White clover mosaic virus-induced gene silencing in pea

Yukari Ido · Kenji S. Nakahara · Ichiro Uyeda

Y. Ido · K. S. Nakahara (✉) · I. Uyeda

Pathogen-Plant Interactions Group, Plant Breeding Science, Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

e-mail: knakahar@res.agr.hokudai.ac.jp

tel: 011-706-2490

fax: 011-706-2483

Total text pages: 15

The numbers of tables and figures: 1 table and 3 figures

Abstract

Double-stranded RNAs formed in secondary structures and replicative intermediates of viral genomes are thought to strongly elicit RNA silencing. This phenomenon is known as virus-induced gene silencing (VIGS). VIGS is a powerful tool for modifying gene expression in host plants. We constructed a virus vector based on *White clover mosaic virus* (WClMV) and demonstrated VIGS of phytoene desaturase (PDS) in pea. Photobleaching of tissues, caused by VIGS of PDS, was observed in restricted areas of upper leaves and stems. We confirmed that the PDS mRNA and subgenomic RNAs of WClMV were reduced in the photobleached tissues.

Keywords:

Pea, RNA silencing, Potexvirus

Post-transcriptional or transcriptional gene silencing, RNA silencing, or RNA interference (RNAi) serves to control not only expression of endogenous genes but also virus infections in plants (Ratcliff et al. 1999). Because most plant viruses have RNA genomes, which potentially form dsRNA intramolecularly or as a replicative intermediate during their replication, plant viruses are thought to be an inducer and target of RNAi, which is triggered by double-stranded RNA (dsRNA). This process is called virus-induced gene silencing (VIGS). Taking advantage of VIGS, expression of some specific (endogenous) genes can be repressed by infection with a recombinant virus vector, in which an engineered viral genome is integrated with the partial nucleotide sequences of the target genes without disrupting viral pathogenicity. In fact, VIGS has been used to silence a wide variety of genes in plants, and is especially useful for silencing genes in which loss-of-function mutants are lethal (Becker and Lange 2010; Robertson 2004). A number of recombinant virus vectors to induce VIGS have been developed based on several viruses, including *Tobacco etch virus* (TEV), *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX), *Tobacco rattle virus* (TRV), and *Tomato golden mosaic virus* (Robertson 2004). The effect and durability of VIGS differs among virus-host plant combinations (Becker and Lange 2010; Robertson 2004), and thus virus vectors for VIGS have generally been developed empirically.

In legumes, a virus vector based on *Pea early-browning virus* has previously been used for VIGS (Constantin et al. 2004). Yoshikawa and colleagues recently developed an *Apple latent spherical virus* (ALSV)-based vector for efficient VIGS in a broad range of plants, including legumes (Igarashi et al. 2009). In this study, to develop a virus vector for VIGS in pea, we selected *White clover mosaic virus* (WClMV). WClMV belongs to the *Potexvirus* genus, in which PVX has been used for VIGS in some species (Robertson 2004), and is distributed worldwide (ICTVdB Management 2006). In Japan,

WClMV has been isolated from *Astragalus sinicus*, white clover, red clover, and pea (Iizuka and Iida 1965; Matsumoto et al. 1989; Tsuchizaki et al. 1981). In New Zealand, the WClMV strains WClMV-M and -O were isolated and their genome sequences were determined (Beck et al. 1990; Forster et al. 1988). WClMV has a single strand of 5.8 kbp plus a sense RNA genome with a cap structure and poly A tail at the 5′- and 3′-terminal, respectively. Five open reading frames (ORF), of which ORF1-5 encodes 147 kDa, 26 kDa, 13 kDa, 7 kDa, and 26 kDa proteins, have been found in the WClMV genome; the proteins encoded by ORF1 are RNA-dependent RNA polymerases, those encoded by ORF2-4 are triple gene block (TGB) proteins, and the ORF5 protein is a capsid protein (CP). Beck et al. (1990) established an infectious clone of WClMV-O, and a modified infectious clone expressing green fluorescent protein (GFP) in infected plants was later developed (Lough et al. 2000). However, VIGS induced by WClMV has not yet been reported. In the present study, we first constructed the infectious clone of WClMV-RC, which was previously isolated from red clover in Yubari, Hokkaido, Japan (Tsuchizaki et al. 1981), and whose full genome sequence has recently been determined (Nakabayashi et al. 2002). Then we examined WClMV-mediated VIGS of the PDS gene in pea.

The full-length cDNA clone of WClMV-RC after the 35S promoter and followed by the 20 nucleotides (nt) of the poly A tail was constructed and the following experiments with the clone were done as described in the supplemental materials and methods. The nucleotide sequence of the full-length cDNA clone was determined (DDBJ accession number: AB669182) and 11 substitutions were found in the 5,843 nt of the WClMV-RC genome compared to the sequence reported (Nakabayashi et al. 2002). Among them, five were non-synonymous in the deduced amino acid sequences of viral proteins. We tentatively designated the full-length cDNA clone pWClMV-RCi.

When pWClMV-RCi was biolistically inoculated into broad bean cv. Kawachi-issun, the upper leaves showed symptoms 1 week after inoculation, indicating the infectivity of the plasmid clone. Using the upper leaves as inoculum, four pea lines were inoculated and chlorosis, mosaic, and leaf deformation symptoms were observed in infected upper leaves 20 days after inoculation (dai) (Supplemental Fig. S1a). There was no significant difference in symptoms between WClMV-RCi-infected and WClMV-RC-infected leaves, suggesting that the virulence of the two are comparable in pea. Infections were confirmed by RT-PCR (Supplemental Fig. S1b).

A transgene was planned to be expressed by taking advantage of the subgenomic RNA of WClMV for translation of the CP protein, as previously described for WClMV-O (Lough et al. 2000). First, to construct the WClMV vector, the transcription initiation site of the CP subgenome was investigated. The initiation site was determined to be the guanine at nucleotide 5156 from the 5′-terminal of the viral genome (-8 nucleotides from the initiation site of CP translation, Fig. 1a), which was expected based on a comparison with other potexviruses (Skryabin et al. 1988). Then we inserted the multi-cloning site accompanied by a putative promoter sequence for transcription of the CP subgenomic RNA to translate the CP protein into pWClMV-RCi at the site just downstream of the subgenome initiation site (Fig. 1a). We did not know the length of the upstream sequence necessary for efficient promoter activity to transcribe the CP subgenomic RNA. Hence, we prepared three WClMV vectors possessing promoters 50, 75, or 100 nt upstream sequences from the subgenome initiation site, as described above (pWClMV-RCi-50, -75, and -100). The CP protein is required for systemic infection of most plant viruses, including PVX (Chapman et al. 1992), and thus presumably for WClMV in legumes. If the inserted putative promoters function properly to translate CP in infected plants, then WClMV would infect systemically. We prepared GFP-tagged

recombinant WClMVs with the putative promoters pWClMV-RCi-GFP50, -GFP75, and -GFP100 (Fig. 1a), and investigated their pathogenicity by observing GFP signals in inoculated broad bean and pea plants.

The infectious plasmids pWClMV-RCi-GFP-50, -GFP75, and -GFP100 were inoculated into three plants of broad bean cv. Kawachi-issun at the third leaf from the plant base. GFP fluorescence was detected in the seventh leaves in plants inoculated with pWClMV-RCi-GFP75 and -GFP100 earlier than that in plants inoculated with pWClMV-RCi-GFP50 (Table 1). Chlorotic and necrotic spots were observed in upper leaves and GFP signals were detected coincidently with these spots. RT-PCR, which amplified WClMV genomic cDNA including the transgene GFP, confirmed infection of recombinant WClMVs and retention of the GFP gene in the recombinant WClMV genomes (Fig. 1b). However, there were a few additional bands, which migrated faster than the expected band, in samples of plants inoculated with pWClMV-RCi-GFP75 and –GFP100, suggesting that the genetic instability of a transgene in the WClMV vector.

Using upper leaves of broad bean inoculated with the recombinant WClMVs as inocula, five plants of either pea PI 226564 or broad bean were inoculated in the third leaf from the base. All broad bean plants showed the GFP fluorescence in the fourth leaves at 5 dai and similar GFP fluorescence in the fifth and upper leaves in the following days. There was no difference in GFP fluorescence among broad bean plants inoculated with the progenies of WClMVs in which the lengths of the promoter sequence differed (data not shown). All pea plants showed GFP fluorescence in the third inoculated and fourth upper leaves from the base at 4 dai (Fig. 2a). Interestingly, GFP fluorescence was disperse in the entire fourth and fifth leaves at 4-6 dai but almost disappeared by 8 dai (Fig. 2a,b). GFP fluorescence was hardly detected in the sixth (Fig. 2a) and seventh leaves. GFP fluorescence was also observed in the tenth and upper

leaves at 16 dai onwards (Fig. 2a); however, the fluorescence was in a more limited area of these leaves than in fourth and fifth leaves at 4 dai. This spatiotemporally restricted disappearance of GFP fluorescence was not observed in infected broad bean. There was no significant difference in GFP fluorescence in terms of intensity and distribution among pea plants inoculated with the progenies of WClMVs possessing promoters of different lengths for transcription of the CP subgenomic RNA (Fig. 2c). Regarding promoter length, these results suggest that even the shortest sequence (50 nt) was sufficient to function as a promoter to transcribe the CP subgenomic RNA, although longer lengths (75 and 100 nt) were more efficient for WClMV infection when broad bean was inoculated with these infectious plasmid DNAs (Table 1). If once the inoculated broad bean was systemically infected with the progeny viruses derived from the plasmid DNAs, these progeny viruses showed similar pathogenicity to pea plants that were inoculated with the progeny viruses of systemic leaves of infected broad bean. Consistently, the PVX vector possessed 77 nt of the promoter sequence for transcription of the CP subgenomic RNA (Chapman et al. 1992). We used pWClMV-RCi-100 to examine its ability for VIGS of an endogenous gene.

We tried to repress PDS expression by VIGS using the WClMV vector expressing the RNA sequence of the PDS gene in pea. PDS is involved in carotenoid biogenesis and thus repression of PDS would reduce carotenoid content, leading to chlorophyll breakdown and photobleaching of tissues (Demmig-Adams and Adams 1996; Kumagai et al. 1995; Pogue et al. 2002; Ratcliff et al. 2001; Turnage et al. 2002). The 470 nt of the nucleotide sequence of the pea PDS gene, in which PDS was repressed by VIGS using the PEBV vector expressing RNA (Constantin et al. 2004), was inserted into pWClMV-RCi-100 to obtain pWClMV-RCi-PDSs and -PDSas. Both of these were biolistically inoculated into broad bean cv. Kawachi-issun and upper leaves expressing

symptoms were used as inocula for mechanical inoculation of pea PI 226564. We inoculated each of the viruses into five pea plants in the second leaves from the base and observed photobleaching caused by the PDS-VIGS in repeated experiments. Severe photobleaching was observed in the sixth or seventh leaves of all plants inoculated with either WClMV-RCi-PDSs or -PDSas (Fig. 3a). When severe photobleaching occurred in the sixth leaf, the seventh leaf showed restricted photobleaching around veins, and the eighth leaf only showed whitening of the vein. The ninth leaf did not show any photobleaching, and the tenth and higher upper leaves only had spots of photobleaching.

Next, we investigated the efficiency of repression of PDS by WClMV-mediated VIGS at the RNA level (Fig. 3b). The PDS mRNA was reduced in the photobleached tissues infected with either WClMV-RCi-PDSs or -PDSas, compared to tissues infected but not photobleached, and those infected with WClMV-RCi-GFP. Interestingly, only the subgenomic RNAs, not genomic RNAs, were reduced below detectable levels in the photobleached tissues, supporting the previous study implying that PVX has developed a method to evade and survive RNAi without encoding a strong RNAi suppressor (van Wezel and Hong 2004). These results suggest that the observed photobleaching was caused by VIGS of PDS, triggered by the recombinant WClMVs. We note that the severe photobleaching in pea inoculated with the WClMV expressing the PDS sequence and the disappearance of GFP fluorescence in pea inoculated with the WClMV expressing GFP seemed to occur coincidently, implying that the disappearance of GFP fluorescence was caused by VIGS against the recombinant WClMV RNA genome with the GFP sequence, although both the genomic and subgenomic RNAs were detected (Fig. 3b, lane 6th GFP100).

Finally, we inoculated VIGS of PDS into *Medicago littoralis* Harbinger, *M. truncatula* Jamelong A-17, and broad bean. Photobleaching was observed in very

limited areas of the infected plant leaves (Fig. 3c).

In this study, we successfully constructed the WClMV vector and demonstrated transgene (GFP) expression and VIGS for an endogenous gene (PDS) using the recombinant WClMV in legumes, in particular pea. However, regarding VIGS, compared to the photobleaching phenotype caused by VIGS of PDS in previous studies using different virus vectors in pea (Constantin et al. 2004; Igarashi et al. 2009), there might still be room to improve our WClMV-RCi vector for use of VIGS for endogenous genes. Photobleaching was observed in almost all of the upper parts of plants infected with the PEBV vector (Constantin et al. 2004) and the ALSV vector (Igarashi et al. 2009), whereas the WClMV-RCi-PDS-infected peas showed photobleaching in a relatively limited area: over the entire sixth or seventh leaf, and veins, or as spots on upper leaves when the plant was inoculated at the second leaf from base. Nevertheless, the WClMV vector has an advantage over the other two virus vectors in terms of the time required for establishment of VIGS in infected peas and the ease of construction of the virus vector for VIGS. It was reported to take 28 days after inoculation for photobleaching in pea infected with PEBV (Constantin et al. 2004), but it took just 8-10 days after inoculation with WClMV. As ALSV proteins were first expressed as a polyprotein and then processed by viral protease, target sequences must be ligated in frame to cloning sites of ALSV (Igarashi et al. 2009). In the WClMV vector, target sequences do not have to be ligated in frame and thus we might be able to obtain high throughput VIGS to screen endogenous genes, as performed previously (Lu et al. 2003) though the flexibility and dispensability of a transgene in the WClMV vector for viral pathogenicity might result in the instability of a transgene integrated in viral genome during virus multiplication in infected plants (Fig. 1b).

The virus vector expressing target sequences as an inverted repeat structure, which

was expected to form dsRNA, was reported to induce more efficient VIGS in infected plants than that expressing the sense or antisense target sequence (Lacomme et al. 2003). Recently, potexvirus vectors deficient in TGB1 in its anti-RNAi activity because of a point mutation (from leucine to proline residue) in which leucine at that position is well-conserved among potexviruses, including WClMV, has been shown to induce more efficient VIGS than that possessing the wild-type TGB1 (Hammond et al. 2010). Hence, to improve the WClMV-RCi vector constructed in this study for VIGS use, it could be worth examining a modified WClMV-RCi vector by inducing a leucine to proline mutation in its TGB1 to attenuate the anti-RNAi activity, or integrate an inverted repeat of the target into the viral genome.

Acknowledgments

We thank Prof. Shigetou Namba (University of Tokyo, Tokyo, Japan) for the WClMV-RC. This work was partly supported by JSPS/MEXT KAKENHI (20688002, 18108001) to K.S.N. and I.U.

References

Beck DL, Forster RLS, Bevan MW, Boxen KA, Lowe SC (1990) Infectious transcripts and nucleotide sequence of cloned cDNA of the potexvirus white clover mosaic virus. Virology 177:152-158

Becker A, Lange M (2010) VIGS--genomics goes functional. Trends Plant Sci 15:1-4

Chapman S, Kavanagh T, Baulcombe D (1992) Potato virus-X as a vector for gene-expression in plants. Plant J 2:549-557

1 Constantin GD, Krath BN, MacFarlane SA, Nicolaisen M, Johansen IE, Lund OS
2 (2004) Virus-induced gene silencing as a tool for functional genomics in a
3 legume species. Plant J 40:622-631
4 Demmig-Adams B, Adams WW (1996) The role of xanthophyll cycle carotenoids in the
5 protection of photosynthesis. Trends in Plant Science 1:21-26
6 Forster RLS, Bevan MW, Harbison SA, Gardner RC (1988) The complete nucleotide
7 sequence of the potexvirus white clover mosaic virus. Nucleic Acids Research
8 16:291-303
9 Hammond J, Lim HS, Vaira AM, Domier LL, Lee SC, Kim HG (2010) Efficiency of
10 VIGS and gene expression in a novel bipartite potexvirus vector delivery system
11 as a function of strength of TGB1 silencing suppression. Virology 402:149-163
12 ICTVdB Management (2006) 00.056.0.01.021. *White clover mosaic virus*. In: ICTVdB -
13 The Universal Virus Database, version 4 edited by Büchen-Osmond, C
14 http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/
15 Igarashi A, Yamagata K, Sugai T, Takahashi Y, Sugawara E, Tamura A, Yaegashi H,
16 Yamagishi N, Takahashi T, Isogai M, Takahashi H, Yoshikawa N (2009) *Apple*
17 *latent spherical virus* vectors for reliable and effective virus-induced gene
18 silencing among a broad range of plants including tobacco, tomato, *Arabidopsis*
19 *thaliana*, cucurbits, and legumes. Virology 386:407-416
20 Iizuka N, Iida W (1965) Studies on the white clover mosaic virus. Ann Phytopath Soc
21 Japan 30:46-53
22 Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK (1995)
23 Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proc
24 Natl Acad Sci USA 92:1679-1683
25 Lacomme C, Hrubikova K, Hein I (2003) Enhancement of virus-induced gene silencing

through viral-based production of inverted-repeats. Plant J 34:543-553

Lough TJ, Netzler NE, Emerson SJ, Sutherland P, Carr F, Beck DL, Lucas WJ, Forster RL (2000) Cell-to-cell movement of potexviruses: evidence for a ribonucleoprotein complex involving the coat protein and first triple gene block protein. Mol Plant Microbe Interact 13:962-974

Lu R, Malcuit I, Moffett P, Ruiz MT, Peart J, Wu AJ, Rathjen JP, Bendahmane A, Day L, Baulcombe DC (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. EMBO J 22:5690-5699

Matsumoto T, Ookawa T, Hashegawa S, Inoue M (1989) Occurrence of viral disease caused by white clover mosaic virus in pea (Abstract in Japanese). Ann Phytopath Soc Japan 55:90

Nakabayashi H, Yamaji Y, Kagiwada S, Ugaki M, Namba S (2002) The complete nucleotide sequence of a Japanese isolate of *White clover mosaic virus* strain RC. J Gen Plant Pathol 68:173-176

Pogue GP, Holzberg S, Brosio P, Gross C (2002) *Barley stripe mosaic virus*-induced gene silencing in a monocot plant. Plant J 30:315-327

Ratcliff F, Martin-Hernandez AM, Baulcombe DC (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. Plant J 25:237-245

Ratcliff FG, MacFarlane SA, Baulcombe DC (1999) Gene silencing without DNA: RNA-mediated cross-protection between viruses. Plant Cell 11:1207-1216

Robertson D (2004) VIGS vectors for gene silencing: many targets, many tools. Annu Rev Plant Biol 55:495-519

Skryabin KG, Morozov SY, Kraev AS, Rozanov MN, Chernov BK, Lukasheva LI, Atabekov JG (1988) Conserved and variable elements in RNA genomes of

potexviruses. FEBS Lett 240:33-40

Tsuchizaki T, Goto T, Fujisawa I, Yoshida K (1981) Virus disease occurring on legumes and vegetables in Hokkaido. Res Bull Hokkaido Natl Agric Exp Stn 131:71-93

Turnage MA, Muangsan N, Peele CG, Robertson D (2002) Geminivirus-based vectors for gene silencing in *Arabidopsis*. Plant J 30:107-114

van Wezel R, Hong Y (2004) Virus survival of RNA silencing without deploying protein-mediated suppression in *Nicotiana benthamiana*. FEBS Lett 562:65-70

Figure legend

Fig. 1 Schematic representation of the construction of the *White clover mosaic virus* (WClMV)-RCi vectors. **a** A multi-cloning site followed by 50, 75, and 100 nucleotides of the putative promoter sequences for transcription of CP translation, indicated by a bar with arrowheads at both ends, was inserted into the infectious clone pWClMV-RCi to construct the WClMV-RCi vector (pWClMV-RCi-50, -75, or -100). The GFP cDNA sequence was inserted into the *Acc*III site of the multi-cloning site to construct the WClMV vector expressing GFP in infected plants (pWClMV-RCi-GFP50, -GFP75, or -GFP100). **b** RT-PCR with primers f (5′-taataggcgtatatcttctagt-3′) and r (5′-aagcgagaggcaagacgtcat-3′) confirmed the infectivity of the WClMV vectors expressing GFP and the retention of the GFP genes in their progeny viruses. The expected sizes of PCR fragments with/without the GFP sequence are indicated by a closed or open arrowhead, respectively

Fig. 2 Observation of GFP fluorescence in pea line PI 226564 inoculated with *White clover mosaic virus* (WClMV)-RCi-GFP50, -GFP75 and -GFP100. **a** The GFP fluorescence in leaves of three plants inoculated with WClMV-RCi-GFP100. **b** Magnified panels of the regions within broken lines. **c** The The GFP fluorescence in pea leaves inoculated with WClMV-RCi-GFP50 and -GFP75. Bar = 3 mm

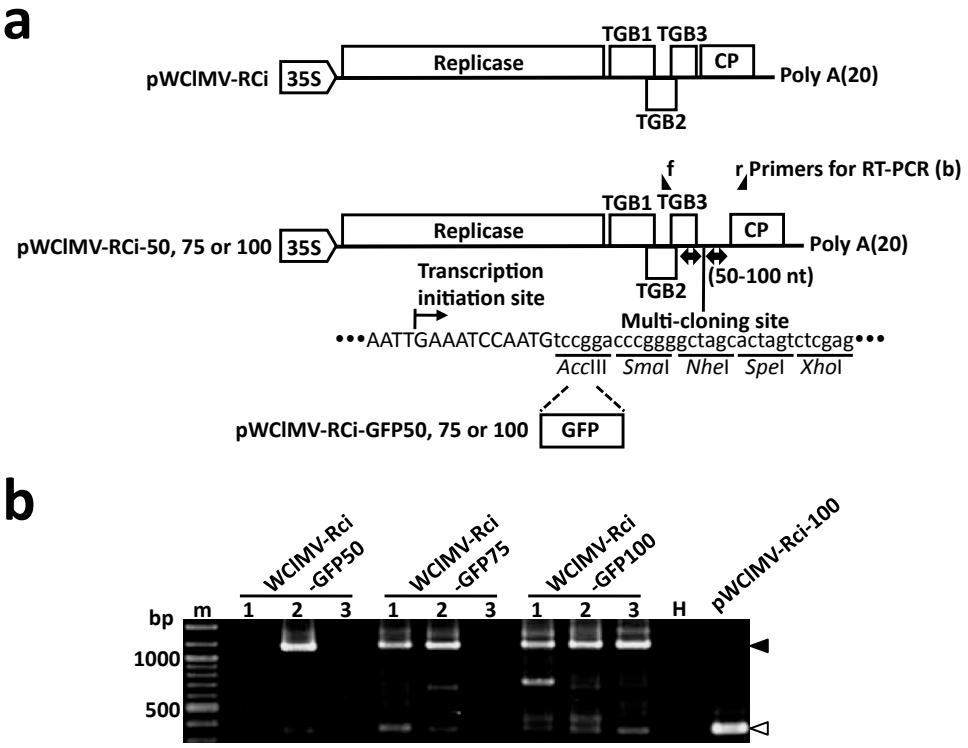
Fig. 3 Observation of the photobleaching phenotype caused by VIGS of the PDS gene in legumes. **a** The photobleaching expressed in upper leaves and stems of pea line PI 226564 inoculated with the *White clover mosaic virus* (WClMV) vectors expressing antisense of the PDS gene (WClMV-RCi-PDSas, PDSas) and sense RNA

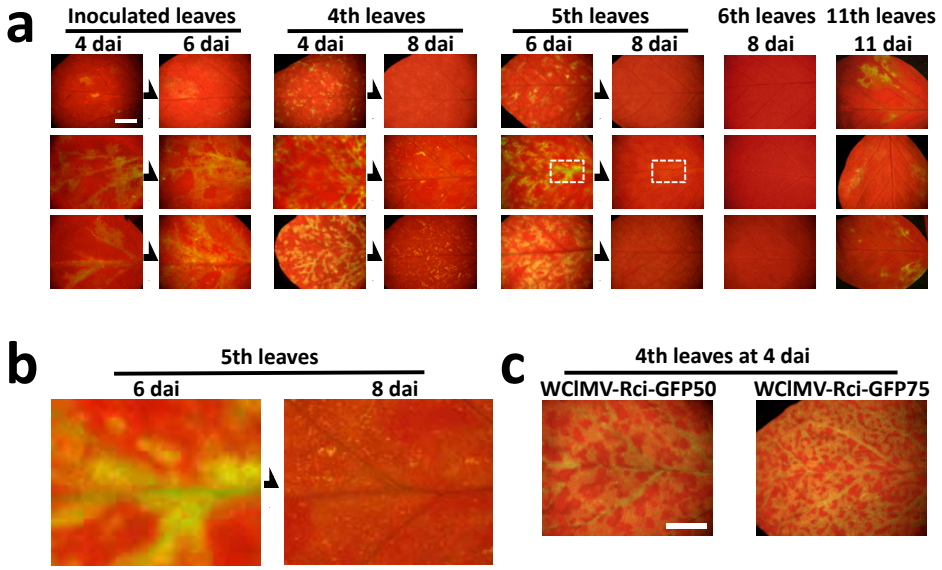
(WClMV-RCi-PDSs, PDSs) at 14 dpi. **b** Northern blotting confirmed VIGS of the PDS gene in pea plants. The DIG-labeled cRNA probes used were complementary to the PDS mRNA and the WClMV CP gene, which was expected to bind to both viral genome (g) and subgenomes (sg, indicated by a slanted line). The PDS mRNA and WClMV genome and subgenomes were detected in RNA extracts from the fourth and sixth leaves. Ribosomal RNAs (rRNA) are shown as loading controls. **c** *Medicago littoralis* Harbinger, *M. truncatula* JamelongA-17 and broad bean cv. Kawachi-issun were also inoculated with WClMV-RCi-PDSs100. The photobleaching expressed in their upper leaves at 30 dpi is shown. Open arrowheads indicate the photobleaching regions

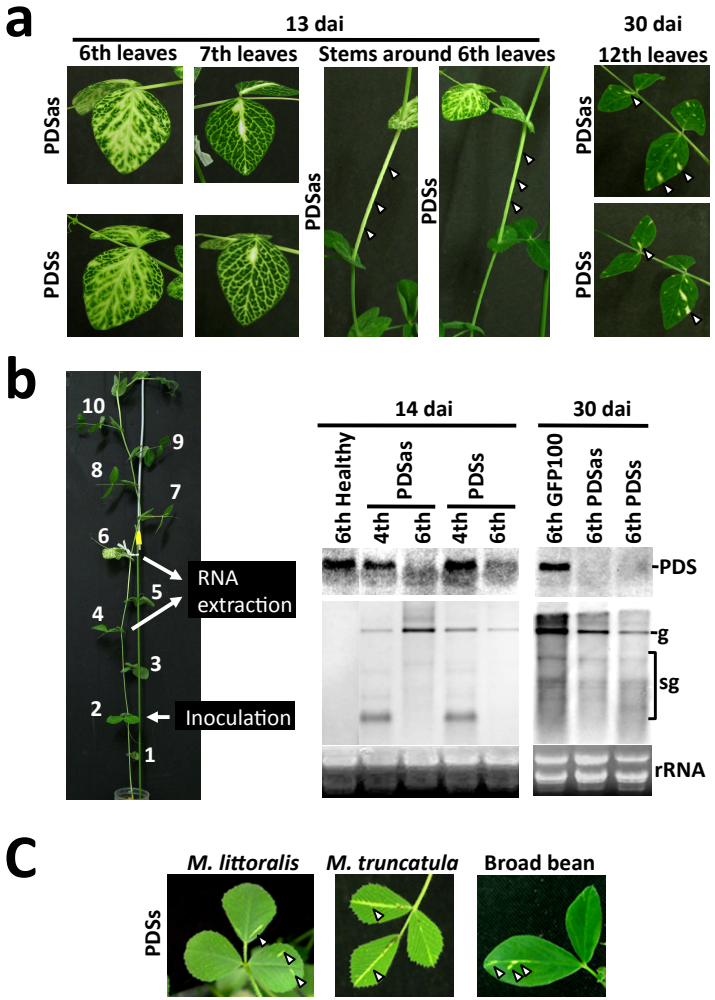
Table 1 Intensity of GFP fluorescence in 7th leaves of broad bean infected with the recombinant *White clover mosaic viruses* (WClMVs)

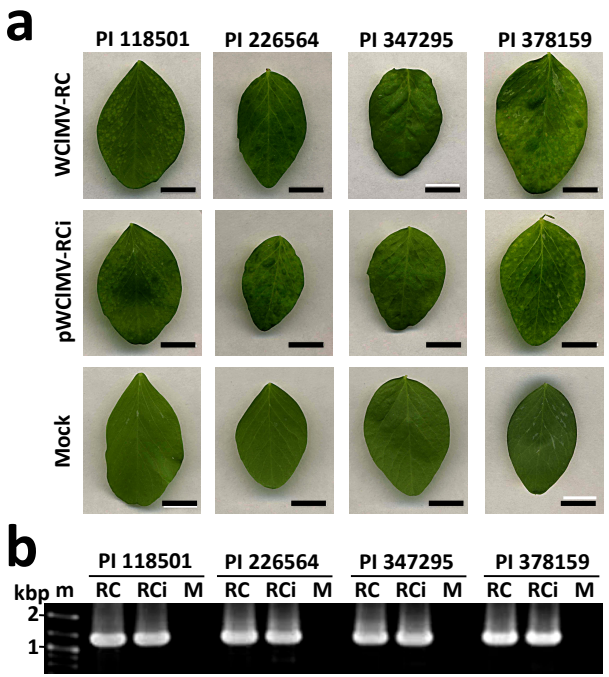
	7	11	14	17	20	24	dai
pWClMV- RCi- GFP50-1	-[a]	-	-	-	-	-	
-2	-	-	-	-	+	+	
-3	-	-	-	-	-	-	
pWClMV- RCi- GFP75-1	-	+	+	+	+	+	
-2	-	-	-	+	+	+	
-3	-	+	+	++	++	+++	
pWClMV- RCi- GFP100-1	-	-	-	++	++	++	
-2	-	+	++	+++	+++	+++	
-3	-	-	+	+	+	+	

[a] -, +, ++, +++ indicate no fluorescence, 1-2 spots of GFP fluorescences in a leaf, 4-5 spots of GFP fluorescences in a leaf, GFP fluorescences across a leaf, respectively.


a
TGB1 TGB3
pWClMV-RCi
35S
Replicase
CP
Poly A(20)
TGB2
f
r Primers for RT-PCR (b)
pWClMV-RCi-50, 75 or 100
Transcription initiation site
(50-100 nt)
Multi-cloning site
•••AATTGAAATCCAATGtccggacccggggctagcactagtctcgag•••
AccIII SmaI NheI SpeI XhoI
pWClMV-RCi-GFP50, 75 or 100
GFP
b
WClMV-Rci -GFP50
WClMV-Rci -GFP75
WClMV-Rci -GFP100
pWClMV-Rci-100
bp
m
1 2 3 1 2 3 1 2 3 H
1000
500


a
Inoculated leaves
4 dai
6 dai
4th leaves
4 dai
8 dai
5th leaves
6 dai
8 dai
6th leaves
8 dai
11th leaves
11 dai
b
5th leaves
6 dai
8 dai
c
4th leaves at 4 dai
WClMV-Rci-GFP50
WClMV-Rci-GFP75


a
13 dai
30 dai
6th leaves
7th leaves
Stems around 6th leaves
12th leaves
PDSas
PDSs
b
10
9
8
7
6
5
4
3
2
1
RNA extraction
Inoculation
14 dai
30 dai
6th Healthy
4th
6th
4th
6th
6th GFP100
6th PDSas
6th PDSs
PDSas
PDSs
-PDS
-g
sg
rRNA
C
M. littoralis
M. truncatula
Broad bean
PDSs



Supplemental Fig. S1 Comparison of virulence of the infectious clone pWClMV-RCi constructed in this study with its parental WClMV-RC in pea plants. a Reactions of pea lines to viral infections in upper leaves at 20 days after inoculation. Bar = 10 mm. b Infections of pea lines were confirmed by RT-PCR with the primers W-kp and CP-Pst. An expected 1.1 kbp band was detected in all samples from four lines inoculated with the virus derived from pWClMV-RCi (RCi) and its parental WClMV-RC (RC). The band was not detected in samples after mock inoculation (M).

Supplemental materials and methods

Plants, virus, and its inoculation into plants

WClMV-RC was previously reported by Namba and colleagues (Nakabayashi et al., 2002) and was kindly provided by Prof. Namba from the University of Tokyo, Japan. Broad bean (*Vicia faba* cv. Kawachi-issun and Wase) and pea (*Pisum sativum*, PI 118501, PI 226564, PI 347295, PI 378159) plants (Andrade et al., 2007) were used in this study. The infectious plasmid clone of WClMV-RC constructed in this study was biolistically inoculated into broad bean as described previously (Andrade et al., 2007). Infected leaves, which contain the recovered virus from the plasmid, were used to mechanically inoculate pea test plants. Test plants were maintained in a growth chamber at 24°C with 16 h of light and 8 h of dark. The green fluorescent protein (GFP) signal in infected plants was observed with an epifluorescence microscope (SZX-12, Olympus) and recorded with a CCD camera (VB-6010; Keyence, Osaka, Japan).

Cloning and sequencing the WClMV-RC genome

Total RNA was isolated from broad bean leaves infected with WClMV-RC using the TRIzol reagent (Invitrogen Carlsbad, CA, USA). The viral genome cDNAs were synthesized using a cloned AMV reverse transcriptase (Invitrogen) and amplified by polymerase chain reaction (PCR) using KOD plus DNA polymerase (Toyobo, Osaka, Japan). The WClMV genome was divided into four fragments, nucleotide numbers 1-761, 756-2033 (EH), 2028-4209 (HH), and 4204-5843 (HB), according to the nucleotide sequence of the WClMV genome (Nakabayashi et al., 2002). Each cDNA of the four fragments was amplified by PCR with primer pairs, fusionF (5′-catttggagagggaaaacaagacgaaacg-3′) and EcoBamR (5′-cgctacggatcctttgaattcatgggagtaggaac-3′), EcoF (5′-gcccatgaattcaaacaactggagtggct-3′) and HinBamR (5′-cgtcaggatccggaagcttggagtagtcgtcaaac-3′), HindF (5′-ggatcgccaagcttcctcaaggctacatcg-3′) and HindR (5′-gcctgaaagctttaattgtggggttggag-3′), or HindF2 (5′-cggtcaaagctttcgcccaattcaaaag-3′) and BamR (5′-gagccgtactggatccttttttttttttttttttttttctgaaattttattaaacagaaagccacac-3′). In addition, the *Cauliflower mosaic virus* 35S promoter fragment in an infectious clone of *Clover yellow*

vein virus (Takahashi et al., 1997) was obtained by PCR with BclSalF (5′-ctcagctgatcagtcgaccttctagagatccgtc-3′) and fusionR (5′-cgtttcgtcttgttttccctctccaaatgaaatgaac-3′). The 35S promoter fragment was fused with the first fragment (1–761) of the WClMV genome with PCR using the primer pair BclSalF and EcoBamR. The fused fragment was named BE. These four cDNA fragments, BE, EH, HH, and HB, which partially overlapped each other and covered the full length of the WClMV-RC genome, were inserted into the pGEM-Teasy plasmid vector (Promega, Madison, WI, USA) and cloned into *Escherichia coli* strain XL10-Gold (Stratagene, La Jolla, CA, USA). The cloned cDNA fragments were sequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Forster, CA, USA).

Construction of the infectious plasmid cDNA clone of WClMV

First, all restriction enzyme sites of the polylinker, except for the *Bam*HI site, on the plasmid vector pBluescript II Sk(-) (Stratagene) were removed to create pBSII/*Bam*HI. The plasmid clone containing BE was digested with restriction enzymes, *Bcl*I and *BamH*I, and the BE fragment was size-fractionated on a slab agarose gel. The purified BE fragment was inserted into pBSII/*Bam*HI at the *Bam*HI site. Then the EH fragment digested with *Eco*RI and *Bam*HI was inserted into pBSII/*Bam*HI containing BE. The HB fragment digested with *Hind*III and *Bam*HI was inserted into pBSII/*Bam*HI containing BE and EH. Finally, the HH fragment digested with *Hind*III was inserted into pBSII/*Bam*HI containing BE, EH, and HB. This plasmid contained full-length cDNA of the WClMV genome following the 35S promoter and was named pWClMV-RCi.

Determination of the transcriptional initiation site of a subgenomic RNA for capsid protein (CP) translation

The initiation site of subgenomic RNA for CP translation was determined using the Gene Racer^{TM} Kit for full-length, RNA ligase-mediated rapid amplification of the 5’- and 3’-ends (RLM-RACE, Invitrogen) according to the manufacturer’s manual.

Construction of the WClMV-RCi vector

The WClMV-RCi vector was constructed based on the infectious clone pWClMV-RCi. A partial cDNA, with the nucleotide number 4472-5166 and a multi-cloning site (MCS) composed of *Acc*III, *Sma*I, *Nhe*I, *Spe*I, and *Xho*I restriction enzyme sites at the 3′-terminal was made by PCR with primers W-Kp (5′-atagggcgaattgggtaccatcctcgcgt-3′) and W-ASNSX (5′-gcaccttgagctcgagactagtgctagccccgggtccggacattggatttcaattag-3′). Both the PCR fragment and pBluescritp II SK(-) plasmid were digested with *Kpn*I and *Xho*I and the digested PCR fragment was inserted into the pBluescript II SK(-) plasmid at the *Kpn*I-*Xho*I site and cloned (pBSII/KpnI-MCS). Next, partial WClMV cDNA fragments 50, 75, and 100 bp of the upstream region from the transcription initiation site of the subgenomic RNA for CP translation were amplified by PCR with primer pairs pro50bp (5′-aagtacctcgagcctcccaaatctca-3′) and CP-Pst (5′-atcccccgggctgcaggcatga-3′) for the cDNA fragment of nucleotide number 5555-6069, pro75bp (5′-cataagttctcgaggcgacaacccggct-3′) and CP-Pst for 5530-6069, and pro100bp (5′-cataagttctcgagggagcttctgttg-3′) and CP-Pst for 5505-6069. These three PCR fragments and pBSII/Kpn-MCS were digested with *Xho*I and *Pst*I, inserted into the digested pBSII/Kpn-MCS, and cloned (pBSII/Kpn-MCS-Promoter-Pst). The region from the KpnI site to the PstI site of pWClMV-RCi was replaced with that of the pBSII/Kpn-MCS-Promoter-Pst to obtain the WClMV-RCi vectors pWClMV-RCi-50, -75, and -100.

The WClMV-RCi vectors expressing GFP in infected plants were constructed as follows. GFP cDNA with attached *Acc*III sites at both terminals was amplified by PCR with primers GFP-head (5′-cctgac tccggaagtaaaggagaagaact-3′) and GFP-tail (5′-cgactccggattcatttgtatagttcatc-3′), using the *clover yellow vein virus* (ClYVV) vector pClYVV/C3-S65T (Sato et al., 2003) as a GFP template, and inserted into pWClMV-RCi plasmids at the *Acc*III sites.

The WClMV-RCi vectors expressing the PDS RNA sequence in pea were prepared. The 470 nt of the partial nucleotide sequence of the PDS gene with attached *Acc*III sites at both terminals was amplified by PCR with primers PDS-head (5′-gtgcaatccggacatcaaccctgatgaa-3′) and PDS-tail (5′-ctgcagtccggatcgtttagctcaattg-3′) using cDNA from the total RNA extract of pea PI 226564 as a template for the PDS sequence, and inserted into the pWClMV-RCi-100 plasmid at *Acc*III sites. The WClMV

vectors possessing the PDS sequence in the sense orientation and antisense orientation were designated pWClMV-RCi-PDSs and -PDSas, respectively.

Northern blot hybridization with a DIG labeled cRNA probe

Northern blotting was performed according to a previous study (Yambao et al., 2008). WClMV and PDS cDNA fragments following the T7 promoter were prepared by PCR with primer pairs PDSnorth-head (5′-ttgaggctcaagatggtgtttctg-3′) and PDSnorth-tailPDS (5′-aattctaatacgactcactatagggggattgccatccaaaaaggcc-3′) for the PDS probe, and CPnorth-head (5′-cttgcctctcgcttgttgggatctg-3′) and CPnorth-tail (5′-aattctaatacgactcactatagggagaaagccacacatattaagtgaa-3′) for the WClMV probe. The DIG-labeled cRNA probe was transcribed from the PCR fragments.

Andrade M, Sato M, Uyeda I (2007) Two resistance modes to *Clover yellow vein virus* in pea characterized by a green fluorescent protein-tagged virus. Phytopathology 97:544-550

Nakabayashi H, Yamaji Y, Kagiwada S, Ugaki M, Namba S (2002) The complete nucleotide sequence of a Japanese isolate of *White clover mosaic virus* strain RC. J Gen Plant Pathol 68:173-176

Sato M, Masuta C, Uyeda I (2003) Natural resistance to *Clover yellow vein virus* in beans controlled by a single recessive locus. Mol Plant Microbe Interact 16:994-1002

Takahashi Y, Takahashi T, Uyeda I (1997) A cDNA clone to clover yellow vein potyvirus genome is highly infectious. Virus Genes 14:235-243

Yambao ML, Yagihashi H, Sekiguchi H, Sekiguchi T, Sasaki T, Sato M, Atsumi G, Tacahashi Y, Nakahara KS, Uyeda I (2008) Point mutations in helper component protease of clover yellow vein virus are associated with the attenuation of RNA-silencing suppression activity and symptom expression in broad bean. Arch Virol 153:105-115