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1 ***White clover mosaic virus-induced gene silencing in pea***

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1 **Abstract**

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

10

11 **Keywords:**

12 Pea, RNA silencing, Potexvirus

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1 Post-transcriptional or transcriptional gene silencing, RNA silencing, or RNA  
2 interference (RNAi) serves to control not only expression of endogenous genes but also  
3 virus infections in plants (Ratcliff et al. 1999). Because most plant viruses have RNA  
4 genomes, which potentially form dsRNA intramolecularly or as a replicative  
5 intermediate during their replication, plant viruses are thought to be an inducer and  
6 target of RNAi, which is triggered by double-stranded RNA (dsRNA). This process is  
7 called virus-induced gene silencing (VIGS). Taking advantage of VIGS, expression of  
8 some specific (endogenous) genes can be repressed by infection with a recombinant  
9 virus vector, in which an engineered viral genome is integrated with the partial  
10 nucleotide sequences of the target genes without disrupting viral pathogenicity. In fact,  
11 VIGS has been used to silence a wide variety of genes in plants, and is especially useful  
12 for silencing genes in which loss-of-function mutants are lethal (Becker and Lange  
13 2010; Robertson 2004). A number of recombinant virus vectors to induce VIGS have  
14 been developed based on several viruses, including *Tobacco etch virus* (TEV), *Tobacco*  
15 *mosaic virus* (TMV), *Potato virus X* (PVX), *Tobacco rattle virus* (TRV), and *Tomato*  
16 *golden mosaic virus* (Robertson 2004). The effect and durability of VIGS differs among  
17 virus-host plant combinations (Becker and Lange 2010; Robertson 2004), and thus virus  
18 vectors for VIGS have generally been developed empirically.

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

1 WCIMV has been isolated from *Astragalus sinicus*, white clover, red clover, and pea  
2 (Iizuka and Iida 1965; Matsumoto et al. 1989; Tsuchizaki et al. 1981). In New Zealand,  
3 the WCIMV strains WCIMV-M and -O were isolated and their genome sequences were  
4 determined (Beck et al. 1990; Forster et al. 1988). WCIMV has a single strand of 5.8  
5 kbp plus a sense RNA genome with a cap structure and poly A tail at the 5'- and  
6 3'-terminal, respectively. Five open reading frames (ORF), of which ORF1-5 encodes  
7 147 kDa, 26 kDa, 13 kDa, 7 kDa, and 26 kDa proteins, have been found in the WCIMV  
8 genome; the proteins encoded by ORF1 are RNA-dependent RNA polymerases, those  
9 encoded by ORF2-4 are triple gene block (TGB) proteins, and the ORF5 protein is a  
10 capsid protein (CP). Beck et al. (1990) established an infectious clone of WCIMV-O,  
11 and a modified infectious clone expressing green fluorescent protein (GFP) in infected  
12 plants was later developed (Lough et al. 2000). However, VIGS induced by WCIMV  
13 has not yet been reported. In the present study, we first constructed the infectious clone  
14 of WCIMV-RC, which was previously isolated from red clover in Yubari, Hokkaido,  
15 Japan (Tsuchizaki et al. 1981), and whose full genome sequence has recently been  
16 determined (Nakabayashi et al. 2002). Then we examined WCIMV-mediated VIGS of  
17 the PDS gene in pea.

18 The full-length cDNA clone of WCIMV-RC after the 35S promoter and followed  
19 by the 20 nucleotides (nt) of the poly A tail was constructed and the following  
20 experiments with the clone were done as described in the supplemental materials and  
21 methods. The nucleotide sequence of the full-length cDNA clone was determined  
22 (DDBJ accession number: AB669182) and 11 substitutions were found in the 5,843 nt  
23 of the WCIMV-RC genome compared to the sequence reported (Nakabayashi et al.  
24 2002). Among them, five were non-synonymous in the deduced amino acid sequences  
25 of viral proteins. We tentatively designated the full-length cDNA clone pWCIMV-RCi.

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

9 A transgene was planned to be expressed by taking advantage of the subgenomic  
10 RNA of WCIMV for translation of the CP protein, as previously described for  
11 WCIMV-O (Lough et al. 2000). First, to construct the WCIMV vector, the transcription  
12 initiation site of the CP subgenome was investigated. The initiation site was determined  
13 to be the guanine at nucleotide 5156 from the 5'-terminal of the viral genome (-8  
14 nucleotides from the initiation site of CP translation, Fig. 1a), which was expected  
15 based on a comparison with other potexviruses (Skryabin et al. 1988). Then we inserted  
16 the multi-cloning site accompanied by a putative promoter sequence for transcription of  
17 the CP subgenomic RNA to translate the CP protein into pWCIMV-RCi at the site just  
18 downstream of the subgenome initiation site (Fig. 1a). We did not know the length of  
19 the upstream sequence necessary for efficient promoter activity to transcribe the CP  
20 subgenomic RNA. Hence, we prepared three WCIMV vectors possessing promoters 50,  
21 75, or 100 nt upstream sequences from the subgenome initiation site, as described above  
22 (pWCIMV-RCi-50, -75, and -100). The CP protein is required for systemic infection of  
23 most plant viruses, including PVX (Chapman et al. 1992), and thus presumably for  
24 WCIMV in legumes. If the inserted putative promoters function properly to translate CP  
25 in infected plants, then WCIMV would infect systemically. We prepared GFP-tagged

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

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

15 Using upper leaves of broad bean inoculated with the recombinant WCIMVs as  
16 inocula, five plants of either pea PI 226564 or broad bean were inoculated in the third  
17 leaf from the base. All broad bean plants showed the GFP fluorescence in the fourth  
18 leaves at 5 dai and similar GFP fluorescence in the fifth and upper leaves in the  
19 following days. There was no difference in GFP fluorescence among broad bean plants  
20 inoculated with the progenies of WCIMVs in which the lengths of the promoter  
21 sequence differed (data not shown). All pea plants showed GFP fluorescence in the  
22 third inoculated and fourth upper leaves from the base at 4 dai (Fig. 2a). Interestingly,  
23 GFP fluorescence was disperse in the entire fourth and fifth leaves at 4-6 dai but almost  
24 disappeared by 8 dai (Fig. 2a,b). GFP fluorescence was hardly detected in the sixth (Fig.  
25 2a) and seventh leaves. GFP fluorescence was also observed in the tenth and upper

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

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

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

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

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

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

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

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

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

11

## 12 **Acknowledgments**

13

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17

## 18 **References**

19

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

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

24 Chapman S, Kavanagh T, Baulcombe D (1992) Potato virus-X as a vector for  
25 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

- 1 through viral-based production of inverted-repeats. *Plant J* 34:543-553
- 2 Lough TJ, Netzler NE, Emerson SJ, Sutherland P, Carr F, Beck DL, Lucas WJ, Forster  
3 RL (2000) Cell-to-cell movement of potexviruses: evidence for a  
4 ribonucleoprotein complex involving the coat protein and first triple gene block  
5 protein. *Mol Plant Microbe Interact* 13:962-974
- 6 Lu R, Malcuit I, Moffett P, Ruiz MT, Peart J, Wu AJ, Rathjen JP, Bendahmane A, Day  
7 L, Baulcombe DC (2003) High throughput virus-induced gene silencing  
8 implicates heat shock protein 90 in plant disease resistance. *EMBO J*  
9 22:5690-5699
- 10 Matsumoto T, Ookawa T, Hashegawa S, Inoue M (1989) Occurrence of viral disease  
11 caused by white clover mosaic virus in pea (Abstract in Japanese). *Ann*  
12 *Phytopath Soc Japan* 55:90
- 13 Nakabayashi H, Yamaji Y, Kagiwada S, Ugaki M, Namba S (2002) The complete  
14 nucleotide sequence of a Japanese isolate of *White clover mosaic virus* strain RC.  
15 *J Gen Plant Pathol* 68:173-176
- 16 Pogue GP, Holzberg S, Brosio P, Gross C (2002) *Barley stripe mosaic virus*-induced  
17 gene silencing in a monocot plant. *Plant J* 30:315-327
- 18 Ratcliff F, Martin-Hernandez AM, Baulcombe DC (2001) Tobacco rattle virus as a  
19 vector for analysis of gene function by silencing. *Plant J* 25:237-245
- 20 Ratcliff FG, MacFarlane SA, Baulcombe DC (1999) Gene silencing without DNA:  
21 RNA-mediated cross-protection between viruses. *Plant Cell* 11:1207-1216
- 22 Robertson D (2004) VIGS vectors for gene silencing: many targets, many tools. *Annu*  
23 *Rev Plant Biol* 55:495-519
- 24 Skryabin KG, Morozov SY, Kraev AS, Rozanov MN, Chernov BK, Lukasheva LI,  
25 Atabekov JG (1988) Conserved and variable elements in RNA genomes of

- 1 potexviruses. FEBS Lett 240:33-40
- 2 Tsuchizaki T, Goto T, Fujisawa I, Yoshida K (1981) Virus disease occurring on  
3 legumes and vegetables in Hokkaido. Res Bull Hokkaido Natl Agric Exp Stn  
4 131:71-93
- 5 Turnage MA, Muangsan N, Peele CG, Robertson D (2002) Geminivirus-based vectors  
6 for gene silencing in *Arabidopsis*. Plant J 30:107-114
- 7 van Wezel R, Hong Y (2004) Virus survival of RNA silencing without deploying  
8 protein-mediated suppression in *Nicotiana benthamiana*. FEBS Lett 562:65-70
- 9
- 10
- 11

1 Figure legend

2

3 **Fig. 1** Schematic representation of the construction of the *White clover mosaic virus*  
4 (WCIMV)-RCi vectors. **a** A multi-cloning site followed by 50, 75, and 100 nucleotides  
5 of the putative promoter sequences for transcription of CP translation, indicated by a bar  
6 with arrowheads at both ends, was inserted into the infectious clone pWCIMV-RCi to  
7 construct the WCIMV-RCi vector (pWCIMV-RCi-50, -75, or -100). The GFP cDNA  
8 sequence was inserted into the *AccIII* site of the multi-cloning site to construct the  
9 WCIMV vector expressing GFP in infected plants (pWCIMV-RCi-GFP50, -GFP75, or  
10 -GFP100). **b** RT-PCR with primers *f* (5'-taataggcgtatatcttctagt-3') and *r*  
11 (5'-aagcgagaggcaagacgtcat-3') confirmed the infectivity of the WCIMV vectors  
12 expressing GFP and the retention of the GFP genes in their progeny viruses. The  
13 expected sizes of PCR fragments with/without the GFP sequence are indicated by a  
14 closed or open arrowhead, respectively

15

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

21

22 **Fig. 3** Observation of the photobleaching phenotype caused by VIGS of the PDS gene  
23 in legumes. **a** The photobleaching expressed in upper leaves and stems of pea line PI  
24 226564 inoculated with the *White clover mosaic virus* (WCIMV) vectors expressing  
25 antisense of the PDS gene (WCIMV-RCi-PDSas, PDSas) and sense RNA

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

11

12

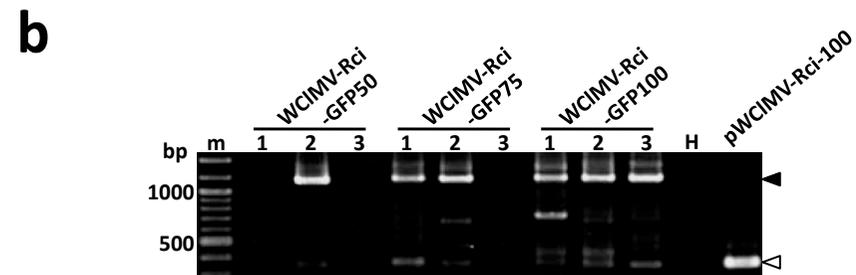
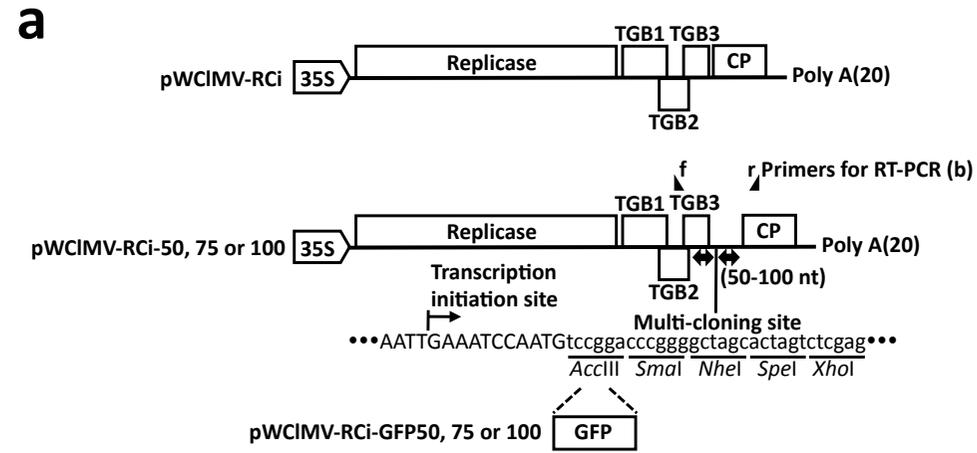
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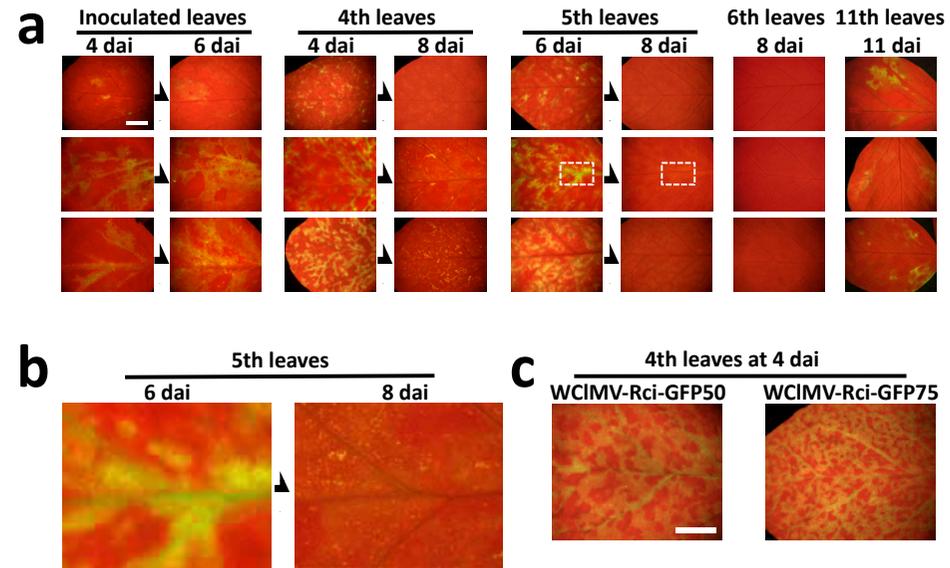
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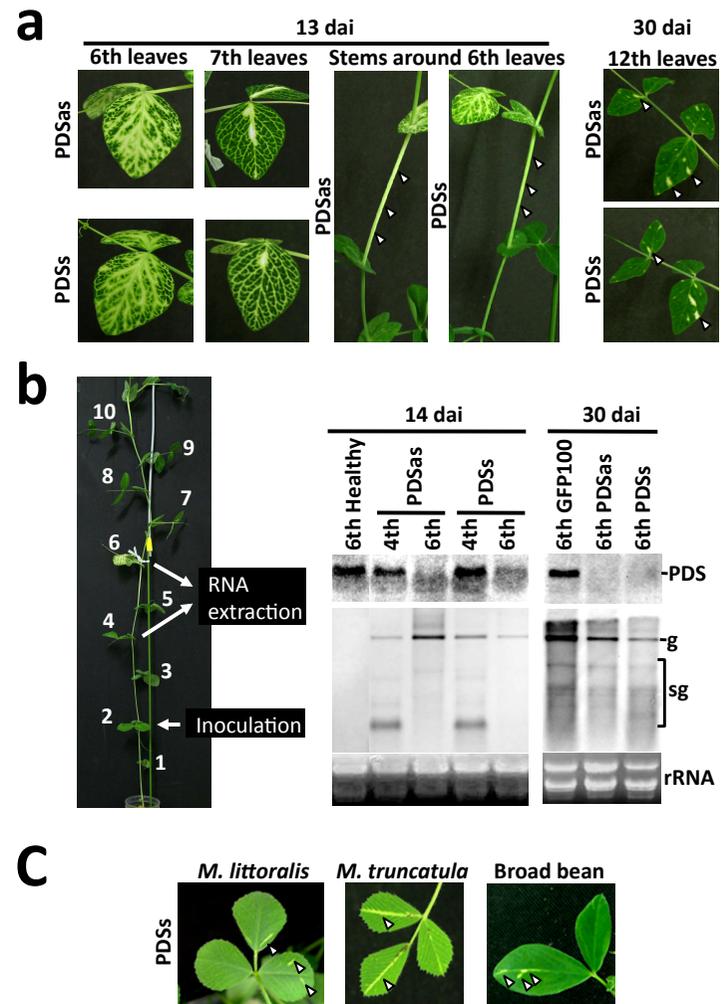
**Table 1** Intensity of GFP fluorescence in 7th leaves of broad bean infected with the recombinant *White clover mosaic viruses* (WCIMVs)

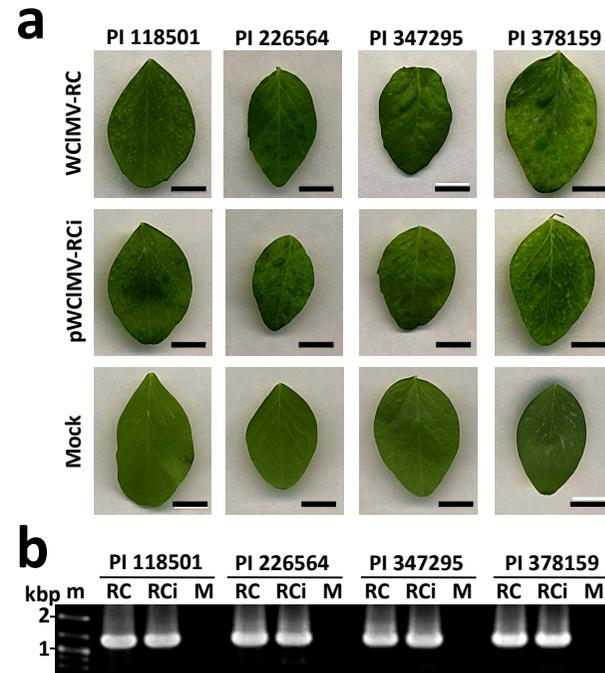
	7	11	14	17	20	24	dai
pWCIMV- RCi- GFP50-1	- <sup>a</sup>	-	-	-	-	-	
-2	-	-	-	-	+	+	
-3	-	-	-	-	-	-	
pWCIMV- RCi- GFP75-1	-	+	+	+	+	+	
-2	-	-	-	+	+	+	
-3	-	+	+	++	++	+++	
pWCIMV- RCi- GFP100-1	-	-	-	++	++	++	
-2	-	+	++	+++	+++	+++	
-3	-	-	+	+	+	+	

<sup>a</sup> -, +, ++, +++ 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.









**Supplemental Fig. S1** Comparison of virulence of the infectious clone pWCIMV-RCi constructed in this study with its parental WCIMV-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 pWCIMV-RCi (RCi) and its parental WCIMV-RC (RC). The band was not detected in samples after mock inoculation (M).

## Supplemental materials and methods

### Plants, virus, and its inoculation into plants

WCIMV-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 WCIMV-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 WCIMV-RC genome

Total RNA was isolated from broad bean leaves infected with WCIMV-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 WCIMV 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 WCIMV 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'-gccatgaattcaacaactggagtggct-3') and HinBamR (5'-cgtcaggatccggaagcttggagtagctgctcaaac-3'), HindF (5'-ggatcgccaagcttctcaaggctacatcg-3') and HindR (5'-gcctgaaagctttaattgtggggttgag-3'), or HindF2 (5'-cggtaaagctttcgccaattcaaaag-3') and BamR (5'-gagccgtactggatccttttttttttttttctgaaattttattaacagaaagccacac-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'-cgttctgtctgttttcctctccaaatgaaatgaac-3'). The 35S promoter fragment was fused with the first fragment (1–761) of the WCIMV 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 WCIMV-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 WCIMV

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 *Bam*HI, 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 WCIMV genome following the 35S promoter and was named pWCIMV-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<sup>TM</sup> 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 WCIMV-RCi vector

The WCIMV-RCi vector was constructed based on the infectious clone pWCIMV-RCi. A partial cDNA, with the nucleotide number 4472-5166 and a multi-cloning site (MCS) composed of *AccIII*, *SmaI*, *NheI*, *SpeI*, and *XhoI* restriction enzyme sites at the 3'-terminal was made by PCR with primers W-Kp (5'-atagggcgaattgggtaccatcctcgcgt-3') and W-ASNSX (5'-gcaccttgagctcgagactagtgctagcccccgggtccggacattggattcaattag-3'). Both the PCR fragment and pBluescript II SK(-) plasmid were digested with *KpnI* and *XhoI* and the digested PCR fragment was inserted into the pBluescript II SK(-) plasmid at the *KpnI-XhoI* site and cloned (pBSII/*KpnI*-MCS). Next, partial WCIMV 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'-aagtacctcgagcctcccaaattctca-3') and CP-Pst (5'-atccccgggctgcaggcatga-3') for the cDNA fragment of nucleotide number 5555-6069, pro75bp (5'-cataagttctcgaggcgacaaccggct-3') and CP-Pst for 5530-6069, and pro100bp (5'-cataagttctcgaggagcttctgttg-3') and CP-Pst for 5505-6069. These three PCR fragments and pBSII/*KpnI*-MCS were digested with *XhoI* and *PstI*, inserted into the digested pBSII/*KpnI*-MCS, and cloned (pBSII/*KpnI*-MCS-Promoter-Pst). The region from the *KpnI* site to the *PstI* site of pWCIMV-RCi was replaced with that of the pBSII/*KpnI*-MCS-Promoter-Pst to obtain the WCIMV-RCi vectors pWCIMV-RCi-50, -75, and -100.

The WCIMV-RCi vectors expressing GFP in infected plants were constructed as follows. GFP cDNA with attached *AccIII* sites at both terminals was amplified by PCR with primers GFP-head (5'-cctgac tccggaagtaaaggagaagaact-3') and GFP-tail (5'-cgactccggattcatttgatagttcatc-3'), using the *clover yellow vein virus* (CIYVV) vector pCIYVV/C3-S65T (Sato et al., 2003) as a GFP template, and inserted into pWCIMV-RCi plasmids at the *AccIII* sites.

The WCIMV-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 *AccIII* sites at both terminals was amplified by PCR with primers PDS-head (5'-gtgcaatccggacatcaaccctgatgaa-3') and PDS-tail (5'-ctgcagtcggatcgtttagctcaattg-3') using cDNA from the total RNA extract of pea PI 226564 as a template for the PDS sequence, and inserted into the pWCIMV-RCi-100 plasmid at *AccIII* sites. The WCIMV

vectors possessing the PDS sequence in the sense orientation and antisense orientation were designated pWCIMV-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). WCIMV and PDS cDNA fragments following the T7 promoter were prepared by PCR with primer pairs PDSnorth-head (5'-ttgaggctcaagatggtgttctg-3') and PDSnorth-tailPDS (5'-aattctaatacagactcactataggggggattgccatccaaaaaggcc-3') for the PDS probe, and CPnorth-head (5'-cttgctctcgttgggatctg-3') and CPnorth-tail (5'-aattctaatacagactcactatagggagaaagccacacatattaagttaa-3') for the WCIMV 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