The functions of the P3 cistron of *Clover yellow vein virus* in resistance breaking and cell-to-cell movement in *Pisum sativum*

(クローバ葉脈黄化ウイルスの P3 遺伝子がエンドウにおける抵抗性打破と細胞間移行に果す役割)

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1. General Introduction

The genus *Potyvirus* is a single strand positive RNA virus and the largest of the plant virus groups recognized by the International Committee on Taxonomy of Viruses (ICTV). Potyviruses cause severe losses of crop yield and quality. These potyviruses invade the cell, where they produce their virulent proteins and interact with host proteins, replicate viral genome, move to adjacent cells, and spread systemically. However, crops also have the capacity to recognize viruses and actively defend themselves from virus invasion with naturally occurring resistance genes: dominant resistance gene and recessive resistance gene. Many of dominant genes recognize avirulence genes and typically trigger hypersensitive response (HR). On the other hand, recessive resistance gene operates by interrupting interactions between viruses and plant factors required for virus life cycle (Díaz-pendón et al. 2004, Robaglia and Caranta, 2006).

Recessive resistance genes to viruses has been identified from crop species (e.g. barely, lettuce, pea, and pepper) and they seem to be more frequent for plant viruses than for other plant pathogens (Díaz-pendón et al, 2004). Moreover, more than half of recessive resistance genes are those for potyviruses (Robaglia and Caranta 2006). Identified recessive resistance genes encode eukaryotic translation initiation factors (eIFs) and inhibit potyvirus replication or cell-to-cell movement such as eIF4E-mutant of *Arabidopsis thaliana* that inhibits *Clover yellow vein virus* (ClYVV) replication or genome expression (Kang 2005b, Sato et al. 2005, Robaglia and Caranta, 2006, Truniger and Aranda, 2009).

ClYVV belongs to the genus *Potyvirus* and is an important pathogen of several leguminous species. It produces 11 functional proteins (P1, HC-Pro, P3, P3N-PIPO,

6K1, CI, 6K2, NIa-VPg, NIa-pro, NIb, CP) and induces yellow and green mosaic, mottle, vein cleaning, vein yellowing, and necrosis on infected plants. In pea (*Pisum sativum*), there are two recessive resistance genes, *cyv1* and *cyv2*, and they were mapped in the linkage group (LG) II and VI (Provvidenti and Hampton 1991), respectively. The *cyv2*-mediated resistance is controlled by eIF4E and is broken by a mutation in P1 cistron of ClYVV (Andrade et al 2009, Nakahara et al. 2010). However, it has not been investigated whether *cyv1* also encodes eIF4E family such as *cyv2*. In addition, which viral functional protein overcomes *cyv1* resistance, and how its resistance is broken by mutations in viral proteins are important subjects to be elucided. These questions let me to focus on elucidating the characteristic of ClYVV with three major objectives as following:

1. Characterization of recessive resistance gene, *cyv1*, of *P. sativum* against ClYVV (chapter I).

2. The P3 cistron is involved in overcoming *cyv1*-mediated resistance in *P. sativum* (chapter II).

3. Functional analysis of the P3 cistron in cell-to-cell movement of ClYVV (chapter III).

Chapter I

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

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 potyviruses 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); *White lupin mosaic virus* (*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 ClYVV (Johansen et al. 2001, Ruffel et al. 2002, Nicaise et al. 2003, Gao et al. 2004b, Kanyuka et al. 2004, Kang et al. 2005a, Stein et al. 2005, Bruun- Rasmussen et al. 2007). Most of these genes encode eukaryotic translation initiation factor 4E (eIF4E) or its isoform eIF(iso)4E, which alone or together control the response to potyviruses (Sato et al. 2005, Ruffel et al. 2006, Andrade et al. 2009, Hwang et al. 2009).

Resistance genes in pea to several potyviruses are closely linked and clustered in the 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 BYMV (*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, which is involved in cell-to-cell movement of PSbMV pathotype P1 (Andrade et al. 2009, Gao et al. 2004b). On the other hand, the resistance genes in LG II, including *cyv1* and *sbm-2*, remain to be identified. Gao et al. (2004a) found that the *eIF(iso)4E* gene was mapped on the same LG II that contains the *sbm-2* gene.

Andrade et al. (2007) 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 resistance 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 that is 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, I 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. 2004a), I examined the possibility that these resistance genes encode eIF(iso)4E in a comparison of the nucleotide sequences of the eIF(iso)4E genes and their expression in resistant and susceptible pea lines.

Materials and Methods

Virus source and plant material

The infectious ClYVV clone pClYVV/C3-S65T-Sal (pCl-no30) was made and modified from pClYVV/C3-S65T of ClYVV isolate no. 30 (hereafter, Cl-no30) in previous studies and contains a coding sequence for the green fluorescent protein (GFP) (Takahashi et al. 1997, Sato et al. 2003). Since the original sequence of ClYVV isolate no. 30 (DDBJ/GenBank/EMBL accession no. AB011819) contains two *Sal*I sites in the VPg and NIb cistrons and the site in VPg was utilized for construction of the chimeric viruses, the site in NIb was eliminated by site-directed mutagenesis prior to construction of the chimeric viruses (Chapter II).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*, two ClYVV resistant pea lines were selected; PI 236439 (Provvidenti 1987) and PI 429853 (Andrade et al. 2007) carrying *cyv1* and *non-cyv2*, respectively (Fig. 1-1), from these 15 lines for further analysis of their reaction to Cl-no30. F1 and F2 progenies were obtained 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 1–5 days post 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 DNA from PI 250438, PI 118501, PI 236493, PI 269818-1, PI 18069-1, and PI 429853 was 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*

For isolating 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 that contained 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 comiso-4eFR, 5′-

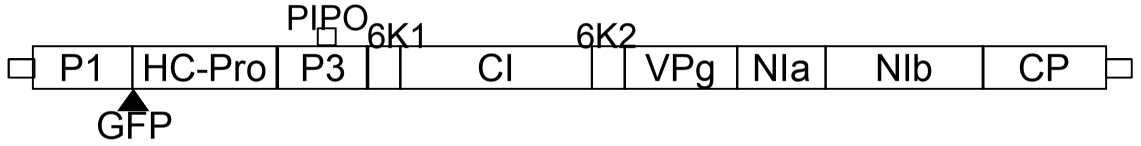
CTTGCTGGGCTTGAATATCTGATC-3′, using Ex-Taq (TaKaRa) and subjected to the following procedure: 95 °C for 3 min, followed by 30 cycles at 95 °C for 0.5 min, 55 °C for 0.5 min, and 68 °C for 4 min, with a final hold at 72 °C for 10 min. The template plasmids were digested with *Dpn*I (Toyobo Biologics, Osaka, 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* DH5a. Plasmids were extracted, and the nucleotide sequences were determined using primers on the vector. On the basis of the determined nucleotide sequences, we designed 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: no30HC30-s, 5′-GAGTCAGATTTGAAGTTTTACAGAGTTGG-3′; no306K150-as, 5′-CATCAAGACTCTGAAATTTGTAGCCGTCN-3′; Ps18SrRNA-F, 5′-CGTTCTTAGTTGGTGGAGCGAT-3′; Ps18SrRNA-R, 5′-CCATAGTCCCTCTAAGAAGCTG-3′; eIF4E(iso)-F, 5′-AACAACAGAACCACTCGTCGAA-3′; eIF(iso)4E-R, 5′-GCCTTGTTTAGGTTTGGATTGG-3′; eIF4E-F,

A

pClYVV/C3-S65T-Sal (pCl-no30)



B

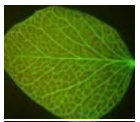
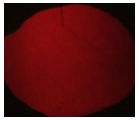
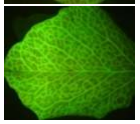
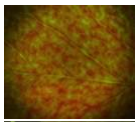
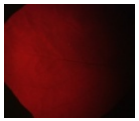
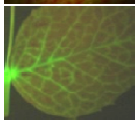
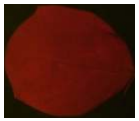
Inoculated plants					Inoculated plants				
Pea line	*r* gene	Infection rate (%)	Infected /Total plants number	GFP filter	Pea line	*r* gene	Infection rate (%)	Infected /Total plants number	GFP filter
PI 250438		100	15/15		**PI 236493**	*cyv1*	0	0/3	
PI 174319-1	*cyv1*	100	3/3		**PI 347420**	*cyv1*	0	0/4	
PI 180669-1	*cyv1*	100	3/3	Necrosis	**PI 347422**	*cyv1*	0	0/5	
PI 378159	*cyv2*	14	2/15		**PI 356851**	*cyv1*	0	0/5	
PI 269818-1	*mo*	100	3/3		**PI 429853**	*non-cyv2*	0	0/7	
PI 391630-1	*mo*	100	3/3	Necrosis	**PI 347295 RS-7**	*non-cyv2*	0	0/3	
					PI 347295 R-18	*non-cyv2*	0	0/9	

FIG 1-1 Several pea lines inoculated with pClYVV/C3-S65T-Sal. A pClYVV/C3-S65T-Sal (pCl-no30) 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 the resistant pea lines carrying *cyv1* or *non-cyv2* until 6 to 37 dpi (right panel). Necrosis means that GFP fluorescence photographs could not be taken.

5′-ATGCGACCCATCTACACTTTCT-3′; and eIF4E-R, 5′-CTGGTATCAGATTTTCCCTTCG-3′.

Results

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

Andrade et al. (2007) showed that several pea lines could be divided into two groups based on 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 the gene tentatively designated *non-cyv2*, were found to be resistant to both ClYVV and BYMV, and not to be controlled by eIF4E (Andrade et al. 2007, 2009). To determine whether *non-cyv2* was *cyv1*, I examined whether pea lines that are known to carry *cyv1* are resistant to Cl-no30 (Fig. 1-1B). 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. 1-1B). PI 236493 was selected and used in the subsequent studies as a representative pea line carrier of *cyv1*. Provvidenti (1987) showed that PI 174319-1 and PI 180669-1 carry *cyv1* and showed resistant to some unspecified strain of ClYVV. However, these two *cyv1*-carrying pea lines showed Cl-no30 susceptibility comparable to that of susceptible pea line PI 250438. Pea lines carrying *mo* (PI 269818-1 and PI 391630-1), 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 *non-cyv2*-carrying

pea lines, PI 347295 and PI 429853, and no GFP fluorescence was observed on upper leaves from them (Fig. 1-1B).

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

In PI 378159, which carries *cyv2*, infection sites of ClYVV indicated that the virus had spread systemically through leaf veins. Conversely in PI 347295, which carries *non-cyv2*, ClYVV replicated at the single-cell level (Andrade et al. 2007). Here the resistance mode of *cyv1* against Cl-no30 was investigated. Viral movement was examined in a *cyv1* pea line (PI 236493). The resistant and susceptible pea lines biolistically inoculated with pCl-no30, virus movement occurred 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. 1-2). 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*

The *non-cyv2* was genetically mapped 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. 1-3A). Since *cyv1* was reported to be located on LG II

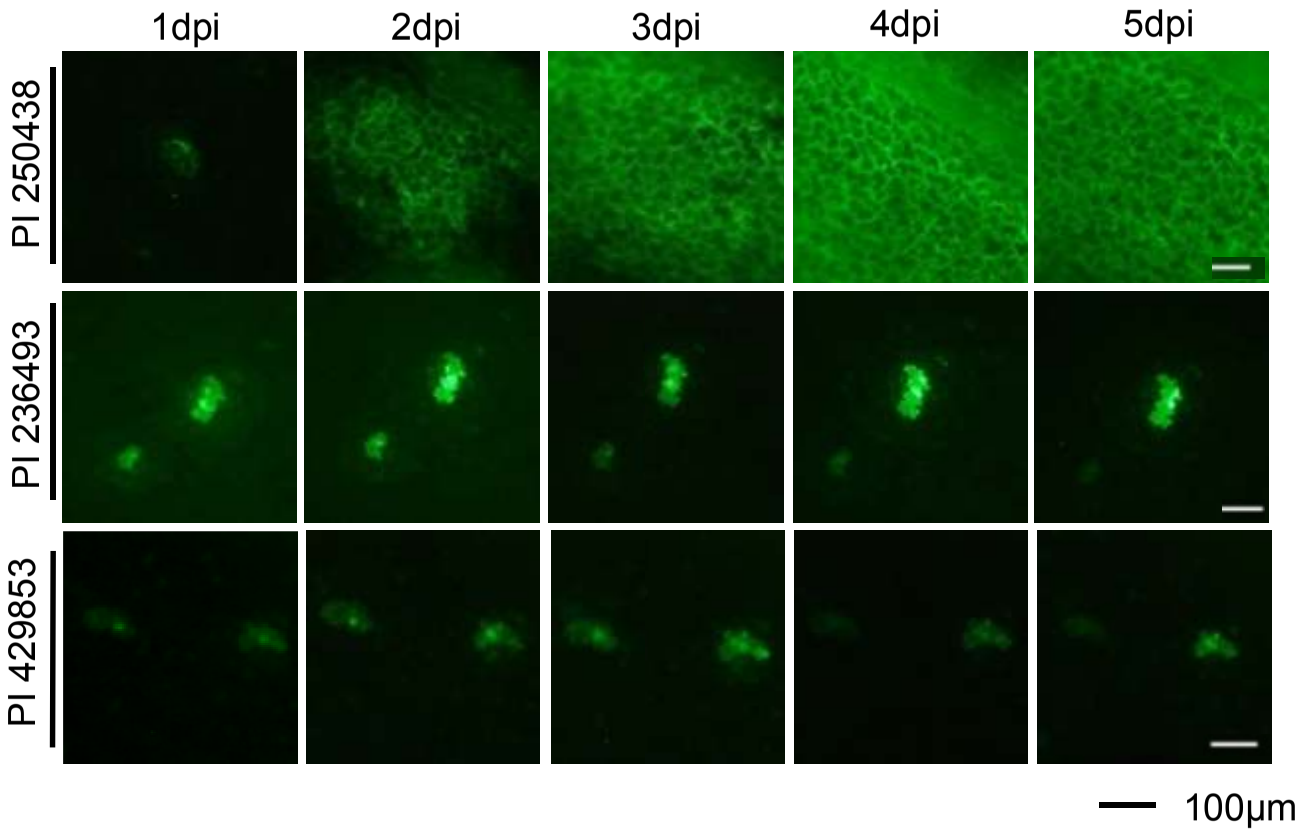


FIG1-2 Inoculation of pCl-no30 using particle bombardment into PI 250438, PI 236493, and PI 429853. Infection by and spread of ClYVV in susceptible pea line PI 250438 and two resistant pea lines carrying *cyv1* and *non-cyv2* were examined by monitoring GFP at 1 to 5 dpi. In PI 250438, ClYVV spread readily 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.

(Provvidenti and Hampton 1991, Weeden et al. 1998), I first investigated the possibility that *non-cyv2* was also on LG II. To develop DNA markers, I 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. 1-3B). These two markers, AB40 and PGM2, were closely linked with each other and with *mo* and *sbm-2* (Weeden et al. 1984, Ellis and Posyer 2002, Aubert et al. 2006). 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 (Fig. 1-4). 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. 2004a), I suspected that both *non-cyv2* and *cyv1* were linked with *eIF(iso)4E*. To investigate whether *non-cyv2* and *cyv1* encode eIF(iso)4E, I 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. 1-1B). Taking advantage of previously reported nucleotide sequences (Gao et al. 2004a), *eIF(iso)4E* genes was obtained from those pea lines. The amplified fragment of about 2,200 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 introns showed difference in the nucleotide sequences of PI 180669-1 and PI 269818-1 at positions of 221-222, 933, and 964. 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 six clones each were sequenced. A sequence of about 2,000 nucleotides upstream of the *eIF(iso)4E* ORF was obtained 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. 1-5A). The mRNA levels of *eIF(iso)4E* in PI 250438 and PI 429853 with/without Cl-no30 infection were compared using real-time PCR. The *eIF(iso)4E* gene seemed to be equivalently expressed in both susceptible and resistant peas (Fig. 1-5B), suggesting that the single nucleotide difference upstream of the eIF(iso)4E coding region did not drastically alter the *eIF(iso)4E* expression (Fig. 1-5B). Taken together, these results suggest that *non-cyv2*, *cyv1*, and *mo* are unlikely to encode eIF(iso)4E.

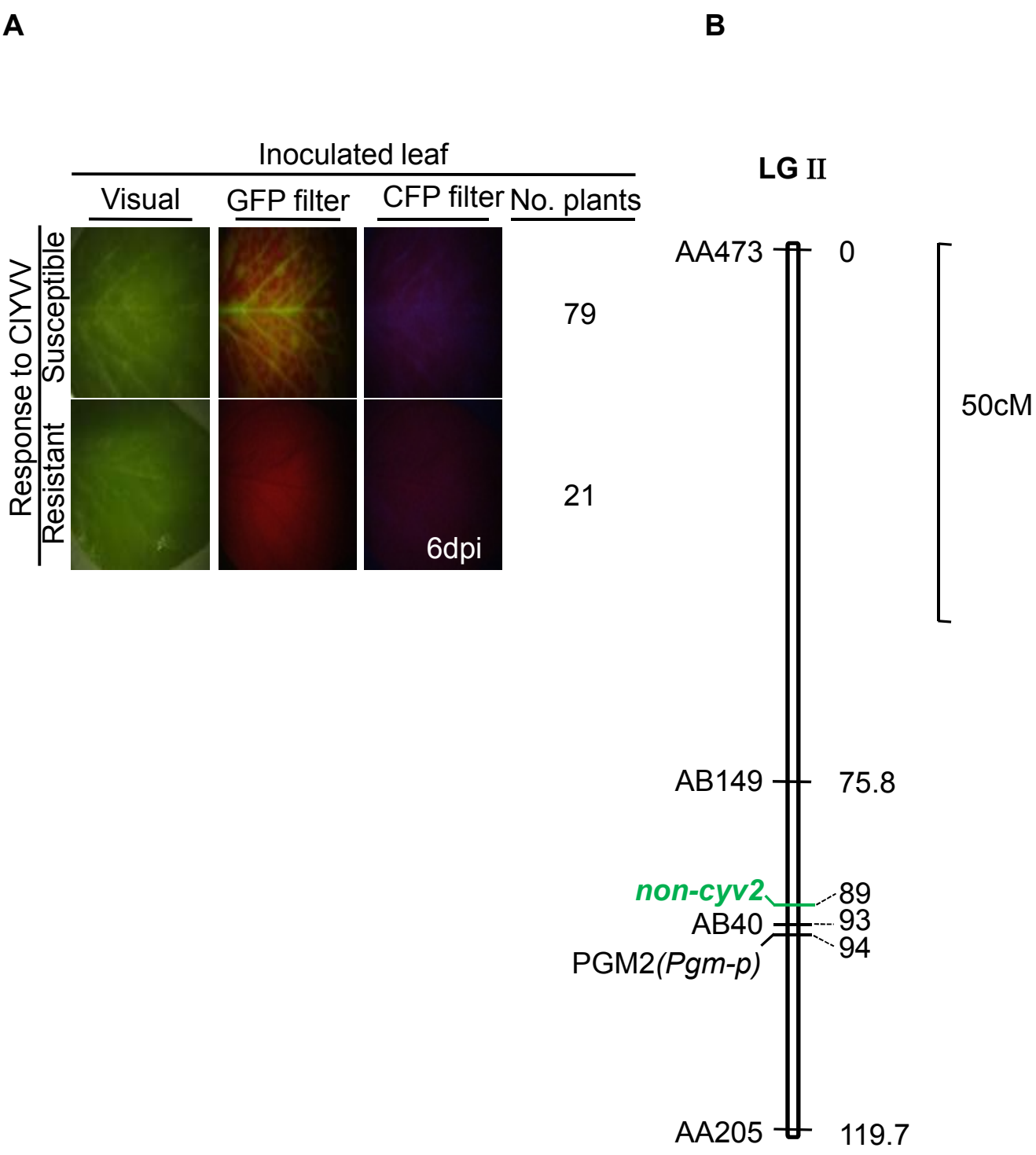


FIG 1-3 Relative position of *non-cyv2* and selected linked reference markers on LG II. The mapping of *non-cyv2* in PI 429853 was determined by analysis of F2 progeny from a cross between PI 250438 and PI 429853 using the QTX map manager program. The 100 F2 progeny segregated at 3:1 (**A**), and the 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

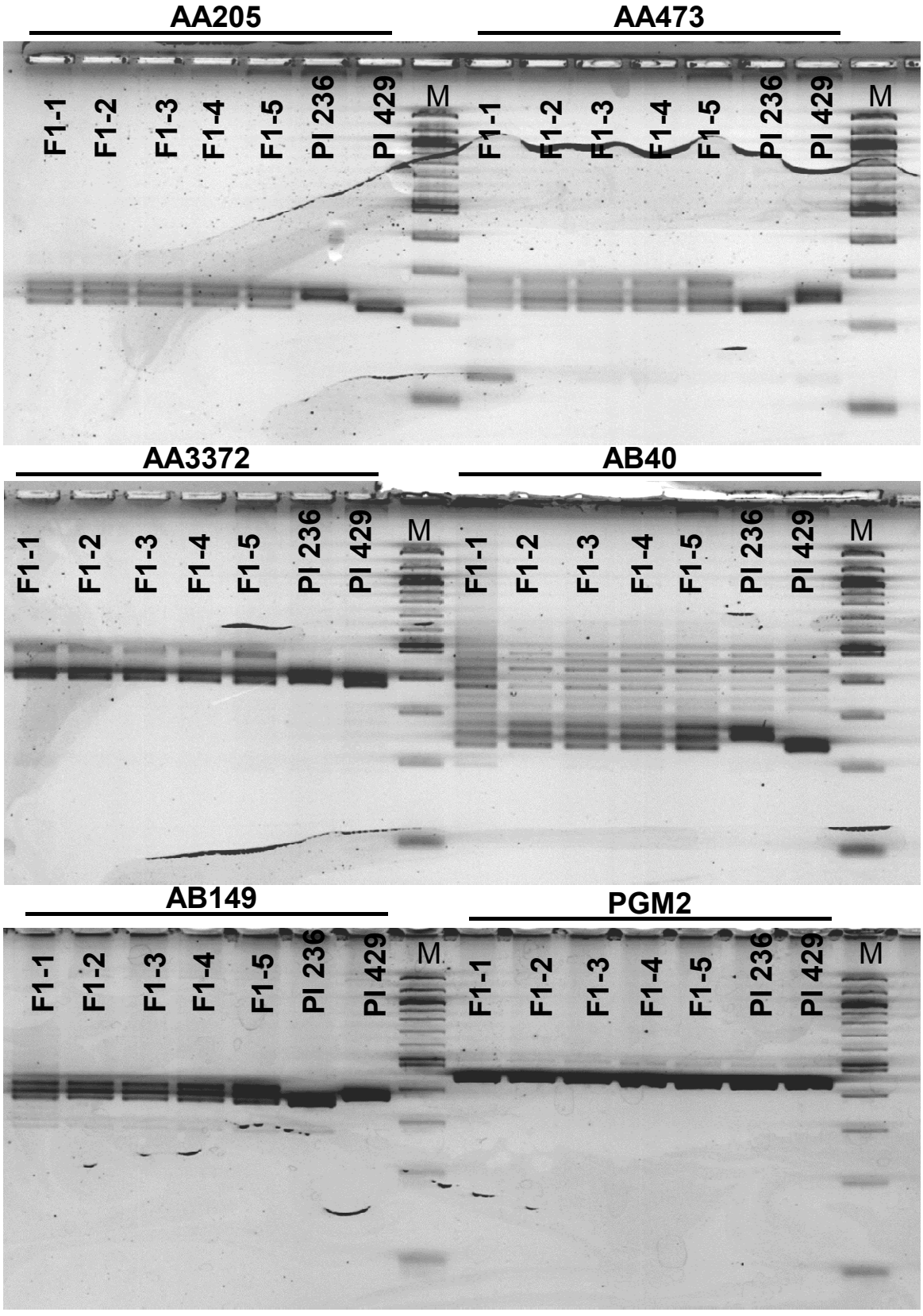


FIG 1-4. Simple Sequence Repeats (SSRs) PCR profile of PI 236493 (PI 236), PI 429853 (PI 429), and F1 progenies between PI 236493 and PI 429853 using 6 primers.

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. 1-2; Andrade et al. 2007); (2) *non-cyv2* should be located near *cyv1* because, when the SSR marker on LG II was used, *non-cyv2* was closely linked with *mo* and *sbm-2* near PGM2 and AB40 (Fig. 1-3B) and *cyv1* was also 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, I 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 all pea cultivars resistant to these potyviruses had 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) as reported *cyv1* peas by Provvidenti (1987). 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 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 (Gao et al. 2004b, Bruum-Rasmussen et al. 2007, Andrade et al. 2009). However, this 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. 1-1). The difference in resistance to Cl-no30 among *cyv1* pea lines was further supported by the recent results that pea lines PI 236493, PI 347420, PI 347422, PI 356851, PI 347295 and PI 429853 responded differentially to several ClYVV isolates (Choi et al. 2013, in chapter II).

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 (Ruffel et al. 2002, Nicaise et al. 2003). 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 (Ruffel et al. 2006, Hwang et al. 2009). I showed that peas carrying *mo* had no resistance against Cl-no30 (Fig. 1-1), 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 on 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, I examined the possibility that these encode eIF(iso)4E. However, the 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

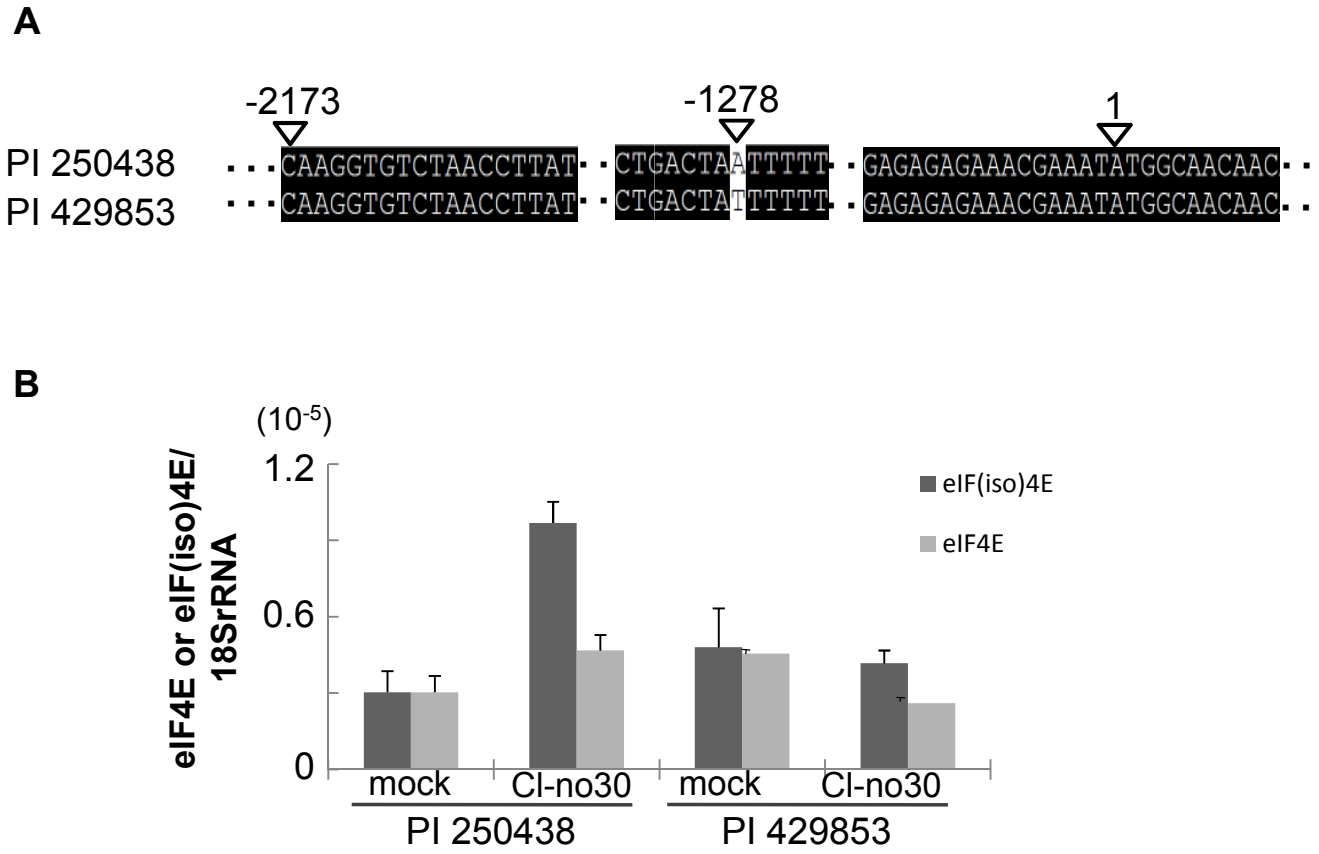


FIG 1-5 Resistance to ClYVV in the resistant pea line carrying *cyv1* does not correspond to *eIF(iso)4E*. A Upstream of *eIF(iso)4E*, a candidate gene for *cyv1*, was isolated from PI 250438 and PI 429853 via a genomic library. About 2 kb upstream from the initiation codon of *eIF(iso)4E*, a substitution 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

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 I 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 (Johansen et al. 2001, Gao et al. 2004a, Hjulsager et al. 2006). It would be interesting to examine whether P3 of ClYVV is involved in breaking resistance controlled by *cyv1* and this possibility was examined and described in chapter II.

TABLE1. Primers evaluated in this chapter.

Primer no.	Name of primer	Sequence (5′-3′)
2361	psiso4eP1	GAAATATGGCAACAACAGAAC
2364	psiso4eM4	TTACACAGTGTATCGAGCCTTTGCA
3250	comiso4eF	GATCAGATATTCAAGCCCAGCAAG
3251	comiso4eR	GTCCACAGCGAAAGTTTATCCTG
3274	comiso4eFR	CTTGCTGGGCTTGAATATCTGATC
3275	comiso4eRF	CAGGATAAACTTTCGCTGTGGAC
3288	2H8_F1	CAAGGTGTCTAACCTTATCAGTCC
2978	no30HC30-s	GAGTCAGATTTGAAGTTTTACAGAGTTGG
3167	no306K150-as	CATCAAGACTCTGAAATTTGTAGCCGTCN
1305	Ps18SrRNA-R	CCATAGTCCCTCTAAGAAGCTG
1419	Ps18SrRNA-F	CGTTCTTAGTTGGTGGAGCGAT
3168	eIF(iso)4E-F	AACAACAGAACCACTCGTCGAA
3169	eIF(iso)4E-R	GCCTTGTTTAGGTTTGGATTGG
3220	eIF4E-F	ATGCGACCCATCTACACTTTCT
3221	eIF4E-R	CTGGTATCAGATTTTCCCTTCG

Chapter II

Quantitative and qualitative involvement of P3N-PIPO in overcoming recessive resistance against *Clover yellow vein virus* in *Pisum sativum* carrying *cyv1*

Introduction

Breeding by transfer of natural resistance genes between varieties of crops and from wild ancestors into crops is one of the major strategies to confer resistance against viruses and other pathogens. However, pathogen mutants that overcome resistance seem to inevitably emerge. Understanding how pathogens overcome host plant resistance is important for developing strategies to confer durable resistance to crops.

As described in chapter I, most of the reported recessive resistance genes in crops have been identified as *eukaryotic initiation factors* (eIFs), such as the eIF4E and eIF4G families of genes that control potyviruses (Truniger and Aranda 2009). In particular, the recessive resistance genes *cyv2* (resistance to ClYVV), *sbm-1*, *-3*, and *-4* (resistance to PSbMV), *wlv* (resistance to BYMV), in the pea, and $mo1^1$ and $mo1^2$ (resistant to LMV) have been identified as identical or allelic genes that encode eIF4E (Gao et al. 2004b, Bruun-Rasmussen et al. 2007, Andrade et al. 2009). eIF4E interacts with the potyviral genomelinked protein VPg at the surface, near a cap-binding pocket (Gao et al. 2004b, Léonard et al. 2004, Ruffel et al. 2004, Kang et al. 2005b, Ruffel et al. 2005, Charron et al. 2008). However, the eIF4Es derived from resistant plants failed to interact with VPg (Wittmann et al. 1997, Léonard et al. 2000, Yeam et al. 2007, Charron et al. 2008,), suggesting that eIF4E and its interaction with VPg are important for potyvirus infection. In fact, *Arabidopsis* knockout mutants of eIF4E and its isoform, eIF(iso)4E, are resistant

against potyviruses (Duprat et al. 2002, Lellis et al. 2002, Sato et al. 2005). Potyvirus mutants that overcame eIF4E-mediated resistance were reported to contain amino acid changes in VPg that restored its ability to bind to the eIF4E produced by the resistant plants (Gao et al. 2004b, Robaglia and Caranta 2006, Yeam et al. 2007, Charron et al. 2008). Besides VPg, other potyvirus proteins, the cylindrical inclusion (CI) protein (Abdul-Razzak et al. 2009, Tavert-Roudet et al. 2012), the helper-component proteases (HC-Pro) (Roudet-Tavert et al. 2007), and P1 (Nakahara et al. 2010) have also been reported to be involved in eIF4E-mediated recessive resistance. There is disagreement as to how eIF4E and these potyviral factors interact with one other in potyvirus infection and how these interaction networks are affected in resistant plants containing the recessive allele of eIF4E (Wang et al. 2012), so these topics require further examination. Tracking of GFP-tagged potyviruses revealed that avirulent potyviruses were defective in cell-to-cell movement at an early stage of virus infection, but it is not yet clear whether the defect was caused by direct inhibition of cell-to-cell movement or by inhibition of viral replication in the resistant plants (Candresse et al. 2002, Nicaise et al. 2003, Gao et al. 2004b, German-Retana et al. 2008, Nakahara et al. 2010).

There are other recessive resistance genes in pea that have not yet been cloned but do not seem to encode eIFs. The recessive resistance genes *cyv1* (resistance to ClYVV), *mo* (resistance to BYMV), and *sbm-2* (resistance to PSbMV) were mapped close to one another in pea LG II (Provvidenti and Hampton 1991, Choi et al. 2012). The *eIF(iso)4E* gene is also found in LG II (Gao et al. 2004a). However, the recessive genes (i.e.,*cyv1*, *mo*, and *sbm-2*) are not likely to be *eIF(iso)4E* because there was no difference in the deduced amino acid sequences of the eIF(iso)4E proteins between susceptible and resistant pea lines (Gao et al. 2004a, Choi et al. 2012). Thus, if virus isolates, which overcome these recessive resistance genes, emerge the viral determinant responsible for

resistant breaking might not be VPg. In fact, the resistance conferred by *sbm-2* was overcome by PSbMV mutants that have mutations corresponding to the N-terminal part of the P3 protein (Johansen et al. 2001, Hjulsager et al. 2006). In this study, the breaking of *cyv1* resistance by ClYVV was attributed to the P3 cistron.

Until recently, the functions of P3 have been poorly understood. The functional P3 protein was found to be recruited to the potyvirus replication complex (Merits et al. 1999) and to be involved in accumulation of the virus (Klein et al. 1994, Suehiro et al. 2004), symptomatology (Jenner et al. 2003, Kim et al. 2010), viral microtubule-related inter- and intracellular movement (Cui et al. 2010), and determination of host range (Suehiro et al. 2004). Few examples of interaction of P3 with host factors have been reported. However, RubisCO directly bound to P3, which led to decreases in plant chlorophyll contents and photosynthesis (Shen et al. 2010, Lin et al. 2011). A small open reading frame (ORF) called PIPO (for Pretty Interesting *Potyviridae* ORF) was found to be embedded in the P3 cistron; this ORF can form a functional protein, P3N-PIPO, comprising the N-terminal amino acids of P3 followed by the amino acids encoded by PIPO, which are added presumably through a +2 (or −1) ribosomal frameshift or transcriptional slippage (Chung et al. 2008, Vijayapalani et al. 2012). Thus, P3N-PIPO has the same N-terminal part (P3N) as P3 and P3N-PIPO of *Turnip mosaic virus* (TuMV) appears to interact with CI in the plasmodesmata of *Nicotiana benthamiana* and facilitate movement of viral RNA to neighboring cells (Wei et al. 2010b, Niehl and Heinlein 2011, Vijayapalani et al. 2012). These studies indicate that both P3 and P3N-PIPO are essential proteins for potyvirus infection and thus have the potential to be the virulence determinant in viruses that can overcome resistance in *cyv1* and *sbm-2* pea.

Previous studies have not revealed whether breaking of the *sbm-2* resistance involves

P3, P3N-PIPO, or both (Hjulsager et al. 2006). Here, I genetically examined whether either P3 or P3N-PIPO was involved in breaking the *cyv1* resistance by ClYVV. I then investigated the contribution of the P3 and P3N-PIPO proteins to breaking the resistance by producing these proteins in *trans*, using either an expression cassette containing the 35S promoter or the *White clover mosaic virus* (WClMV) vector (Ido et al. 2012) and introducing these into plants along with the ClYVV isolate that is normally avirulent to *cyv1* pea.

Materials and Methods

Construction of chimeric viruses and P3 transient expression cassettes

Seven chimeric viruses (Cl-P1HC, Cl-BB, Cl-NS, Cl-SB, RB [resistance breaking construct], RB/P3^{KII}PIPO^{Q62}, and Cl/P3^{RAM}) were generated by replacing regions of Cl-no30 with the corresponding regions of 90-1 Br2. The cDNA sequences of 90-1 Br2 were amplified by reverse transcription (RT)-PCR using KOD-Plus 2 DNA polymerase (Toyobo, Osaka, Japan) with the following primers: UTR3′-s (5′-GGATTGATGTGATATCTCCACTGACGTAAG-3′), HCpro3′-as (5′-GTTGCAAGTTCTCTCGTACC-3′), HCbgl5′-s (5′-ACGTGTACATCAGATCTCAATGTAAT-3′), P3bgl3′-as (5′-TTTTGATGTGAGATCTCACTTGACTC-3′), P3nhe3′-s (5′-TTCTTAATGTGCTAGCAACAATTACG-3′), VPsal5′-as (5′-TAGTCGCGCAAACCACTTATGCGATTTAGA-3′), VPsal5′-s (5′-ACCATACAAGCTGAGGGACTCAAATGTTGT-3′), and Polbam3′-as

(5′-AGCTTGGGATCCTTTTTTTTTTCTCGCTCTATAAAGATCA-3′). Recombinant plasmids Cl-P1HC, Cl-BB, Cl-NS, and Cl-SB (Fig. 2-1) were created by insertion of 90-1 Br2 cDNA sequences into the following restriction sites: for Cl-P1HC, the *Eco*RV site at the 5′untranslated region (UTR) and the *Bgl*II site at the beginning of HC-Pro; for Cl-BB, the *Bgl*II sites within the HC-Pro and P3 coding regions; for Cl-NS, the *Nhe*I site in the center of P3 and the *Sal*I site in the center of VPg; and for Cl-SB, the *Sal*I site in VPg and the *Bam*HI site downstream of the poly(A) sequence.

The chimeric viruses RB, RB/P3^{KII}PIPO^{Q62}, and Cl/P3^{RAM}, which contain the P3 cDNA fragment of 90-1 Br2, were generated by the following steps. To generate RB, the chimeric cDNA fragment composed of HC-Pro of Cl-no30 and P3 of 90-1 Br2 within the *Bgl*II sites on HC-Pro and P3 was made by two-step PCRs with primers no30bgl-s (5′-CAGATCTCAATGTAGTCCAATGTGGTTCTG-3′), no30br2-as (5′-CCAACTCTGTAAAACTTCAAATCTGACTC-3′), no30br2-s (5′-GAGTCAGATTTGAAGTTTTACAGAGTTGG-3′), and br2bgl-as (5′-CAGATCTCACTTGACTCAGAATGC-3′). For the first step, primer pairs no30bgl-s/no30br2-as and no30br2-s/br2bgl-as were used to amplify the cistrons encoding HC-Pro and P3, respectively. Then the cDNAs of HC-Pro and P3 were fused with PCR by using primer pair no30bgl-s and br2bgl-as. The fragment was cloned into a pGEM-T Easy vector (Promega, Madison, WI) to make the chimeric virus RB.

To make RB/P3^{KII}PIPO^{Q62} and Cl/P3^{RAM}, the HC-Pro-P3 fragments were amplified by two-step PCR as described above with the primers, except for br2P3_644-s (5′-GTAACAAGCATCAAGAATCAAGCAATC-3′) and br2P3_644-as (5′-GATTGCTTGATTCTTGATGCTTGTTAC-3′) for RB/P3^{KII}PIPO^{Q62}, and br2RAM-s (5′-GCAACAAGCATTAAAAACCAAGCAATC-3′) and br2RAM-as (5′-GATTGCTTGGTTTTTAATGCTTGTTGC-3′) for Cl/P3^{RAM} instead of no30br2-s

and no30br2-as. These cloned fragments were inserted into vector Cl-no30 after digestion with *Bgl*II.

To express in *trans* the genes for the proteins encoded by the P3 cistrons of Cl-no30 and RB, two different systems were used: the plant transient expression vector pE2113, which contains a 35S promoter for transgene expression (Mitsuhara et al. 1996), and the WClMVvector (Ido et al. 2012). The restriction enzyme sites of *Bam*HI-*Sac*I and *Spe*I-*Xho*I were added to the 5′ and 3′ terminis of cDNA fragments of the P3 cistrons to insert them into the pE2113 and WClMV vectors, respectively (Ido et al. 2012, Mitsuhara et al. 1996). To construct P3 cistrons that exclusively produce the P3N-PIPO protein (designated no30_P3N-PIPO and RB_P3N-PIPO), an adenosine nucleotide (underlined) was inserted at position 464 in G_2A_6, around which a frameshifting is considered to occur (GGAAAAAA→GG<u>A</u>AAAAAA) (Chung et al. 2008). To construct P3 cistrons that exclusively produce the P3 protein (designated no30_P3ΔPIPO and RB_P3ΔPIPO), one nucleotide (underlined) was changed at position 477 to generate a stop codon in the PIPO reading frame (AGA→<u>T</u>GA) without causing an amino acid change in the P3 ORF.

To additionally express P3 or P3N-PIPO from Cl-no30 in *cis*, no30_P3ΔPIPO and no30_P3N-PIPO were inserted into the *Pst*I site of the infectious clone pClYVV-SeIF4E-GFP (Andrade et al. 2009), instead of SeIF4E, as follows. The *Pst*I site followed by the P1 C-terminal fragment and *Pst*I-NIa-Pro digestion site were attached to the 5′ and 3′ termini of the no30-P3ΔPIPO cDNA sequence by two-step PCR as described above using the primers no30_P1-Pst_s (5′-GCGCGCCTGCAGAAAAACTGAAAGTG-3′), Cl-no30P1P3_as (5′-GTCAATGATTTGCCAGAGAATTCT-3′), Cl-no30P1P3_s (5′-AGAATTCTCTGGCAAATCATTGAC-3′), and Cl-no30P3Cter-NIa_as

(5′-GCGCCTGCAGATTGGAAAACAAATTTCATTTCCATGACAAACCACTTTGG TTC-3′). The amplified fragments were inserted into the vector pClYVV-SeIF4E-GFP after digestion with *Pst*I. The resulting construct was designated Cl/P3ΔPIPO (see Fig. 6B).

Cl/P3N-PIPO was also generated using the primers no30_P1-Pst_s, Cl-no30P1P3_as, Cl-no30P1P3_s, and Cl-no30P3NPIPO-NIa_as (5′-GCGCCTGCAGATTGGAAAACAAATTTCATATGCTTGTTACTGAC-3′).

Virus inoculation and detection

I examined the ability of the constructed ClYVV chimeras to systemically infect *cyv1* pea plants as follows. The ClYVV cultures were recovered in broad beans (*Vicia faba*), which were biolistically inoculated with the constructed ClYVV infectious plasmids as described by Andrade et al. (2007). Infected upper leaves of broad bean were stored in a deep freezer and used as inoculum for inoculation of *cyv1* pea plants as described previously (Andrade et al. 2007). Second to fourth upper leaves from bottom of 2 weeks old pea plants carrying *cyv1* were mechanically inoculated. The movement of each virus was monitored by GFP fluorescence, which was observed for 5 days postinoculation (dpi) using an epifluorescence microscope (VB 7010; Keyence, Osaka, Japan). The presence of chimeric viruses in the upper leaves was also detected by RT-PCR, as described in reference Choi et al. (2012). For RT-nested PCR, the first PCR was performed with primers designed based on *NIb* of ClYVV 5′-CTTTAGACCTATGATGGGC-3′ (sense) and 5′-GTTCAAGCCCAATTCTTTG-3′ (antisense). For the second PCR, the sense primer of first PCR and 5′-GTATATATGATCTCCGTGTAC-3′ (antisense) were used to detect ClYVV with greater sensitivity.

I tested the infectivity of the constructed ClYVV chimeras and of Cl-no30 with additional production in *trans* of proteins derived from P3 cistrons as follows. Biolistic inoculation of detached leaves of a *cyv1* pea line (PI 429853) with the ClYVV chimeric clones or coinoculation of the Cl-no30 infectious clone with the pE2113 vectors was performed essentially as described by Andrade et al. (Andrade et al. 2007). Tungsten particles were coated either with 1μg of a ClYVV chimeric plasmid clone or with a mixture of 997 ng pCl-no30 and 3 ng of cloned pE2113 vector containing a P3 cistron (P3, P3N-PIPO, or P3ΔPIPO), and the leaves of cultivar PI 429853 were bombarded with them. The virus infection was monitored by GFP fluorescence from Cl-no30. The GFP fluorescence was observed for 5 dpi, as described above.

I investigated the systemic infection of Cl-no30 coinoculated with WClMV vectors designed to exclusively produce the P3N-PIPO protein in the leaves of *cyv1* pea plants. Biolistic coinoculation of *cyv1* pea was performed with plasmid mixtures containing 800 ng of pCl-no30 and 200 ng of the WClMV expression vector carrying either no30_P3N-PIPO or RB_P3N-PIPO. Infection by Cl-no30 was monitored by using GFP fluorescence and by RT-PCR and RT-nested PCR for ClYVV NIb, as described above. To monitor infection by WClMV, RT-PCR using primers specific for the coat protein (CP) of WClMV (i.e., CP-Nort-head and CP-Norttail) was performed as described by Ido (2012). RT-PCR using primers specific for the multiple-cloning site of WClMV, f (Ido et al. 2012) and Cl-P3-574as (5′-CTCTGGGCTAGTATTTTGGAATAC-3′ [antisense]) confirmed the stability of P3N-PIPO expression from the WClMV vector.

Antibody production and Western blotting

To detect the P3NPIPO protein by immunoblotting, antibodies were raised against the PIPO amino acid sequence fused with the maltose binding protein (MBP), which

was produced by using the expression vector pMAL-c2 (New England BioLabs, Beverley, MA). To generate this protein, the last 186 nucleotides of the P3N-PIPO ORF from Cl-no30, including the stop codon, were inserted downstream from the *malE* gene (which encodes MBP) of pMAL-c2, and the vector was transformed into *E. coli* JM109. The transformants were cultured for 14 h in LB broth containing 50 μg/ml ampicillin. The production of the gene's fusion protein was induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) in fresh LB broth at 35 °C for 4 h. Cell lysates were added to a 10-fold volume of 2×Laemmli buffer, heated for 3 min at 100°C, subjected to SDS-PAGE electrophoresis on a 10% Tris gel for 40 min, and stained with Coomassie brilliant blue. The MBP-fused PIPO protein (around 50 kDa) was used as the PIPO antigen. To confirm that the newly generated antibody was able to detect PIPO, a PIPO cDNA sequence lacking the G_2A_6 motif, which might cause a frameshifting, was inserted downstream of the region encoding glutathione *S*-transferase (GST) in expression vector pGEX (GE Healthcare, Piscataway, NJ). The GST-fused PIPO was produced and subjected to SDS-PAGE as described above. The band, which migrated at around 33 kDa, was specifically detected by immunoblotting with the antibody as described previously (Atsumi et al. 2012), indicating that this antibody is able to detect PIPO (Fig. 2-7B). I later found that the P3N-PIPO antibody could simultaneously detect both P3 and P3N-PIPO. I speculate that a ribosomal frameshift might have occurred in theG_2A_6 motif at the beginning of the PIPO sequence, resulting in the production of an MBP-fused P3 peptide along with the MBP-fused PIPO protein. The motif A_6G_1 has been reported to stimulate a -1 frameshift in *E. coli* (Baranov et al. 2001).

To sensitively detect the P3N-PIPO protein derived from the infecting ClYVV in pea, a urea-containing buffer was used to prepare the samples for immunoblotting, as

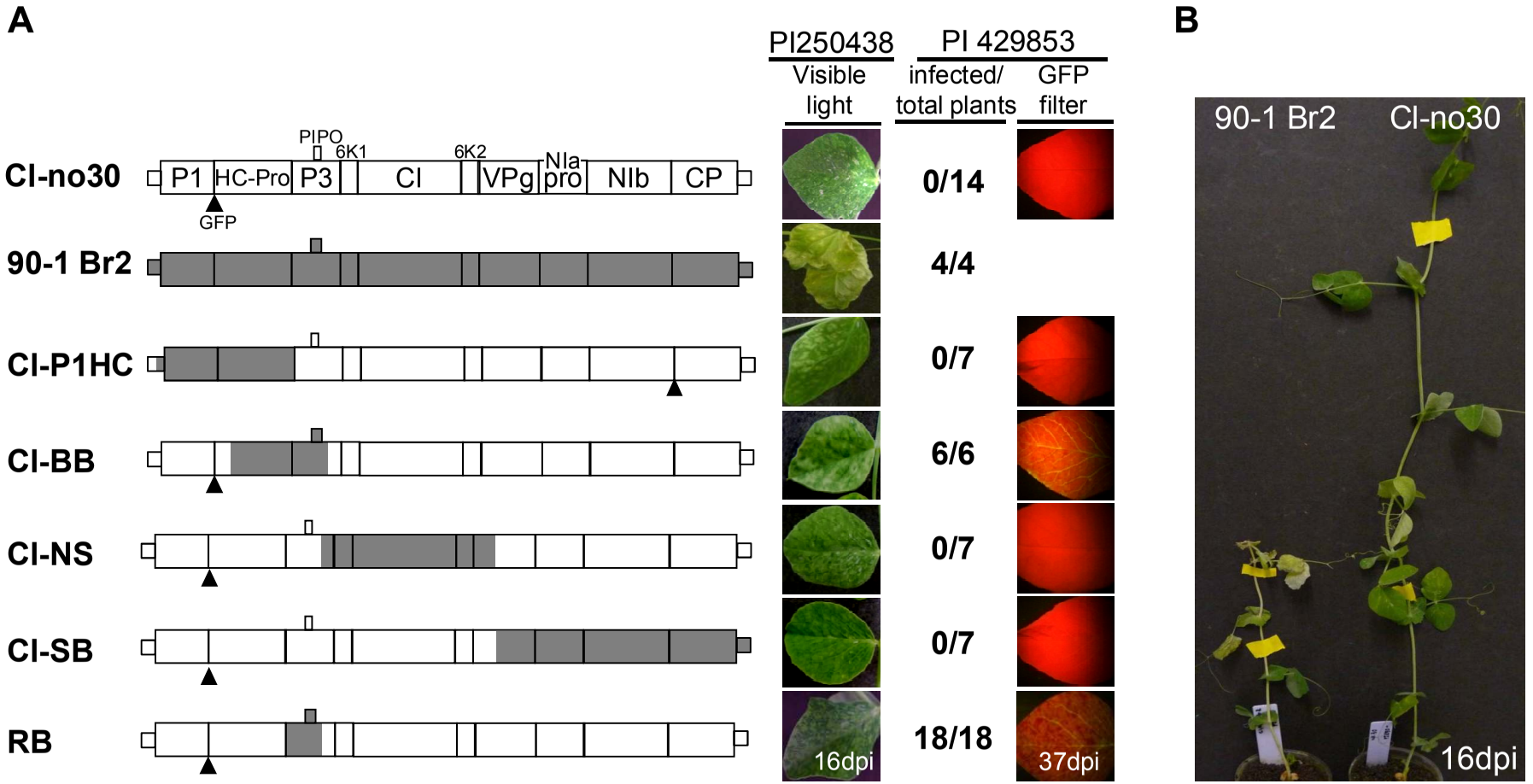


FIG 2-1 Mapping of the ClYVV genomic region responsible for overcoming the *cyv1*-mediated resistance. A. The diagrams on the left illustrate chimeric constructs based on ClYVV isolate no. 30 (Cl-no30; white) containing nucleotide sequences from 90-1 Br2 (dark gray). The pathogenicity of the chimeric constructs on *cyv1* pea plants was investigated by observing the GFP fluorescence of the uninoculated upper leaves for 37 dpi. Pea cultivar PI 429853 carrying *cyv1* (Choi et al 2012) was resistant to Cl-no30. The chimeric viruses designated Cl-BB and RB infected *cyv1* pea systemically, indicating that the P3 cistron of 90-1 BR2 was responsible for breaking the resistance. The figure for PI 429853 indicates the number of infected plants out of the total number of inoculated plants. The infectivity of the constructed chimeric ClYVVs was confirmed by inoculation into a susceptible pea line (PI 250438). Typical symptoms of PI 250438 leaves infected with the constructed chimeras are shown. **B**. Different reactions to Cl-no30 and 90-1 Br2 were observed in a susceptible pea cultivar (PI 250438). The resistance-breaking isolate 90-1 Br2 caused severe dwarfism and mosaic symptoms, whereas Cl-no30 caused only vein yellowing and mild mosaic symptoms.

described previously (Nakahara et al. 2012). Uninoculated upper leaves with symptoms were harvested at 9 dpi, ground in liquid nitrogen, and denatured in 12-fold (wt/vol) urea denaturing buffer containing 4.5 M urea, 1%(vol/vol) Triton X-100, 0.5% dithiothreitol (DTT), 0.00625M Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 5% mercaptoethanol, 5% sucrose, and 0.002% bromophenol blue. The plant lysates were analyzed by SDS-PAGE, as described by Atsumi et al. (2012).

Results

The breaking of the *cyv1* resistance by the ClYVV isolate 90-1 Br2 was attributed to the region encoding the P3 and P3N-PIPO proteins.

Pisum sativum cultivar PI 429853, which carries recessive resistance gene *cyv1*, is resistant to Cl-no30 (Choi et al. 2012). The resistance of *cyv1* can be broken by the isolate 90-1 Br2; *cyv1* pea plants infected with 90-1 Br2 showed chlorotic spots on the upper leaves at 5 weeks postinoculation (wpi). 90-1 Br2 induced more severe symptoms, including dwarfing and necrosis, in susceptible pea cultivar PI 250438 (Andrade et al. 2007) than did Cl-no30 (Fig. 2-1B). To address which gene is responsible for breaking of the *cyv1* resistance, the entire nucleotide sequences of the polyprotein-coding regions were compared between Cl-no30 (AB011819) and 90-1 Br2 (AB732962) and found 483 nucleotide differences dispersed throughout the genomes.

I then created four chimeric viruses in which sequences from the Cl-no30 infectious clone were replaced with the corresponding sequences from 90-1 Br2 (Sato et al. 2003); these chimeras contained the 90-1 Br2 sequence from the 5′ UTR to HC-Pro (Cl-P1HC),

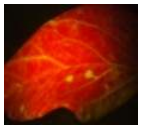
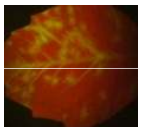
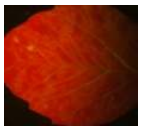
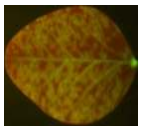
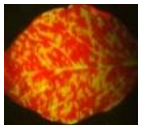
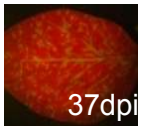
Pea line	Inoculated plants		
	Cl-no30	RB	
	Infection profiles	Infection profiles	GFP filter
PI 236493	R	S	
PI 347420	R	S	
PI 347422	R	S	
PI 429853	R	S	
PI 347295 RS-7	R	S	
PI 347295 R-18	R	S	
PI 116843 R-8	R	S	

FIG 2-2 Pea lines that showed resistance to Cl-no30 were inoculated with RB. PI 236493, PI 347420, PI 347422, and PI 429853 carry *cyv1* (Choi et al 2012); the other lines were presumed to carry *cyv1* because these lines did not carry *cyv2* and RB broke the resistance of all of the tested pea lines around 5 wpi. R, resistant to the inoculated virus; S, susceptible to the inoculated virus.

from HC-Pro to P3 (Cl-BB), from P3 to 6K2 (Cl-NS), and from 6K2 to 3′-poly(A) (Cl-SB) (Fig. 2-1). These four viruses were inoculated into the *cyv1* pea cultivar PI 429853 and observed for 6 weeks post inoculation (wpi). Five wpi, the pea plants inoculated with Cl-BB showed chlorotic spots and GFP fluorescence in the upper leaves (Fig. 2-1), but the other chimeric viruses could not overcome the *cyv1* resistance (Fig. 2-1). The virulence of Cl-BB against *cyv1* appeared to be comparable to that of 90-1 Br2 (Fig. 2-1). Cl-BB contained most of the HC-Pro cistron sequence of 90-1 Br2, but this sequence was also present in Cl-P1HC, which could not overcome the *cyv1*-mediated resistance, so I hypothesized that breaking of the resistance by Cl-BB was caused by the chimeric sequence of the P3 cistron, which was unique to Cl-BB. To test this hypothesis, one more chimeric ClYVV was created, called RB (for resistance breaking), which contained the same part of the P3 cistron from 90-1 Br2 as was present in Cl-BB, and I confirmed that RB broke the *cyv1* resistance (Fig. 2-1 and 2-2), as did 90-1 Br2 and Cl-BB. Thus, I concluded that the P3 cistron of 90-1 Br2 was responsible for its ability to break the resistance conferred by *cyv1*.

Both P3 and P3N-PIPO were involved in the breaking of *cyv1* resistance.

The P3 cistron has been reported to produce two proteins, P3 and P3N-PIPO, which have the same N-terminal sequence (Chung et al. 2008, Vijayapalani et al. 2012). Thus, experiments were designed to test which protein is responsible for breaking the resistance. There were 58 nucleotide differences between the mapped P3 regions of Cl-no30 and RB (Fig. 2-3). A total of 47 were synonymous with respect to both proteins of P3 and PIPO, and 9 caused seven differences in amino acid residues in P3 but not in PIPO (Fig. 2-3A, yellow boxes, and B). (Hereafter, “PIPO” refers to the amino acid region unique to P3N-PIPO.) Two of the nucleotide differences in P3 were synonymous

with the P3 protein but caused two differences in the amino acid sequences of PIPO (Fig. 2-3A, orange boxes, and C). None of the nucleotide differences caused a change in amino acid residue simultaneously in P3 and PIPO. Thus, the nucleotide sequence comparisons did not indicate whether P3N-PIPO, P3, or both are involved in the breaking of *cyv1*-mediated resistance.

I further investigated the possible role of P3N-PIPO in breaking the resistance. To do this, I used two nucleotide sequence differences that caused an amino acid change in PIPO between Cl-no30 and RB but did not affect the amino acid sequence of P3. The PIPO point mutants of Cl-no30 and RB were made by introducing either or both of the nucleotide substitutions into the genomic cDNA of the infectious clones. The point mutants were independently inoculated into *cyv1* pea plants, and the ability of the clones to infect the upper (uninoculated) leaves was tested by means of GFP fluorescence observation, RT-PCR, and RT-nested PCR. These mutations, corresponding to amino acids 38 and 62 of PIPO, altered the virulence of both Cl-no30 and RB in the *cyv1* pea plants. A substitution (underlined) at position 38 in the PIPO amino acid sequence of RB (ATT [isoleucine] → A<u>C</u>T [threonine]; RB/PIPO^{I38T}) dramatically reduced its virulence in *cyv1* pea, but the opposite substitution (threonine to isoleucine) in Cl-no30 (Cl/PIPO^{T38I}) had no effect (Fig. 2-4A). Conversely, a substitution at position 62 (TAA [stop codon] → <u>C</u>AA [glutamine]; Cl/PIPO^{62Q}) partially conferred virulence on Cl-no30, but the opposite substitution (glutamine to a stop codon; RB/PIPO^{Q62}) had no detrimental effect on RB. The mutants in which both substitutions were simultaneously induced (Cl/PIPO$^{T38I, 62Q}$ and RB/PIPO$^{I38T, Q62}$) had similar virulence to the single-point mutants Cl/PIPO^{62Q} and RB/PIPO^{I38T}, respectively (Fig. 2-4A). These results genetically demonstrated the involvement of P3N-PIPO in the breaking of the resistance in *cyv1* pea. In addition to 90-1 Br2, I found three other ClYVV isolates (I89-1

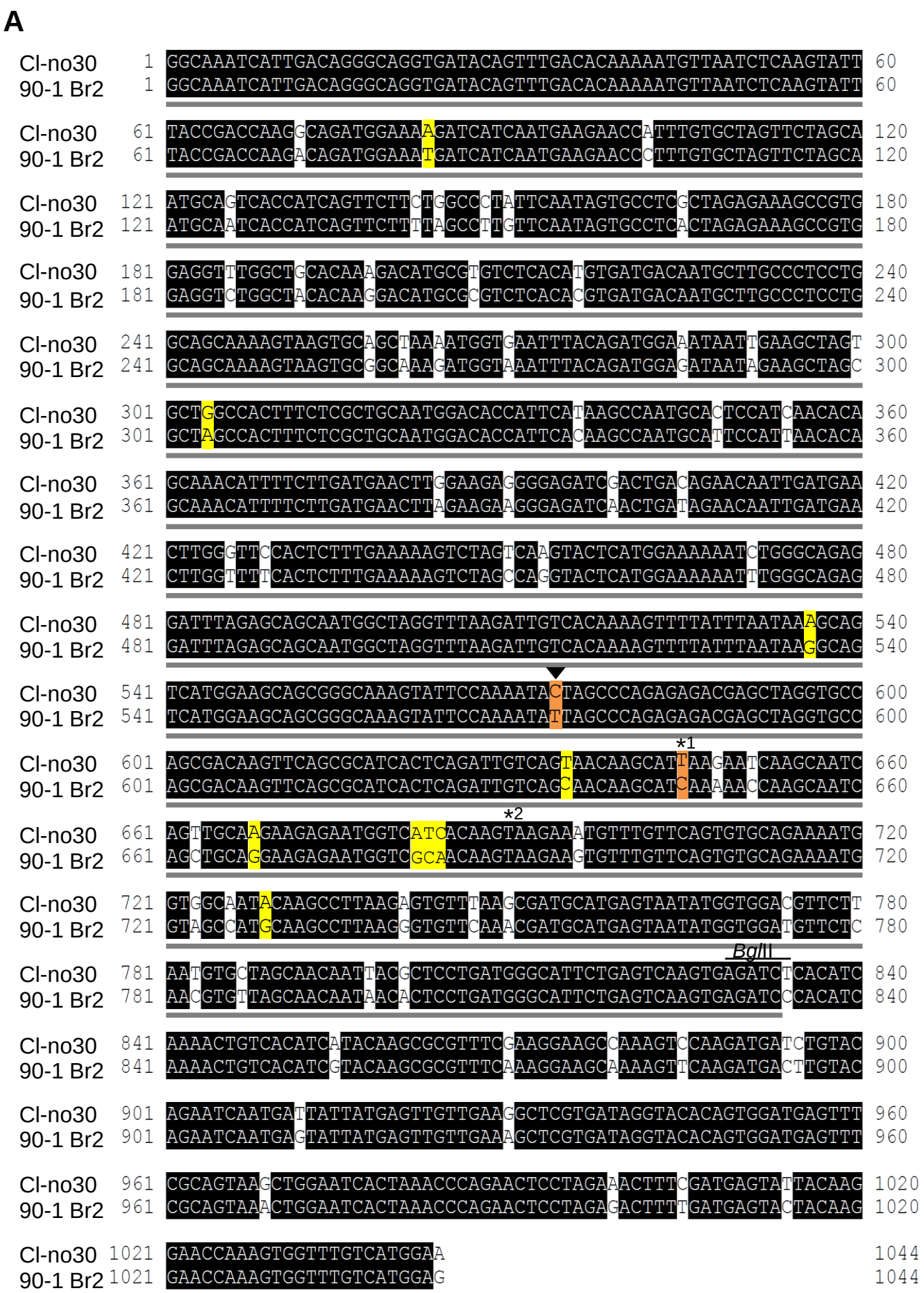


FIG 2-3 Comparison of the nucleotide and amino acid sequences encoded by the P3 cistrons in avirulent (Cl-no30) and resistance-breaking (90-1 Br2) isolates. (Continued)

B

FIG 2-3 Comparison of the nucleotide and amino acid sequences encoded by the P3 cistrons in avirulent (Cl-no30) and resistance-breaking (90-1 Br2) isolates. (Continued)

C

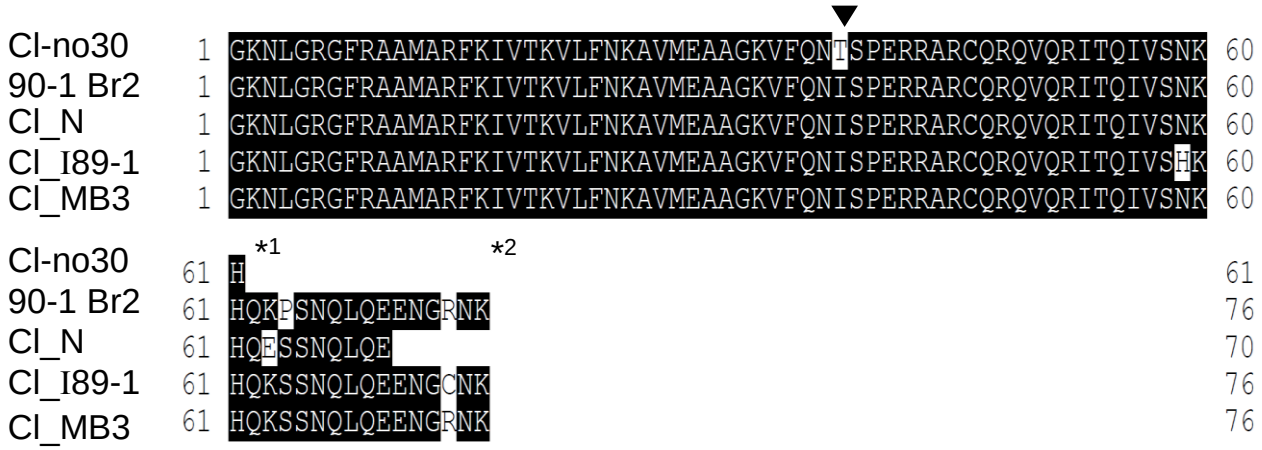


FIG 2-3 Comparison of the nucleotide and amino acid sequences encoded by the P3 cistrons in avirulent (Cl-no30) and resistance-breaking (90-1 Br2) isolates. A. Alignment of the nucleotide sequences of P3 from Cl-no30 and 90-1 Br2. 90-1 Br2 possesses 58 nucleotide differences, including synonymous differences, compared with Cl-no30 in the mapped P3 region (underlined in gray). Yellow boxes indicate nonsynonymous differences with respect to the P3 amino acid sequences but synonymous with respect to the PIPO amino acid sequences. Orange boxes indicate nonsynonymous differences with respect to the PIPO amino acid sequences but synonymous with respect to the P3 amino acid sequences. The black triangle indicates the position of a nonsynonymous difference at position 38 in the PIPO amino acid sequences among ClYVV isolates (see also panel C). Asterisk 1 corresponds to the end of PIPO in Cl-no30 and to position 62 in the 90-1 Br2 PIPO amino acid sequence. Asterisk 2 corresponds to the end of PIPO from 90-1 Br2. RB possesses the genomic sequence of the 90-1 Br2 P3 cistron upstream of the Bgl site (underlined in gray). **B**. Alignment of the P3 amino acid sequences from Cl-no30 and 90-1 Br2. The black triangle, asterisks 1 and 2, and gray underlining are shown in the positions corresponding to those in panel A. **C**. Comparison of the PIPO amino acid sequences from ClYVV isolate Cl-no30, which was avirulent to *cyv1* pea, and others that sometimes broke *cyv1* resistance. Compared with Cl-no30, the *cyv1* resistance-breaking isolates possessed two differences in amino acid sequence: a change from T to I at position 38 (black triangle) and an addition of 9 to 15 amino acids beginning at position 62 (asterisk 1). ClYVV isolates 90-1 Br2,N(accession no. AB732964), I89-1 (AB732963), andMB3(AB732965) induced necrosis or mosaic symptoms in *cyv1* pea when they broke cyv1 resistance (data not shown). Asterisk 2 is shown at the position corresponding to that in panel A (i.e., the end of 90-1 Br2 PIPO).

[AB732963], N [AB732964], and MB3 [AB732965]) that could break the *cyv1* resistance. Among these four isolates, both isoleucine 38 and glutamine 62 were conserved (Fig. 2-3C), supporting the importance of these positions for breaking the *cyv1* resistance. However, with respect to the infection of *cyv1*pea plants, none of the point mutants of Cl-no30 tested here gained virulence that was comparable to that of RB, and none of the point mutants of RB completely lost systemic infectivity to the degree seen in Cl-no30, suggesting the involvement of other parts of the P3N-PIPO protein or of P3 in breaking the resistance.

I thus investigated the possible role of the P3 protein in breaking resistance. *P3N-PIPO* of Cl-no30 shares 644 nucleotide sequences with *P3* of Cl-no30 (Fig. 2-3A and diagram of Cl-no30 and RB on Fig. 2-4A). Only 400 nucleotide sequences of *P3* did not share its sequences with *P3N-PIPO* and it caused 3 non-synonymous in amino acid residues in P3 (Fig. 2-3A and B). Although this region with respect to RB contains 48 nucleotide sequences of both *P3* and *P3N-PIPO* (Fig. 2-3A, from asterisk 1 to asterisk 2), 400 nucleotide sequences were introduced into Cl-no30 and vice versa (Fig. 2-4B). An RB mutant in which nucleic acid substitutions changed 4 amino acid residues in the P3 protein and eliminated the final 15 amino acid residues in the PIPO protein by adding a stop codon (RB/P3^{KII}PIPO^{Q62}) (Fig 2-4B) reduced virulence in *cyv1* pea. Since elimination of the final 15 amino acid residues alone (RB/PIPO^{Q62}) (Fig. 2-4A) did not affect the virulence of RB, the reduced virulence of RB/P3^{KII}PIPO^{Q62} was assumed to be caused by the four amino acid substitutions in the P3 protein. A Cl-no30 mutant containing three amino acid substitutions that only affected the P3 protein (Cl/P3^{RAM}) (Fig. 2-4B), making it more like that of RB, was able to partially break the *cyv1* resistance (Fig. 2-4B). The results indicated that the P3 protein is also involved in breaking the resistance conferred by *cyv1*.

I obtained additional genetic evidence indicating that both P3 and P3N-PIPO were important for breaking the resistance. Pea plants carrying *cyv1* showed resistance to ClYVV isolate 90-1, but it could sometimes overcome *cyv1* resistance. Isolate 90-1 Br2 emerged from systemic leaves of such a *cyv1* pea plant infected with 90-1. Comparing the genomic sequence of the P3 cistron of 90-1 with that of 90-1 Br2, only one point mutation was found that caused a substitution (arginine to methionine) at position 28 (P3 and P3N-PIPO^{R28M}) of the P3 and P3N-PIPO proteins of 90-1, implying that this point mutation enabled 90-1 Br2 to overcome *cyv1*. As expected, a point mutant of RB in which the methionine at position 28 was substituted for with arginine (RB/P3 and P3N-PIPO^{M28R}) failed to overcome the *cyv1* resistance (Fig. 2-4C). However, the difference between RB and Cl-no30 in virulence to *cyv1* pea could not be attributed exclusively to the point mutation at position 28. The amino acid residue at position 28 of P3 in Cl-no30 was found to be lysine, so I introduced mutations into Cl-no30 and RB that changed the lysine of P3 and P3N-PIPO of Cl-no30 to methionine (Cl/P3&P3N-PIPO^{K28M}) and the methionine of these proteins in RB to lysine (RB/P3&P3NPIPO^{M28K}) and inoculated these viruses into *cyv1* pea plants (Fig. 2-4C). In addition, comparing the genomic sequence of the P3 cistron of RB/PIPO^{Q62} and 30 progenies of RB/PIPO^{Q62} from infected *cyv1* peas, 31 nucleotide substitutions were sporadically found at the P3 cistron of 21 progenies (Fig. 2-5). These substitutions did not affect virulence in *cyv1* pea; Cl/P3&P3N-PIPO^{K28M} still failed to overcome the *cyv1*-mediated resistance, and RB/P3&P3N-PIPO^{M28K} and RB/PIPO^{Q62} still infected the *cyv1* pea plants systemically (Fig. 2-4C).

Importance of P3N-PIPO for ClYVV cell-to-cell movement in *cyv1* pea.

I have previously shown that *cyv1* restricts Cl-no30 to single cells (Chapter I, Choi et

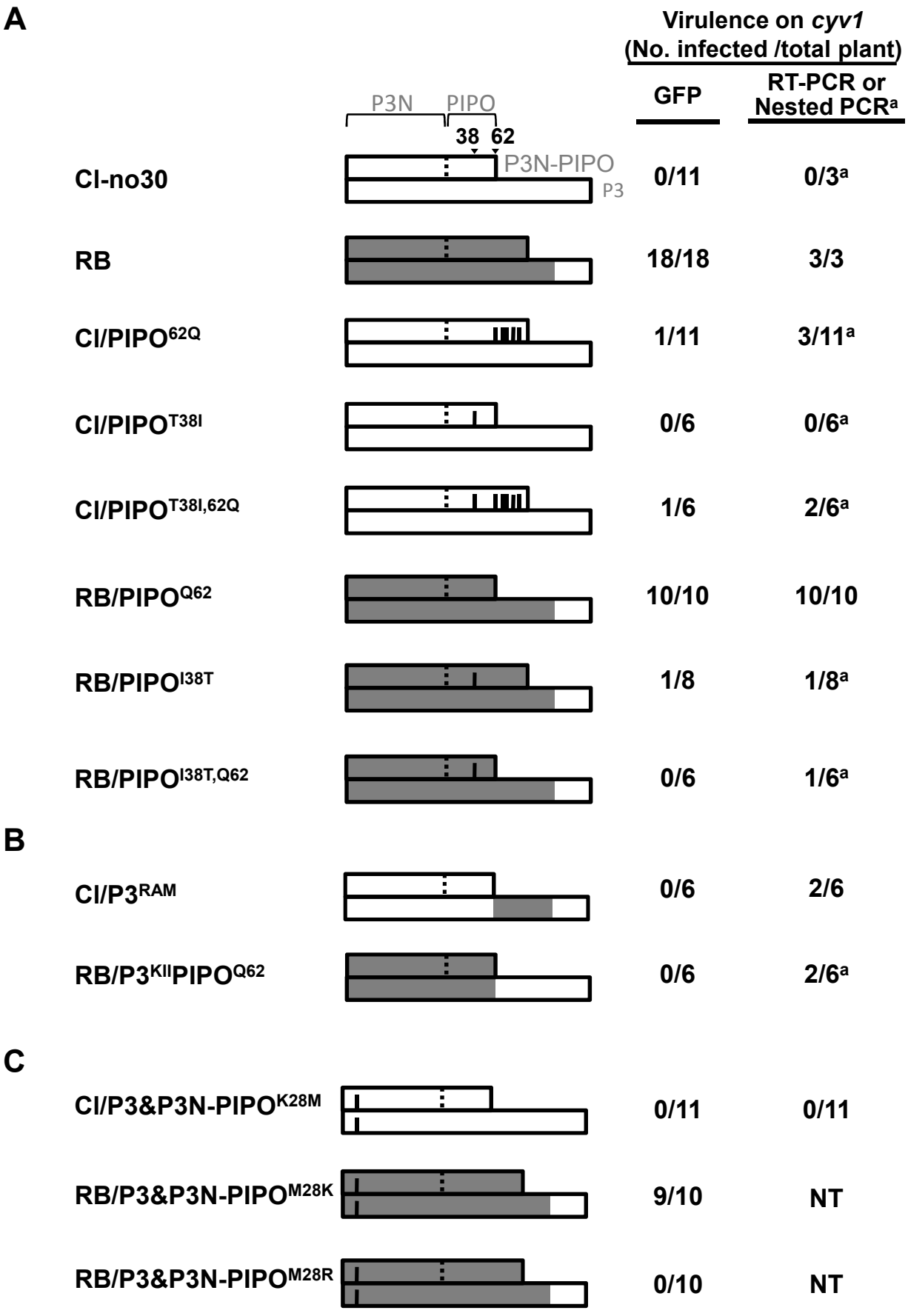


FIG 2-4 Genetic analysis of the roles of P3 and P3N-PIPO in the breaking of *cyv1* resistance (Continued).

FIG 2-4 Genetic analysis of the roles of P3 and P3N-PIPO in the breaking of *cyv1* resistance. A. Examination of the role of P3N-PIPO. Nucleotide differences were introduced into the P3 cistrons of Cl-no30 and RB that caused differences in the PIPO amino acid sequence at amino acids 38 and 62, but did not affect the sequence of P3. The P3N-PIPO of Cl-no30 is 15 amino acid residues shorter than that of RB. Substitutions at one or both positions were introduced into Cl-no30 and RB infectious clones, which were then inoculated into *cyv1* pea plants. The substitution at position 62 of Cl-no30 (TAA [stop codon] $\rightarrow$ CAA [glutamine] in constructs Cl/PIPO$^{T38I,\ 62Q}$ and Cl/PIPO^{62Q}) resulted in four differences in the amino acid sequence of P3N-PIPO (black lines) compared with that of RB. The pathogenicity of these clones was monitored by GFP fluorescence and either RT-PCR or RT-nested PCR (results marked with superscript "a"), which amplified the cDNA sequence of ClYVV NIb in RNA extracts from the upper leaves of inoculated plants at 35 dpi. **B**. Examination of the role of P3 in the breaking of *cyv1* resistance in pea. In RB/P3^{KII}PIPO^{62Q}, the PIPO coding region and upstream region of RB P3 were exchanged with the corresponding region of Cl-no30. In Cl/P3^{RAM}, a region downstream of PIPO of Cl-no30 was replaced with the corresponding sequence from RB. The pathogenicity of the ClYVV mutants was checked as in panel A. **C**. Role of the N-terminal sequence of P3 in the breaking of *cyv1 r*esistance. Isolate 90-1, from which 90-1 Br2 originated, is avirulent to pea lines carrying *cyv1* and possesses arginine (R) at position 28 in the N-terminal region of P3; isolate 90-1 Br2, which is virulent to *cyv1* pea lines, has methionine (M) at this position. Cl-no30 contains lysine (K) at position 28. Nucleotide substitutions were introduced at this position in Cl-no30 and RB and designated Cl/P3&P3N-PIPO^{K28M}, RB/P3&P3N-PIPO^{M28K}, and RB/P3&P3N-PIPO^{M28R}, each containing the indicated substitution. These mutants were independently inoculated onto *cyv1* and their virulence was examined as in panel A. Black bars indicate the amino acid substitution at position 28 in the N terminus of P3. NT, not tested. When the Cl-no30 and RB point mutants used in Fig 2-4 were inoculated into a susceptible pea line (PI 250438), they infected the uninoculated upper leaves at 1 wpi, indicating their similar infectivity in a susceptible pea line.

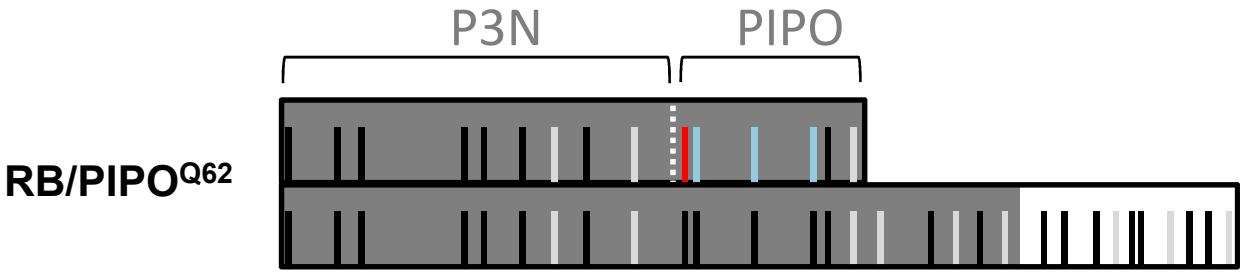


FIG 2-5 Additional genomic mutations found in progenies of RB/PIPO^{Q62} that broke the *cyv1* resistance. The P3 cistron from upper leaf of infected *cyv1* peas (PI 429853) with RB/PIPO^{Q62} was amplified and its sequence was analyzed. 30 progenies from infected *cyv1* peas contains a lot of substitutions on the P3 cistron. Gray bar indicates synonymous substitutions in both P3 and P3N-PIPO. Black bar indicates non-synonymous substitutions in P3 or P3N-PIPO and both of them. Red bar indicates non-synonymous substitution in P3N-PIPO and insertion of adenosine on G_2A_6 motif. This insertion on G_2A_6 motif has showed in 10% rate. Blue indicates non-synonymous substitutions in P3N-PIPO.

al. 2012). Because RB breaks *cyv1* resistance and results in systemic infection, it should no longer be restricted to the initially infected cells. To confirm this assumption, GFP-tagged Cl-no30 and RB were biolistically inoculated into *cyv1* pea plants. The GFP fluorescence of RB around the initially infected cells was able to spread to neighboring cells, but almost no movement was detected for Cl-no30 (Table 2-1). Next, *cyv1* pea plants were biolistically inoculated with the P3N-PIPO point mutants of Cl-no30 and RB. The substitution of isoleucine with threonine at position 38 of PIPO in RB (RB/PIPO^{I38T}) (Fig. 2-4A) impaired its cell-to-cell movement (Table 2-1), whereas the opposite substitution in Cl-no30 (Cl/PIPO^{T38I}) (Fig. 2-4A) had no effect on its cell-to-cell movement (Table 2-1). Conversely, although the substitution at position 62 in RB (RB/PIPO^{Q62}) (Fig. 2-4A) had little effect on its cell-to-cell movement (Table 2-1), the opposite substitution in Cl-no30 (Cl/PIPO^{62Q}) (Fig. 2-4A) increased its efficiency of cell-to-cell movement almost to that of RB (Table 2-1). These results demonstrated that in *cyv1* pea, the cell-to-cell movement rate of the P3N-PIPO point mutants is correlated with the rate of systemic infection, suggesting that if a ClYVV isolate is able to move to adjacent cells in a *cyv1* pea plant, the virus infects systemically.

Quantitative involvement of P3N-PIPO in cell-to-cell and systemic movement of ClYVV in *cyv1* pea.

The genetic analyses described above suggested the involvement of both P3 and P3N-PIPO proteins in breaking the *cyv1* resistance. However, this analysis did not rule out the possibility that the differences between the genomic sequences of RB and Cl-no30, rather than the amino acid sequences of the encoded proteins, were responsible for breaking the resistance. To confirm the involvement of the P3 and P3N-PIPO

TABLE 2-1 Cell-to-cell movement of ClYVV with point mutants in P3NPIPO in the PI 429853 *cyv1* pea line at 5 dpi[a].

Mutant	Moving[a]	Restricted to single cell[b]	Total number of examined infected cells	Cell-to-cell movement rate[c] (%)
Cl-no30	2	40	42	5
Cl/PIPO^{62Q}	15	27	42	36
RB	15	23	38	39
RB/PIPO^{I38T}	5	79	84	6
RB/PIPO^{Q62}	11	33	44	25
Cl/P3ΔPIPO	2	14	16	12
Cl/P3N-PIPO	12	38	50	24

a All mutants are illustrated in Fig. 2-4A and Fig. 2-5B.
b Number of foci where mutants spread to two or more surrounding cells from the initially infected single cells.
c Number of foci where mutants were restricted to the initially infected single cell.
d The percentage of foci where mutants moved to neighboring cells, calculated as (no. of foci where mutants moved to neighboring cells/total no. of foci) ×100.

proteins, the GFP-tagged Cl-no30 infectious clone was biolistically inoculated into *cyv1* pea plants together with one of six transient expression cassettes. These cassettes were expected to produce the P3 protein accompanied by a small amount of P3N-PIPO protein produced from the occasional frameshift (pE2113/P3), the P3N-PIPO protein and a small amount of the P3 protein (pE2113/P3N-PIPO), or the P3 protein only (pE2113/P3ΔPIPO). A cassette of each type was derived from both Cl-no30 and RB. The movement of Cl-no30 was monitored by following the GFP fluorescence. Interestingly, the frequency of cell-to-cell movement of Cl-no30 markedly increased when the P3N-PIPO protein from either Cl-no30 or RB was expressed in *trans* (Fig. 2-6A and Table 2-2). The P3 protein from RB also seemed to enhance the cell-to-cell movement of Cl-no30, although to a lesser extent (Fig. 2-6A and Table 2-2). In particular, the fact that the increased expression of P3N-PIPO, even that from Cl-no30, facilitated the cell-to-cell movement of Cl-no30 suggested a quantitative involvement of the P3N-PIPO protein in breaking the resistance. To confirm the quantitative involvement of P3N-PIPO, I additionally inserted either of the modified Cl-no30 P3 cistrons, which exclusively produced P3N-PIPO (Cl/P3N-PIPO) or P3 (Cl/P3ΔPIPO), into the GFP-tagged Cl-no30 infectious clone (Fig. 2-6B). Cl/P3N-PIPO and Cl/P3ΔPIPO were genetically expected to produce more P3NPIPO and P3, respectively, than the parental clone (Cl-no30) in infected plants. These constructs were biolistically inoculated into *cyv1* pea plants, and their GFP fluorescence was observed for 3 dpi. As expected, Cl/P3N-PIPO moved more frequently to neighboring cells than did Cl/P3ΔPIPO and Cl-no30 (Fig. 2-6B and Table 2-1). This result led me to hypothesize that RB can break *cyv1* resistance, resulting in systemic infection, by producing more P3N-PIPO protein than Cl-no30.

This hypothesis was examined by the following experiments. First, I compared the

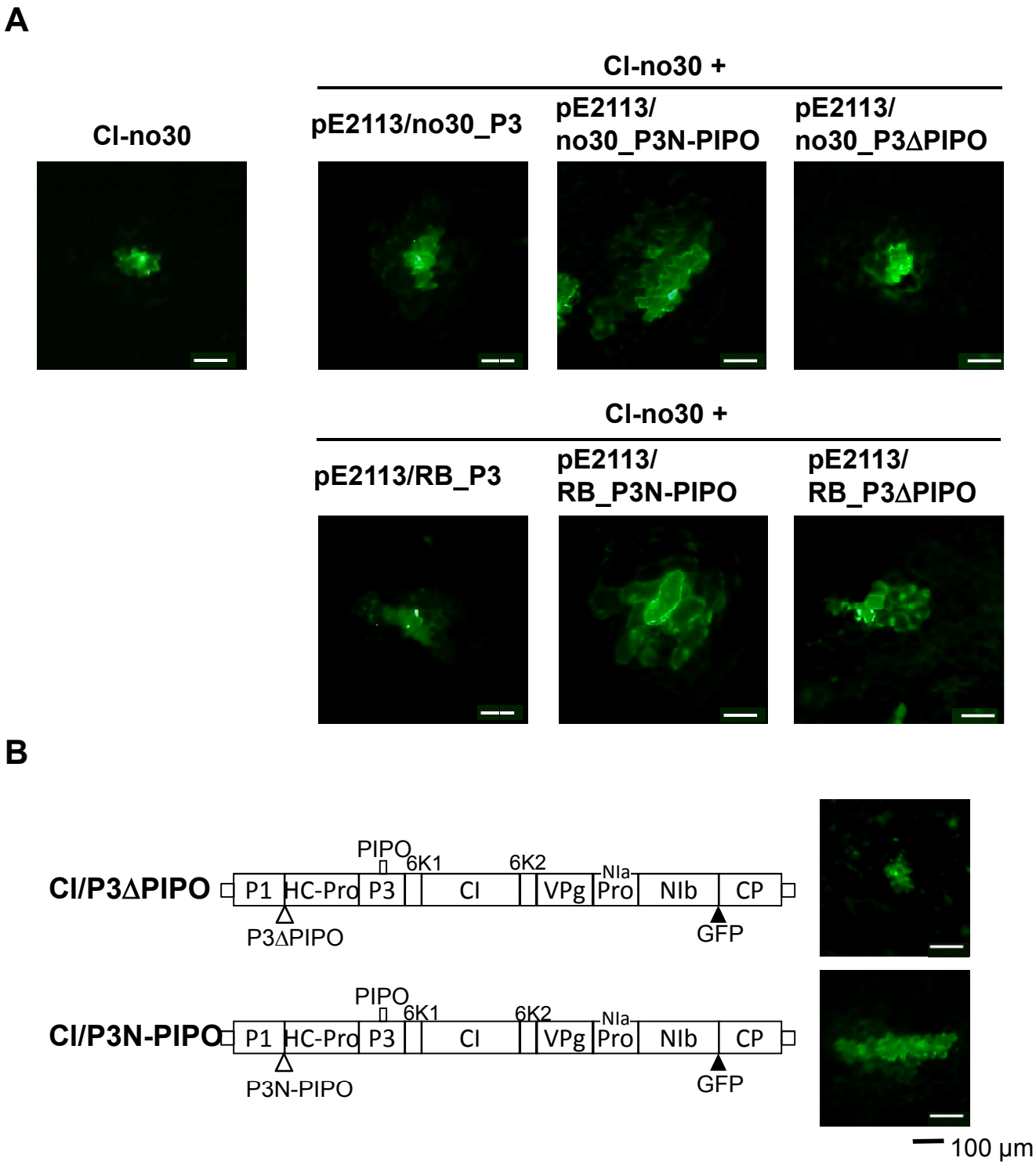


FIG 2-6 Effect of the additional production of P3 and P3N-PIPO in *cis* and *trans* on the cell-to-cell movement of Cl-no30 in *cyv1* pea. FIG 2-6 Effect of the additional production of P3 and P3N-PIPO in *cis* and *trans* on the cell-to-cell movement of Cl-no30 in *cyv1* pea. A. PI 429853 was cobombarded with the Cl-no30 and pE2113 vectors designed to produce P3 and P3N-PIPO from Cl-no30 and RB. The cell-to-cell movement of Cl-no30 was monitored by observing GFP fluorescence at 3 days postbombardment. Constructs with names ending in "P3" were expected to produce the P3 protein plus a small amount of P3N-PIPO. Constructs with names ending in "P3N-PIPO" and "P3PIPO" produced P3N-PIPO and P3, respectively, exclusively. **B**. The P3 cistrons that produced P3 (Cl/P3PIPO) or P3N-PIPO (Cl/P3N-PIPO) exclusively were inserted between P1 and HC-pro of Cl-no30 and used to bombard detached leaves of pea plants carrying *cyv1* (PI 429853). GFP fluorescence was monitored at 3 days postbombardment.

levels of P3N-PIPO at 9 dpi in a susceptible pea line (PI 250438) infected with Cl-no30 or RB. As shown in Fig. 2-7A, P3 and P3N-PIPO were detected at about 40 and 24 kDa, respectively. Compared with P3, which accumulated to comparable levels in both isolates, P3N-PIPO was detected only in the RB-infected pea plants but not in the Cl-no30-infected pea plants (Fig. 2-7A). There were a few faint bands, one of which might have been P3N-PIPO, between 20 and 25 kDa in the sample from Cl-no30 (Fig. 2-7A, experiment 1), but none of them had a comparable intensity to that of RB P3N-PIPO. The accumulation levels of P3N-PIPO produced from the P3 cistrons between Cl-no30 and RB using an *in vitro* translation system were compared (Choi et al. 2013) and the obtained results were consistent with those of *in vivo* analysis (Fig. 2-7A). P3N-PIPO from RB P3 was detected at about 24 kDa, but that from Cl-no30 P3 (at about 21 kDa) was not detected (Choi et al 2013), suggesting a difference in the efficiencies of P3N-PIPO production between Cl-no30 and RB. Although both experiments failed to detect P3N-PIPO from Cl-no30, I believe that Cl-no30 produced P3N-PIPO in the infected pea plants but that its accumulation was not detectable because P3N-PIPO has been reported to be essential for the cell-to-cell movement of potyviruses (Wei et al. 2010b, Wen and Hajimorad 2010), and a point mutant of Cl-no30, which no longer produced P3N-PIPO, failed to systemically infect pea plants inoculated with it (described in chapter III). I therefore conclude that P3N-PIPO accumulated to a higher level in the RB-infected pea plants than in the Cl-no30-infected pea plants.

I then examined whether the presence of additional P3N-PIPO protein would enable Cl-no30 to infect *cyv1* pea plants systemically by using WClMV vectors. The *cyv1* pea plants were coinoculated with Cl-no30 and a WClMV vector designed to produce the P3N-PIPO protein of either Cl-no30 (WCl/no30_P3N-PIPO) or RB

(WCl/RB_P3N-PIPO); thus, the additional P3N-PIPO protein was systemically independent of Cl-no30 in the inoculated *cyv1* pea plants. The expression of P3N-PIPO from WClMV vector was checked by western blotting, however, I could not detect any signal (data not shown). Although I failed to detect any GFP signals and P3N-PIPO protein in the upper leaves of the inoculated *cyv1* pea plants, RT-PCR and RT-nested PCR detected Cl-no30 in the upper leaves of the *cyv1* pea plants inoculated with WClMV that produced the P3N-PIPO protein of either Cl-no30 or RB at 11 dpi (Fig. 2-8, lower panel). Cl-no30 was not able to infect the *cyv1* pea systemically when the *cyv1* pea plants were coinoculated with the WClMV empty vector (Fig. 2-8). Here, I should note that coinoculation of Cl-no30 and WCl/no30_P3N-PIPO or WCl/RB_P3N-PIPO did not always result in systemic infection of *cyv1* pea with both viruses. This failure of systemic infection might be explained by an effect related to RNA-mediated cross protection (Ratcliff et al. 1999) because Cl-no30 is unrelated to WClMV but shares the nucleotide sequence of P3 with WCl/no30_P3N-PIPO and WCl/RB_P3N-PIPO. Even so, Cl-no30 could spread systemically in *cyv1* pea when WCl/no30_P3N-PIPO and WCl/RB_P3N-PIPO spread systemically (Fig. 2-8). These results are consistent with the enhanced cell trafficking of Cl-no30 caused by additional P3N-PIPO protein from either Cl-no30 or RB produced in a pE2113 transient expression vector and Cl/P3N-PIPO (Fig. 2-6 and Tables 2-1 and 2-2).

Discussion

In this study, the region involved in the breaking of *cyv1*-mediated resistance was

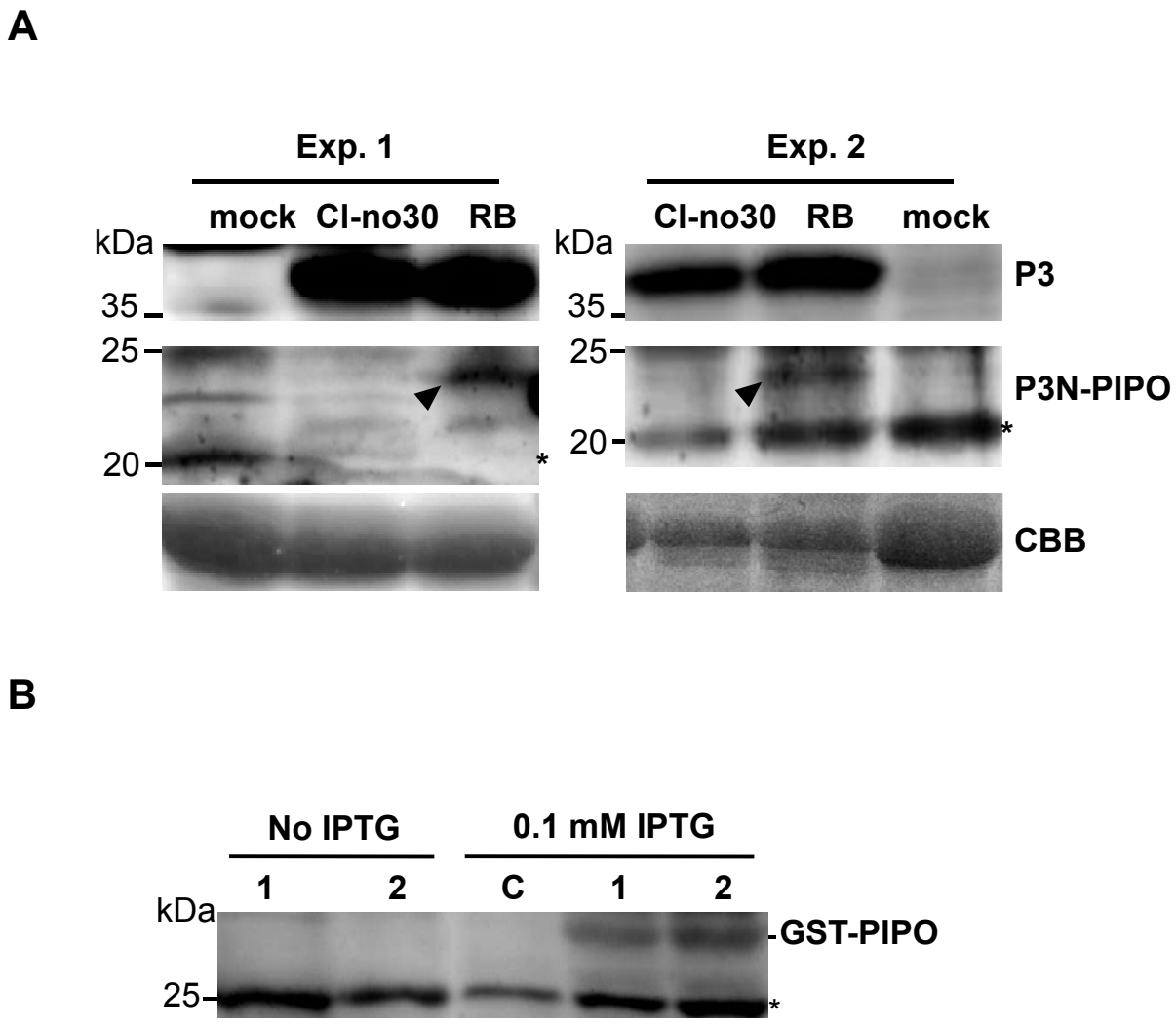


FIG 2-7 P3N-PIPO accumulated to higher levels in a susceptible pea line (PI 250438) infected with RB than in those infected with Cl-no30. A. Western blot analysis of P3 (upper panel) and P3N-PIPO (middle panel), which migrated at around 40 and 24 kDa, respectively, was conducted with samples from the upper leaves of pea plants infected with Cl-no30 and RB. Samples were taken at 9 dpi. The band considered to be P3N-PIPO was only detected in samples from RB-infected pea plants (arrowheads). Asterisks indicate nonspecific signals. The gel stained with Coomassie brilliant blue (CBB) is shown as a loading control (lower panel). Similar results were obtained in independent experiments (experiments 1 and 2). **B**. Confirmation of antibodies raised against PIPO. The expression of GST-fused PIPO (GST-PIPO) was induced with 0.1mMIPTG (see Materials and Methods). Lanes 1 and 2 are samples from *E. coli transformants* that contained the cDNA expressing PIPO fused with GST. Lane C is from a sample transformed with the vector expressing the S8 protein of rice ragged stunt virus fused with GST as a control. The asterisk indicates a nonspecific band (loading control). Arrowheads indicate the bands corresponding to RB P3N-PIPO. Molecular mass markers (kDa) are indicated to the left of the panel.

genetically mapped by using chimeric ClYVVs constructed from isolates that were virulent and avirulent to *cyv1* pea, and I revealed that the P3 cistron was the determinant for breaking of the resistance. Because the P3 cistron had recently been shown to encode two proteins, P3 and P3N-PIPO (Chung et al. 2008), I introduced point mutations, which were synonymous with respect to the P3 protein but non-synonymous with respect to the P3N-PIPO protein, and vice versa, into RB and Cl-no30 infectious clones. Genetic analysis with these point mutants suggested that both P3 and P3N-PIPO were involved in breaking the resistance (Fig. 2-4). Moreover, additional P3N-PIPO protein produced in *cis* and *trans* enabled the simultaneously inoculated Cl-no30 virus to move not only to neighboring cells but also systemically, to other leaves, in *cyv1* pea plants (Fig. 2-6 and 2-8 and Tables 2-1 and 2-2). Therefore, these results early showed that the P3N-PIPO protein is a virulence determinant of ClYVV in *cyv1* pea. The P3 cistron has been shown to be a determinant of the host range of TuMV (Jenner et al. 2003, Suehiro et al. 2004) and of potyvirus virulence in plants with dominant and recessive resistance (Johansen et al. 2001, Hjulsager et al. 2006, Chowda-Reddy et al. 2011). However, whether P3 or P3N-PIPO was responsible for the determination of host range and potyvirus virulence had not been examined. This is the first report showing that the P3N-PIPO protein is a virulence determinant in plants resistant to potyviruses.

Among the known recessive potyvirus resistance genes in pea, *cyv1* and *sbm-2* have not yet been identified (Provvidenti and Hampton 1991, Gao et al. 2004a, Choi et al. 2012). On the other hand, the recessive resistance genes *cyv2* (against ClYVV), *sbm-1*,*-3*, and *-4* (against PSbMV), and *wlv* (against BYMV) are identical or allelic to one another, and all encode pea homologs of eIF4E in LG VI (Provvidenti and Hampton 1991, Gao et al. 2004b, Bruun-Rasmussen et al. 2007, Andrade et al. 2009). As in

TABLE 2-2 Effect of transiently expressed P3N-PIPO on movement of CI-no30 in *cyv1* pea (PI 429853) at 5 dpi.

CI-no30 +	PI 250438[a]			PI 429853		
	Single cell[b]	Moving [c]	Cell-to-cell movement rate[d] (%)	Single cell[b]	Moving [c]	Cell-to-cell movement rate[d] (%)
Empty[e]	9	22	71	40	2	4.7
pE2113/no30_P3	2	77	97	75	3	3.8
pE2113/no30_P3N-PIPO	8	79	91	102	33	32.4
pE2113/no30_P3ΔPIPO	8	81	91	90	7	7.2
pE2113/RB_P3	5	140	97	83	11	13.3
pE2113/RB_P3N-PIPO	3	110	97	145	41	28.3
pE113/RB_P3ΔPIPO	2	12	86	39	4	10.3

a Each cobombardment test was simultaneously carried out with susceptible pea plants of line PI 250438 as a positive control.
b The number of foci where CI-no30 was restricted to the initially infected single cell.
c The number of foci where CI-no30 moved to two or more cells.
d The percentage of foci where CI-no30 moved to neighboring cells, calculated as (number of foci where CI-no30 moved to neighboring cells)/(total number of foci) $\times$100.
e The plants were bombarded with only CI-no30.

pathosystems between other potyviruses and eIF4E family-mediated recessive resistance in plants, in most cases, VPg has been reported to be the virulence determinant of PSbMV and BYMV in these resistant pea lines, although ClYVV P1 was the determinant in *cyv2* pea (Gao et al. 2004b, Bruun-Rasmussen et al. 2007, Nakahara et al. 2010). Regarding the possible relationship and functions of *cyv1*, *sbm-2*, and *mo*, Gao et al. (2004a) reported that a candidate gene, coding for eIF(iso)4E, is located in the same linkage group as *sbm-2* and *mo*. However, because no differences have been detected in the deduced amino acid sequences of eIF(iso)4E between susceptible and resistant pea plants, *cyv1* and *sbm-2* are not likely to encode eIF(iso)4E (Gao et al. 2004a, Choi et al. 2012). Consistent with this, the P3 cistron but not VPg of PSbMV P-2 was associated with the breaking of *sbm-2* resistance in the pea cultivar Dark Skinned Perfection (Johansen et al. 2001, Hjulsager et al. 2006). I also found that the P3 cistron is the virulence determinant of ClYVV in *cyv1* pea plants.

Then what protein does *cyv1* encode and how do P3 and P3N-PIPO interact with it to break *cyv1* resistance? Two mechanisms involving recessive resistance to viruses have been proposed. First, mutations may occur in a plant gene that encodes a repressor of antiviral defense systems. Second, mutations may arise that disrupt the function of an allele encoding a protein that supports viral infection and spread in a plant. The latter case has been well documented for mutant alleles encoding eIFs in several crops showing recessive resistance against potyviruses (Robaglia and Caranta 2006). To my knowledge, there is no report regarding an association of P3 and P3N-PIPO with the repression of antiviral defenses. I thus stand by the latter possibility (i.e., P3 and P3N-PIPO may interact with a *cyv1*-encoding protein that is a coopted host factor for ClYVV infection in pea). Several recent studies have revealed distinct aspects of P3 and P3N-PIPO functions and properties. P3 is localized in the endoplasmic reticulum and

Golgi apparatus and is colocalized into vesicles induced by a 6-kDa potyvirus membrane protein (6K) that contain viral RNA and viral replicase components (Wei et al. 2010a). P3 traffics along actin filaments and plays a role in intra- and intercellular movement (Cui et al. 2010). On the other hand, P3N-PIPO is localized in plasmodesmata and recruits the CI protein to plasmodesmata, which probably facilitates cell-to-cell movement (Wei et al. 2010b). P3N-PIPO has been recently shown to be required for cell-to-cell movement of potyviruses (Wei et al. 2010b, Wen and Hajimorad 2010), and the resistance mode of pea lines carrying *cyv1* and *sbm-2* is to restrict avirulent ClYVV and PSbMV to the initially infected cells (Johansen et al. 2001, Choi et al. 2012). Taken together, these studies raise the possibility that *cyv1* and *sbm-2* are allelic and encode a host factor that is involved in replication and intra- and intercellular movement of potyviruses and interacts with P3 or P3N-PIPO.

ClYVV isolate 90-1 Br2, which broke the resistance of *cyv1*, was more virulent toward susceptible pea line PI 250438 than Cl-no30 (Fig. 2-1B). I have recently mapped the genomic region responsible for the higher virulence in susceptible pea lines using these chimeric ClYVVs shown in Fig. 2-1A and found that the P3 cistron of 90-1 Br2 was responsible for its higher virulence and RB accumulated more and spread more rapidly than did Cl-no30 (G. Atsumi, unpublished data). These results support the possibility that P3 and P3N-PIPO were involved in viral virulence through interaction with a host factor for ClYVV infection. One such host factor is *Arabidopsis* PCaP1, which has been recently identified as the plasma membrane protein that interacts with P3N-PIPO to facilitate cell-to-cell movement of TuMV (Vijayapalani et al. 2012).

The quantitative involvement of the P3N-PIPO protein in the breaking of *cyv1* resistance (Fig. 2-6 to 2-8 and Tables 2-1 and 2-2) is an unexpected characteristic, and interesting findings were made in this study. I initially expected that concurrent

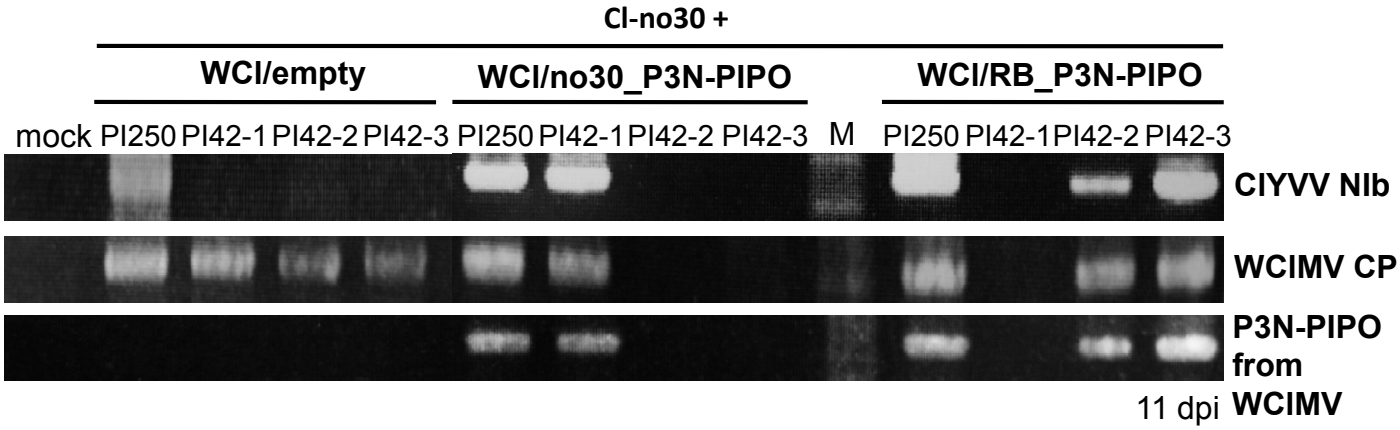


FIG 2-8 Higher levels of P3N-PIPO contribute to the breaking of *cyv1* resistance. Coinoculation with Cl-no30 and WClMV producing P3N-PIPO from Cl-no30 (WCl/no30_P3N-PIPO) or RB (WCl/RB_P3N-PIPO) enabled systemic infection with Cl-no30. The systemic infection of *cyv1* pea plants (PI 429853) with ClYVV (upper panel) and WClMV (middle panel) was detected in samples from uninoculated upper leaves by RT-PCR for WClMV CP and RT-nested PCR for ClYVV NIb. Stability of the transgene P3N-PIPO in the WClMV vectors was confirmed by RT-PCR with primers corresponding to the upstream region of the WClMV multiple-cloning site and 3' terminus of the PIPO open reading frame (lower panel) at 11 dpi. The tests were run in duplicate with three *cyv1* pea plants (PI 429853, written as PI42-1, -2, and -3) per coinoculation test. Essentially similar results were obtained, and one set is shown. PI250, PI 250438, a cultivar susceptible to both ClYVV and WClMV; M, 100-bp DNA ladder marker; Mock, a pea plant (PI 250438) inoculated with 0.01 M phosphate buffer.

expression (in *trans*) of P3N-PIPO derived from the virulent ClYVV RB with avirulent Cl-no30 might contribute to the virulence of Cl-no30 in *cyv1* pea, but that P3N-PIPO from Cl-no30 would have little effect. However, the P3N-PIPO proteins derived from both RB and Cl-no30 facilitated the cell-to-cell movement of Cl-no30 (Fig. 2-6). Moreover, additional systemic production of P3N-PIPO—even that from avirulent Cl-no30—by using a WClMV vector enabled the simultaneously inoculated Cl-no30 to move systemically to upper leaves (Fig. 2-8). These findings suggested that the more P3N-PIPO is produced by ClYVV, the higher its virulence in *cyv1* pea. Indeed, RB produced higher levels of P3N-PIPO protein than did Cl-no30 in susceptible pea lines (Fig. 2-7A, Choi et al 2013), which might explain how RB was able to break *cyv1*-mediated resistance.

How did high levels of P3N-PIPO contribute to breaking of the resistance? P3N-PIPO recruits CI to plasmodesmata in a dose-dependent manner (Wei et al. 2010b), and both CI and P3N-PIPO are essential for cell-to-cell movement of potyvirus (Carrington et al. 1998, Wen and Hajimorad 2010). These studies offer a possible explanation, namely, that P3N-PIPO is defective in recruiting CI to plasmodesmata, perhaps due to inefficient accumulation or localization of P3N-PIPO to the plasmodesmata in *cyv1* pea, but that higher levels of P3N-PIPO produced by RB complement the defect and confer the ability for cell-to-cell movement on RB, thus overcoming *cyv1*-mediated resistance.

The next question concerns how RB produces high levels of P3N-PIPO. Our laboratory colleague showed that the level of P3N-PIPO produced by the P3 cistron derived from RB was higher than that derived from Cl-no30 through an *in vitro* translation assay (Choi et al. 2013). This result is in agreement with the increased accumulation of P3N-PIPO in RB-infected susceptible pea lines (Fig. 2-7A). Although

this *in vitro* assay was insufficient to clarify whether P3N-PIPO is produced by a ribosomal frameshift or transcriptional slippage, several important features of P3N-PIPO expression were revealed: (i) the P3 cistron was sufficient, and other regions of the viral genome were not required for the production of P3N-PIPO, and (ii) the nucleotide differences in the P3 cistron between RB and no30 affected the efficiency of P3N-PIPO production. Together with our other results, we believe that RB acquired virulence to *cyv1* pea in part by producing an increased amount of P3N-PIPO. Using this experimental system, the mechanism of how P3N-PIPO is produced from the potyvirus genome will be elucidated in the near future.

TABLE 2-3 Primers evaluated in this chapter.

Primer no.	Name of primer	Sequence (5′-3′)
1868	UTR3′-s	GGATTGATGTGATATCTCCACTGACGTAAG
3177	HCpro3′-as	GTTGCAAGTTCTCTCGTACC
1839	HCbgl5′-s	ACGTGTACATCAGATCTCAATGTAAT
1840	P3bgl3′-as	TTTTGATGTGAGATCTCACTTGACTC
1825	P3nhe3′-s	TTCTTAATGTGCTAGCAACAATTACG
1805	VPsal5′-as	TAGTCGCGCAAACCACTTATGCGATTTAGA
1806	VPsal5′-s	ACCATACAAGCTGAGGGACTCAAATGTTGT
1817	Polbam3′-as	AGCTTGGGATCCTTTTTTTTTTCTCGCTCTATAAAGATCA
2936	no30bgl-s	CAGATCTCAATGTAGTCCAATGTGGTTCTG
2979	no30br2-as	CCAACTCTGTAAAACTTCAAATCTGACTC
2978	no30br2-s	GAGTCAGATTTGAAGTTTTACAGAGTTGG
2977	br2bgl-as	CAGATCTCACTTGACTCAGAATGC
3306	br2P3_644-s	GTAACAAGCATCAAGAATCAAGCAATC
3305	br2P3_644-as	GATTGCTTGATTCTTGATGCTTGTTAC
3306	br2RAM-s	GCAACAAGCATTAAAAACCAAGCAATC
3307	br2RAM-as	GATTGCTTGGTTTTTAATGCTTGTTGC
1373	no30_P1-Pst_s	GCGCGCCTGCAGAAAAACTGAAAGTG
3794	Cl-no30P1P3_as	GTCAATGATTTGCCAGAGAATTCT
3793	Cl-no30P1P3_s	AGAATTCTCTGGCAAATCATTGAC
3797	Clno30P3Cter-NIa_as	GCGCCTGCAGATTGGAAAACAAATTTCATTTCCATGACAAACCACTTTGGTTC
3798	Cl-no30P3NPIPO-NIa_as	GCGCCTGCAGATTGGAAAACAAATTTCATATGCTTGTTACTGAC
2470	NIb_s	CTTTAGACCTATGATGGGC
2465	NIb_as	GTTCAAGCCCAATTCTTTG
2477	NIb_asnest	GTATATATGATCTCCGTGTAC
3388	Cl-P3-574as	CTCTGGGCTAGTATTTTGGAATAC

General discussion

Plant recessive resistance against viruses, and especially potyviruses, frequently shows inheritance (Díaz-pendón et al. 2004) and the recessive resistance genes in pea to several potyviruses are closely linked and clustered in linkage group (LG) II and VI (Provvidenti and Hampton 1991). The recessive resistance genes in LG VI such as *cyv2* (against ClYVV), *sbm-1*,*-3*, and *-4* (against PSbMV), and *wlv* (against BYMV) are identical or allelic to one another, and all encode pea homologs of eIF4E (Provvidenti and Hampton 1991, Bruun-Rasmussen et al. 2007, Andrade et al. 2009). As in pathosystems between other potyviruses and eIF4E family-mediated recessive resistance in plants, in most cases, VPg has been reported to be the virulence determinant of PSbMV and BYMV in these resistant pea lines, although P1 of ClYVV was the determinant in *cyv2* pea (Gao et al. 2004b, Bruun-Rasmussen et al. 2007, Nakahara et al. 2010).

On other hands, the resistance genes in LG II, including *cyv1* (against ClYVV), *sbm-2* (against PSbMV), and *mo* (against BYMV) have not yet been identified (Provvidenti and Hampton 1991, Gao et al. 2004a, Choi et al. 2012). Regarding the possible relationship and functions of *cyv1*, *sbm-2*, and *mo*, Gao et al. (2004a) reported that a candidate gene, coding for eIF(iso)4E, is located in the same linkage group as *sbm-2* and *mo*. However, because no differences have been detected in the deduced amino acid sequences of eIF(iso)4E between susceptible and resistant pea plants, *cyv1* and *sbm-2* are not likely to encode eIF(iso)4E (Gao et al. 2004a Choi et al. 2012). Consistent with this, the P3 cistron but not VPg of PSbMV P-2, was associated with the breaking of *sbm-2* resistance in the *P. sativum* cultivar Dark Skinned Perfection (Johansen et al. 2001, Hjulsager et al. 2006) and of *cyv1* resistance (Choi et al. 2013).

How does P3 cistron break *cyv1* resistance and what protein does interact with proteins from P3 cistron (P3 and P3N-PIPO)? To successfully move neighboring cells by overcoming host defense, viruses exploit different mechanisms such as microtubules mediated movement and delayed virus replication against a defense response (Mise and Ahlquist 1995, Niehl and Heinlein 2011). The resistance mode of pea lines carrying *cyv1* and *sbm-2* is to restrict avirulent ClYVV and PSbMV to the initially infected cells (Johansen et al. 2001, Choi et al. 2012), but the viruses break resistance by accumulation of amino acid mutations in P3 cistron (Hjulsager et al. 2006, Choi et al. 2013). These mutational adaptations in P3 required to genome amplication (Choi et al. 2005), relying on first strategy of interfering host defense. Sufficient viral RNA for infection form 6-kDa potyvirus membrane protein induced vesicles that contain viral replicase components and localized on endoplasmic reticulum (ER) by C-terminal of P3 cistron (Eiamtanasate et al. 2007, Wei et al. 2010a). P3 cistron except PIPO traffics those vesicles along actin filaments between ER and Golgi (Cui et al. 2010). Virion particles bind to CI and transport to plasmodesmata is mediated by P3N-PIPO and its quantitatively facilitates cell-to-cell movement (Wei et al. 2010b, Niehl and Heinlein 2011, Vijayapalani et al. 2012, Choi et al. 2013), which probably set up a second breaking resistance. Taken together, these results raise the possibility that *cyv1* and *sbm-2* are allelic and encode a coopted host factor that is involved in replication and intra- and intercellular movement of potyviruses and interacts with P3 or P3N-PIPO.

To successfully invade to host plant, plant virus must interact with various host factors essential for their accumulation and inter-/intra-cellular movement. The mechanism that viral movement is the integration of processes from intracellular to whole plant has been elucidated in recent years and some host defense blocks in these steps. Plant virus encodes one or more movement protein (MP) and they are required for

cell-to-cell movement. Until now, potyvirus MP, P3N-PIPO, and MP-associate protein, CI, are reported to be involved in cell-to-cell movement (Wei et al. 2010b, Vijayapalani et al. 2012).This study contributed identification of a new MP, P3N-afs (Hagiwara-Komoda et al. unpublished data), and it facilitates P3N-PIPO-mediated cell-to-cell movement. Also, P3N-afs plays a role in a helper for cell-to-cell movement because P3N-afs showed leaky phenotype in absence of P3N-PIPO and enables P3N-PIPO-mediated cell-to-cell movement spread more to adjacent cells

The cell-to-cell movement by P3 cistron derived proteins functions similarly to TGB of potexvirus does. TGBp3 deficient potexvirus mutant showed leaky phenotype and the impaired cell-to-cell movement is restored by overexpression of TGBp2 (Tamai and Meshi 2001a, b). TGBp3 is localized on ER and targets TGBp2, facilitating cell-to-cell movement as potyvirus P3 cistron produced proteins, hypothesizing that P3 cistron derived proteins are multifunctional machine for plant virus movement.

Plant virus also has the ability to combat host defense mechanisms between developed cells and spread to adjacent cells. *Tobacco etch virus* (TEV, the genus of *Potyvirus*) moves from cell to cell between epidermal cells in the mechanically inoculated potato leaves but can invade a few mesophyll cells (Valkonen and Somersalo, 1996). The plant RNA virus, *Soil-borne wheat mosaic virus* (SBWMV, the genus *Furovirus*) forms distinct size of genetic bottlenecks during cell-to-cell movement. The size of bottlenecks of SBWMV become narrower as RNA virus spread from first cell-to-cell movement to second cell-to-cell movement for effective transmission (Miyashita and Kishino, 2010). It is suggested that P3N-PIPO and P3N-afs also regulate cell-to-cell movement by blocking any host resistance.

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