P3N-PIPO, a Frameshift Product from P3, Pleiotropically Determines the Virulence of Clover Yellow Vein Virus in both Resistant and Susceptible Peas

Author(s)
Atsumi, Go; Suzuki, Haruka; Miyashita, Yuri; Choi, Sun Hee; Hisa, Yusuke; Rihei, Shunsuke; Shimada, Ryoko; Jeon, Eun Jin; Abe, Junya; Nakahara, Kenji S; Uyeda, Ichiro

Citation
Journal of Virology, 90(16): 7388-7404

Issue Date
2016-08

Doc URL
http://hdl.handle.net/2115/64427

Type
article (author version)

File Information
0615Atsumi et al1.pdf
P3N-PIPO, a Frameshift Product from P3, Pleiotropically Determines the Virulence of Clover Yellow Vein Virus in both Resistant and Susceptible Peas

Go Atsumi\textsuperscript{a,b,c}\#, Haruka Suzuki\textsuperscript{a}, Yuri Miyashita\textsuperscript{a}, Sun Hee Choi\textsuperscript{a}, Yusuke Hisa\textsuperscript{a}, Shunsuke Rihei\textsuperscript{a}, Ryoko Shimada\textsuperscript{a}, Eun Jin Jeon\textsuperscript{a}, Junya Abe\textsuperscript{a}, Kenji S. Nakahara\textsuperscript{a,d}\#, Ichiro Uyeda\textsuperscript{a,d}

Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido, Japan\textsuperscript{a}; Iwate Biotechnology Research Center, Kitakami, Iwate, Japan\textsuperscript{b}; National Institute of Advanced Industrial Science and Technology, Sapporo, Hokkaido, Japan\textsuperscript{b}; Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan\textsuperscript{d}

Running Head: ClYVV P3N-PIPO is a pleiotropic virulence determinant

#Address correspondence to Go Atsumi, go-atsumi@aist.go.jp, or Kenji S. Nakahara, knakahar@res.agr.hokudai.ac.jp.

Word count for Abstract: 245
Word count for text (excluding references, table footnotes, and figure legends): 9138
ABSTRACT

Peas carrying the cyv1 recessive resistance gene are resistant to clover yellow vein virus (CiYVV) isolates No. 30 and 90-1 (Cl-No.30 and Cl-90-1), but can be infected by a derivative of Cl-90-1 (Cl-90-1 Br2). The main determinant for the breaking of cyv1 resistance by Cl-90-1 Br2 is P3N-PIPO produced from the P3 gene via transcriptional slippage, and the higher level of P3N-PIPO produced by Cl-90-1 Br2 than by Cl-No.30 contributes to the breaking. Here we show that P3N-PIPO is also a major virulence determinant in susceptible peas that possess another resistance gene, Cyn1, which does not inhibit systemic infection with CiYVV but causes hypersensitive reaction–like lethal systemic cell death. We previously assumed that the susceptible pea cultivar PI 226564 has a weak allele of Cyn1. Cl-No.30 did not induce cell death but Cl-90-1 Br2 killed the plants. Our results suggest that P3N-PIPO is recognized by Cyn1 and induces cell death. Unexpectedly, heterologously strongly expressed P3N-PIPO of Cl-No.30 appears to be recognized by Cyn1 in PI 226564. P3N-PIPO accumulation from the P3 gene of Cl-No.30 was significantly lower than that from Cl-90-1 Br2 in a Nicotiana benthamiana transient assay. Therefore, Cyn1-mediated cell death also appears to be determined by the level of P3N-PIPO. The more efficiently a CiYVV isolate broke cyv1 resistance, the more it induced cell death systemically (resulting in a loss of environment for virus accumulation) in susceptible peas carrying Cyn1, suggesting that antagonistic pleiotropy of P3N-PIPO controls the resistance breaking of CiYVV.

IMPORTANCE

Control of plant viral disease has relied on the use of resistant cultivars; however, emerging mutant viruses have broken many types of resistance. Recently, we revealed that Cl-90-1 Br2 breaks the recessive resistance conferred by cyv1, mainly by accumulating a higher level of P3N-PIPO than the non-breaking isolate Cl-No.30. Here, we show that a susceptible pea line...
recognized the increased P3N-PIPO amount produced by Cl-90-1 Br2 and activated the salicylic-acid-mediated defense pathway, inducing lethal systemic cell death. We found a gradation of virulence among ClYVV isolates in cyvl pea and two susceptible peas. This study suggests a trade-off between breaking of recessive resistance (cyvl) and host viability; the latter is presumably regulated by the dominant Cyn1 gene, which may impose evolutionary constraints upon P3N-PIPO for overcoming resistance. We propose a working model of the host strategy to sustain the durability of resistance and control fast-evolving viruses.
INTRODUCTION

Host plants protect themselves from virus infection by activating defense systems mediated by immune receptors (e.g., nucleotide-binding site (NB)–leucine-rich repeat (LRR) proteins) (1). Plants have many NB-LRR immune receptors, each of which recognizes specific viral proteins. The activated immune response is referred to as a hypersensitive response (HR) and is often accompanied by cell death. When HR is induced, the virus is localized in and around the infection locus. NB-LRR immune receptors are encoded in resistance genes that are genetically dominant.

Another important defense against plant virus infection is genetically recessive resistance (2). The viral life cycle totally relies on the host cells, and viruses require host factors in order to multiply within cells and move to neighboring cells. Therefore, the lack of a specific host co-opted factor required for the viral life cycle leads to host resistance against the virus. Many natural recessive resistances against viruses have been identified in diverse crops (2). Extensive studies have been carried out against the viruses belonging to Potyvirus, the major genus in the Potyviridae family, which is one of the two largest plant virus genera and found in most climatic regions worldwide (3). These viruses infect a broad range of host plants including both monocots and dicots. They cause considerable crop damage, resulting in severe economic losses. Most of the recessive resistance genes against members of genus Potyvirus have been identified as encoding eukaryotic initiation factors such as eIF4E. Host eIF4E binds viral VPg protein that is covalently attached to the 5′ end of viral genomic RNA, and the complex initiates the translation of viral protein (4). For example, cyv2 in pea confers recessive resistance to clover yellow vein virus (CIYVV), which causes severe damage to important legume crops including French bean, broad bean, and pea. A previous study showed that the resistance to CIYVV conferred by cyv2 is mediated by eIF4E (5).
There is another recessive gene, *cyv1*, that confers resistance to ClYVV in pea (6). CIYVV No.30 isolate (Cl-No.30) cannot infect PI 429853 carrying *cyv1*, but CIYVV 90-1 Br isolate (Cl-90-1 Br2) can infect systemically (7) (Table 1). Our previous analysis revealed that P3N-PIPO, which consists of the N-terminal amino acids of P3 followed by a small peptide called PIPO encoded in the +2 reading frame (8-10), is a major determinant for breaking of *cyv1* resistance (7). We suggested that higher accumulation of P3N-PIPO in Cl-90-1 Br2 than in Cl-No.30 contributes to the breaking of resistance (7). P3N-PIPO has an essential role in virus cell-to-cell movement (10). Three independent groups including our own recently showed that P3N-PIPO is produced mainly by transcriptional slippage within the *P3* gene (11-13).

Plant viruses continually evolve and gain virulence, which enables them to inhibit or escape plant defense/resistance systems. Although higher virulence is favorable for virus adaptation, extremely high virulence (i.e., induction of lethal systemic cell death) seems to be a disadvantage for virus survival because the virus loses an environment in which to propagate if the host cells are dead. Several examples of excessively high virulence have been reported. Soybean mosaic virus (SMV) strain G7 induces cell death systemically and kills the host plant (14). The turnip mosaic virus (TuMV) TuR1 isolate induces lethal systemic cell death in *A. thaliana* accession Landsberg erecta (Ler) (15). It was shown that the systemic cell death caused by SMV or TuMV is a form of HR that is regulated by *Rsv1* or *TuNI* in soybean or *A. thaliana* Ler, respectively (16, 17). It was suggested that both *Rsv1* and *TuNI* encode NB-LRR resistance proteins (18, 19). These reports and others lead us to propose that extremely high virulence, an unfavorable state for the virus, is controlled genetically by host plants.

Extremely high virulence of ClYVV has been observed in many pea cultivars: CIYVV systemically induces cell death, resulting in plant death within 2 weeks when used to
infect young plants (20-22) (Table 1). We previously reported that the cell death induced by Cl-No.30 in pea PI 118501 is a form of HR-like response accompanied by the activation of the salicylic acid (SA) pathway, which is one of the hallmarks of HR (20). However, Cl-No.30 infection is not localized and induces cell death systemically. A genetic study in pea suggested that this cell death is controlled by a single dominant locus called Cyn1 (21). Cyn1 is suggested to be an NB-LRR gene whose product recognizes Cl-No.30 and induces HR associated with cell death (20, 21).

We previously revealed that Cl-90-1 Br2, a mutant isolate that originated from Cl-90-1, breaks cyvl recessive resistance in pea (7) (Table 1). In the present study, we revealed that Cl-90-1 Br2 induced lethal systemic cell death and found that P3N-PIPO, but not P3, was a major determinant of the induction of cell death in PI 226564. This was discovered by infection of PI 226564 with chimeric ClYVVVs and by transient assays using white clover mosaic virus (WCIMV) vectors. We showed that P3N-PIPO is quantitatively and/or qualitatively involved in cell death induction in pea by using chimeric and mutant ClYVVVs and by transient assays in N. benthamiana. Chimeric P3N-PIPO expression analysis by using WCIMV indicated that the PIPO peptide was not the sole determinant of cell death induction. Finally, we showed a consistent gradation of virulence among ClYVV isolates in cyvl peas (recessive resistance) and two susceptible peas. This study, combined with our previous studies (7), shows that P3N-PIPO is involved in both breaking of recessive resistance and disease expression. We suggest that the pleiotropic effects of P3N-PIPO in determining ClYVV virulence in pea result in a trade-off between breaking of recessive resistance (cyvl) and maintenance of host viability, which is presumably controlled by a Cyn1-mediated immune response.

MATERIALS AND METHODS
Preparation of plasmid constructs and infectious clones

Sequences of the primers used for vector construction are available upon request. The construction of CIYVV chimeric clones Cl-P1HC, Cl-BB, Cl-NS, Cl-SB, Cl-RB, and Cl-RB\(^{M28R}\) with \(GFP (=RB/P3&P3N-PIPO^{M28R})\) in (7)) was described previously (7). To make Cl-RB without \(GFP\), the \(SalI–BamHI\) fragment of Cl-BB with \(GFP\) was used to replace that of Cl-RB with \(GFP\). To make Cl-RB+P1HC without \(GFP\), the \(SalI–BamHI\) fragment of Cl-BB with \(GFP\) was used to replace that of Cl-RB+P1HC with \(GFP\); Cl-RB+P1HC with \(GFP\) was made by replacing the \(BglII (HC-Pro)–BglII (P3)\) fragment of Cl-BB carrying \(GFP\) with that of Cl-P1HC with \(GFP\). To make Cl-RB without \(GFP\), the \(EcoRV–BglII (HC-Pro)\) fragment of \(pClYVV-Pst/CP\) (23) was used to replace that of Cl-RB+NS with \(GFP\); Cl-RB+NS with \(GFP\) was made by replacing the \(BglII–NheI\) fragment of Cl-RB containing \(HC-Pro\) of Cl-No.30 and \(P3\) of Cl-90-1 Br2 with that of Cl-NS with \(GFP\). To replace the \(BglII–NheI\) fragment, two \(NheI\) sites within the \(BglII–NheI\) fragment of Cl-90-1 Br2 were disrupted by site-directed mutagenesis to generate \(BgNh\Delta Nh\). \(BgNh\Delta Nh\) was amplified with primers no.167 and no.196 using two overlapping fragments amplified with no.167/no.201 and no.202/no.196 as templates. To make Cl-RB+SB without \(GFP\), the \(SalI–BamHI\) fragment of Cl-SB with \(GFP\) was used to replace that of Cl-RB without \(GFP\). To make Cl-90-1 Br2-P3B\(^{No.30}\) without \(GFP\), the \(SalI–BamHI\) fragment of Cl-SB with \(GFP\) was used to replace that of Cl-P1HC with \(GFP\), thus producing Cl-P1HC+SB without \(GFP\), and then the \(NheI–SalI\) fragment of Cl-NS with \(GFP\) was used to replace that of Cl-P1HC+SB without \(GFP\) to produce the final vector. Cl-90-1 Br2-P3B\(^{No.30}\) without \(GFP\) contains an \(NheI\) site located upstream from the \(BglII\) site at the 3′ end of the P3B region, but the sequence of the region from the \(NheI\) site to the \(BglII\) site of Cl-90-1 Br2-P3B\(^{No.30}\) without \(GFP\) is identical to that of Cl-No.30 except for a synonymous mutation (G [Cl-No.30] to A [Cl-90-1 Br2-P3B\(^{No.30}\)]) at nucleotide position 801 of the \(P3\) gene (7).
The WClMV vectors pWCl/P3N-PIPO-RB, pWCl/P3-RB, pWCl/P3ΔPIPO-RB, and pWCl/GFP were constructed previously (24, 25). In this study, we constructed pWCl/P3-No.30, pWCl/P3N-PIPO-No.30, pWCl/P3ΔPIPO-No.30, pWCl/P3N-PIPO-CS, pWCl/P3N<sup>RB</sup>-PIPO<sup>CS</sup>, and pWCl/P3<sup>CS</sup>-PIPO<sup>RB</sup>. For construction of pWCl/P3-No.30, pWCl/P3N-PIPO-No.30, and pWCl/P3ΔPIPO-No.30, pCIYVV/C3-S65T (26) was used as a template. The P3 fragment was obtained by PCR with primers no.3406/no.3412. The P3N-PIPO fragment was amplified using primers no.3406/no.3415 from the mixture of two PCR products amplified with primers no.3406/no.3653 and no.3652/no.3415. The length of P3N-PIPO encoded in Cl-No.30 (647 nucleotides) is shorter than that encoded in Cl-RB (692 nucleotides) (7). We cloned P3N-PIPO of Cl-No.30 as a fragment from the 5' end of P3 to the position corresponding to the stop codon of Cl-RB PIPO (located downstream from stop codon of PIPO frame of Cl-No.30). P3N-PIPO has two mutations: (1) a G insertion in the A<sub>6</sub> sequence within the G<sub>2</sub>A<sub>6</sub> motif which is expected to prevent transcriptional slippage or ribosomal frameshift and translate P3N-PIPO as a zero-frame product, and (2) a G-to-A substitution that introduces a stop codon in the P3 frame but a silent mutation in the PIPO frame (24). The P3ΔPIPO fragment was amplified using primers no.3406/no.3412 from the mixture of two PCR products amplified with primers no.3406/no.3410 and no.3411/no.3412. P3ΔPIPO has a stop codon in the PIPO frame but a silent mutation in the P3 frame (24). For construction of pWCl/P3N-PIPO-CS, a cDNA clone constructed from the BYMV CS isolate (pBY-CS) (27) was used as a template. The P3N-PIPO-CS fragment was amplified using primers no.3621/no.3622 from the mixture of two PCR products amplified with primers no.3621/no.3669 and no.3668/no.3622. We cloned P3N-PIPO of BY-CS as a fragment from 5' to 3' end of P3 gene carrying the same mutations introduced in P3N-PIPO-RB for enabling P3N-PIPO to be translated as a zero-frame product. For construction of pWCl/P3N<sup>RB</sup>-PIPO<sup>CS</sup> and pWCl/P3N<sup>CS</sup>-PIPO<sup>RB</sup>, pCl-RB (7) and pBY-CS (27) were used as templates. The P3N<sup>RB</sup>-
PIPO<sup>CS</sup> fragment was amplified using no.3945/no.3625 from the mixture of P3N of Cl-RB and PIPO of BY-CS amplified with no.3945/no.3885 and no.3955/no.3625, respectively. The P3N<sup>CS</sup>-PIPO<sup>RB</sup> fragment was amplified using no.3946/no.3409 from the mixture of P3N of BY-CS and PIPO of Cl-RB amplified with no.3946/no.3887 and no.3954/no.3409, respectively. For construction of P3N<sup>RB</sup>-PIPO<sup>CS</sup> and P3N<sup>CS</sup>-PIPO<sup>RB</sup>, we cloned from 5' end of P3 to stop codon of PIPO frame. For P3-<sup>No.30</sup>, P3N-<sup>PIPO</sup>-<sup>No.30</sup>, P3Δ<sup>PIPO</sup>-<sup>No.30</sup>, and P3N-<sup>PIPO-CS</sup>, the cDNA fragments were introduced into the pGEM-T Easy plasmid (Promega, Fitchburg, WI), digested with SpeI and XhoI, and inserted into the WCIMV vector (25) cut with the same restriction enzymes. For P3N<sup>CS</sup>-PIPO<sup>RB</sup>, the cDNA fragment introduced into the pGEM-T Easy plasmid was digested with NheI and XhoI and inserted into the WCIMV vector cut with the same restriction enzymes. For P3N<sup>RB</sup>-PIPO<sup>CS</sup>, the cDNA fragment introduced into the pGEM-T Easy plasmid was cut with SacII, and 5' and 3' ends of the plasmid were blunted using T4 DNA polymerase (TaKaRa Bio, Kusatsu, Japan). The blunted fragments were digested with XhoI and inserted into the WCIMV vector cut with SmaI and XhoI.

The pTA/RB-P3(PIPO:FLAG<sup>-1</sup>), pTA/No.30-P3(PIPO:FLAG<sup>-1</sup>), pTA/RB-P3N-PIPO:FLAG<sup>mk</sup>, and pTA/No.30-P3N-PIPO:FLAG<sup>mk</sup> constructs were previously described (13).

All viral fragments were amplified using KOD-plus2 neo DNA polymerase (TOYOBO, Osaka, Japan) according to the manufacturer’s instructions, and their nucleotide sequences were confirmed.

**Plant growth conditions and viral infection**

Pea (Pisum sativum), broad bean (Vicia faba), and N. benthamiana were cultivated in a growth chamber or growth room at 21–23°C with a 16-h photoperiod. Viral inocula were...
prepared as described previously (20). For CIYVV, broad bean was inoculated with each infectious cDNA using particle bombardment. For WCIMV, 1 μg of each WCIMV plasmid was mechanically inoculated onto a susceptible pea line, PI 250438. The upper symptomatic leaves were harvested and ground in an inoculation buffer (0.1 M phosphate buffer, pH 7.0, and 1% 2-mercaptoethanol). The crude sap was mechanically inoculated onto the second and/or third leaves of 2-week-old pea plants. At the same time, all plants were inoculated with inoculation buffer alone as a negative control (mock inoculation).

**Sequence alignment**

P3N-PIPO amino acid (CIYVV, BYMV, and pea seed-borne mosaic virus [PSbMV]) sequences were aligned using MUSCLE (3.8) (http://www.ebi.ac.uk/Tools/msa/muscle/) (28). The amino acid sequences of P3N-PIPO were obtained by translating the sequences from the 5′ end of P3 to the stop codon of P3N-PIPO after introducing an A into the A6–7 region in the G1,2A6–7 motif of each virus to shift the reading frame.

**RNA extraction, reverse transcription, and real-time PCR**

Pea leaves were homogenized in liquid nitrogen, and total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. Each RNA sample was treated with RNase-free DNase I (Roche Diagnostics, Basel, Switzerland), and 1 μg of total RNA was reverse-transcribed using ReverTraAce (TOYOBO). The reaction mixture (20 μl) contained 100 units of ReverTraAce, 1 mM dNTP, 25 pmol random 9-mers, and 1–2 μg total RNA in 1× buffer. Samples were first incubated at 30°C for 10 min, then at 42°C for 30 min, and finally at 99°C for 5 min. Real-time PCR was performed using the DNA Engine Opticon 2 System (Bio-Rad Laboratories, Hercules, CA) as previously described (20). The reaction mixture (25 μl) contained 0.625 U of ExTaq
(TaKaRa), ExTaq buffer, 0.2 mM dNTP, 0.2 μM each of forward and reverse primers, SYBR Green (×30,000 dilution) (Thermo Fisher Scientific), and cDNA obtained by reverse transcribing 12.5 ng of total RNA. Samples were incubated for 5 min at 95°C; followed by 40 cycles of 95°C for 10 s, 53°C for SA-CHI (accession number L37876) or 55°C for HSR203J (AB026296) for 30 s, and 72°C for 20 s. Transcript levels were normalized to that of 18S rRNA (U43011), and means and standard deviations were calculated. The primers used for real-time PCR were as follows: SA-CHI-F and SA-CHI-R for SA-CHI, HSR203J-F and HSR203J-R for HSR203J, and 18S rRNA-F and 18S rRNA-R for 18S rRNA.

* N. benthamiana* leaves were homogenized in liquid nitrogen, and total RNA was isolated by the AGPC (acid guanidinium thiocyanate–phenol/chloroform) extraction method (29), followed by purification with a FARB column (Favorgen Biotech Corp, Ping-Tung, Taiwan). Total RNA was digested with TURBO DNase (Thermo Fisher Scientific) and reverse-transcribed using PrimeScript RTase (TaKaRa) according to the manufacturer’s instructions. Real-time PCR was performed using the StepOnePlus system (Thermo Fisher Scientific). The reaction mixture (10 μl) contained KAPA SYBR FAST qPCR Kit Master Mix ABI Prism (Kapa Biosystems, Wilmington, MA), 0.3 μM each of forward and reverse primer, and cDNA obtained by reverse transcribing 50 ng of total RNA. Samples were incubated for 20 s at 95°C, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Transcript levels of *P3N-PIPO-FLAG* were normalized to that of *NbEF1α* (AY206004). The primers used were as follows: GGS4-3FL-F and GGS4-3FL-R for *P3N-PIPO-FLAG*, NbEF1α-F and NbEF1α-R for *NbEF1α*. Primers were designed in a region of *P3N-PIPO-FLAG* of CI-RB and CI-No.30 with identical nucleotide sequence (linker and FLAG-tag coding sequence).
For virus detection by RT-PCR, we used primer set no.3735/no.3736 for Cl-I89-1 and Cl-90-1 Br2, and no.3908/no.3909 for BY-CS. PCR was done using KOD-FX DNA polymerase (TOYOBO) according to the manufacturer’s instructions.

Sequences of the primers are available upon request.

**Agrobacterium-mediated transient expression**

Agrobacterium-mediated transient expression was conducted as described previously (30). Agrobacterium LBA4404 cells transformed with each construct were suspended in MES buffer [10 mM 2-(N-morpholino)ethanesulfonic acid (MES), 10 mM MgCl₂, pH 5.7], and the suspensions were adjusted to OD₆₀₀ = 1.0. Acetosyringone was added to the suspensions at a final concentration 200 μM, followed by incubation at room temperature for 2–4 h. Each suspension was infiltrated into *N. benthamiana* leaves using needleless syringes. Leaves were sprayed with 30 μM dexamethasone solution containing 0.01% Tween-20 24 h after agroinfiltration (31). Leaves were collected 24 h after dexamethasone treatment and used for western blot and real-time PCR analysis.

**Western blot**

Western blots were conducted as described previously (30, 32). Proteins were resolved in 12% NuPAGE Bis-Tris gel (Thermo Fisher Scientific) using MES-SDS buffer (FLAG-tagged protein detection) or in 10% SDS-PAGE using Tris-glycine buffer (CP detection; (33)), followed by electrotransfer to a PVDF membrane. To detect the FLAG-tagged proteins, monoclonal Anti-FLAG M2–horseradish peroxidase (HRP) (Sigma-Aldrich Corporation, St. Louis, MO) was used at a 1:5000 dilution. To detect CIYVV CP, rabbit polyclonal antibody against CIYVV CP was used as the primary antibody and alkaline-phosphatase-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) was used as the secondary antibody.
Chemiluminescence signals were detected with ECL Prime (GE Healthcare, Little Chalfont, United Kingdom) using a LAS-4000 imaging system (GE Healthcare) for FLAG-tagged protein detection, or with CDP-Star reagent (New England Biolabs, Ipswich, MA) using a LAS-4000-mini imaging system (GE Healthcare) for CP detection. As a loading control for the FLAG-tagged protein experiment, membranes after transfer were stained with 0.1% amido black in 45% methanol and 10% acetic acid followed by destaining in 90% methanol and 2% acetic acid (34).

**GFP fluorescence analysis**

GFP fluorescence of pea plants infected with GFP-tagged viruses (Cl-No.30/GFP and Cl-RB/GFP) was monitored using an MVX10 epifluorescence microscope (Olympus Corporation, Tokyo, Japan). The fluorescent area was measured by using the color thresholding tool of ImageJ software (35).

**Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)**

DAS-ELISA was conducted according to our previous report (32). A single GFP focus derived from virus was excised from inoculated leaves and used for antigens. We used a mouse anti-ClYVV CP IgG as the first antibody and rabbit anti-ClYVV CP as the second antibody. After washing, alkaline-phosphatase-conjugated goat anti-rabbit IgG was added, followed by the substrate solution (disodium Ω-nitrophenyl-phosphate hexahydrate in 10% diethanolamine). The intensity of the signal was measured at an optical density (OD) of 405 nm.

**Determination of full-length ORF sequence of Cl-I89-1**
cDNA was synthesized from total RNA isolated from pea leaves infected with Cl-I89-1. The sequence covering the full-length Cl-I89-1 ORF was amplified using high-fidelity DNA polymerase (KOD-plus2 neo; TOYOBO) into four overlapping fragments using the following primer sets: no.3230/no.3191, no.2978/no.2493, no.2388/no.2471, and no.2470/no.3229. The four PCR products were directly sequenced by the primer-walking method using primers no.157, no.2372, no.2451, no.2464, no.2470, no.2481, no.2491, no.2552, no.2559, no.2621, no.3130, no.3436, and no.3435. The GenBank/ENA/DDBJ accession number for the full-length ORF sequence of Cl-I89-1 is LC096082. Sequences of the primers are available upon request.

**Phylogenetic analysis**

Phylogenetic analysis was performed for full-length nucleotide sequences encoding polyprotein of CIYVV and BYMV. Sequence alignment was conducted using MUSCLE, and a maximum-likelihood tree was inferred using the MEGA6 package (36). The nucleotide substitution models and rates among sites were general time-reversible and gamma distribution. The significance of the nodes was estimated with 1000 bootstrap replicates.

**RESULTS**

**P3 of Cl-90-1 Br2 was the major virulence determinant in PI 226564**

Cl-90-1 Br2 induced lethal systemic cell death in PI 226564 (Fig. 1A, Table 1). To identify the virulence determinant of Cl-90-1 Br2 in PI 226564, chimeric viruses were constructed by swapping parts of Cl-90-1 Br2 and Cl-No.30; the latter virus does not induce cell death in PI 226564 (Fig. 1A, Table 1) (20, 21). These chimeric viruses were based on Cl-No.30 infectious cDNA that we previously constructed and developed for use as a gene expression vector (7, 23, 37, 38). Chimeric viruses tagged with GFP were created that covered almost all
regions of the ClYVV genome: Cl-P1HC/GFP, Cl-BB/GFP, Cl-NS/GFP, and Cl-SB/GFP (Fig. 1B) (7). Symptoms indicated that only the BB region of Cl-90-1 Br2 markedly enhanced the virulence of Cl-No.30 (Fig. 1C). Cl-BB/GFP induced cell death in upper uninoculated leaves (Fig. 1C). The P1HC and SB regions of Cl-90-1 Br2 did not enhance Cl-No.30 virulence (Fig. 1C). The NS region slightly enhanced the virulence: Cl-NS/GFP occasionally induced cell death associated with yellowing in upper uninoculated leaves (Fig. 1C). In contrast to Cl-90-1 Br2, Cl-BB/GFP did not kill completely the plants, but a mosaic pattern associated with cell death developed in the upper uninoculated leaves (Fig. 1C).

Further analysis was focused on the virulence enhancement mediated by the Cl-90-1 Br2 BB region, which included ca. 94% of HC-Pro and ca. 79% of P3 from Cl-90-1 Br2. Cl-P1HC/GFP had the full-length HC-Pro gene of Cl-90-1 Br2 but could not enhance virulence (Fig. 1B and C), suggesting that the P3-containing portion of the BB region (designated P3B as shown in Fig. 1B) was important for high virulence expression. We constructed Cl-RB/GFP, in which the P3B region of Cl-No.30 was replaced by that of Cl-90-1 Br2 (Fig. 1B). Cl-RB/GFP extensively induced cell death in upper uninoculated leaves, comparable to that induced by Cl-BB/GFP (Fig. 1C).

Like Cl-BB/GFP, Cl-RB/GFP did not kill the plants. To investigate whether insertion of GFP (which was present in the first set of chimeric constructs tested) attenuated ClYVV virulence, the symptoms induced by Cl-No.30 with GFP (inserted between P1 and HC-Pro or between Nlb and CP) were compared with those induced by Cl-No.30 without GFP. The results indicated that insertion of GFP weakened the symptoms produced by ClYVV (Fig. D), although virus accumulation was not visibly different as measured by western blotting against CP (Fig. D). To investigate whether the weaker virulence of Cl-RB/GFP relative to Cl-90-1 Br2 was due to GFP insertion, we compared the virulence of Cl-RB without GFP with that of Cl-90-1 Br2 (7) and Cl-No.30 without GFP (37). Cl-RB
without GFP also induced more severe symptoms than Cl-No.30 without GFP, but did not have the level of virulence of Cl-90-1 Br2 (Fig. 1E). We also examined the reciprocal chimera of Cl-RB, Cl-90-1 Br2-P3B<sup>No.30</sup> without GFP, which contained the P3B region from Cl-No.30 and all other regions from Cl-90-1 Br2 (Fig. 1B). Cl-90-1 Br2-P3B<sup>No.30</sup> without GFP induced yellowing and cell death in upper uninoculated leaves, but the timing was delayed in comparison with Cl-RB without GFP (Fig. 1F). It indicated that Cl-90-1 Br2-P3B<sup>No.30</sup> showed less virulence than Cl-RB, and more virulence than Cl-No.30. Together, these results indicated that the P3B region was the main determinant of virulence in PI 226564, though regions outside of P3B also contributed to virulence expression.

To identify the virulence determinant(s) outside the P3B region, we created chimeric viruses without GFP each of which has P1HC, NS, or SB regions of Cl-90-1 Br2 in addition to P3B of Cl-90-1 Br2, so that almost all regions of the CIYVV genome were covered (Fig. 1B). All of the chimeric viruses expressed higher virulence than Cl-RB at 21 days post inoculation (dpi) (Fig. 1G); they induced cell death in both inoculated and upper uninoculated leaves. Plants infected with Cl-RB+P1HC, Cl-RB+NS, or Cl-RB+SB were shorter than those infected with Cl-RB; however, none of the chimeric viruses killed the plants completely (Fig. 1G).

Taken together, these data showed that Cl-No.30 carrying Cl-90-1 Br2 regions outside of P3B (Cl-P1HC, Cl-NS, Cl-SB, and Cl-90-1 Br2-P3B<sup>No.30</sup>) had weaker virulence than Cl-No.30 carrying the P3B region of Cl-90-1 Br2 (Cl-RB) and those in combination with Cl-90-1 Br2 regions (Cl-RB+P1HC, Cl-RB+NS, Cl-RB+SB). This indicated that the effect of virulence was the highest in exchanging the P3B region and the effect of virulence enhancement by regions outside of P3B was higher in virus carrying the P3B region of Cl-90-1 Br2 than in virus carrying the P3B region of Cl-No.30. These symptom observations collectively suggested that, although regions outside of P3 contributed to virulence
expression, the *P3* gene (P3B region) was the major determinant for inducing lethal systemic cell death in PI 226564.

P3N-PIPO, but not P3, of Cl-RB was responsible for cell death induction in PI 226564

*P3* expresses two mature proteins, *P3* and *P3N-PIPO* (8). To dissect which protein induces cell death, *P3, P3ΔPIPO, and P3N-PIPO* from Cl-RB were expressed in PI 226564 by WClMV vectors (designated WCl/P3-RB, WCl/P3ΔPIPO-RB, and WCl/P3N-PIPO-RB, respectively) (24). We have previously shown that WClMV can infect PI 226564 but does not induce cell death (25) (Table 1). The *P3* construct was expected to produce *P3* protein accompanied by a small amount of *P3N-PIPO* protein as a frameshift product (Fig. 2A) (24). *P3ΔPIPO* had a mutation enabling it to produce *P3* but not *P3N-PIPO* (Fig. 2A) (24). *P3N-PIPO* had mutations enabling it to express *P3N-PIPO* in the zero frame but not *P3* frame product (Fig. 2A) (24). We inoculated PI 226564 with the three WClMV vectors and WClMV expressing *GFP* (WCl/GFP) as a negative control.

We found that WCl/P3N-PIPO-RB extensively induced cell death along the veins of inoculated leaves at 5 dpi (Fig. 2B, Table 1). In contrast, infection with WCl/P3-RB, WCl/P3ΔPIPO-RB, and WCl/GFP did not induce cell death (Fig. 2B). These results suggested that *P3N-PIPO*, not *P3*, was the factor responsible for inducing cell death in PI 226564. It should be noted that the nucleotide sequence of *P3N-PIPO* of Cl-RB is the same as that of Cl-90-1 Br2.

P3N-PIPO of Cl-No.30 also induced cell death in PI 226564

Cl-No.30 does not induce cell death in PI 226564, which suggested that *P3N-PIPO* of Cl-No.30 would not induce cell death either (20). We inoculated PI 226564 with WClMV carrying *P3N-PIPO, P3*, or *P3ΔPIPO* of Cl-No.30 (designated WCl/P3-No.30,
WCl/P3ΔPIPO-No.30 and WCl/P3N-PIPO-No.30, respectively). Unexpectedly, WCl/P3N-PIPO-No.30 extensively induced cell death along the veins on the inoculated leaves in PI 226564 (Fig. 2C, Table 1). This symptom was comparable to that induced by WCl/P3N-PIPO-RB (Fig. 2B). WCl/P3-No.30, WCl/P3ΔPIPO-No.30, and WCl/GFP did not induce cell death at 5 dpi in PI 226564 (Fig. 2C).

We constructed a WCLMV vector that expresses P3N-PIPO from the BYMV CS strain (BY-CS), designated WCl/P3N-PIPO-CS, in order to rule out the possibility that cell death was non-specifically caused by overexpression of P3N-PIPO. We expected that P3N-PIPO of BY-CS would not induce cell death because BY-CS has never been reported to induce cell death in pea, including PI 226564 (20, 21). Like CIYVV, however, BY-CS is a member of genus Potyvirus, and the two are closely related (39). The nucleotide sequence identity of P3N-PIPO between BY-CS and CI-90-1 Br2 or CI-No.30 is 64.2% or 61.9%, respectively. The amino acid sequence identity (similarity) of P3N-PIPO between BY-CS and CI-90-1 Br2 or CI-No.30 is 56.5% (73.8%) or 54.9% (71.3%), respectively (Fig. 3). In PI 226564 infected with WCl/P3N-PIPO-CS, cell death was not induced in either inoculated or upper uninoculated leaves even at 14 dpi (Fig. 2D, Table 1), thus ruling out the possibility that overexpression of P3N-PIPO non-specifically induced cell death.

P3N-PIPO of CI-No.30 and RB, but not of BY-CS, activated the SA signaling pathway in PI 226564

One of the possible mechanisms that induces cell death by P3N-PIPO is high activation of the SA signaling pathway. We previously showed that activation of the SA signaling pathway contributes to induction of systemic cell death by CIYVV in susceptible peas PI 118501 and PI 226564 (20). Therefore, we hypothesized that expression of CI-90-1 Br2 P3N-PIPO
activated the SA signaling pathway, which led to induction of systemic cell death in PI 226564.

To test this hypothesis, we analyzed the expression of SA-responsive chitinase gene (SA-CHI) and an HR-related gene homologous to tobacco HSR203J (HSR203J) by real-time PCR. We conducted an expression analysis in leaves of PI 226564 inoculated with Cl-90-1 Br2, Cl-No.30, or BY-CS. The expression level of SA-CHI was significantly higher in leaves inoculated with Cl-90-1 Br2 than in leaves inoculated with either Cl-No.30 or BY-CS (Fig. 4A). There were no significant differences among mock, Cl-No.30, and BY-CS inoculation (Fig. 4A). The expression level of HSR203J was also significantly higher in the leaves inoculated with Cl-90-1 Br2 than in those with mock inoculation (Fig. 4B). These results indicated that Cl-90-1 Br2 infection activated SA and HR-like signaling pathways in PI 226564.

We carried out an expression analysis of SA-CHI in leaves of PI 226564 inoculated with WCl/P3N-PIPO, WCl/P3, or WCl/P3ΔPIPO from Cl-RB; the corresponding set of sequences from Cl-No.30; or WCl/GFP. At 4 dpi, SA-CHI was significantly induced only in leaves inoculated with WCl/P3N-PIPO-RB or WCl/P3N-PIPO-No.30 (Fig. 4C and D). WCl/P3 and WCl/P3ΔPIPO (from both Cl-RB and Cl-No.30), GFP, and mock inoculations did not significantly induce SA-CHI (Fig. 4C and D). We also investigated the expression level of SA-CHI in the leaves inoculated with WCl/P3N-PIPO-CS (from BY-CS) and confirmed that WCl/P3N-PIPO-CS infection did not induce SA-CHI expression (Fig. 4D). The amplitudes of SA-CHI upregulation in plants infected with WCl/P3N-PIPO of Cl-RB and Cl-No.30 (Fig. 4C and D) were several orders of magnitude higher than those in plants infected with Cl-RB (Fig. 4A), possibly because Cl-RB produced lower levels of P3N-PIPO (produced by transcriptional slippage) than did WClMV carrying P3N-PIPO (produced in zero frame).
Lower accumulation of Cl-No.30 P3N-PIPO than Cl-RB P3N-PIPO was presumably the reason for the lower virulence of Cl-No.30

We found that heterologous expression of Cl-No.30 P3N-PIPO could induce cell death in PI 226564 (Fig. 2C, Table 1), which was seemingly inconsistent with the fact that Cl-No.30 does not induce cell death in this same cultivar (Fig. 1A, Table 1) (20, 21). We previously showed that P3N-PIPO could be detected in Cl-RB-infected plants but was below the level of detection in Cl-No.30-infected plants of a susceptible pea cultivar, PI 250438, indicating that the level of P3N-PIPO from Cl-No.30 is significantly lower than that from Cl-90-1 Br2 (7). This same difference was observed when P3N-PIPO was produced from the P3 cistron of each virus using an in vitro translation system with an A. thaliana cell-free system and wheat germ extract (7, 13). In this study, we compared the accumulation of Cl-No.30 P3N-PIPO and Cl-RB P3N-PIPO in a transient expression system, agroinfiltration in N. benthamiana leaf tissues.

We prepared construct P3(PIPO:FLAG−1), in which a FLAG epitope tag sequence was inserted in front of the stop codon of the PIPO coding sequence, as a means to detect PIPO-frame products (Fig. 5A) (13). P3(PIPO:FLAG−1) constructs of Cl-RB and Cl-No.30 were transiently expressed in the same leaf of N. benthamiana, and the production of protein from the PIPO frame and mRNA of P3(PIPO:FLAG−1) was compared by measuring the protein/mRNA ratio. Western blotting using a FLAG antibody indicated that the accumulation of PIPO-frame products of Cl-RB was higher than that of Cl-No.30 in three independent plants (Fig. 5B). Similar results were observed in at least three independent experiments. In contrast, real-time PCR analysis indicated that mRNA level of Cl-No.30 P3(PIPO:FLAG−1) was higher than that of Cl-RB (Fig. 5C). Thus, the protein/mRNA ratio of Cl-No.30 was significantly lower than that of Cl-RB (Fig. 5D). As a control experiment, we
compared the protein/mRNA ratio of \( P3N\text{-PIPO:FLAG}^{mk} \), which has mutations that enable production of \( P3N\text{-PIPO} \) mRNA in the zero frame but does not produce \( P3 \) mRNA, between Cl-RB and Cl-No.30 (Fig. 5A) (13). Western blot analysis indicated that there were no visible differences in protein accumulation between Cl-RB and Cl-No.30 (Fig. 5E). Real-time PCR analysis indicated that the mRNA level of Cl-No.30 \( P3N\text{-PIPO:FLAG}^{mk} \) tended to be higher than that of Cl-RB (Fig. 5F). Thus, the protein/mRNA ratio of Cl-No.30 was lower than that of Cl-RB (Fig. 5G). These results showed that P3N-PIPO production from \( P3 \) cistron of Cl-No.30 was lower than that from \( P3 \) cistron of Cl-RB in a transient expression in \( N. \) benthamiana \) leaf tissues.

We also obtained data supporting the possibility that the increased levels of P3N-PIPO produced by Cl-RB enhance virus accumulation in infected plants. We compared the accumulation of Cl-RB with that of Cl-No.30 by using GFP-tagged versions of each virus. P3N-PIPO is an essential factor for potyviruses to move cell-to-cell in infected leaves (40). Therefore, we anticipated that Cl-RB would accumulate more efficiently than Cl-No.30 in PI 226564 if the level of Cl-RB P3N-PIPO was higher than that of Cl-No.30 P3N-PIPO. Virus accumulation levels were compared between Cl-No.30/GFP and Cl-RB/GFP in PI 226564. We excised infection foci (GFP-expressing areas) from inoculated leaves using an epifluorescence microscope (Fig. 6A) and measured the CP amount of each virus by DAS-ELISA (Fig. 6B). The result showed that Cl-RB/GFP accumulated to higher levels than Cl-No.30/GFP at 5 dpi. By measuring the GFP-fluorescent area, we also found that Cl-RB/GFP spread more rapidly than Cl-No.30/GFP at 5 dpi (Fig. 6C). These results suggested that higher production of P3N-PIPO enhanced the ability of Cl-RB to accumulate in the infected pea, perhaps synergistically increasing the difference in accumulation of P3N-PIPO between pea plants infected with Cl-RB and Cl-No.30.
These results collectively suggest that the lower level of Cl-No.30 P3N-PIPO enabled Cl-No.30 to avoid activating the SA signaling pathway, resulting in the loss of cell death induction in PI 226564.

The increased virulence in Cl-90-1 Br2 relative to Cl-90-1 appears to be caused by a single amino acid difference in P3N-PIPO (P3)

Cl-90-1 Br2, a mutant isolate that originated from Cl-90-1, expressed higher virulence than Cl-90-1, which induced cell death in upper uninoculated leaves of PI 226564 but did not kill the plants (Fig. 7, Table 1). We anticipated that the P3B region was responsible for the virulence enhancement in Cl-90-1 Br2 relative to Cl-90-1. We created a chimeric virus based on Cl-RB/GFP with the P3B region (from Cl-90-1 Br2) replaced by that of Cl-90-1 (Fig. 7A).

There is a single non-synonymous difference between the two sequences (T [Cl-90-1 Br2] vs. G [Cl-90-1]) that causes a substitution of methionine (Cl-90-1 Br2) with arginine (Cl-90-1) at amino acid position 28 of P3 (Fig. 7B); thus, the chimeric virus was designated as Cl-RB^{M28R}/GFP. As expected, the symptoms induced by Cl-RB^{M28R}/GFP were weaker than those induced by Cl-RB/GFP (Fig. 7C and D). Cl-RB^{M28R}/GFP induced yellowing and cell death in upper uninoculated leaves, but the timing was delayed in comparison with Cl-RB/GFP (Fig. 7D). The plants infected with Cl-RB^{M28R}/GFP were reproducibly taller than those infected with Cl-RB/GFP (Fig. 7C). The substitution at aa position 28 of P3N-PIPO (P3) is in the N-terminal region, distant from the PIPO coding region (Fig. 3), suggesting that the substitution did not affect PIPO-frame translation and qualitatively affected virulence through some other mechanism. It should be noted that Cl-RB/GFP can break cyv1 resistance whereas Cl-RB^{M28R}/GFP cannot, indicating that the same single amino acid substitution affected both breaking of cyv1 recessive resistance and symptom severity in susceptible cultivar PI 226564.
The substitution at aa position 28 is also close to the position important for PSbMV virulence in pea carrying sbm-2 recessive resistance (Fig. 3) (41).

**P3N-PIPO also induced cell death in two other pea lines, PI 118501 and PI 429853.**

To investigate whether the P3N-PIPO proteins of Cl-90-1 Br2, Cl-No.30, and BY-CS had the ability to induce cell death in PI 118501 (Cyn1) (21) and PI 429853 (cyv1) (7), these P3N-PIPO proteins were expressed by WClMV vectors in these two lines. P3N-PIPO of Cl-90-1 Br2 and Cl-No.30 extensively induced cell death at 5 dpi in PI 118501, whereas P3N-PIPO of BY-CS did not induce cell death until 8 dpi (Fig. 8A, Table 1). The same pattern was observed for infection of PI 429853 (Fig. 8B and C, Table 1). These results indicated that each P3N-PIPO protein had the ability to induce cell death in both PI 118501 and PI 429853. The results were inconsistent with symptoms in the context of virus infection (Table 1), suggesting that cell death induction was determined by other factors in addition to P3N-PIPO.

**Cell death induction was not determined solely by the PIPO region**

In PI 226564, P3N-PIPO of Cl-RB induced cell death but P3 did not (Fig. 2B). As P3N-PIPO has the same N-terminal region (P3N) as P3, PIPO is the only region distinguishing P3N-PIPO from P3. Therefore, we inferred that the PIPO domain of Cl-RB was responsible for cell death induction in PI 226564. To test this possibility, we created chimeric **P3N-PIPO** genes that had either **P3N** of Cl-RB and **PIPO** of BY-CS (**P3N**\textsuperscript{RB-PIPO\textsuperscript{CS}}) or **P3N** of BY-CS and **PIPO** of Cl-RB (**P3N**\textsuperscript{CS-PIPO\textsuperscript{RB}}); these combinations were chosen because P3N-PIPO of BY-CS did not induce cell death in PI 226564 (Fig. 2D). P3N\textsuperscript{RB-PIPO\textsuperscript{CS}} and P3N\textsuperscript{CS-PIPO\textsuperscript{RB}} were expressed by WClMV vectors in PI 226564. P3N\textsuperscript{CS-PIPO\textsuperscript{RB}} expression induced cell death in the inoculated leaves, but it was markedly weaker than that induced by P3N-PIPO of Cl-RB (Fig. 9A). P3N\textsuperscript{RB-PIPO\textsuperscript{CS}} expression did not induce cell death (Fig. 9A).
We also expressed the chimeric P3N-PIPO proteins in PI 118501 by using the same WCIMV vectors. When we inoculated PI 118501 with WCl/P3N\textsuperscript{RB}-PIPO\textsuperscript{CS} or WCl/P3N\textsuperscript{CS}-PIPO\textsuperscript{RB}, neither one induced cell death at 5 dpi or even at 18 dpi in the inoculated leaves, although both WCl/P3N-PIPO-RB and WCl/P3N-PIPO-CS induced severe cell death at 18 dpi (Fig. 9B).

A consistent gradation of virulence was observed among CIYVV isolates in cyv\textit{l} and susceptible peas

Previous studies using PI 429853 (cyv\textit{l}) indicated that Cl-No.30 never infects this line, Cl-90-1 can produce resistance-breaking mutants, and Cl-90-1 Br2 and Cl-I89-1 can systemically infect this line (Table 1) (7). In this study, we compared the symptoms between Cl-90-1 Br2 and Cl-I89-1. Cl-90-1 Br2 induced no symptoms in PI 429853 (cyv\textit{l}), whereas Cl-I89-1 induced chlorosis and cell death systemically at 25 dpi (Fig. 10A). RT-PCR analysis of upper uninoculated leaves confirmed Cl-I89-1 infection in all three plants and confirmed Cl-90-1 Br2 infection in two out of three plants (Fig. 10A and B). Plants inoculated with BY-CS did not show any symptoms at 25 dpi in PI 429853 (cyv\textit{l}), and RT-PCR analysis indicated that no plants were infected with BY-CS (Fig. 10C). Therefore, the order of virulence in PI 429853 (cyv\textit{l}) was Cl-I89-1 > Cl-90-1 Br2 > Cl-90-1 > Cl-No.30 and BY-CS (Table 1). Similarly, the symptom severity in susceptible pea line PI 226564 was Cl-90-1 Br2 > Cl-90-1 > Cl-No.30 (Fig. 7, Table 1) (20, 42). We compared the symptom severities between Cl-I89-1 and Cl-90-1 Br2, and between Cl-No.30 and BY-CS. The symptoms induced by Cl-I89-1 were more severe than those induced by Cl-90-1 Br2 at 10 dpi in PI 226564 (Fig. 10D). The symptoms induced by Cl-No.30 were more severe than those induced by BY-CS at 10 dpi in PI 226564 (Fig. 10E) These results indicated a consistent
gradation of virulence among these viruses in both PI 429853 (cyv1) and susceptible PI 226564 (Table 1).

Next, we investigated whether the same gradation was observed in another susceptible cultivar, PI 118501, which shows lethal systemic cell death following Cl-No.30 infection but not BY-CS infection (20, 21). The inoculation test indicated that Cl-I89-1, Cl-90-1 Br2, Cl-90-1, and Cl-No.30 similarly induced lethal systemic cell death (Fig. 10F), whereas BY-CS induced only mosaic symptoms in the upper uninoculated leaves at 12 dpi (Fig. 10G and H). Cell death induction by Cl-No.30 was slightly slower than induction by Cl-I89-1, Cl-90-1 Br2, and Cl-90-1. These results indicated that the order of symptom severity in PI 118501 was Cl-I89-1, Cl-90-1 Br2 and Cl-90-1 > Cl-No.30 > BY-CS (Table 1). Taken together, these results indicated that a consistent gradation exists in virulence among CIYVV and BYMV in PI 429853 (cyv1 recessive resistance) and in PI 226564 and PI 118501 (susceptible) (Table 1; Fig. 10I).

Phylogenetic analysis using full-length ORF sequences encoding polyprotein suggested that high-virulence isolates (Cl-90-1, Cl-90-1 Br2, and Cl-I89-1) form a group distinct from the low-virulence isolate Cl-No.30 (Fig. 10J).

DISCUSSION

We revealed that the main determinant of lethal systemic cell death induction by CIYVV was P3N-PIPO. This was determined by analysis using chimeric viruses and transient expression from WCIMV vectors in a susceptible pea cultivar, PI 226564. SMV strain G7 induces lethal systemic cell death in soybean carrying the dominant resistance gene Rsv1 (14). In that virus, P3, not P3N-PIPO, determines virulence (40). TuMV induces lethal systemic cell death in A. thaliana Ler carrying the TuNI gene (15). TuMV P3 expression alone is sufficient for induction of cell death at a single-cell level, and the region required for cell death induction is
upstream of the PIPO coding sequence (43). Our study is the first report to suggest that induction of lethal systemic cell death could be attributed to P3N-PIPO.

As mentioned above, it was unexpected to see that expression of P3N-PIPO from Cl-No.30 by a WClMV vector induced cell death in PI 226564 (Fig. 2C) because Cl-No.30 itself does not induce cell death in this cultivar (Fig. 1A) (20, 21). In N. benthamiana, the Plantago asiatica mosaic virus (PlAMV) Li1 isolate induces systemic necrosis that has HR-like characteristics (44, 45). Transient expression of the PlAMV Li1 isolate RdRp (helicase domain) by agroinfiltration induces cell death in N. benthamiana (46). These results suggest that N. benthamiana recognizes PlAMV RdRp and induces an HR-like response systemically, resulting in systemic necrosis. Interestingly, transient expression of RdRp encoded by the PlAMV Li6 asymptomatic isolate also induces cell death in N. benthamiana (46). RdRp accumulation is higher in the areas infiltrated with Agrobacterium carrying infectious cDNA of the Li1 isolate than when infectious cDNA of the Li6 isolate is used (46). These results suggest that N. benthamiana has the ability to recognize both Li1 and Li6 RdRp, but the difference in their protein accumulation levels determines the induction of systemic necrosis in the context of virus infection. The results reported here can be explained in a similar fashion. Previously, we reported that in susceptible pea PI 250438 infected with Cl-90-1 Br2 or Cl-No.30, the P3N-PIPO of Cl-90-1 Br2 could be detected but that Cl-No.30 was below the level of detection when tested using an antibody against the PIPO peptide (7). Furthermore, we showed that the amount of P3N-PIPO produced from the P3 cistron of Cl-No.30 is significantly less than that of Cl-RB in an in vitro translation system using MM2dL (an extract derived from A. thaliana MM2d cells) and wheat germ extract (7, 13). In this study, we showed evidence that the P3 cistron of Cl-No.30 produced less P3N-PIPO protein than that of Cl-RB in vivo in agroinfiltrated N. benthamiana (Fig. 5B). The protein/mRNA ratio of Cl-No.30 was lower than that of Cl-RB (Fig. 5D). These results suggest that P3N-
PIPO production, or transcriptional slippage efficiency, from Cl-No.30 \(P3(PIPO:FLAG^{-1})\) (which tagged P3N-PIPO produced by frameshift) is lower than that from Cl-RB. On the other hand, the protein/mRNA ratio of Cl-No.30 \(P3N-PIPO:FLAG^{mk}\) (which produced tagged P3N-PIPO as a zero-frame product) was also lower than that of Cl-RB (Fig. 5G). The protein/mRNA ratio of \(P3(PIPO:FLAG^{-1})\) tended to be lower than that of \(P3N-PIPO:FLAG^{mk}\) in repeated experiments, though there was not a statistically significant difference. These findings suggest that the difference in P3N-PIPO accumulation between Cl-No.30 and Cl-RB was determined by a combination of transcriptional slippage efficiency and other factors such as protein stability or translation efficiency. Taken together, these results support the hypothesis that in susceptible pea PI 226564, Cl-90-1 Br2 can produce P3N-PIPO protein sufficient for induction of cell death but Cl-No.30 cannot, even though host cells are able to recognize P3N-PIPO from both isolates.

We showed that each of the tested regions outside of \(P3\) are accessory involved in the virulence enhancement in PI 226564 (Fig. 1G). In previous studies, we revealed that the \(HC-Pro\) gene is indirectly involved in the induction of lethal systemic cell death in PI 118501 (20, 22, 32). We found that a Cl-No.30 mutant with a D to Y substitution in amino acid position 193 of HC-Pro (Cl-D193Y) loses the ability to induce cell death in PI 118501 (20). Potyvirus HC-Pro is an RNA-silencing suppressor required for efficient virus accumulation in host plants (47). The D193Y mutation in HC-Pro markedly reduces its ability to suppress RNA silencing, and Cl-D193Y accumulation is significantly lower than wild-type Cl-No.30 in PI 118501 (20, 22). Heterologous expression of viral suppressors of RNA silencing (tomato bushy stunt virus P19 or cucumber mosaic virus 2b) can complement the virulence of Cl-D193Y in PI 118501 (32). These results suggest that HC-Pro itself is not an elicitor but indirectly affects cell death induction via its RNA-silencing suppressor activity in PI 118501: reduced accumulation of CIYVV leads to reduced accumulation of the elicitor molecules that...
induce cell death. In this study, we found that the elicitor molecule active against PI 118501 was P3N-PIPO (Fig. 8A). Thus, is likely that Cl-D193Y is not lethal in PI 118501 because it cannot produce enough P3N-PIPO to induce cell death. Taken together, the results indicate that lethal systemic cell death in PI 118501 was induced by P3N-PIPO, the production of which was indirectly regulated by the RNA-silencing suppressor activity of HC-Pro. As observed for HC-Pro, viral genes other than P3 might increase the accumulation of P3N-PIPO, enhancing the virulence in PI 226564 (Fig. 1G).

We found that expression of P3N-PIPO from Cl-90-1 Br2, Cl-No.30, and BY-CS by WCIMV vectors induced cell death in PI 226564, PI 118501, and PI 429853, with the exception of BY-CS P3N-PIPO in PI 226564 (Table 1), suggesting that many peas have a common factor that recognizes P3N-PIPO of two different Potyvirus species (CIYVV and BYMV). The Rx gene in potato encodes an NB-LRR–type resistance gene that confers genetically dominant resistance against potato virus X (PVX) (48). Rx specifically recognizes CP of PVX avirulent strains and induces extreme resistance (epistatic to HR) (49). N. benthamiana expressing Rx also shows resistance to PVX (50). Interestingly, Rx is able to recognize CP of three other species in the genus Potexvirus (narcissus mosaic virus, WCIMV, and cymbidium mosaic virus) and to induce HR in N. benthamiana expressing Rx (51). The product of the L4 resistance gene (NB-LRR) isolated from pepper is able to recognize CP of several distant species in the genus Tobamovirus including tomato mosaic virus, TMV, paprika mild mottle virus, and pepper mild mottle virus, and to induce cell death accompanied by HR when both CP and L4 are transiently expressed in N. benthamiana by agroinfiltration (34). Similarly, the product of N' (NB-LRR) isolated from Nicotiana sylvestris is able to recognize CP of tomato mosaic virus, paprika mild mottle virus, and pepper mild mottle virus and induces cell death accompanied by HR when both CP and N' are transiently expressed in N. benthamiana by agroinfiltration (34). These studies suggest
that a single resistance protein has the potential to recognize a wide range of elicitor molecules, at least within the same genus.

In pea, one of the candidate factors to recognize P3N-PIPO is the product of the Cyn1 gene (21). Genetic analysis indicated that the lethal systemic cell death induced by Cl-No.30 in PI 118501 is controlled by the dominant gene Cyn1 (21). Cyn1 is located on linkage group (LG) 3, where many R gene analogs were suggested to be clustered by a synteny study between pea and Medicago truncatula (21). In this study, we showed that expression of P3N-PIPO of Cl-No.30 in a WClMV vector induced cell death in PI 118501 (Fig. 8A), suggesting that Cyn1 recognized P3N-PIPO and induced lethal systemic cell death. We previously showed the possibility that PI 226564 also has a Cyn1 allele that weakly recognizes Cl-No.30 (20). Cl-No.30 does not induce cell death in PI 226564 (Fig. 1A) (20, 21); however, activation of the SA signaling pathway by application of an SA analog, benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), induces systemic cell death in PI 226564 infected with Cl-No.30 (20). In this study, we obtained the supporting result that expression of Cl-No.30 P3N-PIPO by a WClMV vector induced cell death in PI 226564 (Fig. 2C). In contrast, BTH treatment does not induce cell death in PI 226564 infected with BY-CS (20). Consistent with this result, expression of BY-CS P3N-PIPO by WClMV did not induce cell death in PI 226564 (Fig. 2D). These results suggest that PI 226564 has a Cyn1 allele whose product has the potential to specifically recognize Cl-No.30 but not BY-CS. Cyn1 of PI 226564 might be able to recognize P3N-PIPO effectively when P3N-PIPO is accumulated to high levels, e.g., in situations such as overexpression by a WClMV vector or Cl-RB infection, but not when it is at low levels, e.g., in a situation such as Cl-No.30 infection (20).

In PI 429853 (cyv1), P3N-PIPO protein could be recognized when expressed by a WClMV vector, suggesting that PI 429853 has a Cyn1 allele whose product recognizes ClYVV. It was inconsistent with symptoms in the context of virus infection (Table 1). The cyv1-mediated
resistance would inhibit or reduce accumulation of ClYVV and thus the recognition of P3N-PIPO under natural infection conditions (Table 1). In contrast to its lack of effect in PI 226564, P3N-PIPO of BY-CS could induce cell death in PI 118501 and PI 429853, though more slowly than P3N-PIPO of either Cl-90-1 Br2 or Cl-No.30 (Figs. 2 and 8); these data suggest that Cyn1 of PI 118501 and PI 429853 can recognize BY-CS, although less efficiently than Cl-90-1 Br2 and Cl-No.30. It is noted that we could not detect P3N-PIPO expressed via WClMV vector by western blot analysis and thus could not compare the accumulation levels of P3N-PIPO among the three tested pea cultivars. Taken together, these results suggest that the product of Cyn1 recognizes matching P3N-PIPO of ClYVV and BYMV and activates the SA-mediated defense pathway, resulting in systemic cell death induction in many peas (20).

We showed that expression of P3N-PIPO by WClMV induced cell death in PI 226564 but that expression of P3 did not (Fig. 2). One possible explanation is that a pea protein (e.g., the Cyn1 product) recognizes the PIPO peptide, which is part of P3N-PIPO but not P3. However, the PIPO domain of Cl-P3N-PIPO alone did not appear to induce cell death, as shown in the experiment using chimeric P3N-PIPO (constructed from P3N-PIPOs of Cl-RB and BY-CS) in PI 226564 (Fig. 9). Although the PIPO domain contributed to recognition by peas, the overall structure of P3N-PIPO may be important for full activation of the signaling pathway to induce cell death. A second possible explanation is that a pea protein (e.g., the Cyn1 product) recognizes P3N-PIPO–targeted host factor(s) (the guard/decoy model) (52). Recently, it was found that PCaP1 from A. thaliana and its homolog NbDREPP from N. benthamiana interact with P3N-PIPO (53, 54). PCaP1 interacts with P3N-PIPO via the PIPO domain and does not interact with P3, indicating that PCaP1 is specifically targeted by P3N-PIPO (53). Therefore, a pea protein may monitor the target of P3N-PIPO such as PCaP1 and induce cell death. A third explanation is that pea recognizes P3N-PIPO in a
localization-dependent manner. Previous studies reported that P3 localizes to the ER–Golgi interface and that P3N-PIPO localizes to plasmodesmata (9, 55). Therefore, P3 may not be recognized due to its localization.

Our results using five virus isolates (four CIYVV isolates and one BYMV isolate) and three pea genotypes showed that the more efficiently a particular CIYVV isolate broke cvl recessive resistance, the more it expressed virulence in susceptible peas carrying Cynl (Fig. 10I and Table 1). In particular, we observed adaptive evolution from Cl-90-1 to Cl-90-1 Br2, which overcame cvl resistance through attaining a point mutation in the P3N domain (7). This mutation also resulted in Cl-90-1 Br2 gaining higher virulence than Cl-90-1 in susceptible PI 226564 (Fig. 7). Many studies have suggested that trade-offs are observed in plant virus infection across hosts, and antagonistic pleiotropy (when mutations beneficial for infection of one host are deleterious for infection of another one) explains such trade-offs well (56, 57). For example, tobacco etch virus (TEV) infects several solanaceous plants such as N. tabacum in nature, and some non-solanaceous plants (e.g., Helianthus annuus and Spinacia oleracea) are also susceptible under experimental conditions. Analysis using a TEV mutant series indicated that fitness trade-offs due to antagonistic pleiotropy are observed between N. tabacum and non-solanaceous plants (58). Several studies suggest that viruses pay a fitness cost when they overcome dominant resistance due to adaptive mutations with antagonistic pleiotropic effects. In pepper, tobamoviruses that can overcome dominant resistance conferred by L genes are less able to accumulate in susceptible plants, and virus particles of breaking isolates in the soil are less stable than those of wild-type virus (59, 60).

In Brassica napus, TuMV isolates CZE1 and CDN1 are able to overcome dominant resistance conferred by TuRB01 but are outcompeted by avirulent isolate UK1 in susceptible plants (61). Soybeans carrying dominant Rsv1 or Rsv4 alleles are resistant against SMV-N (62) or three strains (SMV-N, SMV-G7, and SMV-G7d) (63), respectively. SMV-N HC-Pro
mutants or SMV P3 mutants of the three strains can overcome these respective resistances, and accumulation of these mutants is reduced in susceptible cultivars (62, 63). These studies indicated that their fitness trade-offs are caused by antagonistic pleiotropy of the viral genes that overcome dominant resistance. Similar observations have also been reported when viruses overcome recessive resistance. Potato virus Y VPg double mutants show more virulence than VPg single mutants in pepper carrying a pvr2\(^j\) recessive resistance gene (64). In contrast, potato virus Y VPg double mutants are less virulent than single mutants in susceptible pepper (64). In rice, rice yellow mottle virus mutants that can overcome rymv1-2 recessive resistance are less virulent than wild-type virus in susceptible cultivars (65).

These studies collectively support the hypothesis that many viruses pay fitness costs to adapt to new hosts or to overcome resistances, and that these across-host trade-offs are caused by adaptive mutations with antagonistic pleiotropic effects. In the case of ClYVV, our results suggest that many susceptible peas carry Cyn1, whose product recognizes ClYVV (or their P3N-PIPO proteins, in particular) that break cyv1 resistance. In Cyn1 peas, these ClYVV induce an HR-like response associated with systemic cell death, resulting in plant death. As noted previously, the more efficiently a particular ClYVV isolate broke cyv1 recessive resistance, the more it induced systemic cell death, resulting in a loss of tissue to support virus accumulation and leading to a reduction of fitness in susceptible peas carrying Cyn1. This observation suggests that there are fitness trade-offs between overcoming cyv1 and reducing recognition by Cyn1 via the antagonistic pleiotropy of P3N-PIPO. The trade-offs shown in previous studies (described above) involve gaining adaptation to a non-host or overcoming a resistant cultivar, resulting in reduction of viral viability or virulence in a susceptible host. This study is unique in terms of showing trade-offs in a virus overcoming two independent defense systems in a single plant species.
We hypothesize the following model of co-evolution between CIYVV and pea, driven by the antagonistic pleiotropy of P3N-PIPO. (1) CIYVV cannot infect pea carrying the *cyv1* recessive resistance gene. (2) Selection favors mutations in the *P3* gene of CIYVV that enable P3N-PIPO to accumulate to higher levels and/or alter its protein structure, enabling the virus to overcome *cyv1* resistance. (3) After overcoming resistance, the virus can infect and accumulate effectively, but now Cyn1 recognizes the more abundant (Cl-No.30 vs Cl-90-1 Br2) and/or adapted (Cl-90-1 vs Cl-90-1 Br2) P3N-PIPO directly or indirectly and induces cell death systemically. (4) Systemic cell death leads to loss of host viability, which is also unfavorable for the virus. (5) Selection favors mutations in CIYVV that reduce the accumulation of P3N-PIPO and/or change the amino acids of P3N-PIPO required for its recognition or function, resulting in loss of the ability to overcome *cyv1* recessive resistance. Based on the proposed model, Cyn1 may have evolved to recognize CIYVVs (or their P3N-PIPO proteins, in particular) that break *cyv1* resistance in susceptible peas. Although Cyn1-mediated activation of the SA defense pathway does not appear to inhibit CIYVV infection efficiently, as observed in authentic HR (20), systemic cell death may oppose the adaptive evolution to overcome *cyv1* resistance because induction of systemic cell death leads to a loss of host viability. We assume that two independent defense mechanisms (recessive resistance and the SA defense pathway) in pea impose antagonistic pleiotropy on P3N-PIPO. Such a trade-off for virus in overcoming paired defense mechanisms may sustain the durability of resistance against fast-evolving viruses (66).

**FUNDING INFORMATION**

This work was supported in part by JSPS KAKENHI grant number 25850030 to G.A. and 25450055 and 16H04879 to K.S.N., the NOVARTIS Foundation Japan for the Promotion of Science (to K.S.N.), and the Asahi Glass Foundation (to K.S.N.).
ACKNOWLEDGMENTS

We thank Kazue Obara and Kami Murakami for technical assistance. We also thank Takashi Aoyama and Nam-Hai Chua for the use of binary vector pTA7001, and we thank Kappei Kobayashi and Kentaro Yoshida for critical reading of the manuscript and useful discussion.


We declare that we have no conflicts of interest.

References


45. Komatsu K, Hashimoto M, Ozeki J, Yamaji Y, Maejima K, Senshu H, Himeno M, Okano Y, Kagiwada S, Namba S. 2010. Viral-induced systemic necrosis in plants involves both programmed cell death and the inhibition of viral multiplication, which are regulated by independent pathways. Mol Plant Microbe Interact 23:283–293.


66. Miyashita, Y, Atsumi, G, Nakahara, KS. 2016. Trade-offs for viruses in overcoming innate immunities in plants. Mol Plant Microbe Interact
http://dx.doi.org/10.1094/MPMI-05-16-0103-CR

Figure legends

FIG 1 Mapping of the virulence determinant of Cl-90-1 Br2 in susceptible pea PI 226564.

(A) Cl-90-1 Br2 or Cl-No.30 was inoculated and the symptoms were observed in inoculated leaf (a–c), upper uninoculated leaf (d–i), and whole plant (j,k). Photographs were taken at 14 (a–i), 17 (j), and 21 (k) days post inoculation (dpi). (B) Schematic representations of chimeric viruses constructed from Cl-90-1 Br2 (yellow) and Cl-No.30 (blue). Symptom severity in PI 226564 is indicated as the number of (+) symbols. We also show the infection profile in PI 429853 carrying recessive gene cyv1, which was reported in (7). Black triangles indicate positions of GFP insertion. (C) A series of chimeric viruses tagged with GFP were inoculated and the symptoms were monitored. Photographs were taken at 12 (a) and 17 (b) and 15 dpi (c; except for Cl-90-1 Br2 at 12 dpi) (D) Effect of GFP insertion into ClYVV on symptom development and virus accumulation. a, Cl-No.30 without GFP, with GFP between P1 and HC-Pro, or with GFP between NLb and CP was inoculated onto PI 226564 (susceptible), and their symptoms were compared. b, Viral CP accumulation levels were compared by western blotting using antiserum against Cl-No.30 CP. The rbc-L band from the SDS-PAGE gel stained with Coomassie Brilliant Blue is shown as a loading control. (E) Comparison of symptoms among Cl-90-1 Br2, Cl-No.30, and Cl-RB without GFP at 21 dpi. (F) Virulence of
Cl-90-1 Br2-P3B<sup>No.30</sup> a Cl-90-1 Br2 mutant in which the P3B region in the 90-1 Br2 isolate was replaced with the corresponding region of Cl-No. 30. None of the viruses illustrated had a GFP insertion. The symptoms in a whole plant (a) and upper uninoculated leaf (b) are shown. Photographs were taken at 19 (a) and 13 (b) dpi. (G) Mapping of virulence determinants outside the P3B region. None of the chimeric viruses had a GFP insertion. The photograph was taken at 21 dpi.

**FIG 2** Expression of P3 and/or P3N-PIPO from Cl-RB, Cl-No.30, and BY-CS in PI 226564 using a heterologous WCIMV vector. (A) Schematic representations of P3, P3N-PIPO, and P3ΔPIPO constructs. The P3 construct was expected to produce P3 accompanied by a small amount of P3N-PIPO as a frameshift product. P3ΔPIPO contained a mutation enabling it to produce P3 but not P3N-PIPO. P3N-PIPO had mutations for expressing P3N-PIPO in the zero frame but no P3. (B, C) P3, P3N-PIPO, and P3ΔPIPO constructs of Cl-RB (B) or Cl-No.30 (C) were expressed by WCIMV vectors. The photographs in (B) and (C) were taken at 5 dpi. (D) P3N-PIPO of BY-CS was expressed by a WCIMV vector. The photographs were taken at 14 dpi.

**FIG 3** Multiple alignment of amino acid sequences of P3N-PIPO. Alignment was performed using the program MUSCLE (3.8) (http://www.ebi.ac.uk/Tools/msa/muscle/). Accession numbers: PSbMV-DPD1 (NC_001671), PSbMV-L1 (AJ252242), PSbMV-NEP1 (AJ311841), PSbMV-NY (X89997), CIYVV-I89-1 (LC096082), CIYVV-No.30 (AB011819), CIYVV-CYVV (HG970870), CIYVV-Gm (KF975894), CIYVV-90-1 Br2 (AB732962), BYMV-CS (AB373203), BYMV-90-2 (AB439731), BYMV-92-1 (AB439732), BYMV-GDD (AY192568), and BYMV-Vfaba2 (JN692500). The amino acid sequences of P3N-PIPO were obtained by translating the sequences from the 5′ end of P3 to the stop codon of P3N-PIPO.
after introducing an A into the A₆₋₋₇ region in the G₁₋₋₂A₆₋₋₇ motif of each virus to shift the reading frame.

**FIG 4** Real-time PCR analysis of defense-associated gene expressions in susceptible PI 226564. (A, B) SA-responsive (SA-CHI) and HR-associated (HSR203J) gene expressions in response to Cl-90-1 Br2, Cl-No.30, and BY-CS infections. Total RNA was extracted from the leaves (n=3) inoculated with Cl-90-1 Br2, Cl-No.30, and BY-CS at 6 dpi. cDNA was synthesized from total RNA and used for real-time PCR analysis. (C, D) SA-CHI expression in response to P3N-PIPO expression via WCIMV. WCIMV vectors carrying P3N-PIPO, P3, or P3ΔPIPO of Cl-RB (C), the corresponding genes from Cl-No.30 (D), P3N-PIPO from BY-CS (D), and GFP (C and D) as negative control were inoculated onto PI 226564. Total RNA was extracted from the inoculated leaves (n=3) at 4 dpi and used to synthesize cDNA for real-time PCR analysis. Expression levels of SA-CHI and HSR203J were normalized to that of 18S rRNA. Fold changes from mock infection are shown. Error bars indicate standard deviations. Statistical analyses were conducted by the Tukey–Kramer method. Different letters above bars indicate statistically significant differences (P < 0.05 [A, B], P < 0.01 [C, D]).

**FIG 5** Comparison of the amount of P3N-PIPO produced from the P3 cistron between Cl-RB and Cl-No.30 using an agroinfiltration assay in *N. benthamiana* leaf tissues. P3(PIPO:FLAG⁻¹) or P3N-PIPO:FLAGmk of Cl-RB and Cl-No.30 were transiently expressed in the same leaf of *N. benthamiana*, and the production of protein from the PIPO frame was compared. (A) Schematic diagrams of plasmids for analyzing P3N-PIPO expression. To detect P3N-PIPO produced via frameshift to the −1 reading frame, we prepared the P3(PIPO:FLAG⁻¹) construct, in which a FLAG epitope tag sequence was inserted in front of the stop codon of
the sequence encoding PIPO. P3N-PIPO:FLAG\textsuperscript{mk} has mutations that enable expression of
P3N-PIPO tagged with FLAG in the zero frame. These modified P3 cistrons were inserted in
a binary vector between a DEX-inducible promoter and a poly(A) addition signal (pAs). (B)
P3N-PIPO-FLAG accumulation was detected by western blotting using an antibody against
FLAG. The numbers below the upper panel are relative band intensities of Cl-No.30
compared with those of Cl-RB in each plant. Arrowheads indicate bands corresponding to
P3N-PIPO. Yellow fluorescent protein was expressed as a negative control. (C) The level of
P3(PIPO:FLAG\textsuperscript{-}) mRNA was compared between Cl-RB and Cl-No.30 by real-time PCR
analysis. The mRNA levels of P3(PIPO:FLAG\textsuperscript{-}) were normalized to those of EF1\textalpha. The
relative value for the P3N-PIPO-FLAG transcript of Cl-No.30 compared with that of Cl-RB
is indicated for each plant. (D) Protein/mRNA ratios were calculated by dividing the relative
value of protein (B) by that of mRNA (C) for each plant. (E–G) P3N-PIPO-FLAGs of Cl-RB
and Cl-No.30 were expressed in the zero frame (P3N-PIPO:FLAG\textsuperscript{mk} of Cl-RB and Cl-No.30,
respectively). The results in (E–G) are presented similarly to those shown in (B)–(D),
respectively. Welch's t-test was applied to the data in D and G. * and ** indicates P<0.05 and
P<0.01, respectively.

FIG 6 Comparison of virus accumulation between Cl-No.30 and Cl-RB by using GFP-tagged
viruses. (A) PI 226564 was inoculated with Cl-No.30/GFP and Cl-RB/GFP, and photographs
of GFP fluorescence were taken at 5 dpi. Scale bar = 1 mm. (B) Comparison of virus
accumulation per single infection focus at 5 dpi. Each sample was collected from 10 spots
from five plants (2 spots/plant) and used for DAS-ELISA. Error bars indicate the standard
deviations. Welch's t-test was applied to the data. *** indicates P < 0.001. (C) Comparison of
virus cell-to-cell movement. GFP-fluorescent areas in inoculated leaves at 5 dpi were
measured by ImageJ software. The areas of 50 spots from five plants (10 spots/plant) were
measured for each virus. Error bars indicate the standard deviations. Welch's $t$-test was applied to the data. *** indicates $P < 0.001$.

**FIG 7** Mapping of the virulence determinant of Cl-90-1 in PI 226564. (A) Schematic representations of chimeric viruses constructed from Cl-No.30 (blue) and either Cl-90-1 Br2 (yellow) or Cl-90-1 (yellow; sequence was not revealed in area with shaded yellow). Black triangles indicate the position of GFP insertion. Cl-RB$^{M28R}$ has the P3B region of Cl-90-1, which contains an amino acid substitution (methionine to arginine) at position 28 of Cl-RB P3 (asterisk). The symptom severity in susceptible PI 226564 is indicated as the number of (+). We also show the infection profile in PI 429853 carrying recessive gene cyv1, which was reported in (7). (B) Alignment of amino acid sequences surrounding aa position 28 in P3. (C, D) Symptoms induced by Cl-RB$^{M28R}$ were compared with those induced by Cl-RB. The photographs were taken at 14 (C) and 12 (D) dpi.

**FIG 8** $P3N$-$PIPO$ expression by WCIMV in PI 118501 and PI 429853. $P3N$-$PIPO$-$RB$, $P3N$-$PIPO$-$No.30$, and $P3N$-$PIPO$-$CS$ were expressed by WCIMV in PI 118501 (A) and PI 429853 (B, C). The photographs were taken at 5 or 8 dpi, as indicated.

**FIG 9** Mapping of the region determining virulence in $P3N$-$PIPO$ in PI 226564 and PI 118501. $P3N^{CS}$-$PIPO^{RB}$, $P3N^{RB}$-$PIPO^{CS}$, $P3N$-$PIPO$-$RB$, $P3N$-$PIPO$-$CS$, and $GFP$ were expressed by WCIMV vectors in PI 226564 (A) and PI 118501 (B). $P3N^{CS}$-$PIPO^{RB}$ has the $P3N$ region of BY-CS and $PIPO$ of Cl-RB; $P3N^{RB}$-$PIPO^{CS}$ has the $P3N$ region of Cl-RB and $PIPO$ of BY-CS. Mock was treated with inoculation buffer only. The photographs of the inoculated leaves were taken at 8 dpi (A), 5 and 18 dpi (B). Lower panel in leftmost panels of
A shows a magnified picture of the area indicated in upper panel. Arrowheads indicate regions in which cell death was induced.

**FIG 10** Gradation of virulence among CIYVV and BYMV in susceptible and *cyv1* (recessive resistance) peas. (A) Symptoms were compared between Cl-90-1 Br2 and Cl-I89-1 in PI 429853 (*cyv1*) at 25 dpi. The presence/absence of virus infection (by RT-PCR shown in B and C) is indicated below the photograph. (B) Cl-I89-1 and Cl-90-1 Br2 infections in upper uninoculated leaves (shown in A) were confirmed by RT-PCR at 25 dpi in PI 429853 (*cyv1*). (C) BY-CS infection in upper uninoculated leaves was confirmed by RT-PCR at 25 dpi in PI 429853 (*cyv1*). For the positive control (p.c.), RT-PCR was done using an upper uninoculated leaf of PI 118501 inoculated with BY-CS. (D) The symptoms were compared between Cl-I89-1 and Cl-90-1 Br2 in PI 226564 at 10 dpi. (E) The symptoms were compared between Cl-No.30 (without GFP) and BY-CS in PI 226564 at 10 dpi. (F–H) Cl-I89-1, Cl-90-1 Br2, Cl-90-1, Cl-No.30, and BY-CS were inoculated onto PI 118501, and their symptoms were compared at 12 dpi. Upper symptomatic leaves of plants inoculated with BY-CS (G) or mock (H) are shown. (I) Gradation of virulence among CIYVV and BYMV in recessive resistant (PI 429853 [*cyv1*, *Cyn1*]) (top graph) and susceptible (PI 226564 [weak *Cyn1*] and PI 118501 [*Cyn1*]) (bottom graph) cultivars. The graphs indicate the consistent gradation observed in this study: the more efficiently a CIYVV isolate broke the resistance conferred by *cyv1* (top), the more it expressed virulence in susceptible peas (bottom). (J) Molecular phylogenetic analysis of full-length nucleotide sequences encoding polyprotein of CIYVV and BYMV. The sequences were aligned using MUSCLE and the maximum-likelihood tree was inferred using the MEGA6 package (36). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. TuMV-Tu-2R1 was set as an outgroup. The significance
of the nodes was estimated with 1000 bootstrap replicates. The accession number of each virus is shown in Fig. 3 except for TuMV-Tu-2R1 (AB105135).
**TABLE 1** Summary of symptoms induced by CIYVV, BYMV, and WClMV expressing P3N-PIPO in pea

<table>
<thead>
<tr>
<th>Pea cultivar (genotype)</th>
<th>CIYVV</th>
<th>BYMV</th>
<th>WClMV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>189-1</td>
<td>90-1 Br2</td>
<td>90-1 No.30</td>
</tr>
<tr>
<td>PI 429853 (Cyn&lt;sup&gt;f&lt;/sup&gt;, cyv&lt;sup&gt;f&lt;/sup&gt;)</td>
<td>SCD&lt;sup&gt;e&lt;/sup&gt;</td>
<td>No/CS&lt;sup&gt;d&lt;/sup&gt;</td>
<td>No infection&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PI 226564 (Cyn&lt;sup&gt;e&lt;/sup&gt;)</td>
<td>LSCD</td>
<td>LSCD</td>
<td>SCD</td>
</tr>
<tr>
<td>PI 118501 (Cyn&lt;sup&gt;f&lt;/sup&gt;)</td>
<td>LSCD</td>
<td>LSCD</td>
<td>LSCD</td>
</tr>
</tbody>
</table>

*SCD, lethal systemic cell death; SCD, systemic cell death; M, mosaic; CS, chlorotic spot; No, no symptoms.*

<sup>a</sup> LSCD, lethal systemic cell death; SCD, systemic cell death; M, mosaic; CS, chlorotic spot; No, no symptoms.

<sup>b</sup> Cell death induction in the inoculated leaves was observed until 8 days after inoculation.

<sup>c</sup> Assumed to carry Cyn<sup>f</sup>

<sup>d</sup> (7).

<sup>e</sup> Putative weak Cyn<sup>f</sup> allele (20).

<sup>f</sup> (20).

<sup>g</sup> (21)
A

P3

\[ \text{GGAAAAAA} \]

P3N

\[ \text{PIPO} \]

\[ \text{FLAG} \]

\text{nonsense mutation in the P3 frame}

P3 (zero frame)

P3N-PIPO

\[ \text{GGAAAAAA} \]

P3N

\[ \text{PIPO} \]

P3N-PIPO (zero frame)

P3ΔPIPO

\[ \text{GGAAAAAA} \]

P3N

\text{nonsense mutation in the PIPO frame}

P3 (zero frame)

B

P3 -RB

P3N-PIPO -RB

P3ΔPIPO -RB

GFP

mock

FIG 2

C

P3 -No.30

P3N-PIPO -No.30

P3ΔPIPO -No.30

GFP

mock

D

P3N-PIPO -CS

P3N-PIPO -RB

GFP

mock
PSbMV vs sbm-2 (41)

Putative start position of PIPO peptide

P3N-PIPO (P3)

FIG 3
FIG 4
**FIG 5**

**A**

![Diagram of P3(PIPO:FLAG⁻¹) and P3N-PIPO:FLAG lak](image)

**B**

<table>
<thead>
<tr>
<th>Plant</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.53</td>
<td>0.77</td>
</tr>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.18</td>
<td>0.77</td>
</tr>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.35</td>
<td>0.77</td>
</tr>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.28</td>
<td>0.77</td>
</tr>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.28</td>
<td>0.77</td>
</tr>
<tr>
<td>Control</td>
<td>1.00</td>
<td>0.28</td>
<td>0.77</td>
</tr>
</tbody>
</table>

**C**

Graph showing Fold change for P3N-PIPO:FLAG⁻¹

**D**

Graph showing Protein / mRNA ratio for P3N-PIPO:FLAG⁻¹

**E**

<table>
<thead>
<tr>
<th>Plant</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.97</td>
<td>1.00</td>
</tr>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.97</td>
<td>1.00</td>
</tr>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.97</td>
<td>1.00</td>
</tr>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.97</td>
<td>1.00</td>
</tr>
<tr>
<td>RB No.30</td>
<td>1.00</td>
<td>0.97</td>
<td>1.00</td>
</tr>
<tr>
<td>Control</td>
<td>1.00</td>
<td>0.97</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**F**

Graph showing Fold change for P3N-PIPO:FLAG⁻¹

**G**

Graph showing Protein / mRNA ratio for P3N-PIPO:FLAG⁻¹
FIG 6
A

<table>
<thead>
<tr>
<th></th>
<th>PI 226564 (susceptible) symptom</th>
<th>PI 429853 (cryv1) infection profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI-90-1</td>
<td>+++++</td>
<td>No infection</td>
</tr>
<tr>
<td>CI-90-1 Br2</td>
<td>++++++</td>
<td>Breaking</td>
</tr>
<tr>
<td>CI-RB&lt;sup&gt;M28R&lt;/sup&gt;</td>
<td>++</td>
<td>No infection</td>
</tr>
<tr>
<td>CI-RB</td>
<td>+++</td>
<td>Breaking</td>
</tr>
<tr>
<td>CI-No.30</td>
<td>+</td>
<td>No infection</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>28aa</th>
<th>90-1</th>
<th>90-1 Br2</th>
<th>No.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSIYRPRQME K IINEEPFVVLV</td>
<td>SSIYRPRQME M IINEEPFVVLV</td>
<td>SSIYRPRQME K IINEEPFVVLV</td>
<td></td>
</tr>
</tbody>
</table>

C

D

FIG 7
FIG 8
FIG 9
FIG 10
FIG S1 Multiple alignment of nucleotide sequences of CIYVV and BYMV P3 genes. Alignment was performed using the program MUSCLE (3.8) (http://www.ebi.ac.uk/Tools/msa/muscle/)(28). Accession numbers: BYMV-CS (AB373203), CIYVV-No.30 (AB011819), CIYVV-I89-1 (LC096082) and CIYVV-90-1 B2 (AB732962). Red underline indicates the G2A motif. Red boxes indicate the stop codon of the PIPO reading frame in each sequence.