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Quantitative and Qualitative Involvement of P3N-PIPO in Overcoming Recessive Resistance against Clover yellow vein virus in Pea Carrying cyv1

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Running title: Role of P3N-PIPO in CIYVV virulence against cyv1 pea

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Abstract

In pea carrying cyv1, a recessive gene for resistance to Clover yellow vein virus (ClYVV), ClYVV isolate Cl-no30 was restricted to the initially infected cells whereas isolate 90-1 Br2 overcame this resistance. We mapped the region responsible for breaking of cyv1-mediated resistance by examining infection of cyv1 peas with chimeric viruses constructed from parts of Cl-no30 and 90-1 Br2. The breaking of resistance was attributed to the P3 cistron, which is known to produce two proteins: P3, from the main open reading frame (ORF), and P3N-PIPO, which has the N-terminal part of P3 fused to amino acids encoded by a small ORF (called PIPO) in the +2 reading frame. We introduced point mutations that were synonymous with respect to the P3 protein but non-synonymous with respect to the P3N-PIPO protein, and vice versa, into the chimeric viruses. Infecting plants with these mutant viruses revealed that both P3 and P3N-PIPO were involved in overcoming cyv1-mediated resistance. Moreover, P3N-PIPO quantitatively affected the virulence of Cl-no30 in cyv1 peas. Additional expression in trans of the P3N-PIPO derived from Cl-no30, using White clover mosaic virus as a vector, enabled Cl-no30 to move to systemic leaves in cyv1 peas. Susceptible pea plants infected with chimeric ClYVV possessing the P3 cistron of 90-1 Br2, and which were therefore virulent toward cyv1 peas, accumulated more P3N-PIPO than did those infected with Cl-no30, suggesting that the higher level of P3N-PIPO in infected cells contributed to the breaking of resistance by 90-1 Br2. This is the first report showing that P3N-PIPO is a virulence determinant in plants resistant to a potyvirus.
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 the resistance seem to inevitably emerge. Understanding how pathogens overcome host plant resistance is important for developing strategies to confer durable resistance to crops.

Plant resistance against viruses, and especially potyviruses, frequently shows recessive inheritance (1). Most of the 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 (2). In particular, the recessive resistance genes cyv2 (resistance to Clover yellow vein virus [ClYVV]; sbm-1, -3, and -4 (resistance to Pea seed-borne mosaic virus [PSbMV]; wlv (resistance to Bean yellow mosaic virus [BYMV]), in pea (Pisum sativum); and mol1 and mol2 (resistant to Lettuce mosaic virus [LMV]) have been identified as identical or allelic genes that encode eIF4E (3-5). eIF4E interacts with the potyviral genome–linked protein VPg at the surface, near a cap-binding pocket (5-10). However, the eIF4Es derived from resistant plants failed to interact with VPg (6, 11-13), 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 (14-16). 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 (5, 6, 13, 17). Besides VPg, other potyvirus proteins, the cylindrical inclusion (CI) protein (18, 19), the helper-component proteases (HC-Pro) (20) and P1 (21), 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 another in potyvirus infection, and how these interaction networks are affected in resistant plants containing the recessive allele of eIF4E (22), 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 (5, 21, 23-25).

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 CIYVV), mo (resistance to BYMV), and sbm-2 (resistance to PSbMV) were mapped close to one another in pea linkage group (LG) II (26, 27). The eIF(iso)4E gene is also found in LG II (28). 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 sequence of the eIF(iso)4E proteins between susceptible and resistant peas (26, 28). Thus, if virus isolates emerge that overcome these recessive resistance genes, the viral determinant responsible 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 (29, 30). In this study, breaking of the cyv1 resistance by CIYVV was attributed to the P3 cistron.

Until recently, the functions of P3 have been poorly understood. Functional P3
protein was found to be recruited to the potyvirus replication complex (31) and to be involved in accumulation of the virus (32, 33), symptomatology (34, 35), viral microtubule-related inter- and intracellular movement (36), and determination of host range (33). 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 (37, 38).

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 (39, 40). 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 (40-42). 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 peas.

Previous studies have not revealed whether breaking of the sbm-2 resistance involves P3, P3N-PIPO, or both (29). Here, we genetically examined whether either P3 or P3N-PIPO was involved in breaking of the cyv1 resistance by CIYVV. We then investigated the contribution of the P3 and P3N-PIPO proteins to breaking of the resistance by producing these proteins in trans, using either an expression cassette containing the 35S promoter or the White clover mosaic virus (WCIMV) vector (43), and introducing these
into plants along with a CIYVV isolate that is normally avirulent to cyv1 pea.

Materials and Methods

Construction of chimeric viruses and P3 transient expression cassettes

The infectious CIYVV clone pClYVV/C3-S65T-Sal (hereafter, Cl-no30) was made and modified from CIYVV isolate no. 30 in previous studies and contains a coding sequence for the green fluorescent protein (GFP) (44, 45). Since the original sequence of CIYVV isolate no. 30 (DDBJ/GenBank/EMBL accession number AB011819) contains two SalI sites in the VPg and NIb cistrons and the site in VPg was utilized for constructing the chimeric viruses, the site in NIb was eliminated by site-directed mutagenesis prior to construction of the chimeric viruses. Seven chimeric viruses (Cl-P1HC, Cl-BB, Cl-NS, Cl-SB, RB, RB/P3KIIPIPOQ62, and Cl/P3RAM) 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 RT-PCR using KOD-Plus 2 DNA polymerase (Toyobo, Osaka, Japan) with the following primers: UTR3′-s (GGATTGATGTGATATCTCCACTGACGTAAG); HCpro3′-as (GTTGCAAGTTCTCTCGTACC); HCBgl5′-s (ACGTGTACATCAGATCTCAA TGTAAT); P3bgl3′-as (TTTTGATGTGAGATCTCACTTGACTC); P3nhe3′-s (TTCTTAATGTGCTAGCAACAATTACG); VPsal5′-as (TAGTCGCGCACAACCACTTATGC ATTGA); VPsal5′-s
(ACCATACAAGCTGAGGGACTCAAATGTTGT); and Polbam3′-as

(AGCTTGGGATCCTTTTTTTTTCTCGCTCTATAAAGATCA). Recombinant plasmids

Cl-P1HC, Cl-BB, Cl-NS, and Cl-SB (Fig. 1) were created by insertion of 90-1 Br2 cDNA

sequences into the following restriction sites: for Cl-P1HC, the EcoRV site at the 5′-UTR

and the BglII site at the beginning of HC-Pro; for Cl-BB, the BglII sites within the HC-Pro

and P3 coding regions; for Cl-NS, the NheI site in the center of P3 and the SalI site in the

center of VPg; and for Cl-SB, the SalI site in VPg and the BamHI site downstream of poly

A sequence.

The chimeric viruses RB, RB/P3KIIPIPO, and Cl/P3RAM, 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

BglII sites on HC-Pro and P3 was made by two-step PCRs with primers no30bgl-s

(CAGATCTCAATGTAGTCCAATGTGGTTCTG), no30br2-as

(CCAACTCTGTAATAACTCTCAATGTGGTTCTG), no30br2-s

(GAGTCAGATTGAAATTTACAGGTTGG), and br2bgl-as

(CAGATCTCAGTTGACTCAGAATGC). 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, cDNAs of HC-Pro and P3 were fused with PCR by

using primer pair no30bgl-s/br2bgl-as. The fragment was cloned into a pGEM-T-Easy

vector (Promega, Madison, WI, USA) for making the chimeric virus RB.

To make RB/P3KIIPIPO and Cl/P3RAM, the HC-Pro–P3 fragments were amplified by

two-step PCR as described above with the primers, except for br2P3_644-s
(GTAACAAGCATCAAGAATCAAGCAATC) and br2P3_644-as
(GATTGCTTGATTCTTGATGCTTGTTAC) for RB/P3^{Kl}PIPO^{Q62}, and br2RAM-s
(GCAACAAAGCATCAAGAATCAAGCAATC) and br2RAM-as
(GATTGCTTGATTCTTGATGCTTGTTAC) for Cl/P3^{RAM}, instead of no30br2-s and
no30br2-as. These cloned fragments were inserted into vector Cl-no30 after digesting with

To express in *trans* the genes for the proteins encoded by the P3 cistrons of
Cl-no30 and RB, we used two different systems: the plant transient expression vector
pE2113, which contains a 35S promoter for transgene expression (46), and the WClMV
vector (43). The restricted enzyme sites of BamHI–SacI and SpeI–XhoI were added to the 5’
and 3’ terminals of cDNA fragments of the P3 cistrons to insert them into the pE2113 and
WClMV vectors, respectively (43, 46). To construct P3 cistrons that exclusively produce
the P3N-PIPO protein (designated no30_P3N-PIPO and RB_P3N-PIPO), an adenosine
nucleotide was inserted at position 464, in the G_{2}A_{6}, around which a frameshifting is
considered to occur, (GGAAAAAA $\rightarrow$ GGAAAAAAA) (39). To construct P3 cistrons that
exclusively produce the P3 protein (designated no30_P3ΔPIPO and RB_P3ΔPIPO), one
nucleotide was changed at position 477 to generate a stop codon in the PIPO reading frame
(AGA $\rightarrow$ TGA) 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 (3), 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′-GCAGGCTGAAAGACTGAAAGGT-3′), 
Cl-no30P1P3_as (5′-GTCAATGATTTGCCAGAGAATTCT-3′), Cl-no30P1P3_s 
(5′-AGAATTCTCTGGAATTCATTGAC-3′), and Cl-no30P3Cter-NIa_as 
(5′-GCGCCTGCAGATTGGAAAACAAATTTCATATGCTTGTTACTGAC-3′). The amplified fragments were inserted into the vector pClYVV-SEIF4E-GFP after 
digestion with Pst1. The resulting construct was designated Cl/P3ΔPIPO (Fig. 5B).

Cl/P3N-PIPO was also generated using the primers no30_P1-Pst_s, 
Cl-no30P1P3_as, Cl-no30P1P3_s, and Cl-no30P3NIPO-NIa_as 
(5′-GCGCCTGCAGATTGGAAAATTTTCATATGCTTGTTACTGAC-3′).

**Virus inoculation and detection**

We examined the ability of the constructed CIYVV chimeras to systemically infect 
cyv1 pea plants as follows. The CIYVV cultures were recovered in broad beans (*Vicia faba*), 
which were biolistically inoculated with the constructed CIYVV infectious plasmids as 
described by Andrade et al (47). Infected upper leaves of broad bean were stored in a deep 
freezer and used as inoculum for inoculation of *cyv1* pea plants as described (47). Second to 
fourth upper leaves from bottom of *cyv1* pea plants two weeks old were mechanically 
inoculated. The movement of each virus was monitored with GFP fluorescence, which was 
observed for 5 days post inoculation (dpi) using an epifluorescence microscope (VB 7010; 
Keyence, Osaka, Japan). The presence of chimeric viruses in upper leaves was also
detected by reverse transcription (RT)-PCR, as described in (26). For RT-nested PCR, the
first PCR was performed with primers designed based on Nib of CIYVV,
5’-CTTTAGACCTATGATGGC-3’ (sense) and 5’-GTTCAGCCCAATTCTTTG-3’
(antisense); for the second PCR, the sense primer of first PCR and
5’-GTATATATGATCTCCGTAC-3’ (antisense) were used to detect CIYVV with
greater sensitivity.

We tested the infectivity of the constructed CIYVV 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 cyvI pea line (PI 429853) with the CIYVV chimeric
clones, or co-inoculation of the Cl-no30 infectious clone with the pE2113 vectors, was
performed essentially as described by Andrade et al (47). Tungsten particles were coated
either with 1 µg of a CIYVV chimeric plasmid clone or with a mixture of 997 ng Cl-no30
and 3 ng of cloned pE2113 vector containing a P3 cistron (P3, P3N-PIPO, or P3ΔPIPO)
and bombarded into leaves of cultivar PI 429853. The virus infection was monitored by
GFP fluorescence from Cl-no30. The GFP fluorescence was observed for 5 dpi, as
described above.

We investigated the systemic infection of Cl-no30 co-inoculated with WCIMV
vectors designed to exclusively produce the P3N-PIPO protein in the leaves of cyvI peas.
Biolistic co-inoculation of cyvI peas was performed with plasmid mixtures containing 800
ng of Cl-no30 and 200 ng of the WCIMV 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 CIYVV 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-Nort-tail) was performed as described by Ido (43).

RT-PCR using primers specific for the multi-cloning site of WClMV, f (43), and Cl-P3-574as (5′-CTCTGGGCTAGTTTTGGAATAC-3′ [antisense]) confirmed the stability of P3N-PIPO expression from the WClMV vector.

Antibody production and western blotting

To detect the P3N-PIPO 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, USA). 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 maleE gene (which encodes MBP) of pMAL-c2, and the vector was transformed into Escherichia coli JM109. The transformants were cultured for 14 h in LB broth including 50 µg/mL ampicillin. The production of the gene’s fusion protein was induced with 0.1 mM IPTG in fresh LB broth at 35°C for 4 h. Cell lysates were added to a ten-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 G2A₆ 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 (48), indicating that this antibody is able to detect PIPO (Fig. S1). We later found that the P3N-PIPO antibody could simultaneously detect both P3 and P3N-PIPO. We speculate that a ribosomal frameshift might have occurred in the G$_2$A$_6$ 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$_6$G$_1$ has been reported to stimulate a $\pm 1$ frameshift in *E. coli* (49).

To sensitively detect the P3N-PIPO protein derived from the infecting ClYVV in peas, a urea-containing buffer was used to prepare the samples for immunoblotting, as described previously (50). Uninoculated upper leaves with symptoms were harvested at 9 dpi, ground in liquid nitrogen, and denatured in 12-fold (w/v) urea denaturing buffer containing 4.5 M urea, 1% (v/v) Triton X-100, 0.5% DTT, 0.00625 M Tris-HCl pH 6.8, 2% (w/v) SDS, 5% mercaptoethanol, 5% sucrose, and 0.002% bromophenol blue. The plant lysates were analyzed by SDS-PAGE, as described by Atsumi et al (48).

*In vitro transcription*

In the plasmids pE2113/no30_P3 and pE2113/RB_P3, the 5′ end of the P3 sequence harbored a *Bam*HI restriction site and the start codon (5′-GGATCCACCATG + P3 sequence), while its 3′ end harbored the stop codon (TAA) and a *Sac*I site (P3 sequence + TAAGAGCTC-3′). The P3 regions of Cl-no30 and RB were cloned into the pSP64 Poly(A)
vector (Promega) using BamHI and SacI. The resulting plasmids, pSP-WT-P3 and pSP-RB-P3, were used as templates for in vitro transcription. The plasmid DNAs were linearized with EcoRI and extracted with phenol/chloroform (1:1 v/v) followed by ethanol precipitation. RNA was synthesized from the linearized DNA using an AmpliCap SP6 High Yield Message Maker Kit (Cellscript Inc., Madison, WI, USA) in the presence of a cap analog, according to the manufacturer’s instructions.

**In vitro translation**

As an in vitro translation system, MM2dL, an extract derived from Arabidopsis MM2d cells (51), was utilized. To obtain MM2dL, the protocol to prepare a tobacco BY-2 cell-derived extract (52, 53) was applied. The translation reaction cocktails (15 µl) for MM2dL included 7.5 µl of MM2dL, 1 µl of 15× Substrate Mix (11.25 mM ATP, 1.5 mM GTP, 375 mM creatine phosphate, 375 µM each of 19 amino acids excluding methionine, and 1.2 mM spermine), 3 µg of creatine phosphokinase (Roche Diagnostics, Indianapolis, IN, USA), 12 U of RNasin, ~500 ng of mRNA, and 0.3 µl of [³⁵S]methionine (43.5 TBq mmol⁻¹; American Radiolabeled Chemicals, St. Louis, MO, USA). The volume was adjusted with TR buffer [30 mM HEPES-KOH (pH 7.4), 80 mM potassium acetate, 1.8 mM magnesium acetate, 2 mM dithiothreitol, and one tablet of Complete Mini protease inhibitor (Roche Diagnostics) per 10 ml of mixture] (52, 53). The mixtures were incubated at 25°C for 120 min.

After incubation, the translation products were separated on a NuPAGE 4–12%
Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) with MES running buffer (Invitrogen). The protein bands were visualized using a FLA-7000 image analyzer (Fuji Photo Film, Tokyo, Japan). The acquired images were processed using MultiGauge (Fuji Photo Film) and Photoshop 12.0.4 (Adobe Systems Inc., San Jose, CA, USA).

Results

The breaking of the cyv1 resistance by CIYVV 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 (26). The resistance of cyv1 can be broken by isolate 90-1 Br2: cyv1 pea infected with 90-1 Br2 showed chlorotic spots on the upper leaves at 5 weeks post inoculation (wpi). 90-1 Br2 induced more severe symptoms, including dwarfing and necrosis, in susceptible pea cultivar PI 250438 (47) than did Cl-no30 (Fig. 1B). To address which gene is responsible for breaking of the cyv1 resistance, we compared the entire nucleotide sequence of the polyprotein-coding region between Cl-no30 (AB011819) and 90-1 Br2 (AB732962) and found 483 nucleotide differences dispersed throughout the genomes.

We then created four chimeric viruses in which sequences from the Cl-no30 infectious clone were replaced with the corresponding sequences from 90-1 Br2 (44); these chimeras contained the 90-1 Br2 sequence from the 5’-UTR to HC-Pro (Cl-P1HC), from
HC-Pro to P3 (Cl-BB), from P3 to 6K2 (Cl-NS), and from 6K2 to 3′-poly A (Cl-SB) (Fig. 1). These four viruses were inoculated into the cyv1 pea cultivar PI 429853 and observed for 6 wpi. After 5 wpi, the peas inoculated with Cl-BB showed chlorotic spots and GFP fluorescence in the upper leaves (Fig. 1) but the other chimeric viruses could not overcome cyv1 resistance (Fig. 1). The virulence of Cl-BB against cyv1 appeared to be comparable to that of 90-1 Br2 (Fig. 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 cyv1-mediated resistance, so we 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, we created one more chimeric ClYVV called Resistance Breaking (RB) which contained the same part of the P3 cistron from 90-1 Br2 as was present in Cl-BB, and we confirmed that RB broke cyv1 resistance (Figs. 1 and 2) as did 90-1 Br2 and Cl-BB. Thus, we 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 breaking of the cyv1 resistance

The P3 cistron has been reported to produce two proteins, P3 and P3N-PIPO, which have the same N-terminal sequence (39, 40). Thus, we designed experiments to test which protein is responsible for breaking of the resistance. There were 58 nucleotide differences between the mapped P3 regions of Cl-no30 and RB (Fig. 3). A total of 47 were synonymous with respect to both proteins, and nine caused seven differences in amino acid
residues in P3 but not in PIPO (Fig. 3A, yellow boxes, and 3B; 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. 3A, orange boxes, and 3C). 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.

We further investigated the possible role of P3N-PIPO in breaking of the resistance. To do this, we 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. We made PIPO point mutants of Cl-no30 and RB 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 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 at position 38 in the PIPO amino acid sequence of RB (ATT [isoleucine] → ACT [threonine]; RB/PIPO<sup>T38T</sup>) dramatically reduced its virulence in cyv1 peas but the opposite substitution (threonine to isoleucine) in Cl-no30 (Cl/PIPO<sup>T38I</sup>) had no effect (Fig. 4A). Conversely, a substitution at position 62 (TAA [stop codon] → CAA [glutamine]; Cl/PIPO<sup>Q62Q</sup>) partially conferred virulence on Cl-no30 but the opposite substitution (glutamine to a stop codon; RB/PIPO<sup>Q62Q</sup>) 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. 4A). These results genetically demonstrated the involvement of P3N-PIPO in the breaking of resistance in cyv1 pea. In addition to 90-1 Br2, we found three other CIYVV isolates (I89-1 [AB732963], N [AB732964], and MB3 [AB732965]) that could break cyv1 resistance. Among these four isolates, both isoleucine-38 and glutamine-62 were conserved (Fig. 3C), supporting the importance of these positions for breaking of 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 of the resistance. We thus investigated the possible role of the P3 protein in breaking of the resistance. 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 (Fig. 4B, RB/P3$_{KII}$PIPO$_{Q62}$), reduced virulence in cyv1 peas. Since elimination of the final 15 amino acid residues alone (Fig. 4A, RB/PIPO$_{Q62}$) 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 (Fig. 4B, Cl/P3$_{RAM}$), making it more like that of RB, was able to partially break cyv1 resistance (Fig. 4B). The results indicated that the P3 protein is also involved in breaking of the resistance conferred by cyv1.
We obtained additional genetic evidence indicating that both P3 and P3N-PIPO were important for breaking of the resistance. Peas 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 infected with 90-1. Comparing the genomic sequence of the P3 cistron of 90-1 with that of 90-1 Br2, we found only one point mutation, which caused a substitution (arginine to methionine) at position 28 (P3&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 with arginine (RB/P3&P3N-PIPO^{M28R}) failed to overcome cyv1 resistance (Fig. 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 we 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&P3N-PIPO^{M28K}), and inoculated these viruses into cyv1 peas (Fig. 4C). These substitutions did not affect virulence in cyv1 peas: Cl/P3&P3N-PIPO^{K28M} still failed to overcome cyv1-mediated resistance and RB/P3&P3N-PIPO^{M28K} still infected the cyv1 peas systemically (Fig. 4C).

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

We have previously shown that cyv1 restricts Cl-no30 to single cells (26). 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 peas. 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 1). Next, we biolistically inoculated cyv1 peas 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\textsuperscript{I38T}, Fig. 4A) impaired its cell-to-cell movement (Table 1), whereas the opposite substitution in Cl-no30 (Cl/PIPO\textsuperscript{T38I}, Fig. 4A) had no effect on its cell-to-cell movement (Table 1). Conversely, although the substitution at position 62 in RB (RB/PIPO\textsuperscript{Q62}, Fig. 4A) had little effect on its cell-to-cell movement (Table 1), the opposite substitution in Cl-no 30 (Cl/PIPO\textsuperscript{62Q}, Fig. 4A) increased its efficiency of cell-to-cell movement almost to that of RB (Table 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 CIYVV isolate is able to move to adjacent cells in a cyv1 pea, the virus infects systemically.

Quantitative involvement of P3N-PIPO in cell-to-cell and systemic movement of CIYVV in cyv1 pea

The genetic analyses described above suggested the involvement of both P3 and P3N-PIPO proteins in breaking of the cyv1 resistance. However, this interpretation did 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 of the resistance. To confirm the involvement of the P3 and P3N-PIPO proteins, the GFP-tagged Cl-no30 infectious clone was biolistically inoculated into cvv1 peas 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/P3-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. 5A and Table 2). The P3 protein from RB also seemed to enhance the cell-to-cell movement of Cl-no30, although to a lesser extent (Fig. 5A and Table 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 of the resistance. To confirm the quantitative involvement of P3N-PIPO, we 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. 5B). Cl/P3N-PIPO and Cl/P3ΔPIPO were genetically expected to produce more P3N-PIPO and P3, respectively, than the parental clone (Cl-no30) in infected plants. These constructs were biolistically inoculated into cvv1 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. 5B
This result led us to hypothesize that RB can break *cvvl* resistance, resulting in systemic infection, by producing more P3N-PIPO protein than Cl-no30.

We examined this hypothesis in the following experiments. First, we compared the levels of P3N-PIPO at 9 dpi in susceptible pea (PI 250438) infected with Cl-no30 or RB. As shown in Fig. 6A, 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 peas but not in the Cl-no30 infected peas (Fig. 6A). 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. 6A, Experiment 1), but none of them had a comparable intensity to that of RB P3N-PIPO. We then compared the accumulation levels of P3N-PIPO produced from the P3 cistrons between Cl-no30 and RB using an *in vitro* translation system and obtained results that were consistent with those of our *in vivo* analysis. P3N-PIPO from RB P3 was detected at about 24 kDa, but that from Cl-no30 P3 (at about 21 kDa) was not detected (Fig. 6C, lanes P3 of Cl-no30 and RB), suggesting a difference in efficiency of P3N-PIPO production between Cl-no30 and RB. Although both experiments failed to detect P3N-PIPO from Cl-no30, we believe that Cl-no30 produced P3N-PIPO in the infected peas but that its accumulation was not detectable because P3N-PIPO has been reported to be essential for the cell-to-cell movement of potyviruses (42, 54), and a point mutant of Cl-no30 that no longer produced P3N-PIPO failed to systemically infect peas inoculated with it (unpublished data). We therefore conclude that P3N-PIPO accumulated to a higher level in the RB-infected pea plants than in the Cl-no30-infected pea plants.
We then examined whether the presence of additional P3N-PIPO protein would enable Cl-no30 to infect cyv1 peas systemically by using WClMV vectors. The cyv1 peas were co-inoculated 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 peas. Although we failed to detect any GFP signals in the upper leaves of the inoculated cyv1 peas, RT-PCR and RT-nested PCR detected Cl-no30 in the upper leaves of the cyv1 peas inoculated with WClMV that produced the P3N-PIPO protein of either Cl-no30 or RB at 11 dpi (Fig. 7). Cl-no30 was not able to infect cyv1 peas systemically when the cyv1 peas were co-inoculated with the WClMV empty vector (Fig. 7). Here, we should note that co-inoculation of Cl-no30 and WCl/no30_P3N-PIPO or WCl/RB_P3N-PIPO did not always result in systemic infection of cyv1 peas with both viruses. This failure of systemic infection might be explained by an effect-related to RNA-mediated cross protection (55) because Cl-no30 is unrelated with 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 peas when WCl/no30_P3N-PIPO and WCl/RB_P3N-PIPO spread systemically (Fig. 7). 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. 5, Table 1 and 2).

Discussion
In this study, we genetically mapped the region involved in the breaking of cyvl-mediated resistance by using chimeric CLYVV\textsubscript{s} constructed from isolates that were virulent and avirulent to cyvl peas, and we 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 (39), we introduced point mutations that 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 of the resistance (Fig. 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 cyvl peas (Figs. 5 and 7 and Tables 1 and 2). Therefore, we clearly showed that the P3N-PIPO protein is a virulence determinant of CLYVV in cyvl peas. The P3 cistron has been shown to be a determinant of the host range of TuMV (33, 34) and of potyvirus virulence in plants with dominant and recessive resistance (29, 30, 56). However, they did not examine whether P3 or P3N-PIPO were responsible for the determination of host range and potyvirus virulence. 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, cyvl and sbm-2 have not yet been identified (26-28). On the other hand, the recessive resistance genes cyv2 (against CLYVV); sbm-1, -3, and -4 [against pea seed-borne mosaic virus (PSbMV)]; and wlv [against bean yellow mosaic virus (BYMV)] are identical or allelic to one another, and
all encode pea homologs of eIF4E in LG VI (3-5, 27). 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
peas, although CIYVV P1 was the determinant in cyv2 peas (4, 5, 21). Regarding the
possible relationship and functions of cyv1, sbm-2, and mo, Gao et al. (28) reported that a
candidate gene, 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 peas, cyv1 and sbm-2 do not likely encode
eIF(iso)4E (26, 28). 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 (29, 30), as we found for the virulence determinants of CIYVV in cyv1 pea
plants.

Then what protein does cyv1 encode and how do P3 and P3N-PIPO interact with it
to break cyv1 resistance? A couple of mechanisms involving recessive resistance to viruses
have been proposed. First, mutations may occur in a plant gene that encodes a repressor of
anti-viral defense systems. Second, mutations may arise that disrupt the function of an
allele-encoding 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 (17). To our knowledge, there is no report
regarding an association of P3 and P3N-PIPO with the repression of anti-viral defenses. We
thus stand by the latter possibility (i.e., P3 and P3N-PIPO may interact with a
cyw1-encoding protein that is a co-opted host factor for CIYVV 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 and is colocalized into vesicles induced by a 6-kDa potyvirus membrane protein (6K) that contain viral RNA and viral replicase components (57). P3 traffics along actin filaments and plays a role in intra- and intercellular movement (36). On the other hand, P3N-PIPO is localized in plasmodesmata and recruits the CI protein to plasmodesmata, which probably facilitates cell-to-cell movement (42). P3N-PIPO has been recently shown to be required for cell-to-cell movement of potyviruses (42, 54), and the resistance mode of pea carrying cyv1 and sbm-2 is to restrict avirulent CIYVV and PSbMV to the initially infected cells (26, 30). Taken together, these studies raise the possibility that cyv1 and sbm-2 are allelic and encode a co-opted host factor that is involved in replication, intra- and intercellular movement of potyviruses and interacts with P3 or P3N-PIPO.

CIYVV isolate 90-1 Br2, which broke the resistance of cyv1, was more virulent toward susceptible pea PI 250438 than Cl-no30 (Fig. 1B). We have recently mapped the genomic region responsible for the higher virulence in susceptible pea using these chimeric CIYVV shown in Fig. 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 co-opted host factor for CIYVV 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 (40).
The quantitative involvement of the P3N-PIPO protein in breaking of the *cvvl* resistance (Figs. 5, 6 and 7, and Tables 1 and 2) is unexpected characteristics, and interesting findings were made in this study. We initially expected that concurrent 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 *cvvl* peas, 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. 5). Moreover, additional systemic production of P3N-PIPO, even that from avirulent Cl-no30, by using a WClMV vector enabled simultaneously inoculated Cl-no30 to move systemically to upper leaves (Fig. 7). These findings suggested that the more P3N-PIPO is produced by ClYVV, the higher its virulence in *cvvl* peas. Indeed, RB produced higher levels of P3N-PIPO protein than did Cl-no30 in susceptible pea (Fig. 6A and 6C), which might explain how RB was able to break *cvvl*-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 (42), and both CI and P3N-PIPO are essential for cell-to-cell movement of potyvirus (54, 58). 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 *cvvl* peas, but that the higher levels of P3N-PIPO produced by RB complement the defect and confer the ability for cell-to-cell movement on RB, thus overcoming *cvvl*-mediated resistance.

The next question concerns how RB produces high levels of P3N-PIPO. We
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 (Fig. 6C). This result is in agreement with the increased accumulation of P3N-PIPO in RB-infected susceptible pea (Fig. 6A). Although our *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: 1) the P3 cistron was sufficient and other regions of the viral genome were not required for the production of P3N-PIPO, and 2) 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* peas 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.

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Figure legends

FIG 1 Mapping of the CIYVV genomic region responsible for overcoming cyv1-mediated resistance. (A) The diagrams on the left illustrate chimeric constructs based on CIYVV isolate no. 30 (CI-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 (26) was resistant to CI-no30. The chimeric viruses designated CI-BB and RB infected cyv1 peas 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 CIYVV was confirmed by inoculation into susceptible peas (PI 250438). Typical symptoms of PI 250438 leaves infected with the constructed chimeras are shown. (B) Different reactions to CI-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 CI-no30 caused only vein yellowing and mild mosaic symptoms.
Pea lines that showed resistance to Cl-no30 were inoculated with RB. PI 236493, PI 347420, PI 347422, and PI 429853 carry cyv1 (26); 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.

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 non-synonymous differences with respect to the P3 amino acid sequences but synonymous with respect to the PIPO amino acid sequences. Orange boxes indicate non-synonymous 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 non-synonymous difference at position 38 in the PIPO amino acid sequences among CIYVV isolates (see also [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 Bg/II 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 (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 number AB732964), I89-1 (AB732963), and MB3 (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 (A) (i.e., the end of 90-1 Br2 PIPO).

**FIG 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. 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 peas. The substitution at position 62 of Cl-no30 [TAA (stop codon) → CAA (glutamine)] in constructs Cl/PIPOT38I,62Q and Cl/PIPO62Q 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 “**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 cyvl resistance in pea. In RB/P3\textsuperscript{KII}PIPO\textsuperscript{62Q}, the PIPO coding region and upstream region of RB P3 were exchanged with the corresponding region of Cl-no30. In Cl/P3\textsuperscript{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 (A). (C) Role of the N-terminal sequence of P3 in the breaking of cyvl resistance. Isolate 90-1, from which 90-1 Br2 originated, is avirulent to pea carrying cyvl and possesses arginine (R) at position 28 in the N-terminal region of P3; isolate 90-1 Br2, which is virulent to cyvl pea, 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 as Cl/P3&P3N-PIPO\textsuperscript{K28M}, RB/P3&P3N-PIPO\textsuperscript{M28K}, and RB/P3&P3N-PIPO\textsuperscript{M28R}, each containing the indicated substitution. These mutants were independently inoculated onto cyvl and their virulence was examined as in (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. 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 5** Effect of the additional production of P3 and P3N-PIPO in cis and trans on the cell-to-cell movement of Cl-no30 in cyvl pea. (A) Cl-no30 and pE2113 vectors designed to produce P3 and P3N-PIPO from Cl-no30 and RB were co-bombarded onto PI 429853. The cell-to-cell movement of Cl-no30 was monitored by observing GFP fluorescence at 3 days post-bombardment. 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 P3ΔPIPO produced P3N-PIPO and P3, respectively, exclusively. (B) The P3 cistrons that produced P3 (Cl/P3ΔPIPO) or P3N-PIPO (Cl/P3N-PIPO) exclusively were inserted between P1 and HC-pro of Cl-no30 and bombarded onto detached leaves of pea carrying cvv1 (PI 429853). GFP fluorescence was monitored at 3 days post-bombardment.

**FIG 6** 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 peas 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 peas (arrowheads). Asterisks indicate non-specific 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 0.1 mM IPTG (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 non-specific band (loading control). (C) *In vitro* analysis of P3N-PIPO production. RNAs were translated in the presence of \(^{35}\text{S}\)methionine. The translation products were separated by 4–12% SDS-PAGE, and the signals were visualized by
autoradiography. The positions of P3 and P3N-PIPO are shown to the right of the panel.

Arrowheads indicate the bands corresponding to RB P3N-PIPO. Molecular mass markers (kDa) are indicated to the left of the panel.

**FIG 7** Higher levels of P3N-PIPO contribute to the breaking of cyv1 resistance.

Co-inoculation with Cl-no30 and WCIMV 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 peas (PI 429853) with ClYVV (upper panel) and WCIMV (middle panel) was detected in samples from uninoculated upper leaves by RT-PCR for WCIMV CP and RT-nested PCR for ClYVV NIb. Stability of the transgene P3N-PIPO in the WCIMV vectors was confirmed by RT-PCR with primers corresponding to the upstream region of the WCIMV multi-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 co-inoculation test. Essentially similar results were obtained, and one set is shown. PI250: PI 250438, a cultivar susceptible to both ClYVV and WCIMV. M: 100-bp DNA ladder marker. Mock: a pea plant (PI 250438) inoculated with 0.01 M phosphate buffer.
TABLE 1  Cell-to-cell movement of ClYVV with point mutants in P3N-PIPO in cyv1 pea PI 429853 at 5 dpi. All mutants are illustrated in Fig. 4A and Fig 5B.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Moving&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Restricted to single cell&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total number of examined infected cells</th>
<th>Cell-to-cell movement rate&lt;sup&gt;c&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl-no30</td>
<td>2</td>
<td>40</td>
<td>42</td>
<td>5</td>
</tr>
<tr>
<td>Cl/PIPO&lt;sup&gt;62Q&lt;/sup&gt;</td>
<td>15</td>
<td>27</td>
<td>42</td>
<td>36</td>
</tr>
<tr>
<td>RB</td>
<td>15</td>
<td>23</td>
<td>38</td>
<td>39</td>
</tr>
<tr>
<td>RB/PIPO&lt;sup&gt;138T&lt;/sup&gt;</td>
<td>5</td>
<td>79</td>
<td>84</td>
<td>6</td>
</tr>
<tr>
<td>RB/PIPO&lt;sup&gt;Q62&lt;/sup&gt;</td>
<td>11</td>
<td>33</td>
<td>44</td>
<td>25</td>
</tr>
<tr>
<td>Cl/P3ΔPIPO</td>
<td>2</td>
<td>14</td>
<td>16</td>
<td>12</td>
</tr>
<tr>
<td>Cl/P3N-PIPO</td>
<td>12</td>
<td>38</td>
<td>50</td>
<td>24</td>
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</tbody>
</table>

<sup>a</sup> Number of foci where mutants spread to two or more surrounding cells from the initially infected single cells.

<sup>b</sup> Number of foci where mutants were restricted to the initially infected single cell.

<sup>c</sup> The percentage of foci where mutants moved to neighboring cells, calculated as [(number of foci where mutants moved to neighboring cells) / (total number of foci)] × 100.
**TABLE 2** The effect of transiently expressed P3N-PIPO on movement of Cl-no30 in *cvv1* pea (PI 429853) at 5 dpi.

<table>
<thead>
<tr>
<th>Cl-no30 +</th>
<th>PI 250438&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PI 429853</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single cell&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Moving&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Empty&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>pE2113/no30_P3</td>
<td>2</td>
<td>77</td>
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<tr>
<td>pE2113/no30_P3N-PIPO</td>
<td>8</td>
<td>79</td>
</tr>
<tr>
<td>pE2113/no30_P3ΔPIPO</td>
<td>8</td>
<td>81</td>
</tr>
<tr>
<td>pE2113/RB_P3</td>
<td>5</td>
<td>140</td>
</tr>
<tr>
<td>pE2113/RB_P3N-PIPO</td>
<td>3</td>
<td>110</td>
</tr>
<tr>
<td>pE113/RB_P3ΔPIPO</td>
<td>2</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each co-bombardment test was simultaneously carried out with susceptible pea, PI 250438, as a positive control.

<sup>b</sup> The number of foci where Cl-no30 was restricted to the initially infected single cell.

<sup>c</sup> The number of foci where Cl-no30 moved to two or more cells.

<sup>d</sup> The percentage of foci where Cl-no30 moved to neighboring cells, calculated as (number of foci where Cl-no30 moved to neighboring cells) / (total number of foci) × 100.

<sup>e</sup> Only Cl-no30 was bombarded into the plants.
A

<table>
<thead>
<tr>
<th>Strain</th>
<th>P1</th>
<th>HC-Pro</th>
<th>P3</th>
<th>CI</th>
<th>VPg</th>
<th>Nla-pro</th>
<th>Nlb</th>
<th>CP</th>
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<tbody>
<tr>
<td>Cl-no30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>90-1 Br2</td>
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<td>Cl-SB</td>
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</tr>
<tr>
<td>RB</td>
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<table>
<thead>
<tr>
<th>Strain</th>
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<th>PI250438</th>
<th>Visible</th>
<th>infected/total plants</th>
<th>GFP filter</th>
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<td>Cl-P1HC</td>
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<td>Cl-NS</td>
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<td>0/7</td>
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<td></td>
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<tr>
<td>Cl-SB</td>
<td></td>
<td>0/7</td>
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<td></td>
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</tr>
<tr>
<td>RB</td>
<td></td>
<td>18/18</td>
<td></td>
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</tr>
</tbody>
</table>

B

FIG. 1

90-1 Br2 Cl-no30

GFP

Visible light

infected/total plants

GFP filter

infected/total plants

GFP filter

90-1 Br2 Cl-no30

16dpi

37dpi

16dpi
<table>
<thead>
<tr>
<th>Pea line</th>
<th>Inoculation profiles</th>
<th>Infection profiles</th>
<th>GFP filter</th>
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<tbody>
<tr>
<td>PI 236493</td>
<td>R</td>
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</tr>
<tr>
<td>PI 347420</td>
<td>R</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>PI 347422</td>
<td>R</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>PI 429853</td>
<td>R</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>PI 347295 RS-7</td>
<td>R</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>PI 347295 R-18</td>
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<td>PI 116843 R-8</td>
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</table>
**A**

Cl-no30
- P3N-PIPO
- Cl/P3

<table>
<thead>
<tr>
<th>Cl-no30</th>
<th>GFP</th>
<th>RT-PCR or Nested PCR</th>
<th>Virulence on cyv1 (No. infected /total plant)</th>
</tr>
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<tbody>
<tr>
<td>0/11</td>
<td>0/3</td>
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RB
- P3N-PIPO

<table>
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<th>RB</th>
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<th>RT-PCR or Nested PCR</th>
<th>Virulence on cyv1 (No. infected /total plant)</th>
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<td>18/18</td>
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Cl/PIPO

<table>
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<tr>
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<th>GFP</th>
<th>RT-PCR or Nested PCR</th>
<th>Virulence on cyv1 (No. infected /total plant)</th>
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<tr>
<td>1/11</td>
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Cl/PIPO

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<th>Virulence on cyv1 (No. infected /total plant)</th>
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<tbody>
<tr>
<td>0/6</td>
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Cl/PIPO

<table>
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<tr>
<th>Cl/PIPOT38I,62Q</th>
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<th>RT-PCR or Nested PCR</th>
<th>Virulence on cyv1 (No. infected /total plant)</th>
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<tbody>
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RB

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<thead>
<tr>
<th>RB/PIPOQ62</th>
<th>GFP</th>
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<tbody>
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RB

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<th>RB/PIPO38T</th>
<th>GFP</th>
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<th>Virulence on cyv1 (No. infected /total plant)</th>
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RB

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**B**

Cl/P3

<table>
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<tr>
<th>Cl/P3RAM</th>
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<th>RT-PCR or Nested PCR</th>
<th>Virulence on cyv1 (No. infected /total plant)</th>
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RB

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<th>RT-PCR or Nested PCR</th>
<th>Virulence on cyv1 (No. infected /total plant)</th>
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<tbody>
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</table>

**C**

Cl/PIPO

<table>
<thead>
<tr>
<th>Cl/P3&amp;PIPOK28M</th>
<th>GFP</th>
<th>RT-PCR or Nested PCR</th>
<th>Virulence on cyv1 (No. infected /total plant)</th>
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<tr>
<td>0/11</td>
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RB

<table>
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<tr>
<th>RB/P3&amp;P3N-PIPOM28K</th>
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<th>RT-PCR or Nested PCR</th>
<th>Virulence on cyv1 (No. infected /total plant)</th>
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<tbody>
<tr>
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RB

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<th>RT-PCR or Nested PCR</th>
<th>Virulence on cyv1 (No. infected /total plant)</th>
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<tr>
<td>0/10</td>
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</table>
**FIG. 5**

A

<table>
<thead>
<tr>
<th>Cl-no30 +</th>
<th>Cl-no30 +</th>
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<tbody>
<tr>
<td>pE2113/no30_P3</td>
<td>pE2113/no30_P3N-PIPO</td>
</tr>
<tr>
<td>pE2113/no30_P3ΔPIPO</td>
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</tbody>
</table>

<table>
<thead>
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<th>Cl-no30 +</th>
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</thead>
<tbody>
<tr>
<td>pE2113/RB_P3</td>
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<tr>
<td>pE2113/RB_P3N-PIPO</td>
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<tr>
<td>pE2113/RB_P3ΔPIPO</td>
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</tbody>
</table>

B

**Cl/P3ΔPIPO**

**Cl/P3N-PIPO**

500 µm
Figure 6

(A) Exp. 1 and Exp. 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mock</th>
<th>Cl-no30</th>
<th>RB</th>
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<tbody>
<tr>
<td>kDa</td>
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<td>25</td>
<td>20</td>
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</table>

- P3
- P3N-PIPO
- CBB

(B) No IPTG and 0.1 mM IPTG

<table>
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<tr>
<th>Condition</th>
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<tbody>
<tr>
<td>kDa</td>
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<td>25</td>
</tr>
</tbody>
</table>

- GST-PIPO

(C) Cl-no30 and RB

- P3
- P3ΔPIPO
- P3N-PIPO
- RB_P3N-PIPO
- Cl-no30_P3N-PIPO
FIG. 7

CI-no30 +

<table>
<thead>
<tr>
<th></th>
<th>WCI/empty</th>
<th>WCI/no30_P3N-PIPO</th>
<th>WCI/RB_P3N-PIPO</th>
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<tbody>
<tr>
<td>mock</td>
<td>mock</td>
<td>mock</td>
<td>mock</td>
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<td>PI250</td>
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CIYVV Nib
WCIMV CP
P3N-PIPO from WCIMV

11 dpi