challenge with PVX construct is presented. c, Real-time RT-PCR determination of normalized relative amounts of *pds* mRNA levels in silenced and control leaves from in vitro grown *S. tuberosum* cv Desiree plants challenged with PVX.PDS_{AS} or PVX.GFP constructs. Leaves were harvested after 4 weeks post challenge (28 dpi, subculture 1) or after 12 weeks post challenge (more than 80 dpi, subculture 3). As before, sampled leaves were cut in half for either RNA extraction (real-time RT-PCR) or phytoene isolation (HPLC analysis). Values are expressed in percentage of normalized *pds* mRNA related to PVX.GFP control. Values represent the means of at least three leaves from five different in vitro plants per construct per experiment ±SE. For each sample, real-time RT-PCR was carried out in triplicate. d, Quantification of phytoene accumulation in leaves of in vitro *S. tuberosum* cv Desiree challenged with PVX.PDS_{AS} or PVX.GFP. Phytoene accumulation was monitored in norflurazon-treated in vitro plants. Values for 15 cis-phytoene and trans-phytoene are HPLC area units (arbitrary units) and represent the means of at least three leaves from different plants per construct per experiment (SE < 20%). e, Quantification of phytoene accumulation in tubers (approximately 15 weeks after challenge with PVX constructs) from silenced (PVX.PDS_{AS} challenged) and control (PVX.GFP challenged) *S. tuberosum* cv Desiree and Stirling. Values for 15 cis-phytoene and trans-phytoene are HPLC area units (arbitrary units) and represent at least ten tubers from at least 2 different plants per construct per experiment (SE < 20%). f, Quantification of phytoene accumulation in in vitro generated microtubers from silenced and control *S. tuberosum* cv Desiree, after 8 weeks (subculture 1) or 12 weeks (subculture 2) initial challenge with PVX constructs. Values for 15 cis-phytoene and trans-phytoene are HPLC area units (arbitrary units) and represent 15 to 20 microtubers generated from 4 to 6 different in vitro grown plants per construct per experiment (SE < 20%).

cis- and trans-phytoene was observed in both norflurazon-treated and PVX.PDS_{AS} silenced plants (Fig. 4d), ranging from 3-fold (trans-phytoene) to 8-fold (15 cis-phytoene) in comparison with control plants challenged with PVX.GFP (Fig. 4d) after 3 cycles of micropropagation. This indicates that *pds* VIGS characteristics are similar both in glasshouse and in vitro conditions and confirmed the relative stability of the VIGS phenotype observed with in vitro grown plants.

Although VIGS proved effective in potato leaves, much research in potato is directed at investigating the tuber life-cycle, improving storage organ quality and resistance to phytopathogens. Therefore, it was important to determine whether gene silencing was observed in tubers. However, a major drawback is the

variability in tuberization time, and the glasshouse space required in making such reverse genetics approach. In vitro grown potato offers an interesting alternative, as in vitro microtuberization is synchronized and controlled (Fig. 4b; Xu et al., 1998). Indeed, fully developed microtubers were obtained by 9 weeks of culture ("Materials and Methods," Fig. 4b), whereas in glasshouse conditions, fully developed tubers were obtained by 12 to 15 weeks post-sowing.

The systemic nature of the VIGS phenotype in tuber tissues from both glasshouse-grown plants and in vitro generated microtubers was investigated. Fully developed tubers from glasshouse plants were obtained by 3 months post-challenge with either PVX.GFP or PVX.PDS$_{AS}$. Similarly, mature in vitro generated microtubers derived from in vitro control and silenced plants challenged by PVX.GFP or PVX.PDS$_{AS}$ were collected and analyzed by HPLC to monitor the extent of accumulation of phytoene in these organs. HPLC phytoene profiles from tubers harvested from *S. tuberosum* L. cvs Desiree and Stirling plants challenged with PVX.PDS$_{AS}$ indicated, respectively, up to a 2- to 5-fold increase in phytoene accumulation in comparison to control PVX.GFP infected plants (Fig. 4e). In contrast, PVX.PDS$_{AS}$ challenged microtubers showed an accumulation of up to 20-fold more phytoene than control microtubers infected by PVX.GFP (Fig. 4f). A more marginal but significant difference (2-fold) in phytoene increase was still detected in the next generation of subcultured microtubers (subculture 2), indicating a less sustained VIGS phenotype in these tissues and experimental conditions (Fig. 4f) than observed in foliar tissues of in vitro micropropagated plants. These results indicate that systemic silencing of endogenous genes in potato, exemplified here by *pds*, does not only extend to foliar tissues but potentially spreads through the whole plant, including tubers. This silencing state can be transmitted and detected for several generations through vegetative propagation.

This study represents the first demonstration of VIGS-mediated down-regulation of gene expression in both diploid and tetraploid Solanum species. Here we report that the carotenoid biosynthetic pathway, where PDS is one of the early steps, can be manipulated by extending the use of a previously described PVX VIGS vector to a different plant host tolerating sufficient levels of PVX accumulation. In parallel, a previously described tobacco rattle virus (TRV, Ratcliff et al., 2001) vector was also tested. However, it did not reach detectable accumulation levels nor did it induce VIGS in the selected tetraploid *S. tuberosum* cultivars (data not shown). In contrast, recombinant PVX.PDS$_{AS}$ vector was detected by RT-PCR in silenced microtubers and in all others silenced organs, including leaves and tubers (data not shown). This emphasizes the prerequisite of a threshold of virus and or dsRNA accumulation to generate an effective VIGS response.

The microtuberization system, in conjunction with VIGS, has a number of potential benefits compared with analysis of tubers produced conventionally in glasshouse conditions. Microtubers develop rapidly in a relatively synchronous manner under controlled tissue-culture conditions. This should enable easier identification of tuber phenotype alterations, making it more amenable for characterization of gene function.

The effectiveness of VIGS in potato tubers opens the way for high throughput analysis of gene function to identify genes involved in important traits such as tuber development, metabolism, and pathogen resistance. Furthermore, this reverse genetic VIGS-approach should be particularly powerful in combination with analyses of the transcriptome and metabolome.

MATERIALS AND METHODS

Construction of PVX-Derived Vectors

The PVX vector (pGR106, Jones et al., 1999; Lu et al., 2003b) used in this study was obtained from David Baulcombe (Sainsbury Laboratory, Norwich, UK). PVX.GFP was generated by cloning a PCR fragment amplified from a *gfp* cDNA template (GenBank accession number U62637; Crameri et al., 1996) using specific oligonucleotide primers incorporating *Asc*I and *Not*I restrictions sites respectively at the 5′- and 3′- termini for cloning into pGR106. The construct PVX.PDS$_{AS}$ was generated by cloning in antisense orientation into pGR106 a *Not*I-*Asc*I 412-bp *pds* cDNA fragment, corresponding to nucleotides 1,133 to 1,529 from *Solanum tuberosum pds* cDNA (GenBank accession number AY484445).

Agrobacterium Infection of Plants

Agrobacterium tumefaciens strain LB4404, carrying the helper plasmid pSoup (Hellens et al., 2000) was transformed with constructs PVX.GFP or PVX.PDS$_{AS}$. Agroinfiltration of *N. benthamiana* and Solanum species with PVX vector was performed as previously described (Lu et al., 2003a). For in vitro agroinoculation, plated individual agrobacteria transformed with PVX.GFP or PVX.PDS$_{AS}$ constructs were picked with a sterile tip and punched onto a leaf of a 2-week old potato plant (Takken et al., 2000).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from frozen control and silenced leaves using the Qiagen RNeasy Plant Mini kit (Qiagen, Valencia, CA), following the manufacturer's instructions. DNAseI treatment and first strand cDNA synthesis were as previously described (Lacomme et al., 2003).

Immunoblot Analysis

Protein extraction and western-blot analysis were as previously described (Lacomme and Santa Cruz, 1999). Membranes were probed with rabbit polyclonal antiserum, raised against the PVX CP as previously described (Santa Cruz et al., 1996).

RT-PCR and SYBR Real-Time RT-PCR Experiments

For RT-PCR analysis, primers that anneal outside the region of the *pds* cDNA cloned into the virus vectors to trigger silencing (nucleotides 1,126–1,516) were used to ensure that only the endogenous *pds* mRNA is reverse-transcribed as indicated in Figure 1a. Potato *ubiquitin* cDNA (GenBank accession no. BQ045862) was used as an internal constitutively expressed control. First-strand cDNA was used as a template for PCR amplification through 20, 25, 30, 40, and 50 cycles. As 30 cycles of amplification was within the log-linear phase of *pds* PCR product amplification in the nonsilenced control samples (data not shown), these conditions were selected for comparison of relative accumulation of both *pds* and ubiquitin mRNAs in all samples. The following primers were used: RTPoPDSfor (5′-CTC GAG GTC GTC TTC TTT GG-3′); RTPoPDSrev (5′-GTT TAG TTG GGC GTG GAG AA-3′); RTPoUBIfor (5′-GCA GTT GGA

GGA CGG AC-3′); and RTPoUBIrev (5′-GGC CAT CTT CCA ACT GTT TC-3′). Similarly, for SYBR real-time RT-PCR (QuantiTect SYBR Green PCR kit, Qiagen, Crawley, UK) experiments, primer pairs were designed outside the region of the *S. tuberosum pds* cDNA targeted for silencing (Fig. 1a) and for the internal control *ubiquitin* cDNA using the Primer Express software supplied with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) following the manufacturer's guideline for primer design. The following primers were used: StPDSfwd (5′-CCA AGA CCA GAG CTA GAC AAT ACA GT-3′); StPDSrev (5′-CCA CCC AAA CCT GCA-3′); StUBIfwd (5′-ACA CCA TTG ATA ATG TCA AGG CTA AG-3′); and StUBIrev (5′-GCC ATC CTC CAA TTG CTT TC-3′). The GenBank accession numbers for *N. benthamiana pds* and *ubiquitin* cDNA are as previously mentioned (Lacomme et al., 2003). Primer concentrations giving the lowest threshold cycle (C_t) value were selected for further analysis. Detection of real-time RT-PCR products, calculations and statistical analysis were performed as previously described (Lacomme et al., 2003).

Plant Material and Growth Conditions

All work involving virus-infected material was carried out in containment glasshouses under SEERAD license GM/180/2003. Potato cultivars Stirling, Bintje, and Desiree were micropropagated in sterile conditions by removing 5 cm of young potato stems from virus-tested potato plants from the Scottish Agricultural Science Agency (SASA, Edinburgh), removing leaves and dividing the stem into individual pieces, each containing a node with an axillary bud. Six stem pieces were cultivated per petri dish containing 20 mL of Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 20% Suc, 0.8% bacto agar (DIFCO Laboratories, Detroit) and adjusted to pH 5.8, sealed with Nescofilm (Bando Chemical, Kobe, Japan). Plants were then grown at 22°C with 16 h photoperiod and 110 μE m^{-2} s^{-1}. When potato plants were 3 to 4 weeks old, they were then transferred to soil in controlled environment chambers with a 16 h photoperiod (22°C, light intensity ranging from 400–1000 μE m^{-2} s^{-1}).

In Vitro Microtuberization

After 28 d culture, potato plants were divided into single nodes and placed on Murashige and Skoog medium supplemented with 60% Suc, 7 mM chlorocholine chloride (CCC), 8 μM benzylaminopurine (BAP), and 0.8% agar (DIFCO). CCC and BAP were added aseptically after autoclaving by filter sterilizing. Fifteen nodes were placed in each dish and the dishes were sealed with Nescofilm. The cultures were then placed in an incubator in an 8 h photoperiod at 80 μE m^{-2} s^{-1} at 16°C for 7 d prior to a total darkness at the same temperature for a further 28 d. The microtubers were then harvested and frozen for further analyses.

Extraction and HPLC Analysis of Phytoene from Transfected Plants

Phytoene was extracted from infected leaves, tubers and microtubers. The method used was as described in Holzberg et al. (2002). A total of 50 mg of freeze-dried leaves (3 leaves from different plants), 100 mg of freeze-dried tubers (representing at least 10 mature tubers), or 100 mg of freeze-dried in vitro generated microtubers (representing 15–20 microtubers deriving from at least 4 independent in vitro grown plants) were extracted in 100% methanol. The samples were then centrifuged for 5 min at 4,000 rpm at 4°C. The supernatants were dried under a stream of nitrogen. Residues were redissolved in 500 μL of 100% methanol and 10 μL were separated by HPLC (Surveyor system, Thermo Finnigan, Bellefonte, PA) with a Phenomenex 2 × 250 mm C-18 column using acetonitrile/methanol/2-propanol (85:10:5, v/v) at a flow rate of 300 μL × min^{-1}. Phytoene was detected with a photodiode array detector using UV absorption at 285 nm and identified by comparing peak retention times with norflurazon-treated and untreated potato plants (Fraser et al., 2000; Holzberg et al., 2002).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY484445.

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