Characterization of the recessive resistance gene *cyv1* of *Pisum sativum* against *Clover yellow vein virus*

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Abstract

Two recessive resistance genes against *Clover yellow vein virus* (ClYVV), *cyv1* and *cyv2*, have been previously reported. We recently screened resistant peas from a separate set of pea lines and classified them into two groups according to their distinct resistant modes. We later revealed that one group carries *cyv2*, encoding eukaryotic translation initiation factor 4E (eIF4E), in linkage group (LG) VI. We explored the possibility that the resistance gene, tentatively designated *non-cyv2*, that confers resistance on the other group, was actually *cyv1*. We found that PI 236493, which carries *cyv1*, showed restriction of ClYVV cell-to-cell movement similar to that in *non-cyv2* peas including PI 429853. PI 429853 was crossed with susceptible line PI 250438. Mapping of F2 progeny revealed that *non-cyv2* was 4 cM from the simple sequence repeat marker AB40, whose loci are close to *cyv1*, *mo*, and *sbm-2* mapped in LG II, which mediates resistance to other potyviruses. Moreover, PI 429853 crossed with PI 236493 produced F1 progeny resistant to ClYVV, raising the possibility that *non-cyv2* is allelic to *cyv1*. Because *mo* was previously mapped with *eIF(iso)4E* in LG II, we examined the possibility that *non-cyv2*, *cyv1*, and *mo* encoded eIF(iso)4E. However, there was no difference in the nucleotide sequence of the eIF(iso)4E-coding region between susceptible and resistant pea lines. The *eIF(iso)4E* gene was equivalently expressed in both PI 429853 and PI 250438 before and after ClYVV infection. Our results suggest that these resistance genes are unlikely to encode eIF(iso)4E on LG II.

Keywords: *Clover yellow vein virus*, *cyv1*, *sbm-2*, pea resistance, eIF(iso)4E

Introduction

Many plant genes for resistance to various plant pathogens, including viruses, have been identified in crops. When sorted by mode of inheritance, about 40% of the known resistance genes against viruses in crops are recessive. Recessive genes against potyviruses are more frequent than those against viruses of other families (Díaz-Pendón et al. 2004; German-Retana et al. 2008). Recessive resistance in crops inhibits various steps in the viral infection cycle, from virus replication at the single cell level to cell-to-cell movement of a virus (Truniger and Aranda 2009).

Several recessive genes resistant to potyvirus have been identified in crops: *pvr1*, *2*, and *6* in pepper against *Tobacco etch virus* (TEV), *Potato virus Y* (PVY), and *Chilli veinal mottle virus* (ChiVMV); *mo1*^{1} and *mo1*^{2} in lettuce against *Lettuce mosaic virus* (LMV); *sbm-1* and *sbm-2* in pea against *Pea seed-borne mosaic virus* (PSbMV); *wlv* in white lupin against *Bean yellow mosaic virus* (BYMV); *rym4/5/6* in barley against *Barley yellow mosaic virus* (BaYMV) and *Barley mild mosaic virus* (BaMMV); and *cyv1* and *cyv2* in pea against *Clover yellow vein virus* (ClYVV) (Bruun-Rasmusse et al. 2007; Gao et al. 2004a; Johansen et al. 2001; Kang et al. 2005a; Kanyuka et al. 2004; Nicaise et al. 2003; Ruffel et al. 2002; Stein et al. 2005). Most of these genes encode eukaryotic translation initiation factor 4E (eIF4E) or its isoform eIF(iso)4E, which alone or together control the reaction to potyviruses (Andrade et al. 2009; Hwang et al. 2009; Ruffel et al. 2006; Sato et al. 2005).

Resistance genes in pea to several potyviruses are closely linked and clustered in linkage groups (LG) II and VI (Provvidenti and Hampton 1991). LG II includes *bcm*, *cyv1*, *mo*, *pmv*, and *sbm-2*, which confer resistance to *Bean common mosaic virus*

(BCMV), ClYVV, BYMV, *Pea mosaic virus* (PMV), and PSbMV pathotype P2, respectively. LG VI includes genes conferring resistance to ClYVV (*cyv2*), PSbMV pathotype P1 (*sbm-1*), pathotype L1 (*sbm-3*), and pathotype P4 (*sbm-4*), and *White lupin mosaic virus* (*wlv*) (Provvidenti and Hampton 1991). The *sbm-1*, *cyv2*, and *wlv* resistance genes were recently shown to encode the same pea homolog of the eIF4E involved in cell-to-cell movement of PSbMV P1 (Andrade et al. 2009; Gao et al. 2004a). On the other hand, the resistance genes in LG II, including *cyv1* and *sbm-2*, remain to be identified. Gao et al. (2004b) found that the *eIF(iso)4E* gene mapped on the same LG II that contains the *sbm-2* gene.

We have also recently screened additional pea lines resistant to ClYVV and found two distinct modes of resistance to isolate no. 30 of ClYVV (Cl-no30). Screened pea lines were divided into two groups according to their resistant modes. In one group of pea lines biolistically inoculated with the infectious plasmid of ClYVV, the virus was restricted within a single cell, whereas in the other group it spread to neighboring cells (Andrade et al. 2007). Resistant pea lines in the latter group were later shown to carry *cyv2*, which encodes eIF4E that controls resistance against ClYVV. In the other group, pea lines including PI 347295 and PI 429853, whose resistance gene has not yet been reported, were tentatively designated as *non-cyv2* lines with resistance was not controlled by eIF4E (Andrade et al. 2009). Which gene in *non-cyv2* pea lines controls resistance against ClYVV and whether *non-cyv2* is an allele of *cyv1* remains to be investigated. In this study, we characterized the resistance mode of *cyv1* against ClYVV, compared it with that of *non-cyv2*, and examined the genetic relationship between *non-cyv2* and *cyv1*. Because *eIF(iso)4E* is close to *non-cyv2*, *cyv1*, and *mo* in LG II (Gao et al. 2004b), we examined the possibility that these resistance genes encode eIF(iso)4E by comparing nucleotide sequences of *eIF(iso)4E* genes and their expression in resistant and susceptible pea lines.

Materials and methods

Virus source and plant material

pClYVV/C3-S65T carrying green fluorescent protein (GFP) was named pCl-no30, and was used as the viral source (Sato et al. 2003). The Cl-no30 virus culture was recovered from pCl-no30. Fifteen pea lines (*Pisum sativum*) were provided by Dr. C. Coyne (Western Regional Plant Introduction Station, Washington State University). To characterize *cyv1*, we selected two ClYVV resistant pea lines—PI 236439 (Provvidenti 1987) and PI 429853 (Andrade et al. 2007) carrying *cyv1* and *non-cyv2*, respectively (Fig. 1)— from these 15 lines for further analysis of their reaction to Cl-no30. We obtained F1 and F2 progeny from crosses between PI 429853 and PI 236493 or PI 250438.

Screening of resistant pea lines and particle bombardment

Fifteen pea lines and F1 and F2 progeny of the crosses were inoculated with Cl-no30, and the infection was examined by monitoring GFP fluorescence, as described by Andrade et al. (2007). pCl-no30 was used to bombard PI 250438, PI 236493, or PI 429853 as described by Andrade et al. (2007), and GFP fluorescence was monitored for 1 to 5 days after inoculation (dpi) using an epifluorescence microscope (VB 7010; Keyence, Osaka, Japan).

DNA markers for analysis and mapping procedure

Genomic DNA of each inbred line was extracted, and polymerase chain reaction (PCR) was carried out for simple sequence repeat (SSR) amplification and the isozyme-related DNA marker, phosphoglucomutase (PGM)-2 (Harrison et al. 2000; Loridon et al. 2005) in a C1000 thermal cycler (Biorad, Hercules, CA, USA) as described by Ravelo et al. (2007). One hundred of the F2 progeny were used for genetic mapping and genotype scores were entered in the mapping software Map Manager QTX (Manly et al. 2001).

Isolation and nucleotide sequencing of *eIF(iso)4E*

Genomic DNAs from PI 250483, PI 118501, PI 236493, PI 269818-1, PI 18069-1, and PI 429853 were extracted from 4 g of pea leaves using DNA Plantzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The DNA samples were overlaid on a $CsCl_2$ density gradient and purified with ultracentrifugation. The primers used to isolate the open reading frame (ORF) of *eIF(iso)4E* were 5′-GAAATATGGCAACAACAGAAC-3′ (sense) and 5′-TTACACAGTGTATCGAGCCTTTGCA-3′ (antisense), designed based on *eIF(iso)4E* of Bonneville (*P. sativum*; GenBank accession DQ778078.1). The full-length *eIF(iso)4E* ORF was amplified using 100 ng of genomic DNA in a 20 μL reaction mixture containing sense and antisense primers and EX-Taq (TaKaRa, Ohtsu, Japan). The sample was incubated for 3 min at 95°C, followed by 35 cycles at 95°C for 0.5 min, 60°C for 0.5 min, 72°C for 3 min, and finally held at 72°C for 10 min. The products were inserted into the pGEM-T-easy vector system (Promega, Madison, WI, USA) and sequenced using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA) as described by Andrade et al. (2009).

Identification of the upstream region of *eIF(iso)4E*

To isolate the upstream regulatory region of the *eIF(iso)4E* gene, purified genomic DNA from another susceptible pea line, PI 118501 (Ravelo et al. 2007), was inserted into the pSTV/28 vector (TaKaRa). About 1 million clones were separated into a thousand pools, each of which was comprised of a thousand of clones containing about 5-kb genomic DNA. *Escherichia coli* transformants with each pool of the clones were cultured as a bulk and their plasmids were extracted using QIAprep Spin Miniper (Qiagen, Dusseldorf, Germany). The pools including the clones containing the upstream of *eIF(iso)4E* were screened with Go-Taq green mastermix (Promega) containing a pair of primers that were homologous to *pvr6* mRNA, which encodes eIF(iso)4E in pepper, and the central part of *eIF(iso)4E*, comiso4eF, 5′-GATCAGATATTCAAGCCCAGCAAG-3′, and comiso4eR, 5′-GTCCACAGCGAAAGTTTATCCTG-3′. For cloning plasmids containing the upstream region of *eIF(iso)4E* from the screened pool, 1 μl of plasmids was amplified inversely from the central domain of the *eIF(iso)4E* ORF with a pair of primers, comiso4eRF, 5′-CAGGATAAACTTTCGCTGTGGAC-3′, and comiso4eFR, 5′-CTTGCTGGGCTTGAATATCTGATC-3′, using Ex-Taq (TaKaRa) and subjected to the following procedure: 95°C for 3 min, followed by 30 cycles at 95°C for 0.5 min, 55°C for 0.5 min, and 68°C for 4 min, with a final hold at 72°C for 10 min. The template plasmids were digested with *Dpn*I (Toyobo Biologics, Osaka, Japan), PCR products resistant to *Dpn*I were fractionated on a 0.8% (w/v) agarose gel and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), and then reacted at 37°C for 2 h with T4 polynucleotide kinase (TaKaRa). The PCR products were circularized by adding T4 DNA ligase (Promega), and the circularized PCR products were used to transform *E. coli* DH5α. Plasmids were extracted, and the nucleotide sequences were determined using primers on the vector. Based on the determined nucleotide sequences, we designed a primer, 2H8F1, 5′-CAAGGTGTCTAACCTTATCAGTCC-3′ specifically for isolating the upstream region of *eIF(iso)4E* ORF and isolated the upstream sequences from PI 250438 and PI 429853 using primer pair 2H8F1 and comiso4eR.

RNA extraction, RT-PCR, and real-time PCR

Total RNA was isolated from leaves inoculated with Cl-no30 and RT-PCR and real-time PCR were performed (Andrade et al. 2007; Atsumi et al. 2009). The following primers were used for RT-PCR and real-time PCR: no30HC3′-s, 5′-GAGTCAGATTTGAAGTTTTACAGAGTTGG-3′; no306K15′-as, 5′-CATCAAGACTCTGAAATTTGTAGCCGTCN-3′; Ps18SrRNA-F, 5′-CCATAGTCCCTCTAAGAAGCTG-3′; Ps18SrRNA-R, 5′-CCATAGTCCCTCTAAGAAGCTG-3′; eIF4E(iso)-F, 5′-AACAACAGAACCACTCGTCGAA-3′; eIF(iso)4E-R, 5′-GCCTTGTTTAGGTTTGGATTGG-3′; eIF4E-F, 5′-ATGCGACCCATCTACACTTTCT-3′; and eIF4E-R, 5′-CTGGTATCAGATTTTCCCTTCG-3′.

Results

Reactions of *non-cyv2-, cyv1-* and *mo*-carrying pea lines to isolate no. 30 of ClYVV

Andrade et al. (2007) showed that several pea lines were divided into two groups by their resistance modes, which were represented in pea lines PI 347295 and PI 378159. PI 378159 was shown to carry *cyv2* that encodes eIF4E (Andrade et al. 2009). Additionally, PI 347295 and PI 429853, which carry tentatively designated *non-cyv2*, were found to be resistant to both ClYVV and BYMV, and to not be controlled by eIF4E (Andrade et al. 2007; 2009). To determine whether *non-cyv2* was *cyv1*, we examined whether pea lines that are known to carry *cyv1* are resistant to Cl-no30. Six *cyv1* pea lines that were reported to be resistant to a strain of ClYVV by Provvidenti (1987) were mechanically inoculated with Cl-no30. Four of the six lines, including PI 236493, showed resistance to Cl-no30 (Fig. 1). PI 236493 was selected and used in the subsequent studies as a representative pea line carrier of *cyv1*. Two *cyv1*-carrying pea lines showed Cl-no30 susceptibility comparable to that of susceptible pea line PI 250438. Pea lines carrying *mo*, which is closely linked with *cyv1* on LG II and confers resistance against another potyvirus (BYMV), showed no resistance to Cl-no30. As expected, Cl-no30 was not able to infect this line, and no GFP fluorescence was observed on upper leaves from inoculated pea lines PI 347295 and PI 429853 carrying *non-cyv2* (Fig. 1).

Comparison of the resistance mode of *cyv1* with that of *non-cyv2*

In PI 378159, which carries *cyv2*, sites with ClYVV infection indicated that the virus had spread systemically through the leaf vein. Conversely in PI 347295, which carries *non-cyv2*, ClYVV replicated on a single-cell level (Andrade et al. 2007). Here we investigated the resistance mode of *cyv1* against Cl-no30. Viral movement was examined in a *cyv1* pea line (PI 236493). Resistant and susceptible pea lines biolistically inoculated with pCl-no30, and the virus movement was observed from 1 to 5 dpi. In susceptible pea line PI 250438, Cl-no30 moved readily from the infected single cells to neighboring cells and spread systemically through the veins. Cl-no30 moved to a few adjacent cells at 1 dpi in the *cyv1*-carrying pea line PI 236493, but the virus no longer moved by 5 dpi. In the *non-cyv2*-carrying pea line PI 429853, Cl-no30 was restricted to the infected single cells, and few viruses had moved to adjacent cells, yielding a ratio of two infection sites per 40 inoculated sites by 5 dpi (Fig. 2). These results indicate that the resistance mode of *cyv1* against ClYVV is quite similar to that of *non-cyv2*.

The genetic relationship between *non-cyv2* and *cyv1*

We genetically mapped *non-cyv2* on the pea genome. The *non-cyv2* pea line PI 429853 was crossed with PI 250438, and 100 F2 progeny were inoculated with Cl-no30. The analysis of F2 revealed a 3:1 segregation of susceptibility versus resistance to Cl-no30 (79 versus 21 plants, respectively and supported by χ^2 of 0.853), indicating that the resistance gene in PI 429853 is recessively inherited and that a single gene controls resistance (Fig. 3a). Since *cyv1* was reported to be located on LG II (Provvidenti and Hampton 1991; Weeden et al. 1998), we first investigated the possibility that *non-cyv2* was also on LG II. To develop DNA markers, we screened 14 SSR markers (Loridon et al. 2005) and the isozyme-related DNA marker, PGM-2, (Harrison et al. 2000) on LG II for polymorphisms in PI 250438 and PI 429853. Among 15 markers, five were developed for the mapping of *non-cyv2*: AA205, AA473, AB149, AB40, and PGM2. Linkage analysis of 100 F2 plants showed that the recessive resistance gene in PI

429853 was about 4 and 5 cM from AB40 and PGM2, respectively, and was located in LG II (Fig. 3b). These two markers, AB40 and PGM2, were closely linked with each other and with *mo* and *sbm-2* (Aubert et al. 2006; Ellis and Posyer 2002; Weeden et al. 1984). Because *cyv1* was previously reported to be closely linked with *mo* and *sbm-2* (Weeden et al. 1998), *non-cyv2* was also expected to be linked with them and with *cyv1*.

The *non-cyv2* pea PI 429853 was crossed with the *cyv1* pea PI 236493. Molecular analysis with the SSR marker indicated that five F1 plants were successfully crossed (data not shown). Among the five F1 plants, three plants were used to determine susceptibility to Cl-no30. Inoculated F1 plants showed no GFP fluorescence with GFP-tagged Cl-no30 inoculation at 28 dpi. RT-PCR analysis confirmed that none of the F1 plants were infected (data not shown), indicating that *non-cyv2* may be an allele of *cyv1*.

The possibility of *non-cyv2* and *cyv1* encoding eIF(iso)4E

Because the *mo* locus was mapped close to *eIF(iso)4E* on LG II (Gao et al. 2004b), we suspected that both *non-cyv2* and *cyv1* were linked with *eIF(iso)4E*. To investigate whether *non-cyv2* and *cyv1* encode eIF(iso)4E, we looked for differences in the nucleotide sequences of the *eIF(iso)4E* cDNAs of the Cl-no30-susceptible PI 250438, PI 118501, PI 180669-1 (*cyv1*), and PI 269818-1 (*mo*); and the resistant PI 429853 (*non-cyv2*) and PI 236493 (*cyv1*) (see Fig. 1). Taking advantage of previously reported nucleotide sequences (Gao et al. 2004b), we obtained *eIF(iso)4E* genes from those pea lines. The amplified fragment of about 2200 bp of *eIF(iso)4E* contained five exons and four introns and showed no difference in the nucleotide sequences of susceptible and resistant pea lines (data not shown) except for PI 180669-1 and PI 269818-1

(DDBJ/EMBL/GenBank accession numbers AB 691237, AB 691238, AB 691239, and AB 691240). The cDNA sequences from PI 180669-1 and PI 269818-1 had no difference in exons from the other lines. To investigate mutations upstream of *eIF(iso)4E*, we generated a genomic library of pea line PI 118501 as representative of ClYVV-susceptible pea line (Ravelo et al. 2007), and screened for genomic clones that carry the *eIF(iso)4E* sequence. Based on these sequences, those of PI 250438 and PI 429853 were cloned, and the six clones each were sequenced. We obtained a sequence of about 2000 nucleotides upstream of the *eIF(iso)4E* ORF and found only one nucleotide difference between PI 250438 and PI 429853 (DDBJ/EMBL/GenBank accession numbers AB 646248 and AB 646249), where adenosine in PI 250438 was altered to thymine in PI 429853, 1.2 kb upstream from the initiation codon (Fig. 4a). We compared the mRNA levels of *eIF(iso)4E* in PI 250438 and PI 429853 with/without Cl-no30 infection using real-time PCR. The *eIF(iso)4E* gene seemed to be equivalently expressed in both susceptible and resistant peas (Fig. 4b), suggesting that the single nucleotide difference upstream of the eIF(iso)4E coding region did not drastically alter the *eIF(iso)4E* expression (Fig. 4b). Taken together, these results suggest that *non-cyv2*, *cyv1*, and *mo* are unlikely to encode eIF(iso)4E.

Discussion

In regard to *non-cyv2*, this study revealed that (1) the resistance modes in PI 347295 and PI 429853 carrying *non-cyv2* were similar to that of PI 236493 carrying *cyv1* (Fig. 2; Andrade et al. 2007); (2) *non-cyv2* should be located near *cyv1* because, when using the SSR marker on LG II, *non-cyv2* was closely linked with *mo* and *sbm-2* near PGM2 and AB40 (Fig. 3b) and *cyv1* was also previously reported to be close to *mo* and *sbm-2*

(Weeden et al. 1998); and (3) *non-cyv2* pea PI 429853 crossed with *cyv1* pea PI 236493 produced F1 progeny resistant to ClYVV, implying that *non-cyv2* is an allele of *cyv1*. However, we cannot rule out the possibility that the locus of *non-cyv2* is different from that of *cyv1*, and that PI 236493, which crossed with the *non-cyv2* pea PI429853, possessed both *non-cyv2* and *cyv1*. Nevertheless, Provvidenti (1987) examined the response of pea cultivars to ClYVV and BYMV and found that in all pea cultivars resistance to these potyviruses was a monogenetic recessive trait. Provvidenti (1987) also reported that all *cyv1* peas were resistant to both ClYVV and BYMV, whereas *cyv2* peas were resistant to ClYVV but susceptible to BYMV. Although exceptions exist, including JI1405, which carries *wlv* (*cyv2*) making it resistant to BYMV-W but susceptible to BYMV-S (Bruun-Rasmussen et al, 2007), the *non-cyv2* peas were resistant to both BYMV and ClYVV (Andrade et al. 2007). Taken together, these results demonstrate the close relationship between *non-cyv2* and *cyv1*. Identifying what gene *non-cyv2* or *cyv1* encodes is one of the challenging future tasks for determining whether *non-cyv2* is an allele of *cyv1*.

Resistance genes against other potyviruses cluster around both loci: *cyv1*, *sbm-2* and *mo* or *cyv2*, *sbm-1* and *wlv*, conferring monogenic resistance to ClYVV, PSbMV, and BYMV, respectively (Provvidenti and Hampton 1991). Interestingly, *cyv2*, *sbm-1*, and *wlv* were shown to be the same allele of the *eIF4E* gene (Andrade et al. 2009; Bruum-Rasmussen et al. 2007; Gao et al. 2004a). However, our study implies that *cyv1*, *sbm-2* and *mo* are not the same allele. All pea lines carrying *cyv1* were resistant to ClYVV that Provvidenti used in the previous study (Provvidenti 1987), but not all *cyv1* pea lines showed resistance to Cl-no30 in the present study (Fig. 1). The difference in resistance to Cl-no30 among *cyv1* pea lines was further supported by recent results that pea lines, PI 236493, PI 347420, PI 347422, PI 356851, PI 347295 and PI 429853, reacted differently to several ClYVV isolates (Choi et al. unpublished results).

Multiple alleles of the same locus were previously reported to mediate resistance to one potyvirus of different pathotypes: *mo1*^{1} and *mo1*^{2} on the *mo* locus in lettuce, and *pvr2*^{1} and *pvr2*^{2} on the *pvr2* locus in pepper (Nicaise et al. 2003; Ruffel et al. 2002). Two pairs of alleles on distinct loci (*pvr2* and *pvr6*, *pvr1*^{2} and *pvr6*) in pepper are also simultaneously necessary for resistance to *Pepper veinal mottle virus* (PVMV) and ChiVMV, respectively (Hwang et al. 2009; Ruffel et al. 2006). We showed that peas carrying *mo* had no resistance against Cl-no30 (Fig. 1), suggesting that *non-cyv2*, *cyv1*, *sbm-2*, and *mo* could be allelic or different loci. Their relationship is an open question to be addressed. Further analysis is needed to elucidate whether or not multiple alleles of the same locus mediate resistance to different potyvirus species.

Since *non-cyv2*, *cyv1*, and *mo* were mapped to a region near the *eIF(iso)4E* gene, we examined the possibility that these encode eIF(iso)4E. However, our results showed that there was no difference in the eIF(iso)4E-encoding sequences and that eIF(iso)4E is equivalently expressed in both susceptible and resistance peas, suggesting that these resistance genes encode proteins other than eIF(iso)4E. Nevertheless, previously identified host factors interacting with potyviruses in naturally resistant crops have only been in the translation initiation factor families, eIF4E and eIF4G. These factors are involved in some steps of the potyvirus infection cycle through their interaction with VPg (Kang et al. 2005b; Nicaise et al. 2007). Therefore, the resistant host plants are thought to have mutations in the required translation initiation factor, which no longer binds to VPg, and hence resistance-breaking viruses are thought to have mutations in VPg, which leads to the restoration of the affinity with the host translation initiation factor. Actually, most resistance-breaking viruses are reported to have critical mutations in VPg, with few exceptions (Abdul-Razzak et al. 2009; Nakahara et al. 2010).

Although we do not know whether *sbm-2* and *cyv1* encode the same gene, the viral determinant of *sbm-2*-resistance-breaking PSbMVs was not VPg, but P3 (Gao et al. 2004b; Hjulsager et al. 2006; Johansen et al. 2001). It would be interesting to examine whether P3 of ClYVV is involved in breaking resistance controlled by *cyv1*.

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Figure Legends

Fig. 1 Several pea lines inoculated with pClYVV/C3-S65T. **a** pClYVV derivatives carrying GFP (Sato et al. 2003). **b** Results of inoculation of resistant pea lines with ClYVV. Vein yellowing or necrosis symptoms were observed on upper leaves of pea lines susceptible to ClYVV at 5 or 6 dpi (left panel), but not in resistant pea lines carrying *cyv1* or *non-cyv2* until 6 to 37 dpi (right panel). Necrosis meant that GFP fluorescence photographs could not be taken

Fig. 2 Inoculation of pCl-no30 using particle bombardment into PI 250438, PI 236493, and PI 429853. Infection by, and spread of, ClYVV in susceptible pea line PI 250438 and two resistant pea lines carrying *cyv1* and *non-cyv2* were observed by monitoring

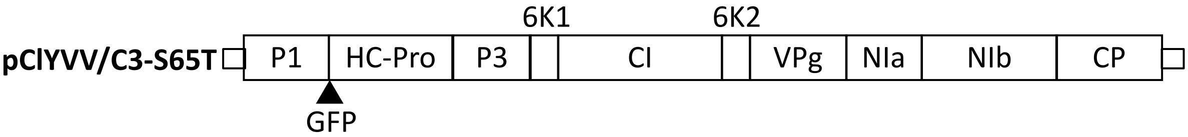
GFP at 1 to 5 dpi. In PI 250438, ClYVV spread readily at 1 dpi. Cl-no30 was restricted in PI 236493 carrying *cyv1* and PI 429853 carrying *non-cyv2*, although the virus was more motile in PI 236493 than in PI 429853

Fig. 3 Relative position of *non-cyv2* and selected linked reference markers on LG II. The mapping of *non-cyv2* in PI 429853 was determined by analysis of F2 progeny from a cross between PI 250438 and PI 429853 using the QTX map manager program. The 100 F2 progeny segregated at 3:1 (a), and the recessive resistance gene was located on LG II 4 cM and 5 cM from the AB40 and PGM2 markers (b), respectively. Map distances are in Kosambi cM

Fig. 4 Resistance to ClYVV in the resistant pea line carrying *cyv1* does not correspond to *eIF(iso)4E*. (a) Upstream of *eIF(iso)4E*, a candidate gene for *cyv1*, was isolated from PI 250438 and PI 429853 via a genomic library. About 2 kb upstream from the initiation codon of *eIF(iso)4E*, a substitution of A to T is present in PI 429853. (b) Quantitative expression analysis of *eIF4E* or *eIF(iso)4E* in resistant (PI 429853 carrying *cyv1*) and susceptible (PI 250438) pea lines. After inoculation with ClYVV, the amount of mRNA was measured with real-time PCR. The relative amounts of mRNA for the two genes were approximately the same in PI 429853 pea plants and did not differ in ClYVV-inoculated plants

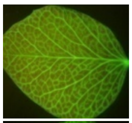
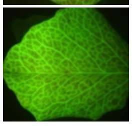
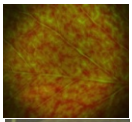
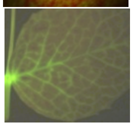
Choi et al. 2012 Fig. 1

a

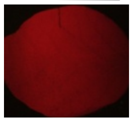
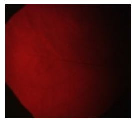
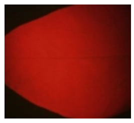
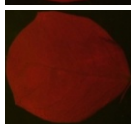


b

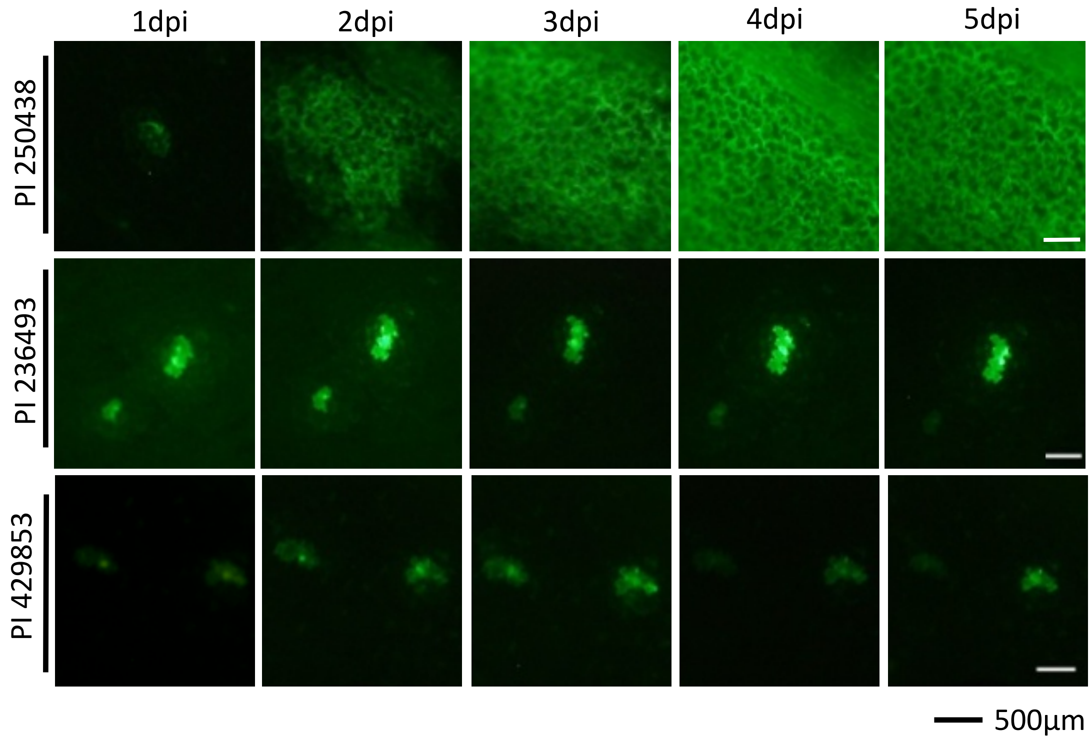
Response to ClYVV

Pea line	*r* gene	Infection rate (%)	Infected /Total plants number	GFP filter
PI 250438		100	15/15	
PI 174319-1	*cyv1*	100	3/3	
PI 180669-1	*cyv1*	100	3/3	Necrosis
PI 378159	*cyv2*	14	2/15	
PI 269818-1	*mo*	100	3/3	
PI 391630-1	*mo*	100	3/3	Necrosis

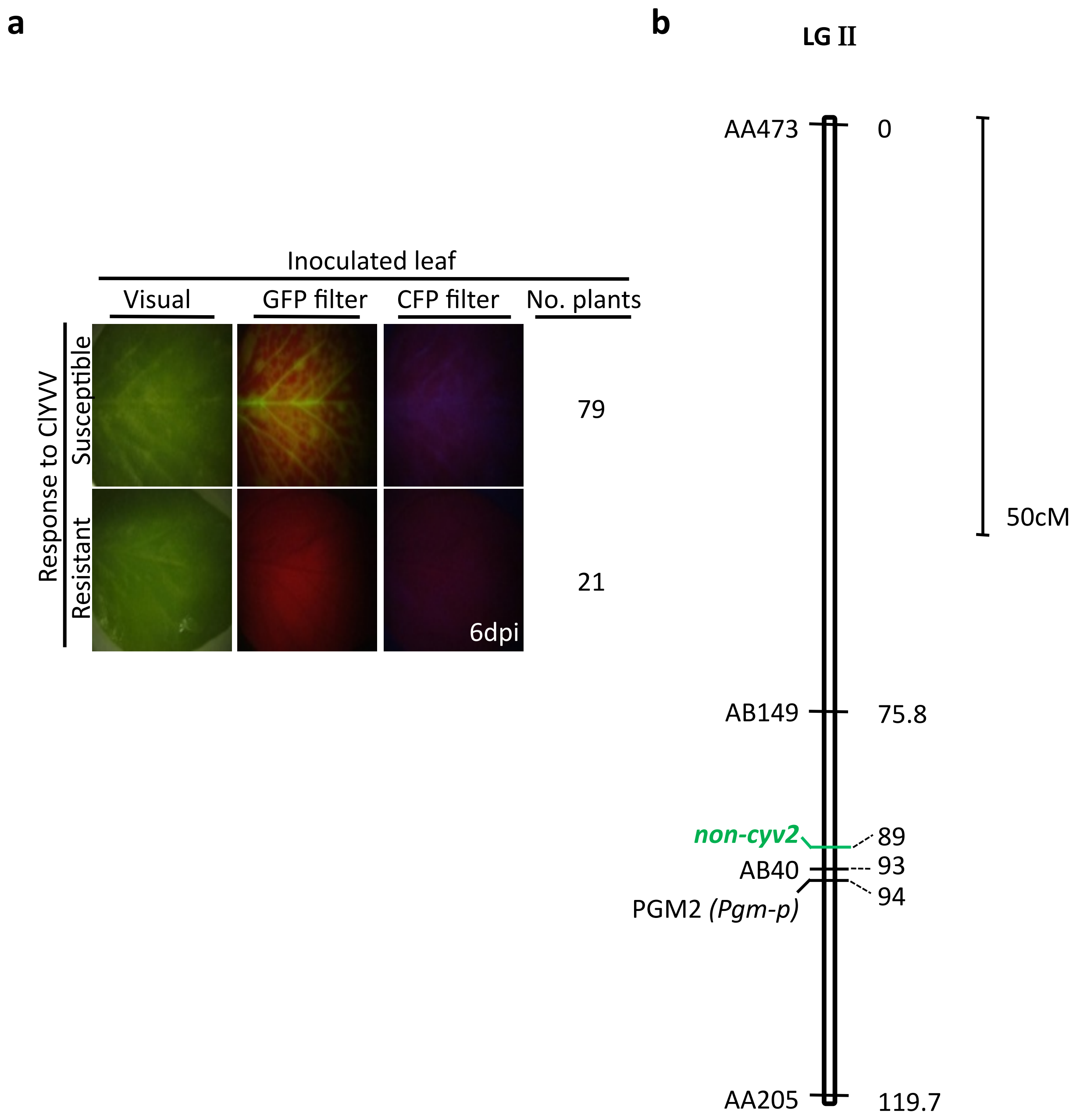
Response to ClYVV

Pea line	*r* gene	Infection rate (%)	Infected /Total plants number	GFP filter
PI 236493	*cyv1*	0	0/3	
PI 347420	*cyv1*	0	0/4	
PI 347422	*cyv1*	0	0/5	
PI 356851	*cyv1*	0	0/5	
PI 429853	*non-cyv2*	0	0/7	
PI 347295 RS-7	*non-cyv2*	0	0/3	
PI 347295 R-18	*non-cyv2*	0	0/9	

Choi et al. 2012 Fig. 2



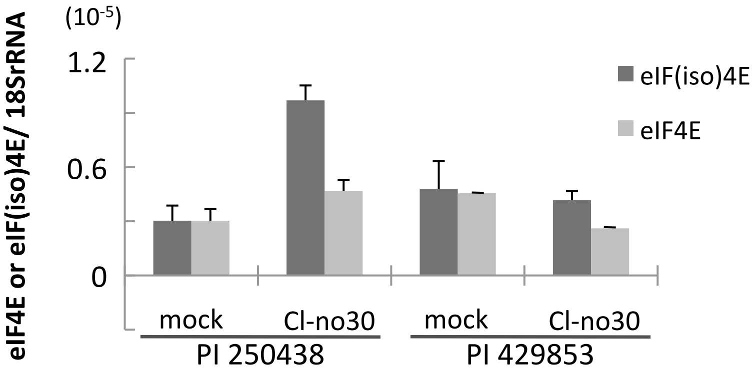
Choi et al. 2012 Fig.3



a


-2173
-1278
1
PI 250438
PI 429853
CAAGGTGTCTAACCTTAT
CTGACTAATTTTT
GAGAGAGAAACGAAATATGGCAACAAC
CAAGGTGTCTAACCTTAT
CTGACTATTTTTT
GAGAGAGAAACGAAATATGGCAACAAC

b


eIF4E or eIF(iso)4E/ 18SrRNA
(10-5)
1.2
0.6
0
eIF(iso)4E
eIF4E
mock
Cl-no30
mock
Cl-no30
PI 250438
PI 429853