Virology 386 (2009) 407–416

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Apple latent spherical virus vectors for reliable and effective virus-induced gene silencing among a broad range of plants including tobacco, tomato, *Arabidopsis thaliana*, cucurbits, and legumes

Aki Igarashi [a], Kousuke Yamagata [a], Tomokazu Sugai [a], Yukari Takahashi [a], Emiko Sugawara [a], Akihiro Tamura [a], Hajime Yaegashi [b], Noriko Yamagishi [a], Tsubasa Takahashi [c], Masamichi Isogai [a], Hideki Takahashi [d], Nobuyuki Yoshikawa [a,b,c,*]

[a] *Plant Pathology Laboratory, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan*
[b] *The United Graduate School of Agricultural Sciences, Iwate University, Morioka 020-8550, Japan*
[c] *The 21st Century Center of Excellence Program, Iwate University, Morioka 020-8550, Japan*
[d] *Department of Life Science, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan*

ARTICLE INFO

Article history:
Received 17 November 2008
Returned to author for revision
20 December 2008
Accepted 10 January 2009
Available online 25 February 2009

Keywords:
Apple latent spherical virus vector
Virus-induced gene silencing
Arabidopsis thaliana
Tobacco
Cucurbit
Legume

ABSTRACT

Apple latent spherical virus (ALSV) vectors were evaluated for virus-induced gene silencing (VIGS) of endogenous genes among a broad range of plant species. ALSV vectors carrying partial sequences of a subunit of magnesium chelatase (*SU*) and phytoene desaturase (*PDS*) genes induced highly uniform knockout phenotypes typical of *SU* and *PDS* inhibition on model plants such as tobacco and *Arabidopsis thaliana*, and economically important crops such as tomato, legume, and cucurbit species. The silencing phenotypes persisted throughout plant growth in these plants. In addition, ALSV vectors could be successfully used to silence a meristem gene, proliferating cell nuclear antigen and disease resistant *N* gene in tobacco and *RCY1* gene in *A. thaliana*. As ALSV infects most host plants symptomlessly and effectively induces stable VIGS for long periods, the ALSV vector is a valuable tool to determine the functions of interested genes among a broad range of plant species.

Introduction

Virus infection induces an RNA-mediated defense mechanism that targets viral RNAs in a nucleotide sequence-specific manner in plants, commonly referred to as RNA silencing (Waterhouse et al., 2001; Vance and Vaucheret 2001; Voinnet 2005). When the virus carries sequences of plant genes, virus infection triggers virus-induced gene silencing (VIGS) that results in the degradation of endogenous mRNA homologous to the plant genes through a homology-dependent RNA degradation mechanism (Lu et al., 2003a,b; Waterhouse and Helliwell, 2002). VIGS has been shown to have great potential as a reverse-genetics tool for studies of gene functions in plants, and it has several advantages compared with other functional genomics approaches (Burch-Smith et al., 2004). For example, VIGS can knock down the expression of a gene without the need to generate transgenic plants, and it can identify a loss-of-function phenotype for a specific gene within a single generation. In addition, VIGS can overcome the problem of redundancy by using a target sequence from the highly conserved region of a gene family. Thus, VIGS offers an easy way to determine the functions of the genes in a short time, and it can also be applied to high throughput functional genomics in plants (Benedito et al. 2004; Burch-Smith et al., 2004; Lu et al., 2003a,b; Godge et al., 2008).

So far, several virus vectors designed for VIGS in plants have been developed (Godge et al., 2008), including vectors of *Tobacco mosaic virus* (TMV) (Kumagai et al., 1995), *Potato virus X* (PVX) (Chapman et al., 1992; Ruiz et al., 1998), *Tomato golden mosaic virus* (TGMV) (Peele et al., 2001), and *Tomato yellow leaf curl China virus* satellite DNA (Tao and Zhou, 2004). These VIGS vectors have successfully silenced endogenous genes like phytoene desaturase (PDS) in *Nicotiana benthamiana* plants. *Tobacco rattle virus* (TRV)-derived vectors can be used for VIGS in *Solanum* species including tomato, potato, *N. benthamiana*, and *Arabidopsis thaliana* (Brigneti et al., 2004; Burch-Smith et al., 2006; Cai et al., 2006; Fu et al., 2005; Liu et al., 2002a; Ratcliff et al., 2001). The modified TRV-vectors are compatible with the GATEWAY system allowing restriction- and ligation-free cloning and large-scale functional genomics screening (Liu et al., 2002a). *Cabbage leaf curl virus* (CbLCV) for VIGS in *A. thaliana* (Turnage et al.,

* Corresponding author. Plant Pathology Laboratory, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan. Fax: +81 196 23 6150.
E-mail address: yoshikawa@iwate-u.ac.jp (N. Yoshikawa).

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.01.039

2002), *Turnip yellow mosaic* virus (TYMV) in *A. thaliana* (Pflieger et al., 2008), satellite TMV (STMV) in *N. tabacum* (Gossele et al., 2002), *Barley stripe mosaic virus* (BSMV) in barley (Holzberg et al., 2002; Lacomme et al., 2003), *Pea early browning virus* (PEBV), *Bean pod mottle virus* and Cucumber mosaic virus in legume species (Constantin et al., 2004; Nagamatsu et al., 2007; Zhang and Ghabrial, 2006), *African cassava mosaic virus* (ACMV) in cassava (Fofana et al., 2004), and *Brome mosaic virus* in monocotyledonous plants (Ding et al., 2006) were reported as vectors for other hosts.

One of the limitations of VIGS technology is that the most reliable and effective VIGS vectors have a limited host range and are functional only in some plant species, especially in the model plant *N. benthamiana* (Goodin et al., 2008). Moreover, some of the viruses used in VIGS induce symptoms that confuse the phenotype caused by silencing of the target gene. So, the development of reliable VIGS vectors for additional plant species will be very useful for the development of plant genomics (Burch-Smith et al., 2004).

Apple latent spherical virus (ALSV), classified into a newly established genus *Cheravirus* (Le Gall et al., 2007), has isometric virus particles *c.* 25 nm in diameter, and it contains two ssRNA species (RNA 1 and RNA 2) and three capsid proteins (Vp25, Vp20 and Vp24) (Li et al., 2000). ALSV is composed of two components, M and B, which are thought to contain two molecules of RNA 2 and a single molecule of RNA 1, respectively (Li et al., 2000). RNA 1 (6813nt excluding the 3′ poly (A) tail) has a single open reading frame (ORF) encoding a polypeptide of 243K, which contains the consensus motifs of the protease cofactor, the NTP-binding helicase, the cysteine protease and the RNA polymerase from the N-terminus (Li et al., 2000). RNA 2 (3385nt excluding the 3′ poly (A) tail) also has a single ORF encoding a polypeptide of 119K/108K containing a 53K/42K movement protein (MP) on the N-terminal side and three capsid proteins in the C-terminal region (Li et al., 2000). Recently, we constructed infectious cDNA clones of ALSV-RNAs and modified them into viral vectors for the expression of foreign genes in plants (Li et al., 2004). ALSV vectors expressing fluorescence proteins have been used to trace the cell-to-cell movement of ALSV in infected plant tissues and for analyzing the distribution of identical virus populations labeled with different fluorescence proteins in co-infected plants (Yoshikawa et al., 2006; Takahashi et al., 2007). An ALSV vector expressing green fluorescent protein (GFP-ALSV) was also used for analysis of VIGS of a transgene in tobacco plants expressing GFP (Yaegashi et al., 2007).

Here, we describe how ALSV vectors effectively induce reliable VIGS of endogenous genes among a broad range of plants, i.e., *Nicotiana* species (*N. tabacum*, *N. occidentalis*, *N. glutinosa*, and *N. benthamiana*), *Solanum lycopersicum*, *A. thaliana*, cucurbit species (*Cucumis sativus*, *C. melo*, *Cucurbita pepo*, *Citrullus lanatus*, *Luffa cylindrical*, and *Lagenaria siceraria*), and legume species (*Glycine max*, *Pisum sativum*, *Vigna angularis*, and *V. unguiculata*). As ALSV does not induce any obvious symptoms in most host plants, ALSV vectors can be used for functional genomics in the host plants. This is also the first report on virus vectors for VIGS in cucurbit species.

Results

Symptomless infection of ALSV vectors in most host plant species

We previously reported that ALSV infected *Chenopodium quinoa*, *Tetragonia expansa*, *C. amaranticolor*, and *Beta vulgaris*, the latter two symptomlessly (Li et al., 2000). Further examination of the ALSV host range showed that the virus could systemically infect the following herbaceous plants: *Gomphrena globosa* and *Celosia cristata* in the family *Amaranthaceae*; *A. thaliana* in *Brassicaceae*; *C. sativus*, *C. melo*, *C. pepo*, *C. lanatus*, *L. cylindrical*, and *L. siceraria* in *Cucurbitaceae*; *G. max*, *P. sativum*, and *V. angularis*, *V. unguiculata* in *Leguminaceae*; *Linaria maroccana* in *Scrophulariaceae*; and *N. benthamiana*, *N. occidentalis*, *N. glutinosa*, *N. tabacum*, *S. lycopersicum*, and *Petunia hybrida* in *Solanaceae*. Among these plants, soybean, cowpea, and cucurbit plants initially developed chlorotic spots in a few upper leaves, followed by the development of symptomless leaves. Other plants showed no obvious symptoms on both inoculated and upper leaves.

Silencing of endogenous genes in Nicotiana species and tomato with ALSV vectors

To test whether ALSV vectors could act as effective inducers for the silencing of endogenous genes in *Nicotiana* species, we first targeted the tobacco sulfur (*SU*) gene that encodes a magnesium chelatase subunit required for chlorophyll production. The *SU* gene fragment (321 nt) was inserted into an ALSV-RNA2 vector (Fig. 1), and the resulting virus (tSU-ALSV) was inoculated into the plants (6 to 8 true leaf stage) of four *Nicotiana* species (*N. benthamiana*, *N. tabacum*, *N. glutinosa*, and *N. occidentalis*). The inoculated plants started to develop yellow leaf symptom along the veins on upper uninoculated leaves from 7 to 10 days post inoculation (dpi) depending on the species, and then newly developed leaves, petioles and stems of infected plants showed a highly uniform yellowing which is a knockout phenotype typical of *Su* inhibition, indicating that the *SU* gene had been silenced by tSU-ALSV (Fig. 2A, Table 1). The *SU* silencing persisted for more than three months in four *Nicotiana* species, and the recovery of the silencing phenotype was never observed during the experiments. A semi-quantitative PCR analysis indicated that the levels of *SU* mRNA were reduced in the silenced leaves (Fig. 3A). Mechanical inoculation of *Nicotiana* plants with tSU-ALSV resulted in 100% infection in separate experiments, and all plants developed the same knockout phenotype. The plants inoculated with a wild-type (wt) ALSV did not show any viral symptoms nor a change of leaf color (data not shown).

Inoculation of tSU-ALSV into tomato plants also resulted in a highly uniform yellowing phenotype typical of *Su* inhibition (Fig. 2B), and

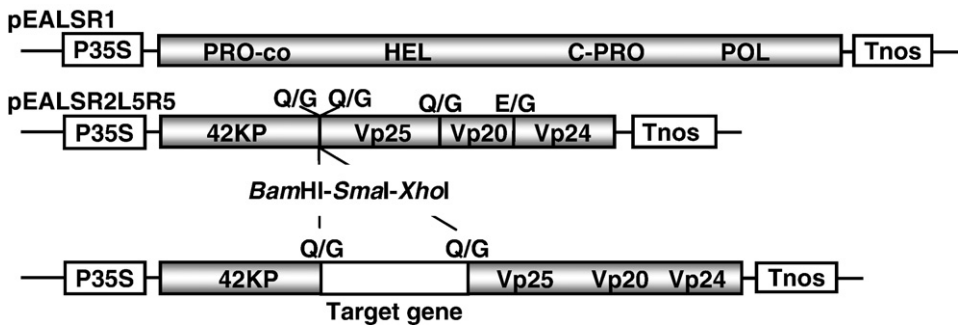

Fig. 1. Schematic representation of the infectious cDNA clones of ALSV-RNA1 (pEALSR1) and ALSV-RNA2 vector (pEALSR2L5R5) with artificial processing sites by duplicating the Q/G cleavage site between 42KP and VP25. A target gene can be inserted between 42KP and VP25 using Xho I, Sma I and Bam HI restriction sites. P35S, Cauliflower mosaic virus 35S promoter; Tnos, nopaline synthase terminator; PRO-co, protease cofactor; HEL, NTP-binding helicase; C-PRO, cysteine protease, POL, RNA polymerase; 42KP, 42K movement protein; Vp25, Vp20, and Vp24, capsid proteins.

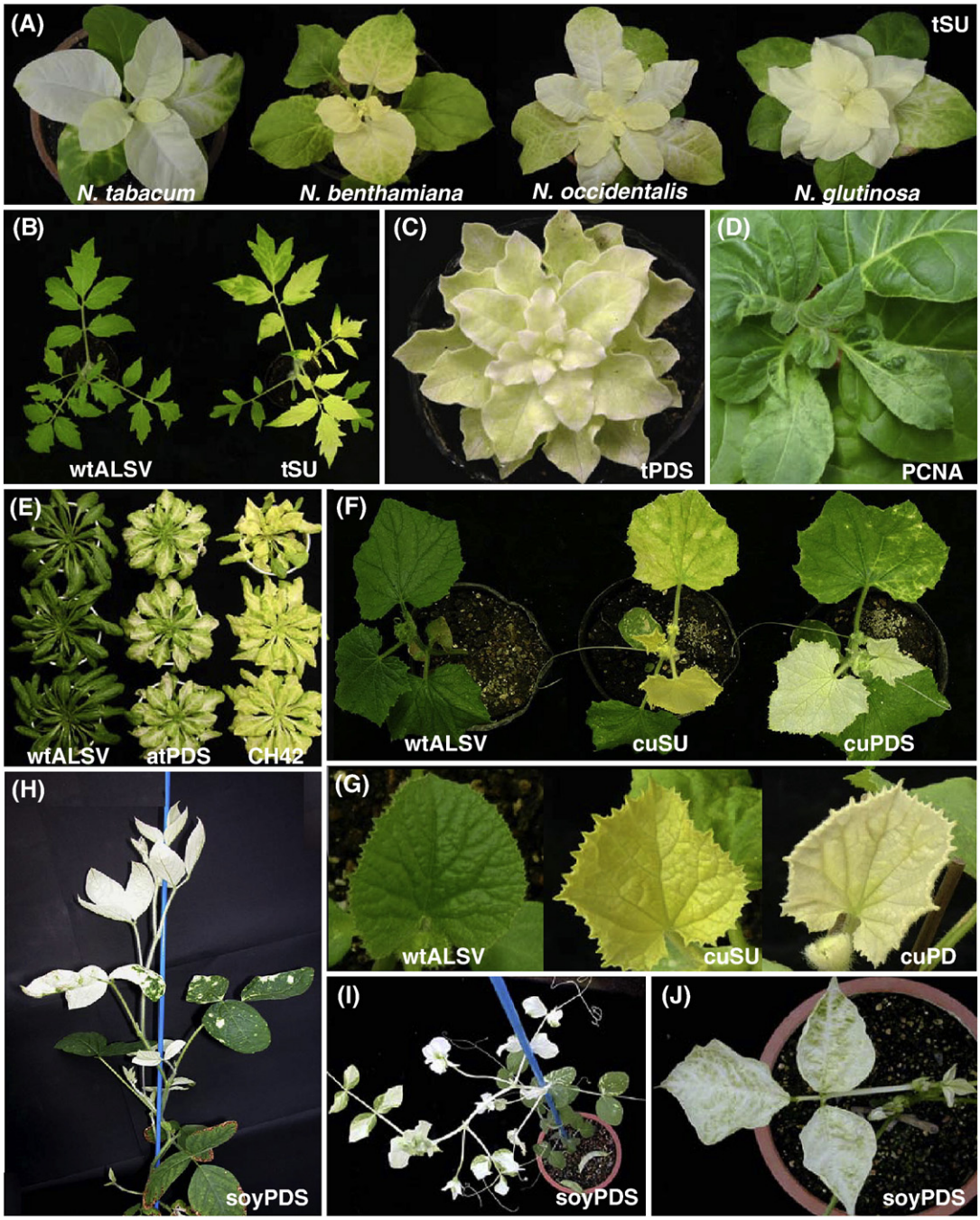

Fig. 2. Virus-induced gene silencing in plants infected with ALSV vectors. (A) Silencing of *SU* gene in tobacco cv Xanthi nc (30 dpi), *N. benthamiana* (14 dpi), *N. occidentalis* (23 dpi), and *N. glutinosa* (24 dpi) infected with tSU-ALSV. (B) Tomato (cv. Oogata fukuju) infected with wtALSV (left) and tSU-ALSV (right) 31 dpi. (C) Silencing of *PDS* gene in tobacco infected with tPDS-ALSV 75 dpi. (D) Leaf malformation of tobacco infected with PCNA-ALSV 30 dpi. (E) *A. thaliana* Col plants infected with wtALSV (left, three plants), atPDS-ALSV (center), and CH42-ALSV (right) 30 dpi. (F) *C. sativus* cv. Tsubasa infected with wtALSV (left), cuSU-ALSV (center), and cuPDS-ALSV (right) 27 dpi. (G) *C. melo* cv. Earis Knight infected with wtALSV (left), cuSU-ALSV (center), and cuPDS-ALSV (right) 31 dpi. (H) *G. max* cv. Suzukari infected with soyPDS-ALSV 50 dpi. (I) *P. sativum* cv. Denkou infected with soyPDS-ALSV 15 dpi. (J) *V. angilaris* cv. Benidainagon infected with soyPDS-ALSV 51 dpi.

the *SU* silencing phenotype was maintained for more than one month after the start of silencing.

We next targeted a phytoene desaturase (*PDS*) gene essential for the production of carotenoid pigments. All four *Nicotiana* species inoculated with tPDS201-ALSV containing a 201 bp fragment of tobacco *PDS* gene developed a white photo-bleached phenotype typical of *PDS* inhibition from 8 to 14 dpi depending on which *Nicotiana* species it was. Then, a uniform white photo-bleached phenotype appeared on newly developed leaves, and the phenotype was maintained for more than two months after the start of silencing (Fig. 2C, Table 1). A semi-quantitative PCR analysis indicated that *PDS* mRNA was strikingly reduced in the silenced white leaves (Fig. 3A).

To investigate whether ALSV vectors can be used to silence the genes expressed in meristematic tissues, we tested the proliferating cell nuclear antigen (*PCNA*) gene used to evaluate the silencing of a meristem-expressed gene (Peele et al., 2001; Tao and Zhou, 2004). When ALSV containing a fragment (291nt) of *PCNA* gene (PCNA-ALSV) was inoculated to tobacco, chlorosis, malformation, and severe dwarfing appeared on newly developed leaves (Fig. 2D), indicating that the PCNA gene in tobacco plants was effectively silenced.

Table 1
Plant species in which VIGS of trans- and endogenous genes was effectively induced by ALSV vectors

Plant species	Symptoms by virus infection[a]	Genes silenced[b]
Brassicaceae		
Arabidopsis thaliana	−	*CH42*, *PDS*, *RCY1*
Leguminaceae		
Glycine max	±	*PDS*
Pisum sativum	−	*PDS*
Vigna angularis	−	*PDS*
V. unguiculata	±	*PDS*
Cucurbitaceae		
Cucumis sativus	±	*SU*, *PDS*
Cucurbita pepo	±	*SU*, *PDS*
Citrullus lanatus	±	*SU*, *PDS*
Luffa cylindrical	±	*SU*, *PDS*
Lagenaria siceraria	±	*SU*, *PDS*
Solanaceae		
Nicotiana benthamiana	−	*SU*, *PDS*, *PCNA*
N. glutinosa	−	*SU*, *PDS*, *PCNA*
N. occidentalis	−	*GFP*, *SU*, *PDS*, *PCNA*
N. tabacum	−	*GFP*, *SU*, *PDS*, *PCNA*, *N*
Solanum lycopersicum	−	*SU*

[a] −, no symptoms. ±, appearance of faint chlorotic spots on a few leaves followed by development of normal symptomless leaves.
[b] *CH42*, *Chlorata 42* gene encoding aprotoporphyrin–IX Mg-chelatase; *PDS*, phytoene desaturase; *RCY1*, a resistant gene to an yellow strain of *Cucumber mosaic virus* in *A. thaliana*; *SU*, sulfur gene that encodes a magnesium chelatase subunit; *PCNA*, proliferating cell nuclear antigen; GFP, green fluorescence protein; *N*, a resistant gene to *Tobacco mosaic virus* in *N. glutinosa*. Silencing of GFP gene was reported by Yaegashi et al. (2007).

Malformation of leaves and severe dwarfing of the plants were observed similarly in all four *Nicotiana* species tested (Table 1).

Silencing of CH42 and PDS genes in A. thaliana with ALSV vectors

In order to determine whether ALSV vectors are functional as effective silencing inducers of endogenous genes in *A. thaliana*, the fragments (201 bp) of a *Chlorata42* (*CH42*) gene encoding aprotoporphyrin–IX Mg-chelatase and a *PDS* gene from *A. thaliana* were inserted into ALSV-RNA2 vectors, and the resulting viruses were inoculated into *A. thaliana* plants at the 8 true-leaves stage. All plants inoculated with ALSV containing a *CH42* gene fragment (CH42-ALSV) started to develop yellow phenotype along the veins on uninoculated upper leaves 10 dpi, and then newly developed leaves, petioles and stems showed a highly uniform knockout phenotype of *SU* (Fig. 2E). The silencing was maintained for more than 1 month. Infection with ALSV containing *A. thaliana PDS* gene (AtPDS-ALSV) also resulted in the white photo-bleached silencing phenotype 10 dpi, and the silencing was maintained for more than 1 month (Fig. 2E). A semi-quantitative PCR analysis indicated that the levels of both *CH42*- and *PDS*-mRNAs in the silenced leaves were strikingly reduced (Fig. 3B). On the other hand, plants infected with wtALSV did not show any obvious symptoms nor a change of leaf color (Fig. 2E).

Silencing of PDS and SU genes in cucurbit species with ALSV vectors

The *PDS* and *SU* gene fragments (300 bp) amplified from mRNAs of cucumber leaves were inserted into ALSV-RNA2 vectors. The resulting viruses (cuPDS-ALSV and cuSU-ALSV) were inoculated to cotyledons of cucumber (*C. sativus*), melon (*C. melo*), zucchini (*C. pepo*), watermelon (*C. lanatus*), sponge gourd (*L. cylindrical*), and bottle gourd (*L. siceraria*). All species inoculated with cuPDS-ALSV developed white leaves that have a highly uniform *PDS* knockout phenotype, indicating that the *PDS* gene had been silenced by cuPDS-ALSV (Figs. 2F, G, Table 1). Similarly, the plants infected with cuSU-ALSV developed yellow leaves typical of *Su* inhibition, indicating that the *SU* gene had been silenced by cuSU-ALSV (Figs. 2F, G, Table 1). The growth of silenced plants was severely suppressed due to the loss of chlorophyll. As ALSV infection was restricted to the inoculated leaves of pumpkin plants (*Cucurbita moschata*), no silencing phenotypes appeared on either inoculated or uninoculated upper leaves of plants inoculated with cuPDS-ALSV or cuSU-ALSV. A semi-quantitative PCR analysis indicated that the levels of a *SU*-mRNA and a *PDS*-mRNA in the silenced leaves were strikingly reduced in the silenced leaves of melon and sponge gourd plants (Figs. 3C, D).

Silencing of PDS gene in legume species with ALSV vectors

The 300-bp fragment of a *PDS* gene from soybean plants was inserted into ALSV-RNA2 vectors, and the resulting viruses (soyPDS-ALSV) were inoculated to primary leaves of soybean, pea, Adzuki bean, and cowpea plants. Inoculated soybean, pea and Adzuki bean plants initiated the development of white spots on the third trifoliate true leaf 10 to 14 dpi, and then showed highly uniform white photo-bleached phenotype in the fourth or fifth true leaves of the plants, indicating that the *PDS* gene had been silenced (Figs. 2H, I, J). The *PDS* silencing on these plants persisted for a month, though the growth of plants was severely suppressed due to loss of chlorophyll. In cowpea plants inoculated with soyPDS-ALSV, infected leaves showed yellow color instead of bleached white phenotype (data not shown).

Silencing of R genes in N. tabacum and A. thaliana with ALSV vectors

The tobacco *N* gene confers resistance to TMV and encodes a protein belonging to a Toll-interleukin-1 receptor – nucleotide binding site – leucine-rich repeat (TIR-NSB-LRR) class (Whitham et al., 1994). We constructed ALSV vectors containing fragments of *N* gene from *N. tabacum* cv. Xanthi nc and tested whether the vectors can be used to silence the *N* gene in *N. tabacum*. The wtALSV and ALSV containing fragments of *N* gene (TIRN-ALSV and TIRF-ALSV) were mechanically inoculated onto three leaves (the first to third true leaves of four-leaf stage) of tobacco (cv. Xanthi nc). The SU-ALSV was also inoculated to tobacco to monitor the progress of VIGS in each leaf (Fig. 4A-a). After 30 days, the upper leaves (the tenth and eleventh leaves) of tobacco plants infected with wtALSV (wtALSV-tobacco), TIRN-ALSV (TIRN-ALSV-tobacco), and TIRF-ALSV (TIRF-ALSV-tobacco) were then inoculated with purified *Tomato mosaic virus* (ToMV) (100 ng/ml). In wtALSV-tobacco plants inoculated with ToMV, local necrotic lesions appeared on the inoculated leaves at 2 dpi (Fig. 4A-b). ToMV had localized near local lesions and never moved to upper uninoculated leaves. On the other hand, in TIRN-ALSV- and TIRF-ALSV-tobacco inoculated with ToMV, necrotic lesions started to develop at 3 dpi, and the lesion numbers were about half of that in wtALSV-tobacco (Table 2 and Fig. 4A-c,d). These plants then developed necrosis on the stem and petioles of newly developed upper leaves and stunted severely (Fig. 4A-e,f), indicating that ToMV moved to upper uninoculated leaves from the inoculated leaves. Enzyme linked immunosorbent assay of ToMV-inoculated leaves showed that ToMV was distributed on green tissues among necrotic lesions in TIRN-ALSV- or TIRF-ALSV-tobacco plants, in contrast to ToMV which was detected only on tissues containing local lesions in wtALSV-tobacco plants.

We next examined whether ALSV vectors can be used for the silencing of the R gene in *A. thaliana*. The *RCY1* gene found in *A. thaliana* ecotype C24 is an R gene to a yellow strain of *Cucumber mosaic virus* (CMV-Y) and is characterized by the development of a necrotic local lesion at the site of infection that restricts viral spread (Takahashi et al., 1994, 2002). ALSV vectors containing 300-bp fragments of *RCY1* gene (RCYN-ALSV and RCYCEN-ALSV) were constructed and tested whether the vectors can be used to silence *RCY1* gene in transgenic *A. thaliana* ecotype Columbia (Col-RCY1) (Takahashi et al., 2002). RCYN-ALSV and RCYCEN-ALSV were mechanically inoculated onto two true leaves (three-true leaf stages) of Col-RCY1 plants. After 14 days, the upper leaves (the fifth and sixth

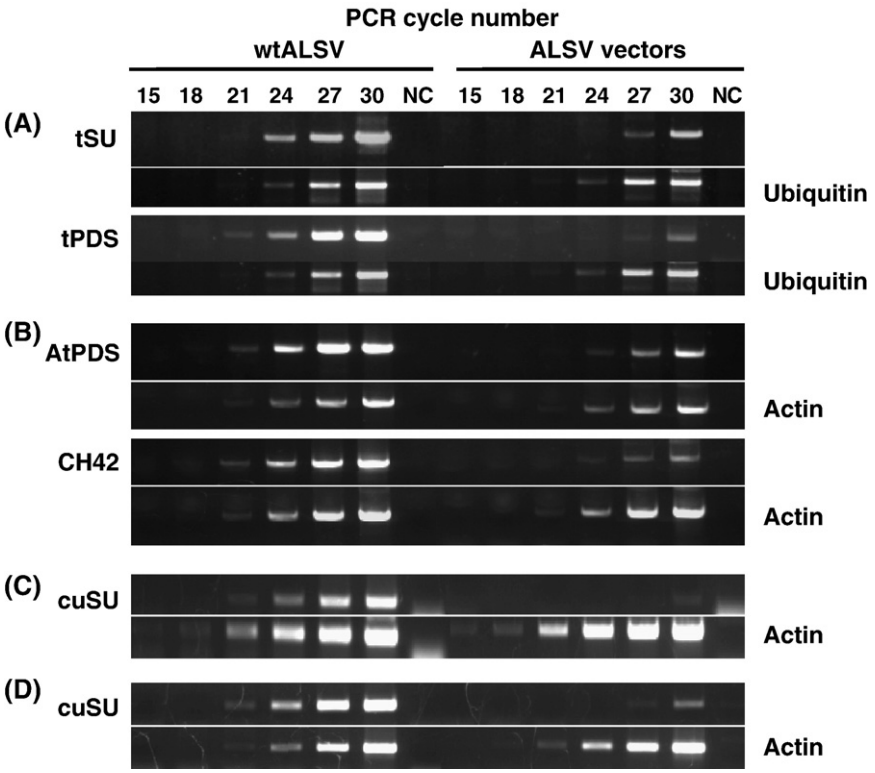

Fig. 3. RT-PCR analysis of *SU*, *PDS* or *CH42* mRNA levels in silenced (ALSV vectors-infected) and non-silenced (wtALSV-infected) leaves of (A) tobacco, (B) *A. thaliana*, (C) *C. melo*, and (D) *L. cylindrical* cv. Oonagahechima. Ubiquitin gene in tobacco and actin genes in *A. thaliana*, *C. melo*, and *L. cylindrical* were used as internal controls. Lane NC represents the controls in which the reverse transcriptase-free RT reaction mix was used as a template in the reaction (30 cycles).

true leaves) of Col-RCY1 plants infected with RCYN-ALSV, RCYCEN-ALSV, or wtALSV were then inoculated with CMV-Y and analyzed regarding the systemic movement of CMV-Y in inoculated plants. In most Col-RCY1 plants pre-inoculated with wt ALSV, CMV induced necrotic local lesion in inoculated leaves and did not move to upper uninoculated leaves (Table 3 and Fig. 4B). In contrast, CMV-Y systemically infected Col-RCY1 plants and the infected plants showed severe stunting (Table 3 and Fig. 4B). Immunoblot and tissue blot analyses confirmed that CMV-Y was systemically distributed throughout the plants previously inoculated with RCYN-ALSV or RCYCEN-ALSV (Fig. 4C). Thus, ALSV vectors containing fragments of *RCY1* gene successfully silenced RCY1 gene, and the plants showed a loss of resistance to CMV-Y.

Influence of insert length on efficiency and stability of VIGS

To investigate the influence of insert length on the efficiency and the stability of VIGS, ALSV vectors carrying different lengths of a *PDS* gene (Fig. 5A) were inoculated to the second to fourth true leaves of tobacco plants (5-true leaf stage) and were investigated regarding their efficiencies and the stability of VIGS. The plants infected with PDS108′-, PDS102-, PDS201-, PDS300-, or PDS408-ALSV vectors showed a white photo-bleached phenotype typical of *PDS* inhibition on the sixth to ninth true leaves. Interestingly, tobacco plants inoculated with PDS108-ALSV always developed yellow green leaves in contrast to white color leaves induced by other vectors (Fig. 5B). Tobacco plants inoculated with PDS156-ALSV also developed cream color leaves (Fig. 5B). Measurements of chlorophyll contents clearly showed the difference among white, yellow green, and cream color phenotypes (Fig. 5C). These results indicated that the length and/or positions of inserted sequences affected the efficiencies of VIGS. Another striking difference among vectors carrying different lengths of a *PDS* gene was found on the stability of VIGS. Plants inoculated with PDS408-ALSV and PDS300-ALSV initially developed white colored leaves followed by the development of leaves showing mosaic phenotypes consisting of white and green islands after 30 to 40 dpi. RT-PCR analysis indicated that ALSV vectors in green islands had lost PDS sequences, in contrast to viruses in the white area which maintained their sequences. Plants infected with PDS108-ALSV, PDS102-ALSV, PDS108′-ALSV, PDS156-ALSV, and PDS201-ALSV showed highly uniform knockout phenotypes of *PDS* for more than two months (Fig. 2), indicating that the ALSV vector carrying ~200-bp PDS sequences is suitable for long term silencing.

Discussion

We previously reported that the infection of ALSV expressing GFP (GFP-ALSV) induced VIGS of a transgene (*GFP* gene) in transgenic tobacco plants, and the VIGS persisted for more than three months (Yaegashi et al., 2007). Transgenes are known to often be more susceptible to VIGS than endogenous plant genes are. In the present study, we investigated whether ALSV vectors could act as effective inducers for the silencing of endogenous genes in plants. Our results showed that ALSV vectors carrying plant endogenous gene sequences effectively induced VIGS of the genes in plants including *A. thaliana*, four *Nicotiana* species (*N. tabacum*, *N. occidentalis*, *N. glutinosa*, *N. benthamiana*), tomato, four legume species (*G. max*, *P. sativum*, *V. angularis*, and *V.unguiculata*), and six cucurbit species (*C. sativus*, *C. melo*, *C. pepo*, *C. lanatus*, *L. cylindrical*, and *L. siceraria*) (Table 1). To the best of our knowledge, this is the first report on virus vectors useful for VIGS in cucurbit species (Godge et al., 2008).

Compared to other VIGS vectors reported so far, ALSV seems to have several advantages for VIGV in plants. Firstly, ALSV is a latent virus that infects most host plants without showing any symptoms. This is one of the requirements for reliable and effective plant virus vectors used for VIGS to assess gene functions (Burch-Smith et al. 2004; Godge et al., 2008). Second, ALSV induces a highly uniform knockout phenotype on the entire area of most leaves in infected plants as shown in Fig. 2. The

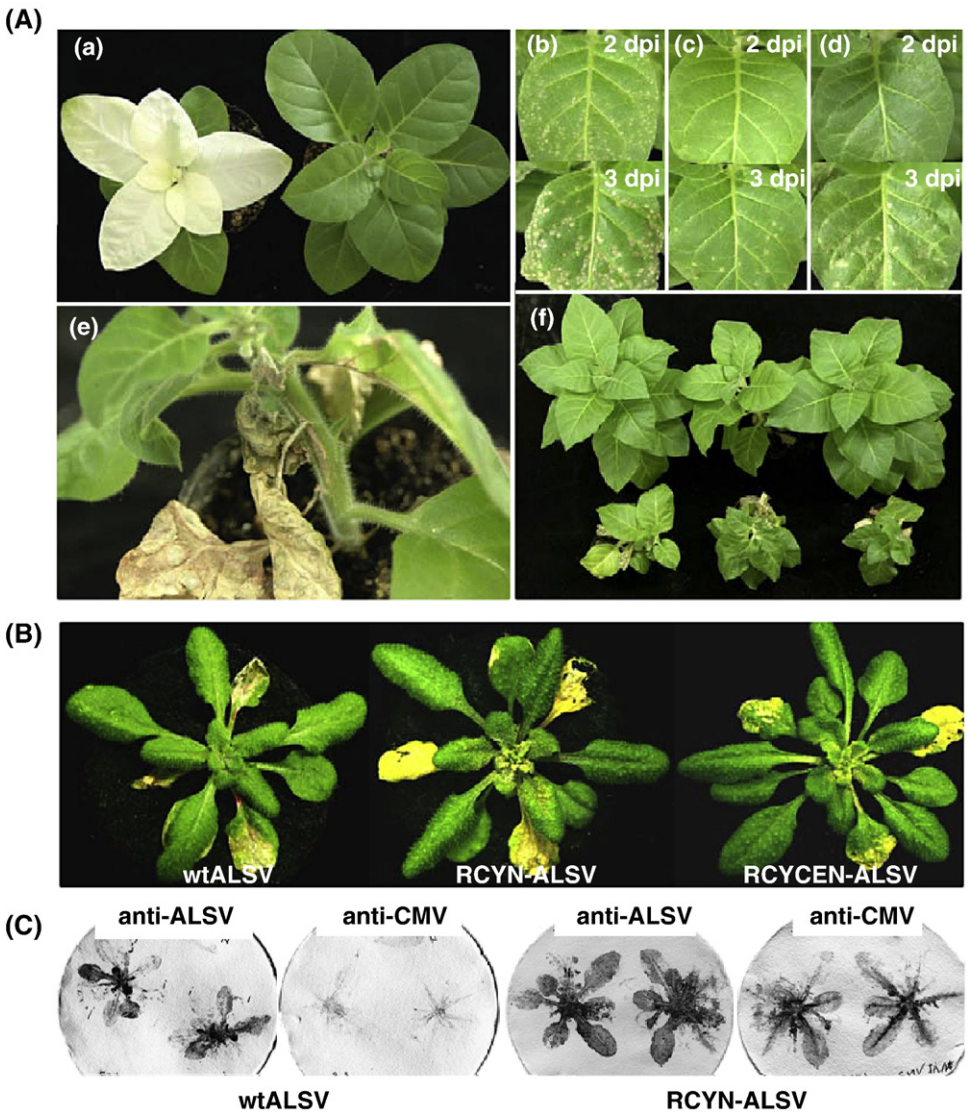

Fig. 4. Silencing of *N* gene in tobacco (A) and *RCY1* gene in *A. thaliana* (B and C). (A) (a) Tobacco plants infected with Su-ALSV (left) and TIRF-ALSV (right) 30 dpi. (b–d) Appearance of necrotic lesions on ToMV-inoculated leaves of plants pre-infected with wtALSV (b), TIRN-ALSV (c), and TIRF-ALSV (d). (e) Necrosis on the stem and petiole of upper leaves of tobacco plants pre-infected with TIRF-ALSV 24 days after ToMV inoculation. (f) Severe stunting of tobacco plants pre-infected with TIRF-ALSV (lower three plants) compared with normal development of plants pre-infected wtALSV (upper three plants) after 24 days after ToMV inoculation. (B) Symptoms of CMV-Y-inoculated *A. thaliana* (Col-RCY1) plants pre-infected with wtALSV (left), RCYN-ALSV (center), and RCYCEN-ALSV (right). (C) Tissue blot analysis of CMV-Y-inoculated Col-RCY1 plants pre-infected with wtALSV and RCYN-ALSV. Whole CMV-Y-inoculated plants pre-infected with wtALSV or RCYN-ALSV were placed and crushed between two layers of filter paper, and one paper was probed with an antiserum against ALSV (anti-ALSV) and the other with an antiserum against CMV (anti-CMV).

ALSV vectors also have high stability and the VIGS persisted throughout plant growth in infected plants, though the stability of VIGS depends on the kind and the length of inserted genes. Thirdly, an ALSV vector can also be used to successfully silence a meristem gene (PCNA) in *Nicotiana* species, similar to a TRV vector that is able to penetrate growing points (Ratcliff et al., 2001). It is reported that PVX and TMV vectors are excluded from meristematic tissues and therefore may not be effective in assessing the function of genes involved in shoot, leaf, flower, and fruit development (Burch-Smith et al., 2004). Fourthly, ALSV vectors can facilitate efficient VIGS among a broad range of plants including model plants, *A. thaliana* and *Nicotiana* species including tobacco, and economically important crops such as tomato, cucurbits, and legumes. Most plant virus vectors for VIGS are

Table 2
Number of local lesions induced by ToMV in leaves of tobacco (cv. Xanthi nc) pre-inoculated with wtALSV, TIRN-ALSV, and TIRF-ALSV

Virus vectors	Number of inoculated leaves	Number of local lesions[a] ± S.D.
wt-ALSV	18	14.97 ± 4.88
TIRN-ALSV	15	5.97 ± 3.67
TIRF-ALSV	15	7.41 ± 3.86

[a] Average number of local lesions ± standard deviation per 1 cm^2 of inoculated leaves.

Table 3
Systemic infection of CMV-Y in *A. thaliana* Col-RCY1 pre-inoculated with ALSV vectors containing *RCY1* gene

Virus vectors	Number of plants systemically infected with CMV-Y/inoculated plants
wtALSV	2[a]/13
RCYCEN-ALSV	20/20
RCYN-ALSV	9/10

[a] Two plants without showing any symptoms were found to be systemically infected with CMV-Y by immunoblot analysis, though the signals were so weak compared with those of plants pre-inoculated with RCYN-ALSV and RCYCEN-ALSV.

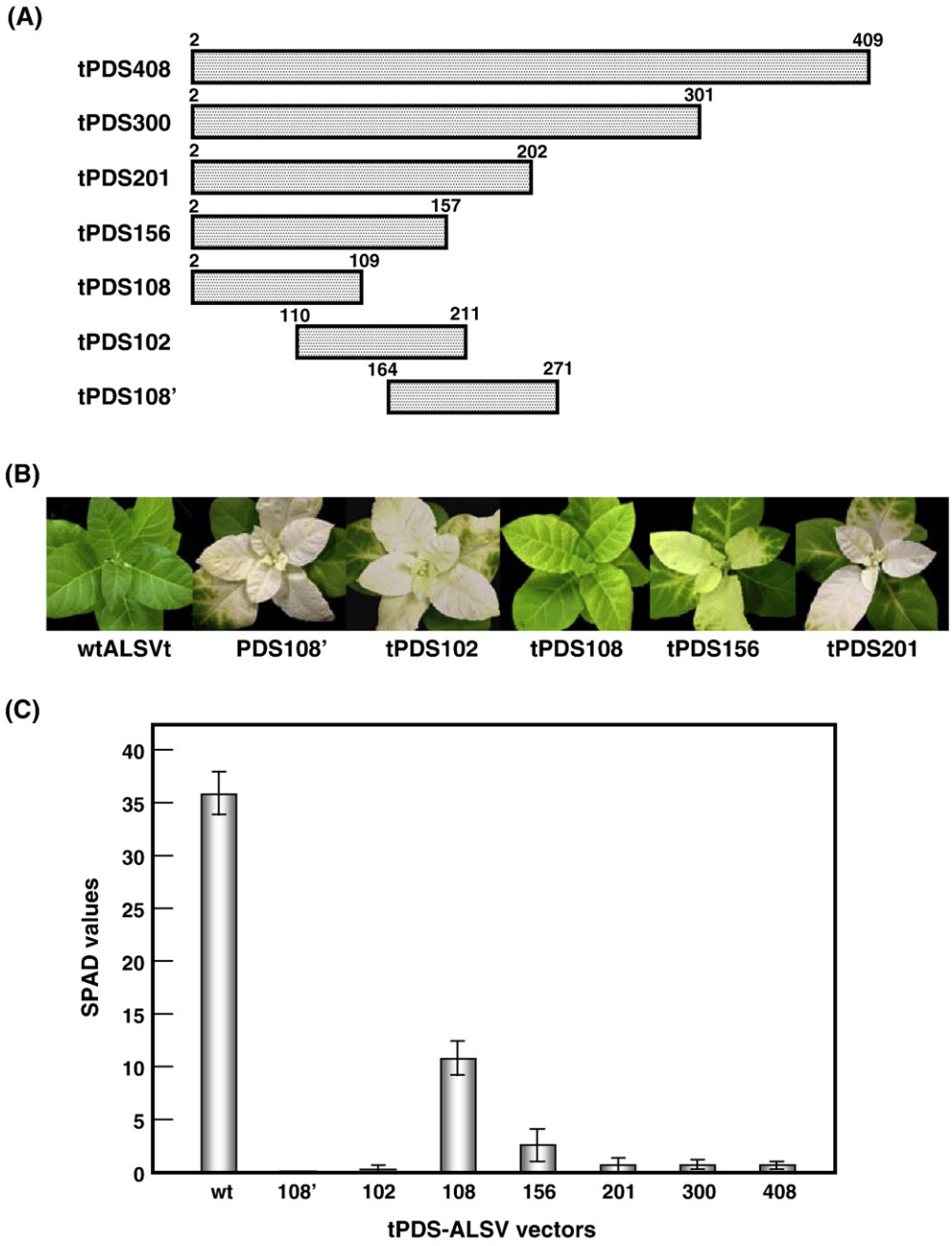

Fig. 5. (A) Schematic representation of the positions and length of *PDS* genes inserted into ALSV vectors. The number above the shaded box is a nucleotide number of the tobacco *PDS* gene (Genebank accession no. AJ571699). (B) Photo-bleached phenotypes of PDS inhibition on tobacco cv Xanthi nc infected with tPDS-ALSV vectors containing different lengths of *PDS* genes shown in (A). (C) Relative chlorophyll contents (SPAD values) in non-silenced leaves infected with wtALSV (wt) and in silenced leaves infected with tPDS-ALSV vectors containing different lengths of *PDS* genes shown in (A).

functional only in some plant species, especially in *N. benthamiana*. For example, tobacco, one of most widely used species in plant biology, has proven recalcitrant to the use of most VIGS vectors besides a satellite virus-induced silencing system (SVISS) by the satellite tobacco mosaic virus (Burch-Smith et al., 2004; Godge et al., 2008; Gossele et al., 2002). As described in this and a previous paper (Yaegashi et al., 2007), ALSV vectors effectively induced VIGS of several genes (*GFP*, *SU*, *PDS*, and *PCNA*) in tobacco and other *Nicotiana* species. In addition, we could successfully silence the *N* gene in tobacco cv. Xanthi nc, and ToMV moved into upper uninoculated leaves in silenced tobacco plants. On the tobacco *N* gene, it was shown that *Rar1*, *EDS1* and *NPR1*/*NIM1* like genes are required for N-mediated resistance in transgenic *N. benthamiana* containing the tobacco *N* gene using TRV-vectors (Liu et al., 2002b). We believe that ALSV vectors can be used for functional analysis of several genes associated with the *N*-mediated signaling pathway in original tobacco plants.

It is worth noting that tobacco plants infected with PDS108-ALSV developed yellow green leaves that are clearly distinguished from white leaves induced by PDS102-ALSV and PDS108′-ALSV (Fig. 5B). This indicates that a nucleotide sequence and/or a position on a target gene inserted into an ALSV-RNA2 vector affects the phenotype induced by VIGS, even if the length of the inserted sequence was equivalent. This is inconsistent with the results showing that in BSMV vectors caring fragments of *PDS* ranging from 128 to 584 nucleotides, the insert length has been reported to influence stability but not efficiency of VIGS (Bruun-Rasmussen et al., 2007). When ALSV vectors were used for VIGS of a specific gene, the length and position of a target sequence should be carefully selected.

One of the disadvantages of an ALSV vector comes from its gene expression strategy of virus genome. As the proteins encoded by ALSV genome were expressed by polyprotein synthesis followed by proteolytic processing, it is necessary to ligate target sequences in frame to the cloning sites of the ALSV vector. This makes it difficult to apply an ALSV vector for high throughput functional genomics as reported by other vectors (Burch-Smith et al., 2004; Lu et al., 2003a,b). There have also been reports on effective and convenient methods for inoculation of the VIGS vectors into plants (Godge et al., 2008). These are based on methods using *Agrobatererium tumefaciens*, e.g., agroinfiltration and "Agrodrench" (Fu et al., 2005; Ryu et al., 2004). At present, it is necessary to prepare ALSV-infected leaves as inocula for inducing VIGS in plants, because direct inoculation of infectious ALSV-cDNA clones was less efficient. Studies on convenient methods for delivery of ALSV vectors into plants are under way.

Nevertheless, we think that ALSV vectors are a valuable tool to determine the functions of interested genes in plants because of their effective VIGS inducers among a broad range of plant species. In particular, ALSV vectors are useful for functional genomics of cucurbits and legumes in which systems to generate transgenic plants are not fully established.

Materials and methods

Construction of ALSV vectors

The sequences of tobacco *SU*, *PDS*, and *PCNA* genes were amplified from mRNA samples from tobacco leaves using primer pairs; SU31 (+) (5′-gagggattagaatcccagtt-3′ corresponding to nt positions 31–50) and SU351(−) (5′-ctggatctgaattgaacgga-3′ complementary to nt positions 332–351) for *SU* gene (Genebank accession no. AJ571699), tPDS2(+) (5′-gcactcaactttataaaccc-3′ corresponding to nt positions 2–21) and tPDS409(−) (5′-cttcagttttctgtcaaacc-3′ complementary to nt positions 390–409) for *PDS* gene (AJ616724), and PCNA61(+) (5′-cctaaccctaatttccccag-3′ corresponding to nt positions 61–80) and PCNA480(−) (5′-tcactgtcaatgtccatccag-3′ complementary to nt positions 461–480) (AF305075), respectively. The amplified DNAs were cloned into pGEM^{R}-T Easy vectors, and all cloned sequences were confirmed by automated dye-terminator sequencing using an ABI 310 sequencer. To construct a plasmid for tSU-ALSV, a DNA fragment was amplified by using a cloned SU-DNA as a template and a primer pair tSU33Xho(+) and tSU350Bam(−) (Table 4). The DNA product was double-digested with Xho I and Bam HI and ligated to pEALSR2L5R5GFP restricted with the same enzymes (Fig. 1) (Yaegashi et al., 2007).

To construct ALSV vectors containing different sizes of *PDS* gene, DNA fragments were amplified using primer pairs, tPDS2Xho(+) and tPDS109Bam(−), tPDS157Bam(−), tPDS202Bam(−), tPDS301Bam (−), or tPDS409Bam(−) (Table 4). The DNA products were inserted to a RNA2 vector as described above, and the resulting viruses were designated tPDS108-ALSV, tPDS156-ALSV, tPDS201-ALSV, tPDS300-ALSV, and tPDS408-ALSV, respectively (Fig. 5). PDS102-ALSV and PDS108′-ALSV were also constructed using primer pairs, tPDS110Xho (+) and tPDS 211Bam(−) and tPDS164Xho(+) and tPDS 271Bam (−), respectively.

An ALSV vector carrying tobacco *PCNA* gene (PCNA-ALSV) was constructed using a primer pair tPCNA113Xho(+) and tPCNA403Bam (−) as described above.

The sequences of cucumber *SU* and *PDS* genes were amplified from mRNA samples from cucumber leaves using primer pairs, cuSU1(+) (5′-ACAGCATTGGAAGAGAATTGG-3′ corresponding to nt positions 1–21) and cuSU507(−) (5′-CCCACGCCCAGTATCTTTAA-3′ complementary to nt positions 488–507) for a *SU* gene (DQ641092), and cuPDS31(+) (TTTGGGGCTTATCCCAATGT corresponding to nt positions 31–50) and cuPDS900(−) (TTCCAACATGGACTGGTTTG complementary to nt positions 881–900) for a PDS gene (EF159942), respectively. The amplified DNAs were cloned into pGEM^{R}-T Easy vectors for sequencing. To construct plasmids for cuSU-ALSV and cuPDS-ALSV, DNA fragments were amplified using cloned DNAs as templates and primer pairs cuSU1Xho(+) and cuPDS330Bam(−) (Table 4) for *SU* gene and cuPDS31Xho(+) and cuSU300Bam(−) (Table 4) for PDS gene, respectively. The DNA product was double-digested with Xho I and Bam HI and ligated to pEALSR2L5R5GFP restricted with the same enzymes.

Table 4
List of primers used to construct ALSV vectors for VIGS

Name	Sequences (5′–3′)
tSU33Xho(+)	ccctcgaggggattaggggaatccca
tSU350Bam(−)	cgggatcctggatctgaattgaacgg
tPDS2Xho(+)	ccctcgaggcactcaactttataaaac
tPDS110Xho(+)	agctcgagaatcctcctgagagactt
tPDS164Xho(+)	tacatctcgagggccaagtcagactaaac
tPDS109Bam(−)	cgggatccaccatctaaaaaggccat
tPDS157Bam(−)	cgggatcctgactcaatatgttcaac
tPDS202Bam(−)	cgggatccctcaatctttttttattcg
tPDS211Bam(−)	gaggatccctcattgagctcaatctt
tPDS271Bam(−)	tacatggatccaaaagcatctccttttaat
tPDS301Bam(−)	cgggatcccttgaagatatccactgg
tPDS409Bam(−)	cgggatcccttcagttttctgtcaaa
tPCNA113Xho(+)	ccctcgagttggaattacggcttgtt
tPCNA403Bam(−)	ccggatccggtgtcactgccatc
cuSU1Xho(+)	tacatctcgagacagcattggaagagaat
cuSU300Bam(−)	tacatggatccataacaaacatcactcat
cuPDS31Xho(+)	tacatctcgagtttggggcttatcccaat
cuPDS330Bam(−)	tacatggatcctctcatccactcttgcac
soyPDS494Xho(+)	tactactcgagtctccgcgtcctctaaaa
soyPDS793Bam(−)	tacatggatcctccaggcttatttggcat
NTIR52Xho(+)	ccgctcgagatggcatcttcttcttcttc
NTIR252Bam(−)	cgggatccttgagactcttctatagctt
NTIR528Sma(−)	tcccccggggtcacgattatcacaggagc
RCY1-1Xho(+)	aaactcgagatggctgaaggatttgtg
RCY1-300Bam(−)	tttggatccgcgagcaagtcgtctcac
RCY1-1300Xho(+)	aaactcgagtccaagatatataggcat
RCY1-1599Bam(−)	tttggatccatgtatgctgagtctgcg

+: sense primer, −; antisense primer.
Restriction sites are underlined.

The sequence of soybean *PDS* gene was amplified from soybean leaves using a primer pair soyPDS1(+) (5′-GAATTCCTTCTACG-TACTGC-3′ corresponding to nt positions 1–20) and soyPDS1020(−) (5′-ACCTCATCAGCTACCCGTTC-3′) designed from soybean PDS gene (AF305075). The amplified DNAs were cloned and sequenced as described above. To construct an ALSV vector containing soybean *PDS* (soyPDS-ALSV), a DNA fragment was then amplified by using cloned soyPDS-DNA as a template and a primer pair, soyPDS494Xho(+) and soyPDS793Bam(−) (Table 4). The DNA product was double-digested with Xho I and Bam HI and ligated to pEALSR2L5R5GFP restricted with the same enzymes.

The partial sequence of tobacco *N* gene was amplified from mRNA samples from *N. glutinosa* leaves using a primer pair, Ngene-TIR(+) (5′-ATGGCATCTTCTTCTTCTTC-3′ corresponding to nt positions 52–71) and Ngene-TIR(−) (5′-TTGTCACGATTATCACAGGA-3′ complementary to nt positions 411–530) designed from *N* gene (U15605). The amplified DNAs were cloned and sequenced as described above. To construct ALSV vectors containing N gene (TIRN-ALSV and TIRF-ALSV), DNA fragments were amplified using cloned DNAs as templates and primer pairs NTIR52Xho(+) and NTIR252Bam(−) (Table 4) for TIRN-ALSV, and NTIR52Xho(+) and NTIR528Sma(−) (Table 4) for TIRF-ALSV. The DNA products were ligated to an ALSV-RNA2 vector as described above.

ALSV vectors containing *RCY1* gene (RCYN-ALSV and RCYCEN-ALSV) were constructed as follows: RCY1-DNA fragments were amplified using RCY1 full-length cDNA clone #1 as a template and primer pairs RCY1-1Xho(+) and RCY1-300Bam(−) (Table 4) for RCYN-ALSV and RCY1-1300Xho(+) and RCY1-1599Bam(−) (Table 4) for RCYCEN-ALSV. The DNA products were ligated to an ALSV-RNA2 vector as described above.

The constructed vectors were purified from large-scale cultures of *Escherichia coli* JM109 using a QIAGEN plasmid Maxi kit (QIAGEN, Japan) and then mechanically inoculated to *C. quinoa* plants (Li et al., 2004). After two to three weeks, leaves with symptoms were homogenized in 3 volumes of extraction buffer (0.1 M Tris–HCl, pH7.8, 0.1 M NaCl, 5 mM $MgCl_2$) and reinoculated to *C. quinoa* plants. The infected leaves were used as inocula for induction of VIGS in plants.

Plant materials and growing conditions

The following plants were used for VIGS of endogenous genes by ALSV vectors: *A. thaliana* ecotype Columbia (Col), *N. tabacum* cv. Xanthi nc, *N. occidentalis*, *N. glutinosa*, *N. benthamiana*, *S. lycopersicon* cvs. Kouju and Oogata fukuju, *G. max* cv. Suzukari, *P. sativum* cv. Denkou, *V. angularis* cv. Benidainagon, *V. unguiculata* cv. Akadanesan-jakuoonaga, *C. sativus* cv.Tubasa, *C. melo* cv. Earis Knight, *C. pepo* cv Diner, *C. lanatus* cv. Zuisyo, *L. cylindrical* cv. Oonagahechima, and *L. siceraria* cv. Oonagayuugao.

N.tabacum, *N. glutinosa*, and *N. benthamiana* plants inoculated with ALSV vectors were grown in a growth chamber under conditions at 25 °C and daylength of 16 h. Inoculated *A. thaliana* and *N. occidentalis* plants were grown in a chamber under conditions at 22 °C and daylength of 8 h. Other plants were grown in a greenhouse at natural conditions at a minimum temperature of 20 °C with supplementary light (daylength of 16 h) in the winter season.

RNA extraction and semi-quantitative RT-PCR

Silenced and non-silenced leaves (0.1 g) were homogenized with 500 μl of 0.1 M Tris–HCl, 10 mM EDTA, pH8.0, 0.1 M LiCl, 1% SDS, 500 μl of a phenol/chloroform (1:1). After centrifugation, the supernatants were re-extracted with chloroform and then mixed with an equal volume of 4 M LiCl. The pellets were collected by centrifugation, washed with 70% ethanol, and dissolved in 50 μl of TE buffer (0.1 M Tris, 10 mM EDTA, pH8.0). The nucleic acid samples were then treated with RNase free DNase I (TAKARA), and the resulting RNAs were finally dissolved in distilled water at a concentration of 1 μg/μl.

First strand cDNA was synthesized using 2 μg of RNA, oligo(dT) primer, and Rever Tra Ace reverse transcriptase (TOYOBO). Semi-quantitative RT-PCR was conducted as described by Burton et al. (2000). PCR amplifications were performed for 15, 18, 21, 27, and 30 cycles. Tobacco ubiquitin gene (Genebank accession no. NTU66267) in *Nicotiana* species, *A. thaliana* actin gene (U39449) in *A. thaliana*, cucumber actin gene (AB010922) in cucurbit species, and pea actin gene (X67666) in legume species were used as internal controls.

Measurements of chlorophyll contents in PDS-silenced leaves

Relative chlorophyll contents in silenced leaves infected with ALSV vectors containing *PDS* genes were determined using SPAD-502 (MINOLTA Co. Ltd). Measurements were made at fifteen spots (5 spots per leaf) on three leaves per plant.

VIGS of R genes in tobacco and A. thaliana

The *N* gene tobacco (*N. tabacum* cv. Xanthi nc) and transgenic *A. thaliana* Col expressing a *RCY1* gene (Col-RCY1) (Takahashi et al., 2002) were also used for VIGS of R genes against ToMV and CMV-Y, respectively.

TIRN-ALSV, TIRF-ALSV, or wt ALSV was inoculated to the first to third true leaves of tobacco plants (four-leaf stage). After 30 days, upper leaves (the ninth to eleventh true leaves) of infected tobacco plants were inoculated with ToMV (100 μg ml^{-1}), and the plants were grown in a growth chamber for 1 month.

In the experiment of VIGS in Col-RCY1, inocula of ALSV vectors (RCYN-ALSV, RCYCEN-ALSV, and wtALSV) were prepared as follows: Infected *C. quinoa* leaves (10 g) were homogenized with 30 ml of an extraction buffer, clarified by bentonite, precipitated by polyethylene glycol, and finally dissolved in 1 ml of a extraction buffer (Li et al., 2000). ALSV vectors were inoculated to the first and second true leaf of Col-RCY1 (three-leaf stage). After 14 days, upper leaves (the fifth to seventh true leaf) of infected Col-RCY1 (eight-leaf stage) were inoculated with CMV-Y (100 μg ml^{-1}), and the plants were grown in a growth chamber in controlled conditions as described above.

Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay was conducted as described previously (Takahashi et al., 2007).

Tissue blot analysis

Direct tissue immunoblotting analysis was conducted as described previously (Yaegashi et al., 2007) using antisera against ALSV and CMV.

Acknowledgments

We thank Prof. N. Kawai, Iwate University for his helpful advice. This work was supported in part by Grant-in-Aids for KAKENHI (no. 19380026, 18380196, 20380025) and the 21st Century of Excellence Program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Benedito, V., Visser, P.B., Angenebt, G.C., Krens, F.A., 2004. The potential of virus-induced gene silencing for speeding up functional characterization of plant genes. Genet. Mol. Res. 3, 323–341.
Brigneti, G., Martin-Hernandez, A.M., Jin, H., Chen, J., Baulcombe, D.C., Baker, B., Jones, J.D.G., 2004. Virus-induced gene silencing in *Solanum* species. Plant J. 39, 264–272.
Bruun-Rasmussen, M., Madsen, C.T., Jessing, S., Albrechtsen, M., 2007. Stability of Barley stripe mosaic virus-induced gene silencing in barley. Mol. Plant-Microbe Interact. 20, 1323–1331.
Burch-Smith, T.M., Anderson, J.C., Martin, G.B., Dinesh-Kumar, S.P., 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J. 39, 734–746.
Burch-Smith, T.M., Schiff, M., Liu, Y., Dinesh-Kumar, S.P., 2006. Efficient virus-induced gene silencing in *Arabidopsis*. Plant Physiol. 142, 21–27.
Burton, R.A., Gibeaut, D.M., Bacic, A., Findlay, K., Roberts, K., Hamilton, A., Baulcombe, D.C., Fincher, G.B., 2000. Virus-induced silencing of a plant cellulose synthase gene. Plant Cell 12, 691–705.
Cai, X.-Z., Xu, Q.-F., Wang, C.-C., Zheng, Z., 2006. Development of a virus-induced gene-silencing system for functional analysis of the RPS2-dependent resistance signaling pathways in *Arabidopsis*. Plant Mol. Biol. 62, 223–232.
Chapman, S.N., Kavenagh, T.A., Baulcombe, D.C., 1992. Potato virus X as a vector for gene expression in plants. Plant J. 2, 549–557.
Constantin, G.D., Krath, B.N., MacFarlane, S.A., Nicolaisen, M., Johansen, I.E., Lund, O.S., 2004. Virus-induced gene silencing as a tool for functional genomics in a legume species. Plant J. 40, 622–631.
Ding, X.S., Schneider, W.L., Chaluvadi, S.R., Mian, M.A.R., Nelson, R.S., 2006. Characterization of a *Brome mosaic virus* strain and its use as a vector for gene silencing in monocotyledonous hosts. Mol. Plant-Microbe Interact. 11, 1229–1239.
Fofana, I.B.F., Sangare, A., Collier, R., Taylor, C., Fauquet, C.M., 2004. A geminivirus-induced gene silencing system for gene function validation in cassava. Plant Mol. Biol. 56, 613–624.
Fu, D.-Q., Zhu, B.-Z., Zhu, H.-L., Jiang, W.-B., Luo, Y.-B., 2005. Virus-induced gene silencing in tomato fruit. Plant J. 43, 299–308.
Godge, M.R., Purkayastha, A., Dasgupta, I., Kummar, P.P., 2008. Virus-induced gene silencing for functional analysis of selected genes. Plant Cell Rep. 27, 209–219.
Goodin, M.M., Zaitlin, D., Naidu, R.A., Lommel, S.A., 2008. *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. Mol. Plant-Microbe Interact. 21, 1015–1026.
Gossele, V., Fache, I., Meulewaeter, F., Cornelissen, M., Metzlaff, M., 2002. SVISS — a novel transient gene silencing system for gene function discovery and validation in tobacco plants. Plant J. 32, 859–866.
Holzberg, S., Brosio, P., Gross, C., Pogue, G.P., 2002. Barley stripe mosaic virus-induced gene silencing in a monocot plant. Plant J. 30, 315–327.
Kumagai, M.H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K., Grill, L.K., 1995. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proc. Natl. Acad. Sci. USA 92, 1670–1683.
Lacomme, C., Hrubikova, K., Hein, I., 2003. Enhancement of a virus-induced gene silencing through viral-based production of inverted-repeats. Plant J. 34, 543–553.

Le Gall, O., Sanfacon, H., Ikegami, M., Iwanami, T., Jones, T., Karasev, A., Lehto, K., Wellink, J., Wetzel, T., Yoshikawa, N., 2007. *Cheravirus* and *Sadwavirus*: two unassigned genera of plant positive-sense single-stranded RNA viruses formerly considered atypical members of the genus *Nepovirus* (family *Comoviridae*). Arch. Virol. 152, 1767–1774.

Li, C., Yoshikawa, N., Takahashi, T., Ito, T., Yoshida, K., Koganezawa, H., 2000. Nucleotide sequence and genome organization of Apple latent spherical virus: a new virus classified into the family Comoviridae. J. Gen. Virol. 81, 541–547.

Li, C., Sasaki, N., Isogai, M., Yoshikawa, N., 2004. Stable expression of foreign proteins in herbaceous and apple plants using Apple latent spherical virus RNA2 vectors. Arch. Virol. 149, 1541–1558.

Liu, Y., Schliff, M., Dinesh-Kumar, S.P., 2002a. Virus-induced gene silencing in tomato. Plant J. 31, 777–786.

Liu, Y., Schliff, M., Marathe, R., Dinesh-Kumar, S.P., 2002b. Tobacco *Rar1*, *EDS1* and *NPR1*/*NIM1* like genes are required for *N*-mediated resistance to tobacco mosaic virus. Plant J. 30, 415–429.

Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I., Baulcombe, D.C., 2003a. Virus-induced gene silencing in plants. Methods 30, 296–303.

Lu, R., Malcuit, I., Moffett, P., Ruiz, M.T., Peart, J., Wu, A.-J., Rathjen, J.P., Bendahmane, A., Day, L., Baulcombe, D.C., 2003b. High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. EMBO J. 22, 5690–5699.

Nagamatsu, A., Masuta, C., Senda, M., Matsuura, H., Kasai, A., Hong, J.S., Kitamura, K., Abe, J., Kanazawa, A., 2007. Functional analysis of soybean genes involved in flavonoid biosynthesis by virus-induced gene silencing. Plant Biotechnol. J. 5, 778–790.

Peele, C., Jordan, C.V., Muangsan, N., Turnage, M., Egelkrout, E., Eagle, P., Hanley-Bowdoin, L., Robertson, D., 2001. Silencing of a meristematic gene using geminivirus-derived vectors. Plant J. 27, 357–366.

Pflieger, S., Blanchet, S., Camborde, L., Drugeon, G., Rousseau, A., Noizet, M., Planchais, S., Jupin, I., 2008. Efficient virus-induced gene silencing in *Arabidopsis* using a 'one-step' TYMV-derived vector. Plant J. (doi:10.1111/j.1365-313x.2008.03620.x).

Ratcliff, F., Martin-Hernandez, A.M., Baulcombe, D.C., 2001. Tobacco rattle virus as a vector for analysis of gene function by silencing. Plant J. 25, 237–245.

Ruiz, M.T., Voinnet, O., Baulcombe, D.C., 1998. Initiation and maintenance of virus-induced gene silencing. Plant J. 10, 937–946.

Ryu, C.-M., Anand, A., Kang, L., Mysore, K.S., 2004. Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. Plant J. 40, 322–331.

Takahashi, H., Goto, N., Ehara, Y., 1994. Hypersensitive response in cucumber mosaic virus-inoculated *Arabidopsis thaliana*. Plant J. 6, 369–377.

Takahashi, H., Miller, J., Nozaki, Y., Sukamto, Takeda, M., Shah, J., Hase, S., Ikegami, M., Ehara, Y., Dinesh-Kumar, S.P., 2002. RCY1, an *Arabidopsis thaliana RPP8*/*HRT* family resistance gene, conferring resistance to cucumber mosaic virus requires salicylic acid, ethylene and a novel signal transduction mechanism. Plant J. 32, 655–667.

Takahashi, T., Sugawara, T., Yamatsuta, T., Isogai, M., Natsuaki, T., Yoshikawa, N., 2007. Analysis of the spatial distribution of identical and two distinct virus populations differently labeled with cyan and yellow fluorescent proteins in coinfected plants. Phytopathology 97, 1200–1206.

Tao, X., Zhou, X., 2004. A modified viral satellite DNA that suppresses gene expression in plants. Plant J. 38, 850–860.

Turnage, M.A., Muangsan, N., Peele, C.G., Robertson, D., 2002. Geminivirus-based vectors for gene silencing in *Arabidopsis*. Plant J. 30, 107–114.

Vance, V., Vaucheret, H., 2001. RNA silencing in plants-defense and counterdefense. Science 292, 2277–2280.

Voinnet, O., 2005. Induction and suppression of RNA silencing: insights from viral infections. Nature Rev. Genet. 6, 206–220.

Waterhouse, P.M., Helliwell, C.A., 2002. Exploring plant genomes by RNA-induced gene silencing. Nature Rev. Genet. 4, 29–38.

Waterhouse, P.M., Wang, M.-B., Lough, T., 2001. Gene silencing as an adaptive defense against viruses. Nature 411, 834–842.

Whitham, S., Dinesh-Kumer, S.P., Choi, D., Hehl, R., Corr, C., Baker, B., 1994. The product of the tobacco mosaic virus resistance gene *N*: similarity to Toll and the interleukin-1 receptor. Cell 78, 1101–1115.

Yaegashi, H., Yamatsuta, T., Takahashi, T., Li, C., Isogai, M., Kobori, T., Ohki, S., Yoshikawa, N., 2007. Characterization of virus-induced gene silencing in tobacco plants infected with apple latent spherical virus. Arch. Virol. 152, 1839–1849.

Yoshikawa, N., Okada, K., Asanuma, K., Watanabe, K., Igarashi, A., Li, C., Isogai, M., 2006. A movement protein and three capsid proteins are all necessary for the cell-to-cell movement of apple latent spherical cheravirus. Arch. Virol. 151, 837–848.

Zhang, C., Ghabrial, S.A., 2006. Development of Bean pod mottle virus-based vectors for stable protein expression and sequence-specific virus-induced gene silencing in soybean. Virology 344, 401–411.