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1 **Title**

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

4

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13 Running Head: CIYVV P3N-PIPO is a pleiotropic virulence determinant

14

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21 **ABSTRACT**

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

40

41 **IMPORTANCE**

42 Control of plant viral disease has relied on the use of resistant cultivars; however, emerging
43 mutant viruses have broken many types of resistance. Recently, we revealed that CI-90-1 Br2
44 breaks the recessive resistance conferred by *cyvI*, mainly by accumulating a higher level of
45 P3N-PIPO than the non-breaking isolate CI-No.30. Here, we show that a susceptible pea line

46 recognized the increased P3N-PIPO amount produced by CI-90-1 Br2 and activated the
47 salicylic-acid-mediated defense pathway, inducing lethal systemic cell death. We found a
48 gradation of virulence among CIYVV isolates in *cyv1* pea and two susceptible peas. This
49 study suggests a trade-off between breaking of recessive resistance (*cyv1*) and host viability;
50 the latter is presumably regulated by the dominant *Cyn1* gene, which may impose
51 evolutionary constraints upon *P3N-PIPO* for overcoming resistance. We propose a working
52 model of the host strategy to sustain the durability of resistance and control fast-evolving
53 viruses.

54

55

56 **INTRODUCTION**

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

64 Another important defense against plant virus infection is genetically recessive
65 resistance (2). The viral life cycle totally relies on the host cells, and viruses require host
66 factors in order to multiply within cells and move to neighboring cells. Therefore, the lack of
67 a specific host co-opted factor required for the viral life cycle leads to host resistance against
68 the virus. Many natural recessive resistances against viruses have been identified in diverse
69 crops (2). Extensive studies have been carried out against the viruses belonging to *Potyvirus*,
70 the major genus in the *Potyviridae* family, which is one of the two largest plant virus genera
71 and found in most climatic regions worldwide (3). These viruses infect a broad range of host
72 plants including both monocots and dicots. They cause considerable crop damage, resulting
73 in severe economic losses. Most of the recessive resistance genes against members of genus
74 *Potyvirus* have been identified as encoding eukaryotic initiation factors such as eIF4E. Host
75 eIF4E binds viral VPg protein that is covalently attached to the 5' end of viral genomic
76 RNA, and the complex initiates the translation of viral protein (4). For example, *cyv2* in pea
77 confers recessive resistance to clover yellow vein virus (CIYVV), which causes severe
78 damage to important legume crops including French bean, broad bean, and pea. A previous
79 study showed that the resistance to CIYVV conferred by *cyv2* is mediated by eIF4E (5).

80 There is another recessive gene, *cyvI*, that confers resistance to CIYVV in pea (6).
81 CIYVV No.30 isolate (CI-No.30) cannot infect PI 429853 carrying *cyvI*, but CIYVV 90-1 Br
82 isolate (CI-90-1 Br2) can infect systemically (7) (Table 1). Our previous analysis revealed
83 that P3N-PIPO, which consists of the N-terminal amino acids of P3 followed by a small
84 peptide called PIPO encoded in the +2 reading frame (8-10), is a major determinant for
85 breaking of *cyvI* resistance (7). We suggested that higher accumulation of P3N-PIPO in CI-
86 90-1 Br2 than in CI-No.30 contributes to the breaking of resistance (7). P3N-PIPO has an
87 essential role in virus cell-to-cell movement (10). Three independent groups including our
88 own recently showed that P3N-PIPO is produced mainly by transcriptional slippage within
89 the *P3* gene (11-13).

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

103 Extremely high virulence of CIYVV has been observed in many pea cultivars:
104 CIYVV systemically induces cell death, resulting in plant death within 2 weeks when used to

105 infect young plants (20-22) (Table 1). We previously reported that the cell death induced by
106 CI-No.30 in pea PI 118501 is a form of HR-like response accompanied by the activation of
107 the salicylic acid (SA) pathway, which is one of the hallmarks of HR (20). However, CI-
108 No.30 infection is not localized and induces cell death systemically. A genetic study in pea
109 suggested that this cell death is controlled by a single dominant locus called *Cyn1* (21). *Cyn1*
110 is suggested to be an NB-LRR gene whose product recognizes CI-No.30 and induces HR
111 associated with cell death (20, 21).

112 We previously revealed that CI-90-1 Br2, a mutant isolate that originated from CI-
113 90-1, breaks *cyv1* recessive resistance in pea (7) (Table 1). In the present study, we revealed
114 that CI-90-1 Br2 induced lethal systemic cell death and found that P3N-PIPO, but not P3, was
115 a major determinant of the induction of cell death in PI 226564. This was discovered by
116 infection of PI 226564 with chimeric CIYVVs and by transient assays using white clover
117 mosaic virus (WCIMV) vectors. We showed that P3N-PIPO is quantitatively and/or
118 qualitatively involved in cell death induction in pea by using chimeric and mutant CIYVVs
119 and by transient assays in *N. benthamiana*. Chimeric P3N-PIPO expression analysis by using
120 WCIMV indicated that the PIPO peptide was not the sole determinant of cell death induction.
121 Finally, we showed a consistent gradation of virulence among CIYVV isolates in *cyv1* peas
122 (recessive resistance) and two susceptible peas. This study, combined with our previous
123 studies (7), shows that P3N-PIPO is involved in both breaking of recessive resistance and
124 disease expression. We suggest that the pleiotropic effects of P3N-PIPO in determining
125 CIYVV virulence in pea result in a trade-off between breaking of recessive resistance (*cyv1*)
126 and maintenance of host viability, which is presumably controlled by a *Cyn1*-mediated
127 immune response.

128

129 **MATERIALS AND METHODS**

130 **Preparation of plasmid constructs and infectious clones**

131 Sequences of the primers used for vector construction are available upon request. The
132 construction of CIYVV chimeric clones CI-P1HC, CI-BB, CI-NS, CI-SB, CI-RB, and CI-
133 RB^{M28R} with *GFP* (=RB/P3&P3N-PIPO^{M28R} in (7)) was described previously (7). To make CI-
134 RB without *GFP*, the *Sall*–*Bam*HI fragment of CI-BB with *GFP* was used to replace that of
135 CI-RB with *GFP*. To make CI-RB+P1HC without *GFP*, the *Sall*–*Bam*HI fragment of CI-BB
136 with *GFP* was used to replace that of CI-RB+P1HC with *GFP*; CI-RB+P1HC with *GFP* was
137 made by replacing the *Bgl*III (*HC-Pro*)–*Bgl*III (*P3*) fragment of CI-BB carrying *GFP* with that
138 of CI-P1HC with *GFP*. To make CI-RB+NS without *GFP*, the *Eco*RV–*Bgl*III (*HC-Pro*)
139 fragment of pCIYVV-Pst/CP (23) was used to replace that of CI-RB+NS with *GFP*; CI-
140 RB+NS with *GFP* was made by replacing the *Bgl*III (*HC-Pro*)–*Nhe*I (*P3*) fragment of CI-RB
141 containing *HC-Pro* of CI-No.30 and *P3* of CI-90-1 Br2 with that of CI-NS with *GFP*. To
142 replace the *Bgl*III–*Nhe*I fragment, two *Nhe*I sites within the *Bgl*III–*Nhe*I fragment of CI-90-1
143 Br2 were disrupted by site-directed mutagenesis to generate BgNhΔNh. BgNhΔNh was
144 amplified with primers no.167 and no.196 using two overlapping fragments amplified with
145 no.167/no.201 and no.202/no.196 as templates. To make CI-RB+SB without *GFP*, the *Sall*–
146 *Bam*HI fragment of CI-SB with *GFP* was used to replace that of CI-RB without *GFP*. To
147 make CI-90-1 Br2-P3B^{No.30} without *GFP*, the *Sall*–*Bam*HI fragment of CI-SB with *GFP* was
148 used to replace that of CI-P1HC with *GFP*, thus producing CI-P1HC+SB without *GFP*, and
149 then the *Nhe*I–*Sall* fragment of CI-NS with *GFP* was used to replace that of CI-P1HC+SB
150 without *GFP* to produce the final vector. CI-90-1 Br2-P3B^{No.30} without *GFP* contains an *Nhe*I
151 site located upstream from the *Bgl*III site at the 3' end of the P3B region, but the sequence of
152 the region from the *Nhe*I site to the *Bgl*III site of CI-90-1 Br2-P3B^{No.30} without *GFP* is
153 identical to that of CI-No.30 except for a synonymous mutation (G [CI-No.30] to A [CI-90-1
154 Br2-P3B^{No.30}]) at nucleotide position 801 of the *P3* gene (7).

155 The WCIMV vectors pWCI/P3N-PIPO-RB, pWCI/P3-RB, pWCI/P3 Δ PIPO-RB,
156 and pWCI/GFP were constructed previously (24, 25). In this study, we constructed pWCI/P3-
157 No.30, pWCI/P3N-PIPO-No.30, pWCI/P3 Δ PIPO-No.30, pWCI/P3N-PIPO-CS,
158 pWCI/P3N^{RB}-PIPO^{CS}, and pWCI/P3N^{CS}-PIPO^{RB}. For construction of pWCI/P3-No.30,
159 pWCI/P3N-PIPO-No.30, and pWCI/P3 Δ PIPO-No.30, pCIYVV/C3-S65T (26) was used as a
160 template. The *P3* fragment was obtained by PCR with primers no.3406/no.3412. The *P3N-*
161 *PIPO* fragment was amplified using primers no.3406/no.3415 from the mixture of two PCR
162 products amplified with primers no.3406/no.3653 and no.3652/no.3415. The length of *P3N-*
163 *PIPO* encoded in CI-No.30 (647 nucleotides) is shorter than that encoded in CI-RB (692
164 nucleotides) (7). We cloned *P3N-PIPO* of CI-No.30 as a fragment from the 5' end of *P3* to
165 the position corresponding to the stop codon of CI-RB *PIPO* (located downstream from stop
166 codon of *PIPO* frame of CI-No.30). *P3N-PIPO* has two mutations: (1) a G insertion in the A₆
167 sequence within the G₂A₆ motif which is expected to prevent transcriptional slippage or
168 ribosomal frameshift and translate P3N-PIPO as a zero-frame product, and (2) a G-to-A
169 substitution that introduces a stop codon in the *P3* frame but a silent mutation in the *PIPO*
170 frame (24). The *P3 Δ PIPO* fragment was amplified using primers no.3406/no.3412 from the
171 mixture of two PCR products amplified with primers no.3406/no.3410 and no.3411/no.3412.
172 *P3 Δ PIPO* has a stop codon in the *PIPO* frame but a silent mutation in the *P3* frame (24). For
173 construction of pWCI/P3N-PIPO-CS, a cDNA clone constructed from the BYMV CS isolate
174 (pBY-CS) (27) was used as a template. The *P3N-PIPO-CS* fragment was amplified using
175 primers no.3621/no.3622 from the mixture of two PCR products amplified with primers
176 no.3621/no.3669 and no.3668/no.3622. We cloned *P3N-PIPO* of BY-CS as a fragment from
177 5' to 3' end of *P3* gene carrying the same mutations introduced in *P3N-PIPO-RB* for enabling
178 P3N-PIPO to be translated as a zero-frame product. For construction of pWCI/P3N^{RB}-PIPO^{CS}
179 and pWCI/P3N^{CS}-PIPO^{RB}, pCI-RB (7) and pBY-CS (27) were used as templates. The *P3N^{RB-}*

180 *PIPO*^{CS} fragment was amplified using no.3945/no.3625 from the mixture of *P3N* of CI-RB
181 and *PIPO* of BY-CS amplified with no.3945/no.3885 and no.3955/no.3625, respectively. The
182 *P3N*^{CS}-*PIPO*^{RB} fragment was amplified using no.3946/no.3409 from the mixture of *P3N* of
183 BY-CS and *PIPO* of CI-RB amplified with no.3946/no.3887 and no.3954/no.3409,
184 respectively. For construction of *P3N*^{RB}-*PIPO*^{CS} and *P3N*^{CS}-*PIPO*^{RB}, we cloned from 5' end of
185 *P3* to stop codon of *PIPO* frame. For *P3-No.30*, *P3N-PIPO-No.30*, *P3ΔPIPO-No.30*, and
186 *P3N-PIPO-CS*, the cDNA fragments were introduced into the pGEM-T Easy plasmid
187 (Promega, Fitchburg, WI), digested with *SpeI* and *XhoI*, and inserted into the WCIMV vector
188 (25) cut with the same restriction enzymes. For *P3N*^{CS}-*PIPO*^{RB}, the cDNA fragment
189 introduced into the pGEM-T Easy plasmid was digested with *NheI* and *XhoI* and inserted into
190 the WCIMV vector cut with the same restriction enzymes. For *P3N*^{RB}-*PIPO*^{CS}, the cDNA
191 fragment introduced into the pGEM-T Easy plasmid was cut with *SacII*, and 5' and 3' ends
192 of the plasmid were blunted using T4 DNA polymerase (TaKaRa Bio, Kusatsu, Japan). The
193 blunted fragments were digested with *XhoI* and inserted into the WCIMV vector cut with
194 *SmaI* and *XhoI*.

195 The pTA/RB-P3(PIPO:FLAG⁻¹), pTA/No.30-P3(PIPO:FLAG⁻¹), pTA/RB-P3N-
196 PIPO:FLAG^{mk}, and pTA/No.30-P3N-PIPO:FLAG^{mk} constructs were previously described
197 (13).

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

201

202 **Plant growth conditions and viral infection**

203 Pea (*Pisum sativum*), broad bean (*Vicia faba*), and *N. benthamiana* were cultivated in a
204 growth chamber or growth room at 21–23°C with a 16-h photoperiod. Viral inocula were

205 prepared as described previously (20). For CIYVV, broad bean was inoculated with each
206 infectious cDNA using particle bombardment. For WCIMV, 1 µg of each WCIMV plasmid
207 was mechanically inoculated onto a susceptible pea line, PI 250438. The upper symptomatic
208 leaves were harvested and ground in an inoculation buffer (0.1 M phosphate buffer, pH 7.0,
209 and 1% 2-mercaptoethanol). The crude sap was mechanically inoculated onto the second
210 and/or third leaves of 2-week-old pea plants. At the same time, all plants were inoculated
211 with inoculation buffer alone as a negative control (mock inoculation).

212

213 **Sequence alignment**

214 P3N-PIPO amino acid (CIYVV, BYMV, and pea seed-borne mosaic virus [PSbMV])
215 sequences were aligned using MUSCLE (3.8) (<http://www.ebi.ac.uk/Tools/msa/muscle/>) (28).
216 The amino acid sequences of P3N-PIPO were obtained by translating the sequences from the
217 5' end of *P3* to the stop codon of *P3N-PIPO* after introducing an A into the A_{6,7} region in the
218 G_{1,2}A_{6,7} motif of each virus to shift the reading frame.

219

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

221 Pea leaves were homogenized in liquid nitrogen, and total RNA was isolated using TRIzol
222 reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's
223 instructions. Each RNA sample was treated with RNase-free DNase I (Roche Diagnostics,
224 Basel, Switzerland), and 1 µg of total RNA was reverse-transcribed using ReverTraAce
225 (TOYOBO). The reaction mixture (20 µl) contained 100 units of ReverTraAce, 1 mM dNTP,
226 25 pmol random 9-mers, and 1–2 µg total RNA in 1× buffer. Samples were first incubated at
227 30°C for 10 min, then at 42°C for 30 min, and finally at 99°C for 5 min. Real-time PCR was
228 performed using the DNA Engine Opticon 2 System (Bio-Rad Laboratories, Hercules, CA)
229 as previously described (20). The reaction mixture (25 µl) contained 0.625 U of ExTaq

230 (TaKaRa), ExTaq buffer, 0.2 mM dNTP, 0.2 μ M each of forward and reverse primers, SYBR
231 Green ($\times 30,000$ dilution) (Thermo Fisher Scientific), and cDNA obtained by reverse
232 transcribing 12.5 ng of total RNA. Samples were incubated for 5 min at 95°C; followed by 40
233 cycles of 95°C for 10 s, 53°C for *SA-CHI* (accession number L37876) or 55°C for *HSR203J*
234 (AB026296) for 30 s, and 72°C for 20 s. Transcript levels were normalized to that of *18S*
235 *rRNA* (U43011), and means and standard deviations were calculated. The primers used for
236 real-time PCR were as follows: SA-CHI-F and SA-CHI-R for *SA-CHI*, HSR203J-F and
237 HSR203J-R for *HSR203J*, and 18S rRNA-F and 18S rRNA-R for *18S rRNA*.

238 *N. benthamiana* leaves were homogenized in liquid nitrogen, and total RNA was
239 isolated by the AGPC (acid guanidinium thiocyanate–phenol/chloroform) extraction method
240 (29), followed by purification with a FARB column (Favorgen Biotech Corp, Ping-Tung,
241 Taiwan). Total RNA was digested with TURBO DNase (Thermo Fisher Scientific) and
242 reverse-transcribed using PrimeScript RTase (TaKaRa) according to the manufacturer's
243 instructions. Real-time PCR was performed using the StepOnePlus system (Thermo Fisher
244 Scientific). The reaction mixture (10 μ l) contained KAPA SYBR FAST qPCR Kit Master
245 Mix ABI Prism (Kapa Biosystems, Wilmington, MA), 0.3 μ M each of forward and reverse
246 primer, and cDNA obtained by reverse transcribing 50 ng of total RNA. Samples were
247 incubated for 20 s at 95°C, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.
248 Transcript levels of *P3N-PIPO-FLAG* were normalized to that of *NbEF1 α* (AY206004). The
249 primers used were as follows: GGS4-3FL-F and GGS4-3FL-R for *P3N-PIPO-FLAG*,
250 *NbEF1 α* -F and *NbEF1 α* -R for *NbEF1 α* . Primers were designed in a region of *P3N-PIPO-*
251 *FLAG* of Cl-RB and Cl-No.30 with identical nucleotide sequence (linker and FLAG-tag
252 coding sequence).

253 For virus detection by RT-PCR, we used primer set no.3735/no.3736 for CI-I89-1
254 and CI-90-1 Br2, and no.3908/no.3909 for BY-CS. PCR was done using KOD-FX DNA
255 polymerase (TOYOBO) according to the manufacturer's instructions.

256 Sequences of the primers are available upon request.

257

258 ***Agrobacterium*-mediated transient expression**

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

268

269 **Western blot**

270 Western blots were conducted as described previously (30, 32). Proteins were resolved in
271 12% NuPAGE Bis-Tris gel (Thermo Fisher Scientific) using MES-SDS buffer (FLAG-
272 tagged protein detection) or in 10% SDS-PAGE using Tris-glycine buffer (CP detection;
273 (33)), followed by electrotransfer to a PVDF membrane. To detect the FLAG-tagged proteins,
274 monoclonal Anti-FLAG M2–horseradish peroxidase (HRP) (Sigma-Aldrich Corporation, St.
275 Louis, MO) was used at a 1:5000 dilution. To detect CIYVV CP, rabbit polyclonal antibody
276 against CIYVV CP was used as the primary antibody and alkaline-phosphatase-conjugated
277 goat anti-rabbit IgG (Thermo Fisher Scientific) was used as the secondary antibody.

278 Chemiluminescence signals were detected with ECL Prime (GE Healthcare, Little Chalfont,
279 United Kingdom) using a LAS-4000 imaging system (GE Healthcare) for FLAG-tagged
280 protein detection, or with CDP-Star reagent (New England Biolabs, Ipswich, MA) using a
281 LAS-4000-mini imaging system (GE Healthcare) for CP detection. As a loading control for
282 the FLAG-tagged protein experiment, membranes after transfer were stained with 0.1%
283 amido black in 45% methanol and 10% acetic acid followed by destaining in 90% methanol
284 and 2% acetic acid (34).

285

286 **GFP fluorescence analysis**

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

291

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

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

300

301 **Determination of full-length ORF sequence of CI-I89-1**

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

311

312 **Phylogenetic analysis**

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

318

319 **RESULTS**

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

321 CI-90-1 Br2 induced lethal systemic cell death in PI 226564 (Fig. 1A, Table 1). To identify
322 the virulence determinant of CI-90-1 Br2 in PI 226564, chimeric viruses were constructed by
323 swapping parts of CI-90-1 Br2 and CI-No.30; the latter virus does not induce cell death in PI
324 226564 (Fig. 1A, Table 1) (20, 21). These chimeric viruses were based on CI-No.30
325 infectious cDNA that we previously constructed and developed for use as a gene expression
326 vector (7, 23, 37, 38). Chimeric viruses tagged with GFP were created that covered almost all

327 regions of the CIYVV genome: CI-P1HC/GFP, CI-BB/GFP, CI-NS/GFP, and CI-SB/GFP
328 (Fig. 1B) (7). Symptoms indicated that only the BB region of CI-90-1 Br2 markedly
329 enhanced the virulence of CI-No.30 (Fig. 1C). CI-BB/GFP induced cell death in upper
330 uninoculated leaves (Fig. 1C). The P1HC and SB regions of CI-90-1 Br2 did not enhance CI-
331 No.30 virulence (Fig. 1C). The NS region slightly enhanced the virulence: CI-NS/GFP
332 occasionally induced cell death associated with yellowing in upper uninoculated leaves (Fig.
333 1C). In contrast to CI-90-1 Br2, CI-BB/GFP did not kill completely the plants, but a mosaic
334 pattern associated with cell death developed in the upper uninoculated leaves (Fig. 1C).

335 Further analysis was focused on the virulence enhancement mediated by the CI-90-
336 1 Br2 BB region, which included ca. 94% of *HC-Pro* and ca. 79% of *P3* from CI-90-1 Br2.
337 CI-P1HC/GFP had the full-length *HC-Pro* gene of CI-90-1 Br2 but could not enhance
338 virulence (Fig. 1B and C), suggesting that the *P3*-containing portion of the BB region
339 (designated P3B as shown in Fig. 1B) was important for high virulence expression. We
340 constructed CI-RB/GFP, in which the P3B region of CI-No.30 was replaced by that of CI-90-
341 1 Br2 (Fig. 1B). CI-RB/GFP extensively induced cell death in upper uninoculated leaves,
342 comparable to that induced by CI-BB/GFP (Fig. 1C).

343 Like CI-BB/GFP, CI-RB/GFP did not kill the plants. To investigate whether
344 insertion of *GFP* (which was present in the first set of chimeric constructs tested) attenuated
345 CIYVV virulence, the symptoms induced by CI-No.30 with *GFP* (inserted between *P1* and
346 *HC-Pro* or between *N1b* and *CP*) were compared with those induced by CI-No.30 without
347 *GFP*. The results indicated that insertion of *GFP* weakened the symptoms produced by
348 CIYVV (Fig. D), although virus accumulation was not visibly different as measured by
349 western blotting against CP (Fig. D). To investigate whether the weaker virulence of CI-
350 RB/GFP relative to CI-90-1 Br2 was due to *GFP* insertion, we compared the virulence of CI-
351 RB without *GFP* with that of CI-90-1 Br2 (7) and CI-No.30 without *GFP* (37). CI-RB

352 without *GFP* also induced more severe symptoms than CI-No.30 without *GFP*, but did not
353 have the level of virulence of CI-90-1 Br2 (Fig. 1E). We also examined the reciprocal
354 chimera of CI-RB, CI-90-1 Br2-P3B^{No.30} without *GFP*, which contained the P3B region from
355 CI-No.30 and all other regions from CI-90-1 Br2 (Fig. 1B). CI-90-1 Br2-P3B^{No.30} without
356 *GFP* induced yellowing and cell death in upper uninoculated leaves, but the timing was
357 delayed in comparison with CI-RB without *GFP* (Fig. 1F). It indicated that CI-90-1 Br2-
358 P3B^{No.30} showed less virulence than CI-RB, and more virulence than CI-No.30. Together,
359 these results indicated that the P3B region was the main determinant of virulence in PI
360 226564, though regions outside of P3B also contributed to virulence expression.

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

369 Taken together, these data showed that CI-No.30 carrying CI-90-1 Br2 regions
370 outside of P3B (CI-P1HC, CI-NS, CI-SB, and CI-90-1 Br2-P3B^{No.30}) had weaker virulence
371 than CI-No.30 carrying the P3B region of CI-90-1 Br2 (CI-RB) and those in combination
372 with CI-90-1 Br2 regions (CI-RB+P1HC, CI-RB+NS, CI-RB+SB). This indicated that the
373 effect of virulence was the highest in exchanging the P3B region and the effect of virulence
374 enhancement by regions outside of P3B was higher in virus carrying the P3B region of CI-90-
375 1 Br2 than in virus carrying the P3B region of CI-No.30. These symptom observations
376 collectively suggested that, although regions outside of *P3* contributed to virulence

377 expression, the *P3* gene (P3B region) was the major determinant for inducing lethal systemic
378 cell death in PI 226564.

379

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

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

391 We found that WCI/P3N-PIPO-RB extensively induced cell death along the veins
392 of inoculated leaves at 5 dpi (Fig. 2B, Table 1). In contrast, infection with WCI/P3-RB,
393 WCI/P3ΔPIPO-RB, and WCI/GFP did not induce cell death (Fig. 2B). These results
394 suggested that P3N-PIPO, not P3, was the factor responsible for inducing cell death in PI
395 226564. It should be noted that the nucleotide sequence of *P3N-PIPO* of CI-RB is the same
396 as that of CI-90-1 Br2.

397

398 **P3N-PIPO of CI-No.30 also induced cell death in PI 226564**

399 CI-No.30 does not induce cell death in PI 226564, which suggested that P3N-PIPO of CI-
400 No.30 would not induce cell death either (20). We inoculated PI 226564 with WCIMV
401 carrying *P3N-PIPO*, *P3*, or *P3ΔPIPO* of CI-No.30 (designated WCI/P3-No.30,

402 WCI/P3 Δ PIPO-No.30, and WCI/P3N-PIPO-No.30, respectively). Unexpectedly, WCI/P3N-
403 PIPO-No.30 extensively induced cell death along the veins on the inoculated leaves in PI
404 226564 (Fig. 2C, Table 1). This symptom was comparable to that induced by WCI/P3N-
405 PIPO-RB (Fig. 2B). WCI/P3-No.30, WCI/P3 Δ PIPO-No.30, and WCI/GFP did not induce cell
406 death at 5 dpi in PI 226564 (Fig. 2C).

407 We constructed a WCIMV vector that expresses *P3N-PIPO* from the BYMV CS
408 strain (BY-CS), designated WCI/P3N-PIPO-CS, in order to rule out the possibility that cell
409 death was non-specifically caused by overexpression of *P3N-PIPO*. We expected that P3N-
410 PIPO of BY-CS would not induce cell death because BY-CS has never been reported to
411 induce cell death in pea, including PI 226564 (20, 21). Like CIYVV, however, BY-CS is a
412 member of genus *Potyvirus*, and the two are closely related (39). The nucleotide sequence
413 identity of *P3N-PIPO* between BY-CS and CI-90-1 Br2 or CI-No.30 is 64.2% or 61.9%,
414 respectively. The amino acid sequence identity (similarity) of P3N-PIPO between BY-CS
415 and CI-90-1 Br2 or CI-No.30 is 56.5% (73.8%) or 54.9% (71.3%), respectively (Fig. 3). In PI
416 226564 infected with WCI/P3N-PIPO-CS, cell death was not induced in either inoculated or
417 upper uninoculated leaves even at 14 dpi (Fig. 2D, Table 1), thus ruling out the possibility
418 that overexpression of *P3N-PIPO* non-specifically induced cell death.

419

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

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

426 activated the SA signaling pathway, which led to induction of systemic cell death in PI
427 226564.

428 To test this hypothesis, we analyzed the expression of SA-responsive chitinase gene
429 (*SA-CHI*) and an HR-related gene homologous to tobacco *HSR203J* (*HSR203J*) by real-time
430 PCR. We conducted an expression analysis in leaves of PI 226564 inoculated with CI-90-1
431 Br2, CI-No.30, or BY-CS. The expression level of *SA-CHI* was significantly higher in leaves
432 inoculated with CI-90-1 Br2 than in leaves inoculated with either CI-No.30 or BY-CS (Fig.
433 4A). There were no significant differences among mock, CI-No.30, and BY-CS inoculation
434 (Fig. 4A). The expression level of *HSR203J* was also significantly higher in the leaves
435 inoculated with CI-90-1 Br2 than in those with mock inoculation (Fig. 4B). These results
436 indicated that CI-90-1 Br2 infection activated SA and HR-like signaling pathways in PI
437 226564.

438 We carried out an expression analysis of *SA-CHI* in leaves of PI 226564 inoculated
439 with WCI/P3N-PIPO, WCI/P3, or WCI/P3ΔPIPO from CI-RB; the corresponding set of
440 sequences from CI-No.30; or WCI/GFP. At 4 dpi, *SA-CHI* was significantly induced only in
441 leaves inoculated with WCI/P3N-PIPO-RB or WCI/P3N-PIPO-No.30 (Fig. 4C and D).
442 WCI/P3 and WCI/P3ΔPIPO (from both CI-RB and CI-No.30), GFP, and mock inoculations
443 did not significantly induce *SA-CHI* (Fig. 4C and D). We also investigated the expression
444 level of *SA-CHI* in the leaves inoculated with WCI/P3N-PIPO-CS (from BY-CS) and
445 confirmed that WCI/P3N-PIPO-CS infection did not induce *SA-CHI* expression (Fig. 4D).
446 The amplitudes of *SA-CHI* upregulation in plants infected with WCI/P3N-PIPO of CI-RB and
447 CI-No.30 (Fig. 4C and D) were several orders of magnitude higher than those in plants
448 infected with CI-RB (Fig. 4A), possibly because CI-RB produced lower levels of P3N-PIPO
449 (produced by transcriptional slippage) than did WCIMV carrying *P3N-PIPO* (produced in
450 zero frame).

451

452 **Lower accumulation of CI-No.30 P3N-PIPO than CI-RB P3N-PIPO was presumably the**
453 **reason for the lower virulence of CI-No.30**

454 We found that heterologous expression of CI-No.30 P3N-PIPO could induce cell death in PI
455 226564 (Fig. 2C, Table 1), which was seemingly inconsistent with the fact that CI-No.30
456 does not induce cell death in this same cultivar (Fig. 1A, Table 1) (20, 21). We previously
457 showed that P3N-PIPO could be detected in CI-RB-infected plants but was below the level of
458 detection in CI-No.30-infected plants of a susceptible pea cultivar, PI 250438, indicating that
459 the level of P3N-PIPO from CI-No.30 is significantly lower than that from CI-90-1 Br2 (7).
460 This same difference was observed when P3N-PIPO was produced from the *P3* cistron of
461 each virus using an *in vitro* translation system with an *A. thaliana* cell-free system and wheat
462 germ extract (7, 13). In this study, we compared the accumulation of CI-No.30 P3N-PIPO
463 and CI-RB P3N-PIPO in a transient expression system, agroinfiltration in *N. benthamiana*
464 leaf tissues.

465 We prepared construct $P3(PIPO:FLAG^{-1})$, in which a *FLAG* epitope tag sequence
466 was inserted in front of the stop codon of the *PIPO* coding sequence, as a means to detect
467 PIPO-frame products (Fig. 5A) (13). $P3(PIPO:FLAG^{-1})$ constructs of CI-RB and CI-No.30
468 were transiently expressed in the same leaf of *N. benthamiana*, and the production of protein
469 from the PIPO frame and mRNA of $P3(PIPO:FLAG^{-1})$ was compared by measuring the
470 protein/mRNA ratio. Western blotting using a FLAG antibody indicated that the
471 accumulation of PIPO-frame products of CI-RB was higher than that of CI-No.30 in three
472 independent plants (Fig. 5B). Similar results were observed in at least three independent
473 experiments. In contrast, real-time PCR analysis indicated that mRNA level of CI-No.30
474 $P3(PIPO:FLAG^{-1})$ was higher than that of CI-RB (Fig. 5C). Thus, the protein/mRNA ratio of
475 CI-No.30 was significantly lower than that of CI-RB (Fig. 5D). As a control experiment, we

476 compared the protein/mRNA ratio of *P3N-PIPO:FLAG^{mk}*, which has mutations that enable
477 production of *P3N-PIPO* mRNA in the zero frame but does not produce *P3* mRNA, between
478 CI-RB and CI-No.30 (Fig. 5A) (13). Western blot analysis indicated that there were no visible
479 differences in protein accumulation between CI-RB and CI-No.30 (Fig. 5E). Real-time PCR
480 analysis indicated that the mRNA level of CI-No.30 *P3N-PIPO:FLAG^{mk}* tended to be higher
481 than that of CI-RB (Fig. 5F). Thus, the protein/mRNA ratio of CI-No.30 was lower than that
482 of CI-RB (Fig. 5G). These results showed that P3N-PIPO production from *P3* cistron of CI-
483 No.30 was lower than that from *P3* cistron of CI-RB in a transient expression in *N.*
484 *benthamiana* leaf tissues.

485 We also obtained data supporting the possibility that the increased levels of P3N-
486 PIPO produced by CI-RB enhance virus accumulation in infected plants. We compared the
487 accumulation of CI-RB with that of CI-No.30 by using GFP-tagged versions of each virus.
488 P3N-PIPO is an essential factor for potyviruses to move cell-to-cell in infected leaves (40).
489 Therefore, we anticipated that CI-RB would accumulate more efficiently than CI-No.30 in PI
490 226564 if the level of CI-RB P3N-PIPO was higher than that of CI-No.30 P3N-PIPO. Virus
491 accumulation levels were compared between CI-No.30/GFP and CI-RB/GFP in PI 226564.
492 We excised infection foci (GFP-expressing areas) from inoculated leaves using an
493 epifluorescence microscope (Fig. 6A) and measured the CP amount of each virus by DAS-
494 ELISA (Fig. 6B). The result showed that CI-RB/GFP accumulated to higher levels than CI-
495 No.30/GFP at 5 dpi. By measuring the GFP-fluorescent area, we also found that CI-RB/GFP
496 spread more rapidly than CI-No.30/GFP at 5 dpi (Fig. 6C). These results suggested that
497 higher production of P3N-PIPO enhanced the ability of CI-RB to accumulate in the infected
498 pea, perhaps synergistically increasing the difference in accumulation of P3N-PIPO between
499 pea plants infected with CI-RB and CI-No.30.

500 These results collectively suggest that the lower level of CI-No.30 P3N-PIPO
501 enabled CI-No.30 to avoid activating the SA signaling pathway, resulting in the loss of cell
502 death induction in PI 226564.

503

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

506 CI-90-1 Br2, a mutant isolate that originated from CI-90-1, expressed higher virulence than
507 CI-90-1, which induced cell death in upper uninoculated leaves of PI 226564 but did not kill
508 the plants (Fig. 7, Table 1). We anticipated that the P3B region was responsible for the
509 virulence enhancement in CI-90-1 Br2 relative to CI-90-1. We created a chimeric virus based
510 on CI-RB/GFP with the P3B region (from CI-90-1 Br2) replaced by that of CI-90-1 (Fig. 7A).
511 There is a single non-synonymous difference between the two sequences (T [CI-90-1 Br2] vs.
512 G [CI-90-1]) that causes a substitution of methionine (CI-90-1 Br2) with arginine (CI-90-1) at
513 amino acid position 28 of P3 (Fig. 7B); thus, the chimeric virus was designated as CI-
514 RB^{M28R}/GFP. As expected, the symptoms induced by CI-RB^{M28R}/GFP were weaker than those
515 induced by CI-RB/GFP (Fig. 7C and D). CI-RB^{M28R}/GFP induced yellowing and cell death in
516 upper uninoculated leaves, but the timing was delayed in comparison with CI-RB/GFP (Fig.
517 7D). The plants infected with CI-RB^{M28R}/GFP were reproducibly taller than those infected
518 with CI-RB/GFP (Fig. 7C). The substitution at aa position 28 of P3N-PIPO (P3) is in the N-
519 terminal region, distant from the PIPO coding region (Fig. 3), suggesting that the substitution
520 did not affect PIPO-frame translation and qualitatively affected virulence through some other
521 mechanism. It should be noted that CI-RB/GFP can break *cyv1* resistance whereas CI-
522 RB^{M28R}/GFP cannot, indicating that the same single amino acid substitution affected both
523 breaking of *cyv1* recessive resistance and symptom severity in susceptible cultivar PI 226564

524 (7). The substitution at aa position 28 is also close to the position important for PSbMV
525 virulence in pea carrying *sbm-2* recessive resistance (Fig. 3) (41).

526

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

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

537

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

539 In PI 226564, P3N-PIPO of CI-RB induced cell death but P3 did not (Fig. 2B). As P3N-PIPO
540 has the same N-terminal region (P3N) as P3, PIPO is the only region distinguishing P3N-
541 PIPO from P3. Therefore, we inferred that the PIPO domain of CI-RB was responsible for
542 cell death induction in PI 226564. To test this possibility, we created chimeric *P3N-PIPO*
543 genes that had either *P3N* of CI-RB and *PIPO* of BY-CS (*P3N^{RB}-PIPO^{CS}*) or *P3N* of BY-CS
544 and *PIPO* of CI-RB (*P3N^{CS}-PIPO^{RB}*); these combinations were chosen because P3N-PIPO of
545 BY-CS did not induce cell death in PI 226564 (Fig. 2D). *P3N^{RB}-PIPO^{CS}* and *P3N^{CS}-PIPO^{RB}*
546 were expressed by WCIMV vectors in PI 226564. *P3N^{CS}-PIPO^{RB}* expression induced cell
547 death in the inoculated leaves, but it was markedly weaker than that induced by P3N-PIPO of
548 CI-RB (Fig. 9A). *P3N^{RB}-PIPO^{CS}* expression did not induce cell death (Fig. 9A).

549 We also expressed the chimeric P3N-PIPO proteins in PI 118501 by using the same
550 WCIMV vectors. When we inoculated PI 118501 with WCI/P3N^{RB}-PIPO^{CS} or WCI/P3N^{CS}-
551 PIPO^{RB}, neither one induced cell death at 5 dpi or even at 18 dpi in the inoculated leaves,
552 although both WCI/P3N-PIPO-RB and WCI/P3N-PIPO-CS induced severe cell death at 18
553 dpi (Fig. 9B).

554

555 **A consistent gradation of virulence was observed among CIYVV isolates in *cyv1* and**
556 **susceptible peas**

557 Previous studies using PI 429853 (*cyv1*) indicated that CI-No.30 never infects this line, CI-
558 90-1 can produce resistance-breaking mutants, and CI-90-1 Br2 and CI-I89-1 can
559 systemically infect this line (Table 1) (7). In this study, we compared the symptoms between
560 CI-90-1 Br2 and CI-I89-1. CI-90-1 Br2 induced no symptoms in PI 429853 (*cyv1*), whereas
561 CI-I89-1 induced chlorosis and cell death systemically at 25 dpi (Fig. 10A). RT-PCR analysis
562 of upper uninoculated leaves confirmed CI-I89-1 infection in all three plants and confirmed
563 CI-90-1 Br2 infection in two out of three plants (Fig. 10A and B). Plants inoculated with BY-
564 CS did not show any symptoms at 25 dpi in PI 429853 (*cyv1*), and RT-PCR analysis
565 indicated that no plants were infected with BY-CS (Fig. 10C). Therefore, the order of
566 virulence in PI 429853 (*cyv1*) was CI-I89-1 > CI-90-1 Br2 > CI-90-1 > CI-No.30 and BY-CS
567 (Table 1). Similarly, the symptom severity in susceptible pea line PI 226564 was CI-90-1 Br2
568 > CI-90-1 > CI-No.30 (Fig. 7, Table 1) (20, 42). We compared the symptom severities
569 between CI-I89-1 and CI-90-1 Br2, and between CI-No.30 and BY-CS. The symptoms
570 induced by CI-I89-1 were more severe than those induced by CI-90-1 Br2 at 10 dpi in PI
571 226564 (Fig. 10D). The symptoms induced by CI-No.30 were more severe than those
572 induced by BY-CS at 10 dpi in PI 226564 (Fig. 10E) These results indicated a consistent

573 gradation of virulence among these viruses in both PI 429853 (*cyvI*) and susceptible PI
574 226564 (Table 1).

575 Next, we investigated whether the same gradation was observed in another
576 susceptible cultivar, PI 118501, which shows lethal systemic cell death following CI-No.30
577 infection but not BY-CS infection (20, 21). The inoculation test indicated that CI-I89-1, CI-
578 90-1 Br2, CI-90-1, and CI-No.30 similarly induced lethal systemic cell death (Fig. 10F),
579 whereas BY-CS induced only mosaic symptoms in the upper uninoculated leaves at 12 dpi
580 (Fig. 10G and H). Cell death induction by CI-No.30 was slightly slower than induction by CI-
581 I89-1, CI-90-1 Br2, and CI-90-1. These results indicated that the order of symptom severity in
582 PI 118501 was CI-I89-1, CI-90-1 Br2 and CI-90-1 > CI-No.30 > BY-CS (Table 1). Taken
583 together, these results indicated that a consistent gradation exists in virulence among CIYVVs
584 and BYMV in PI 429853 (*cyvI* recessive resistance) and in PI 226564 and PI 118501
585 (susceptible) (Table 1; Fig. 10I).

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

589

590 **DISCUSSION**

591 We revealed that the main determinant of lethal systemic cell death induction by CIYVV was
592 P3N-PIPO. This was determined by analysis using chimeric viruses and transient expression
593 from WCIMV vectors in a susceptible pea cultivar, PI 226564. SMV strain G7 induces lethal
594 systemic cell death in soybean carrying the dominant resistance gene *RsvI* (14). In that virus,
595 *P3*, not *P3N-PIPO*, determines virulence (40). TuMV induces lethal systemic cell death in *A.*
596 *thaliana* Ler carrying the *TuNI* gene (15). TuMV *P3* expression alone is sufficient for
597 induction of cell death at a single-cell level, and the region required for cell death induction is

598 upstream of the *PIPO* coding sequence (43). Our study is the first report to suggest that
599 induction of lethal systemic cell death could be attributed to P3N-PIPO.

600 As mentioned above, it was unexpected to see that expression of P3N-PIPO from
601 CI-No.30 by a WCIMV vector induced cell death in PI 226564 (Fig. 2C) because CI-No.30
602 itself does not induce cell death in this cultivar (Fig. 1A) (20, 21). In *N. benthamiana*, the
603 *Plantago asiatica* mosaic virus (PIAMV) Li1 isolate induces systemic necrosis that has HR-
604 like characteristics (44, 45). Transient expression of the PIAMV Li1 isolate RdRp (helicase
605 domain) by agroinfiltration induces cell death in *N. benthamiana* (46). These results suggest
606 that *N. benthamiana* recognizes PIAMV RdRp and induces an HR-like response systemically,
607 resulting in systemic necrosis. Interestingly, transient expression of RdRp encoded by the
608 PIAMV Li6 asymptomatic isolate also induces cell death in *N. benthamiana* (46). RdRp
609 accumulation is higher in the areas infiltrated with *Agrobacterium* carrying infectious cDNA
610 of the Li1 isolate than when infectious cDNA of the Li6 isolate is used (46). These results
611 suggest that *N. benthamiana* has the ability to recognize both Li1 and Li6 RdRp, but the
612 difference in their protein accumulation levels determines the induction of systemic necrosis
613 in the context of virus infection. The results reported here can be explained in a similar
614 fashion. Previously, we reported that in susceptible pea PI 250438 infected with CI-90-1 Br2
615 or CI-No.30, the P3N-PIPO of CI-90-1 Br2 could be detected but that CI-No.30 was below
616 the level of detection when tested using an antibody against the PIPO peptide (7).
617 Furthermore, we showed that the amount of P3N-PIPO produced from the *P3* cistron of CI-
618 No.30 is significantly less than that of CI-RB in an *in vitro* translation system using MM2dL
619 (an extract derived from *A. thaliana* MM2d cells) and wheat germ extract (7, 13). In this
620 study, we showed evidence that the *P3* cistron of CI-No.30 produced less P3N-PIPO protein
621 than that of CI-RB *in vivo* in agroinfiltrated *N. benthamiana* (Fig. 5B). The protein/mRNA
622 ratio of CI-No.30 was lower than that of CI-RB (Fig. 5D). These results suggest that P3N-

623 PIPO production, or transcriptional slippage efficiency, from CI-No.30 *P3(PIPO:FLAG⁻¹)*
624 (which tagged P3N-PIPO produced by frameshift) is lower than that from CI-RB. On the
625 other hand, the protein/mRNA ratio of CI-No.30 *P3N-PIPO:FLAG^{mk}* (which produced tagged
626 P3N-PIPO as a zero-frame product) was also lower than that of CI-RB (Fig. 5G). The
627 protein/mRNA ratio of *P3(PIPO:FLAG⁻¹)* tended to be lower than that of *P3N-*
628 *PIPO:FLAG^{mk}* in repeated experiments, though there was not a statistically significant
629 difference. These findings suggest that the difference in P3N-PIPO accumulation between CI-
630 No.30 and CI-RB was determined by a combination of transcriptional slippage efficiency and
631 other factors such as protein stability or translation efficiency. Taken together, these results
632 support the hypothesis that in susceptible pea PI 226564, CI-90-1 Br2 can produce P3N-PIPO
633 protein sufficient for induction of cell death but CI-No.30 cannot, even though host cells are
634 able to recognize P3N-PIPO from both isolates.

635 We showed that each of the tested regions outside of *P3* are accessorially involved in
636 the virulence enhancement in PI 226564 (Fig. 1G). In previous studies, we revealed that the
637 *HC-Pro* gene is indirectly involved in the induction of lethal systemic cell death in PI 118501
638 (20, 22, 32). We found that a CI-No.30 mutant with a D to Y substitution in amino acid
639 position 193 of HC-Pro (CI-D193Y) loses the ability to induce cell death in PI 118501 (20).
640 Potyvirus HC-Pro is an RNA-silencing suppressor required for efficient virus accumulation
641 in host plants (47). The D193Y mutation in HC-Pro markedly reduces its ability to suppress
642 RNA silencing, and CI-D193Y accumulation is significantly lower than wild-type CI-No.30
643 in PI 118501 (20, 22). Heterologous expression of viral suppressors of RNA silencing
644 (tomato bushy stunt virus P19 or cucumber mosaic virus 2b) can complement the virulence of
645 CI-D193Y in PI 118501 (32). These results suggest that HC-Pro itself is not an elicitor but
646 indirectly affects cell death induction via its RNA-silencing suppressor activity in PI 118501:
647 reduced accumulation of CIYVV leads to reduced accumulation of the elicitor molecules that

648 induce cell death. In this study, we found that the elicitor molecule active against PI 118501
649 was P3N-PIPO (Fig. 8A). Thus, is likely that CI-D193Y is not lethal in PI 118501 because it
650 cannot produce enough P3N-PIPO to induce cell death. Taken together, the results indicate
651 that lethal systemic cell death in PI 118501 was induced by P3N-PIPO, the production of
652 which was indirectly regulated by the RNA-silencing suppressor activity of HC-Pro. As
653 observed for *HC-Pro*, viral genes other than *P3* might increase the accumulation of P3N-
654 PIPO, enhancing the virulence in PI 226564 (Fig. 1G).

655 We found that expression of *P3N-PIPO* from CI-90-1 Br2, CI-No.30, and BY-CS
656 by WCIMV vectors induced cell death in PI 226564, PI 118501, and PI 429853, with the
657 exception of BY-CS *P3N-PIPO* in PI 226564 (Table 1), suggesting that many peas have a
658 common factor that recognizes P3N-PIPO of two different *Potyvirus* species (CIYVV and
659 BYMV). The *Rx* gene in potato encodes an NB-LRR–type resistance gene that confers
660 genetically dominant resistance against potato virus X (PVX) (48). *Rx* specifically recognizes
661 CP of PVX avirulent strains and induces extreme resistance (epistatic to HR) (49). *N.*
662 *benthamiana* expressing *Rx* also shows resistance to PVX (50). Interestingly, *Rx* is able to
663 recognize CP of three other species in the genus *Potexvirus* (narcissus mosaic virus, WCIMV,
664 and cymbidium mosaic virus) and to induce HR in *N. benthamiana* expressing *Rx* (51). The
665 product of the *L⁴* resistance gene (NB-LRR) isolated from pepper is able to recognize CP of
666 several distant species in the genus *Tobamovirus* including tomato mosaic virus, TMV,
667 paprika mild mottle virus, and pepper mild mottle virus, and to induce cell death
668 accompanied by HR when both *CP* and *L⁴* are transiently expressed in *N. benthamiana* by
669 agroinfiltration (34). Similarly, the product of *N'* (NB-LRR) isolated from *Nicotiana*
670 *sylvestris* is able to recognize CP of tomato mosaic virus, paprika mild mottle virus, and
671 pepper mild mottle virus and induces cell death accompanied by HR when both *CP* and *N'*
672 are transiently expressed in *N. benthamiana* by agroinfiltration (34). These studies suggest

673 that a single resistance protein has the potential to recognize a wide range of elicitor
674 molecules, at least within the same genus.

675 In pea, one of the candidate factors to recognize P3N-PIPO is the product of the
676 *Cyn1* gene (21). Genetic analysis indicated that the lethal systemic cell death induced by CI-
677 No.30 in PI 118501 is controlled by the dominant gene *Cyn1* (21). *Cyn1* is located on linkage
678 group (LG) 3, where many *R* gene analogs were suggested to be clustered by a synteny study
679 between pea and *Medicago truncatula* (21). In this study, we showed that expression of *P3N-*
680 *PIPO* of CI-No.30 in a WCIMV vector induced cell death in PI 118501 (Fig. 8A), suggesting
681 that *Cyn1* recognized P3N-PIPO and induced lethal systemic cell death. We previously
682 showed the possibility that PI 226564 also has a *Cyn1* allele that weakly recognizes CI-No.30
683 (20). CI-No.30 does not induce cell death in PI 226564 (Fig. 1A) (20, 21); however,
684 activation of the SA signaling pathway by application of an SA analog, benzo (1,2,3)-
685 thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), induces systemic cell death in PI
686 226564 infected with CI-No.30 (20). In this study, we obtained the supporting result that
687 expression of CI-No.30 *P3N-PIPO* by a WCIMV vector induced cell death in PI 226564 (Fig.
688 2C). In contrast, BTH treatment does not induce cell death in PI 226564 infected with BY-CS
689 (20). Consistent with this result, expression of BY-CS *P3N-PIPO* by WCIMV did not induce
690 cell death in PI 226564 (Fig. 2D). These results suggest that PI 226564 has a *Cyn1* allele
691 whose product has the potential to specifically recognize CI-No.30 but not BY-CS. *Cyn1* of
692 PI 226564 might be able to recognize P3N-PIPO effectively when P3N-PIPO is accumulated
693 to high levels, e.g., in situations such as overexpression by a WCIMV vector or CI-RB
694 infection, but not when it is at low levels, e.g., in a situation such as CI-No.30 infection (20).
695 In PI 429853 (*cyv1*), P3N-PIPO protein could be recognized when expressed by a WCIMV
696 vector, suggesting that PI 429853 has a *Cyn1* allele whose product recognizes CIYVV. It was
697 inconsistent with symptoms in the context of virus infection (Table 1). The *cyv1*-mediated

698 resistance would inhibit or reduce accumulation of CIYVV and thus the recognition of P3N-
699 PIPO under natural infection conditions (Table 1). In contrast to its lack of effect in PI
700 226564, P3N-PIPO of BY-CS could induce cell death in PI 118501 and PI 429853, though
701 more slowly than P3N-PIPO of either CI-90-1 Br2 or CI-No.30 (Figs. 2 and 8); these data
702 suggest that *Cyn1* of PI 118501 and PI 429853 can recognize BY-CS, although less
703 efficiently than CI-90-1 Br2 and CI-No.30. It is noted that we could not detect P3N-PIPO
704 expressed via WCIMV vector by western blot analysis and thus could not compare the
705 accumulation levels of P3N-PIPO among the three tested pea cultivars. Taken together, these
706 results suggest that the product of *Cyn1* recognizes matching P3N-PIPO of CIYVV and
707 BYMV and activates the SA-mediated defense pathway, resulting in systemic cell death
708 induction in many peas (20).

709 We showed that expression of *P3N-PIPO* by WCIMV induced cell death in PI
710 226564 but that expression of *P3* did not (Fig. 2). One possible explanation is that a pea
711 protein (e.g., the *Cyn1* product) recognizes the PIPO peptide, which is part of P3N-PIPO but
712 not P3. However, the PIPO domain of CI-P3N-PIPO alone did not appear to induce cell death,
713 as shown in the experiment using chimeric P3N-PIPO (constructed from P3N-PIPOs of CI-
714 RB and BY-CS) in PI 226564 (Fig. 9). Although the PIPO domain contributed to recognition
715 by peas, the overall structure of P3N-PIPO may be important for full activation of the
716 signaling pathway to induce cell death. A second possible explanation is that a pea protein
717 (e.g., the *Cyn1* product) recognizes P3N-PIPO-targeted host factor(s) (the guard/decoy
718 model) (52). Recently, it was found that PCaP1 from *A. thaliana* and its homolog NbDREPP
719 from *N. benthamiana* interact with P3N-PIPO (53, 54). PCaP1 interacts with P3N-PIPO via
720 the PIPO domain and does not interact with P3, indicating that PCaP1 is specifically targeted
721 by P3N-PIPO (53). Therefore, a pea protein may monitor the target of P3N-PIPO such as
722 PCaP1 and induce cell death. A third explanation is that pea recognizes P3N-PIPO in a

723 localization-dependent manner. Previous studies reported that P3 localizes to the ER–Golgi
724 interface and that P3N-PIPO localizes to plasmodesmata (9, 55). Therefore, P3 may not be
725 recognized due to its localization.

726 Our results using five virus isolates (four CIYVV isolates and one BYMV isolate)
727 and three pea genotypes showed that the more efficiently a particular CIYVV isolate broke
728 *cyv1* recessive resistance, the more it expressed virulence in susceptible peas carrying *Cyn1*
729 (Fig. 10I and Table 1). In particular, we observed adaptive evolution from CI-90-1 to CI-90-1
730 Br2, which overcame *cyv1* resistance through attaining a point mutation in the P3N domain
731 (7). This mutation also resulted in CI-90-1 Br2 gaining higher virulence than CI-90-1 in
732 susceptible PI 226564 (Fig. 7). Many studies have suggested that trade-offs are observed in
733 plant virus infection across hosts, and antagonistic pleiotropy (when mutations beneficial for
734 infection of one host are deleterious for infection of another one) explains such trade-offs
735 well (56, 57). For example, tobacco etch virus (TEV) infects several solanaceous plants such
736 as *N. tabacum* in nature, and some non-solanaceous plants (e.g., *Helianthus annuus* and
737 *Spinacia oleracea*) are also susceptible under experimental conditions. Analysis using a TEV
738 mutant series indicated that fitness trade-offs due to antagonistic pleiotropy are observed
739 between *N. tabacum* and non-solanaceous plants (58). Several studies suggest that viruses
740 pay a fitness cost when they overcome dominant resistance due to adaptive mutations with
741 antagonistic pleiotropic effects. In pepper, tobamoviruses that can overcome dominant
742 resistance conferred by *L* genes are less able to accumulate in susceptible plants, and virus
743 particles of breaking isolates in the soil are less stable than those of wild-type virus (59, 60).
744 In *Brassica napus*, TuMV isolates CZE1 and CDN1 are able to overcome dominant
745 resistance conferred by *TuRB01* but are outcompeted by avirulent isolate UK1 in susceptible
746 plants (61). Soybeans carrying dominant *Rsv1* or *Rsv4* alleles are resistant against SMV-N
747 (62) or three strains (SMV-N, SMV-G7, and SMV-G7d) (63), respectively. SMV-N *HC-Pro*

748 mutants or SMV *P3* mutants of the three strains can overcome these respective resistances,
749 and accumulation of these mutants is reduced in susceptible cultivars (62, 63). These studies
750 indicated that their fitness trade-offs are caused by antagonistic pleiotropy of the viral genes
751 that overcome dominant resistance. Similar observations have also been reported when
752 viruses overcome recessive resistance. Potato virus Y *VPg* double mutants show more
753 virulence than *VPg* single mutants in pepper carrying a *pvr2*³ recessive resistance gene (64).
754 In contrast, potato virus Y *VPg* double mutants are less virulent than single mutants in
755 susceptible pepper (64). In rice, rice yellow mottle virus mutants that can overcome *rymv1-2*
756 recessive resistance are less virulent than wild-type virus in susceptible cultivars (65).

757 These studies collectively support the hypothesis that many viruses pay fitness costs
758 to adapt to new hosts or to overcome resistances, and that these across-host trade-offs are
759 caused by adaptive mutations with antagonistic pleiotropic effects. In the case of CIYVV, our
760 results suggest that many susceptible peas carry *Cyn1*, whose product recognizes CIYVVs (or
761 their P3N-PIPO proteins, in particular) that break *cyn1* resistance. In *Cyn1* peas, these
762 CIYVVs induce an HR-like response associated with systemic cell death, resulting in plant
763 death. As noted previously, the more efficiently a particular CIYVV isolate broke *cyn1*
764 recessive resistance, the more it induced systemic cell death, resulting in a loss of tissue to
765 support virus accumulation and leading to a reduction of fitness in susceptible peas carrying
766 *Cyn1*. This observation suggests that there are fitness trade-offs between overcoming *cyn1*
767 and reducing recognition by *Cyn1* via the antagonistic pleiotropy of P3N-PIPO. The trade-
768 offs shown in previous studies (described above) involve gaining adaptation to a non-host or
769 overcoming a resistant cultivar, resulting in reduction of viral viability or virulence in a
770 susceptible host. This study is unique in terms of showing trade-offs in a virus overcoming
771 two independent defense systems in a single plant species.

772 We hypothesize the following model of co-evolution between CIYVV and pea,
773 driven by the antagonistic pleiotropy of P3N-PIPO. (1) CIYVV cannot infect pea carrying the
774 *cyv1* recessive resistance gene. (2) Selection favors mutations in the *P3* gene of CIYVV that
775 enable P3N-PIPO to accumulate to higher levels and/or alter its protein structure, enabling
776 the virus to overcome *cyv1* resistance. (3) After overcoming resistance, the virus can infect
777 and accumulate effectively, but now *Cyn1* recognizes the more abundant (CI-No.30 vs CI-90-
778 1 Br2) and/or adapted (CI-90-1 vs CI-90-1 Br2) P3N-PIPO directly or indirectly and induces
779 cell death systemically. (4) Systemic cell death leads to loss of host viability, which is also
780 unfavorable for the virus. (5) Selection favors mutations in CIYVV that reduce the
781 accumulation of P3N-PIPO and/or change the amino acids of P3N-PIPO required for its
782 recognition or function, resulting in loss of the ability to overcome *cyv1* recessive resistance.
783 Based on the proposed model, *Cyn1* may have evolved to recognize CIYVVs (or their P3N-
784 PIPO proteins, in particular) that break *cyv1* resistance in susceptible peas. Although *Cyn1*-
785 mediated activation of the SA defense pathway does not appear to inhibit CIYVV infection
786 efficiently, as observed in authentic HR (20), systemic cell death may oppose the adaptive
787 evolution to overcome *cyv1* resistance because induction of systemic cell death leads to a loss
788 of host viability. We assume that two independent defense mechanisms (recessive resistance
789 and the SA defense pathway) in pea impose antagonistic pleiotropy on P3N-PIPO. Such a
790 trade-off for virus in overcoming paired defense mechanisms may sustain the durability of
791 resistance against fast-evolving viruses (66).

792

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805 We declare that we have no conflicts of interest.

806

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999

1000

1001 **Figure legends**

1002 **FIG 1** Mapping of the virulence determinant of CI-90-1 Br2 in susceptible pea PI 226564.
1003 (A) CI-90-1 Br2 or CI-No.30 was inoculated and the symptoms were observed in inoculated
1004 leaf (a–c), upper uninoculated leaf (d–i), and whole plant (j,k). Photographs were taken at 14
1005 (a–i), 17 (j), and 21 (k) days post inoculation (dpi). (B) Schematic representations of chimeric
1006 viruses constructed from CI-90-1 Br2 (yellow) and CI-No.30 (blue). Symptom severity in PI
1007 226564 is indicated as the number of (+) symbols. We also show the infection profile in PI
1008 429853 carrying recessive gene *cyv1*, which was reported in (7). Black triangles indicate
1009 positions of *GFP* insertion. (C) A series of chimeric viruses tagged with *GFP* were inoculated
1010 and the symptoms were monitored. Photographs were taken at 12 (a) and 17 (b) and 15 dpi
1011 (c; except for CI-90-1 Br2 at 12 dpi) (D) Effect of *GFP* insertion into CIYVV on symptom
1012 development and virus accumulation. a, CI-No.30 without *GFP*, with *GFP* between *PI* and
1013 *HC-Pro*, or with *GFP* between *Nlb* and *CP* was inoculated onto PI 226564 (susceptible), and
1014 their symptoms were compared. b, Viral CP accumulation levels were compared by western
1015 blotting using antiserum against CI-No.30 CP. The *rbc-L* band from the SDS-PAGE gel
1016 stained with Coomassie Brilliant Blue is shown as a loading control. (E) Comparison of
1017 symptoms among CI-90-1 Br2, CI-No.30, and CI-RB without *GFP* at 21 dpi. (F) Virulence of

1018 CI-90-1 Br2-P3B^{No.30}, a CI-90-1 Br2 mutant in which the P3B region in the 90-1 Br2 isolate
1019 was replaced with the corresponding region of CI-No. 30. None of the viruses illustrated had
1020 a *GFP* insertion. The symptoms in a whole plant (a) and upper uninoculated leaf (b) are
1021 shown. Photographs were taken at 19 (a) and 13 (b) dpi. (G) Mapping of virulence
1022 determinants outside the P3B region. None of the chimeric viruses had a *GFP* insertion. The
1023 photograph was taken at 21 dpi.

1024

1025 **FIG 2** Expression of P3 and/or P3N-PIPO from CI-RB, CI-No.30, and BY-CS in PI 226564
1026 using a heterologous WCIMV vector. (A) Schematic representations of *P3*, *P3N-PIPO*, and
1027 *P3ΔPIPO* constructs. The *P3* construct was expected to produce P3 accompanied by a small
1028 amount of P3N-PIPO as a frameshift product. *P3ΔPIPO* contained a mutation enabling it to
1029 produce P3 but not P3N-PIPO. *P3N-PIPO* had mutations for expressing P3N-PIPO in the
1030 zero frame but no P3. (B, C) *P3*, *P3N-PIPO*, and *P3ΔPIPO* constructs of CI-RB (B) or CI-
1031 No.30 (C) were expressed by WCIMV vectors. The photographs in (B) and (C) were taken at
1032 5 dpi. (D) *P3N-PIPO* of BY-CS was expressed by a WCIMV vector. The photographs were
1033 taken at 14 dpi.

1034

1035 **FIG 3** Multiple alignment of amino acid sequences of P3N-PIPO. Alignment was performed
1036 using the program MUSCLE (3.8) (<http://www.ebi.ac.uk/Tools/msa/muscle/>). Accession
1037 numbers: PSbMV-DPD1 (NC_001671), PSbMV-L1 (AJ252242), PSbMV-NEP1 (AJ311841),
1038 PSbMV-NY (X89997), CIYVV-I89-1 (LC096082), CIYVV-No.30 (AB011819), CIYVV-
1039 CYVV (HG970870), CIYVV-Gm (KF975894), CIYVV-90-1 Br2 (AB732962), BYMV-CS
1040 (AB373203), BYMV-90-2 (AB439731), BYMV-92-1 (AB439732), BYMV-GDD
1041 (AY192568), and BYMV-Vfaba2 (JN692500). The amino acid sequences of P3N-PIPO were
1042 obtained by translating the sequences from the 5' end of *P3* to the stop codon of *P3N-PIPO*

1043 after introducing an A into the $A_{6,7}$ region in the $G_{1-2}A_{6,7}$ motif of each virus to shift the
1044 reading frame.

1045

1046 **FIG 4** Real-time PCR analysis of defense-associated gene expressions in susceptible PI
1047 226564. (A, B) SA-responsive (*SA-CHI*) and HR-associated (*HSR203J*) gene expressions in
1048 response to CI-90-1 Br2, CI-No.30, and BY-CS infections. Total RNA was extracted from the
1049 leaves ($n=3$) inoculated with CI-90-1 Br2, CI-No.30, and BY-CS at 6 dpi. cDNA was
1050 synthesized from total RNA and used for real-time PCR analysis. (C, D) *SA-CHI* expression
1051 in response to P3N-PIPO expression via WCIMV. WCIMV vectors carrying *P3N-PIPO*, *P3*,
1052 or *P3ΔPIPO* of CI-RB (C), the corresponding genes from CI-No.30 (D), *P3N-PIPO* from
1053 BY-CS (D), and *GFP* (C and D) as negative control were inoculated onto PI 226564. Total
1054 RNA was extracted from the inoculated leaves ($n=3$) at 4 dpi and used to synthesize cDNA
1055 for real-time PCR analysis. Expression levels of *SA-CHI* and *HSR203J* were normalized to
1056 that of *18S rRNA*. Fold changes from mock infection are shown. Error bars indicate standard
1057 deviations. Statistical analyses were conducted by the Tukey–Kramer method. Different
1058 letters above bars indicate statistically significant differences ($P < 0.05$ [A, B], $P < 0.01$ [C,
1059 D]).

1060

1061 **FIG 5** Comparison of the amount of P3N-PIPO produced from the *P3* cistron between CI-RB
1062 and CI-No.30 using an agroinfiltration assay in *N. benthamiana* leaf tissues. *P3(PIPO:FLAG⁻¹)*
1063 *¹* or *P3N-PIPO:FLAG^{mk}* of CI-RB and CI-No.30 were transiently expressed in the same leaf
1064 of *N. benthamiana*, and the production of protein from the PIPO frame was compared. (A)
1065 Schematic diagrams of plasmids for analyzing P3N-PIPO expression. To detect P3N-PIPO
1066 produced via frameshift to the -1 reading frame, we prepared the *P3(PIPO:FLAG⁻¹)*
1067 construct, in which a *FLAG* epitope tag sequence was inserted in front of the stop codon of

1068 the sequence encoding PIPO. *P3N-PIPO:FLAG^{mk}* has mutations that enable expression of
1069 P3N-PIPO tagged with FLAG in the zero frame. These modified *P3* cistrons were inserted in
1070 a binary vector between a DEX-inducible promoter and a poly(A) addition signal (pAs). (B)
1071 P3N-PIPO-FLAG accumulation was detected by western blotting using an antibody against
1072 FLAG. The numbers below the upper panel are relative band intensities of CI-No.30
1073 compared with those of CI-RB in each plant. Arrowheads indicate bands corresponding to
1074 P3N-PIPO. Yellow fluorescent protein was expressed as a negative control. (C) The level of
1075 *P3(PIPO:FLAG^{-l})* mRNA was compared between CI-RB and CI-No.30 by real-time PCR
1076 analysis. The mRNA levels of *P3(PIPO:FLAG^{-l})* were normalized to those of *EF1 α* . The
1077 relative value for the *P3N-PIPO-FLAG* transcript of CI-No.30 compared with that of CI-RB
1078 is indicated for each plant. (D) Protein/mRNA ratios were calculated by dividing the relative
1079 value of protein (B) by that of mRNA (C) for each plant. (E–G) P3N-PIPO-FLAGs of CI-RB
1080 and CI-No.30 were expressed in the zero frame (*P3N-PIPO:FLAG^{mk}* of CI-RB and CI-No.30,
1081 respectively). The results in (E–G) are presented similarly to those shown in (B)–(D),
1082 respectively. Welch's *t*-test was applied to the data in D and G. * and ** indicates $P < 0.05$ and
1083 $P < 0.01$, respectively.

1084

1085 **FIG 6** Comparison of virus accumulation between CI-No.30 and CI-RB by using GFP-tagged
1086 viruses. (A) PI 226564 was inoculated with CI-No.30/GFP and CI-RB/GFP, and photographs
1087 of GFP fluorescence were taken at 5 dpi. Scale bar = 1 mm. (B) Comparison of virus
1088 accumulation per single infection focus at 5 dpi. Each sample was collected from 10 spots
1089 from five plants (2 spots/plant) and used for DAS-ELISA. Error bars indicate the standard
1090 deviations. Welch's *t*-test was applied to the data. *** indicates $P < 0.001$. (C) Comparison of
1091 virus cell-to-cell movement. GFP-fluorescent areas in inoculated leaves at 5 dpi were
1092 measured by ImageJ software. The areas of 50 spots from five plants (10 spots/plant) were

1093 measured for each virus. Error bars indicate the standard deviations. Welch's *t*-test was
1094 applied to the data. *** indicates $P < 0.001$.

1095

1096 **FIG 7** Mapping of the virulence determinant of CI-90-1 in PI 226564. (A) Schematic
1097 representations of chimeric viruses constructed from CI-No.30 (blue) and either CI-90-1 Br2
1098 (yellow) or CI-90-1 (yellow; sequence was not revealed in area with shaded yellow). Black
1099 triangles indicate the position of *GFP* insertion. CI-RB^{M28R} has the P3B region of CI-90-1,
1100 which contains an amino acid substitution (methionine to arginine) at position 28 of CI-RB
1101 P3 (asterisk). The symptom severity in susceptible PI 226564 is indicated as the number of
1102 (+). We also show the infection profile in PI 429853 carrying recessive gene *cyvI*, which was
1103 reported in (7). (B) Alignment of amino acid sequences surrounding aa position 28 in P3. (C,
1104 D) Symptoms induced by CI-RB^{M28R} were compared with those induced by CI-RB. The
1105 photographs were taken at 14 (C) and 12 (D) dpi.

1106

1107 **FIG 8** *P3N-PIPO* expression by WCIMV in PI 118501 and PI 429853. *P3N-PIPO-RB*, *P3N-*
1108 *PIPO-No.30*, and *P3N-PIPO-CS* were expressed by WCIMV in PI 118501 (A) and PI
1109 429853 (B, C). The photographs were taken at 5 or 8 dpi, as indicated.

1110

1111 **FIG 9** Mapping of the region determining virulence in *P3N-PIPO* in PI 226564 and PI
1112 118501. *P3N^{CS}-PIPO^{RB}*, *P3N^{RB}-PIPO^{CS}*, *P3N-PIPO-RB*, *P3N-PIPO-CS*, and *GFP* were
1113 expressed by WCIMV vectors in PI 226564 (A) and PI 118501 (B). *P3N^{CS}-PIPO^{RB}* has the
1114 *P3N* region of BY-CS and *PIPO* of CI-RB; *P3N^{RB}-PIPO^{CS}* has the *P3N* region of CI-RB and
1115 *PIPO* of BY-CS. Mock was treated with inoculation buffer only. The photographs of the
1116 inoculated leaves were taken at 8 dpi (A), 5 and 18 dpi (B). Lower panel in leftmost panels of

1117 A shows a magnified picture of the area indicated in upper panel. Arrowheads indicate
1118 regions in which cell death was induced.

1119

1120 **FIG 10** Gradation of virulence among CIYVVs and BYMV in susceptible and *cyvI*
1121 (recessive resistance) peas. (A) Symptoms were compared between CI-90-1 Br2 and CI-I89-1
1122 in PI 429853 (*cyvI*) at 25 dpi. The presence/absence of virus infection (by RT-PCR shown in
1123 B and C) is indicated below the photograph. (B) CI-I89-1 and CI-90-1 Br2 infections in upper
1124 uninoculated leaves (shown in A) were confirmed by RT-PCR at 25 dpi in PI 429853 (*cyvI*).
1125 (C) BY-CS infection in upper uninoculated leaves was confirmed by RT-PCR at 25 dpi in PI
1126 429853 (*cyvI*). For the positive control (p.c.), RT-PCR was done using an upper uninoculated
1127 leaf of PI 118501 inoculated with BY-CS. (D) The symptoms were compared between CI-
1128 I89-1 and CI-90-1 Br2 in PI 226564 at 10 dpi. (E) The symptoms were compared between CI-
1129 No.30 (without *GFP*) and BY-CS in PI 226564 at 10 dpi. (F–H) CI-I89-1, CI-90-1 Br2, CI-
1130 90-1, CI-No.30, and BY-CS were inoculated onto PI 118501, and their symptoms were
1131 compared at 12 dpi. Upper symptomatic leaves of plants inoculated with BY-CS (G) or mock
1132 (H) are shown. (I) Gradation of virulence among CIYVVs and BYMV in recessive resistant
1133 (PI 429853 [*cyvI*, *CynI*]) (top graph) and susceptible (PI 226564 [weak *CynI*] and PI 118501
1134 [*CynI*]) (bottom graph) cultivars. The graphs indicate the consistent gradation observed in
1135 this study: the more efficiently a CIYVV isolate broke the resistance conferred by *cyvI* (top),
1136 the more it expressed virulence in susceptible peas (bottom). (J) Molecular phylogenetic
1137 analysis of full-length nucleotide sequences encoding polyprotein of CIYVV and BYMV.
1138 The sequences were aligned using MUSCLE and the maximum-likelihood tree was inferred
1139 using the MEGA6 package (36). The tree is drawn to scale, with branch lengths measured in
1140 the number of substitutions per site. TuMV-Tu-2R1 was set as an outgroup. The significance

1141 of the nodes was estimated with 1000 bootstrap replicates. The accession number of each
1142 virus is shown in Fig. 3 except for TuMV-Tu-2R1 (AB105135).

1143

1144

1145 **TABLE 1** Summary of symptoms induced by CIYVV, BYMV, and WCIMV expressing

1146 P3N-PIPO in pea^a

Pea cultivar (genotype)	CIYVV				BYMV	WCIMV ^b			GFP
	CIYVV P3N-PIPO		BYMV P3N-PIPO			CS			
	I89-1	90-1 Br2	90-1	No.30	CS		90-1 Br2	No.30	
PI 429853 (<i>CynI</i> ^c , <i>cyvI</i>)	SCD ^d	No/CS ^d	No infection ^d	No infection ^d	No infection	Cell death	Cell death	Delayed cell death	No
PI 226564 (<i>Cyn</i> ^e)	LSCD	LSCD	SCD	M/CS ^{fg}	M/CS ^f	Cell death	Cell death	No	No
PI 118501 (<i>CynI</i>)	LSCD	LSCD	LSCD	LSCD ^{fg}	M/CS ^g	Cell death	Cell death	Delayed cell death	No

1147 ^a LSCD, lethal systemic cell death; SCD, systemic cell death; M, mosaic; CS, chlorotic spot; No, no symptoms.

1148 ^b Cell death induction in the inoculated leaves was observed until 8 days after inoculation.

1149 ^c Assumed to carry *CynI*

1150 ^d (7).

1151 ^e Putative weak *CynI* allele (20).

1152 ^f (20).

1153 ^g (21)

1154

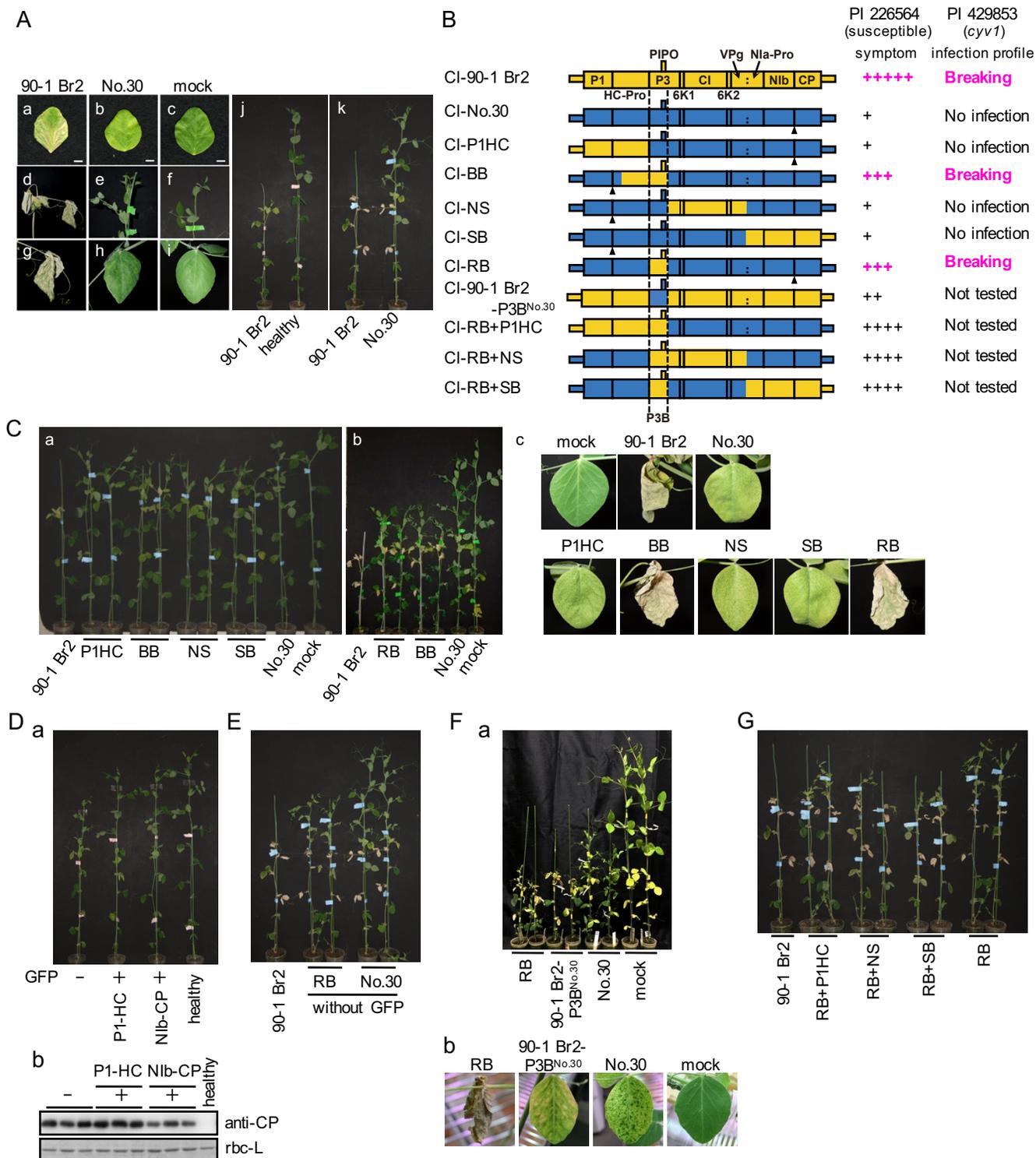


FIG 1

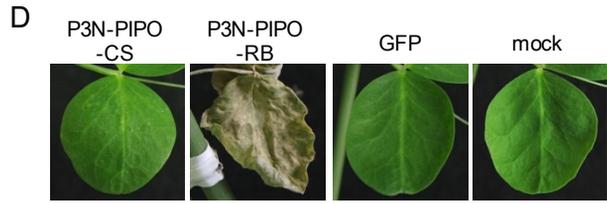
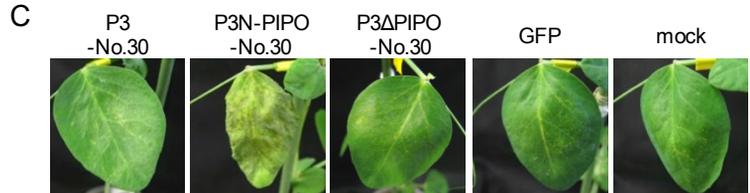
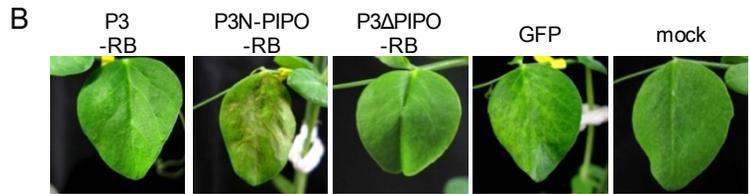
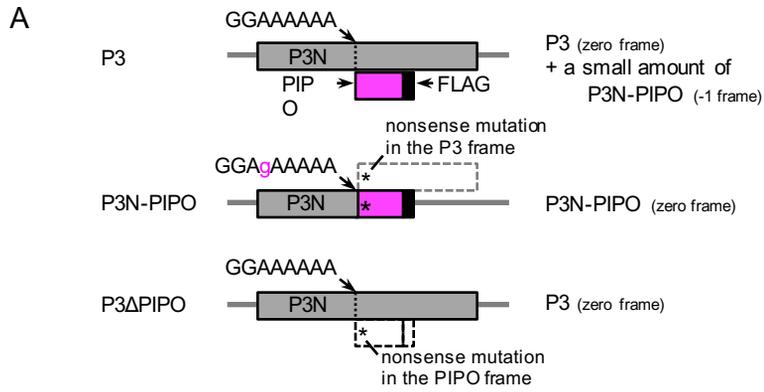


FIG 2

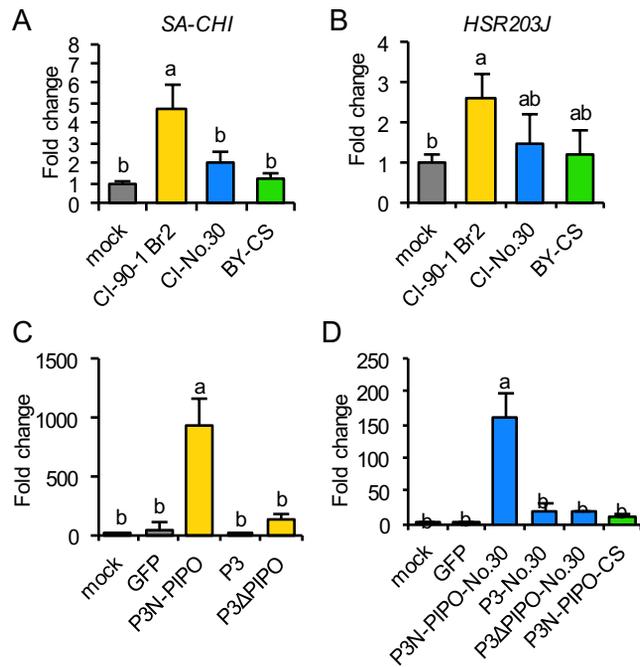


FIG 4

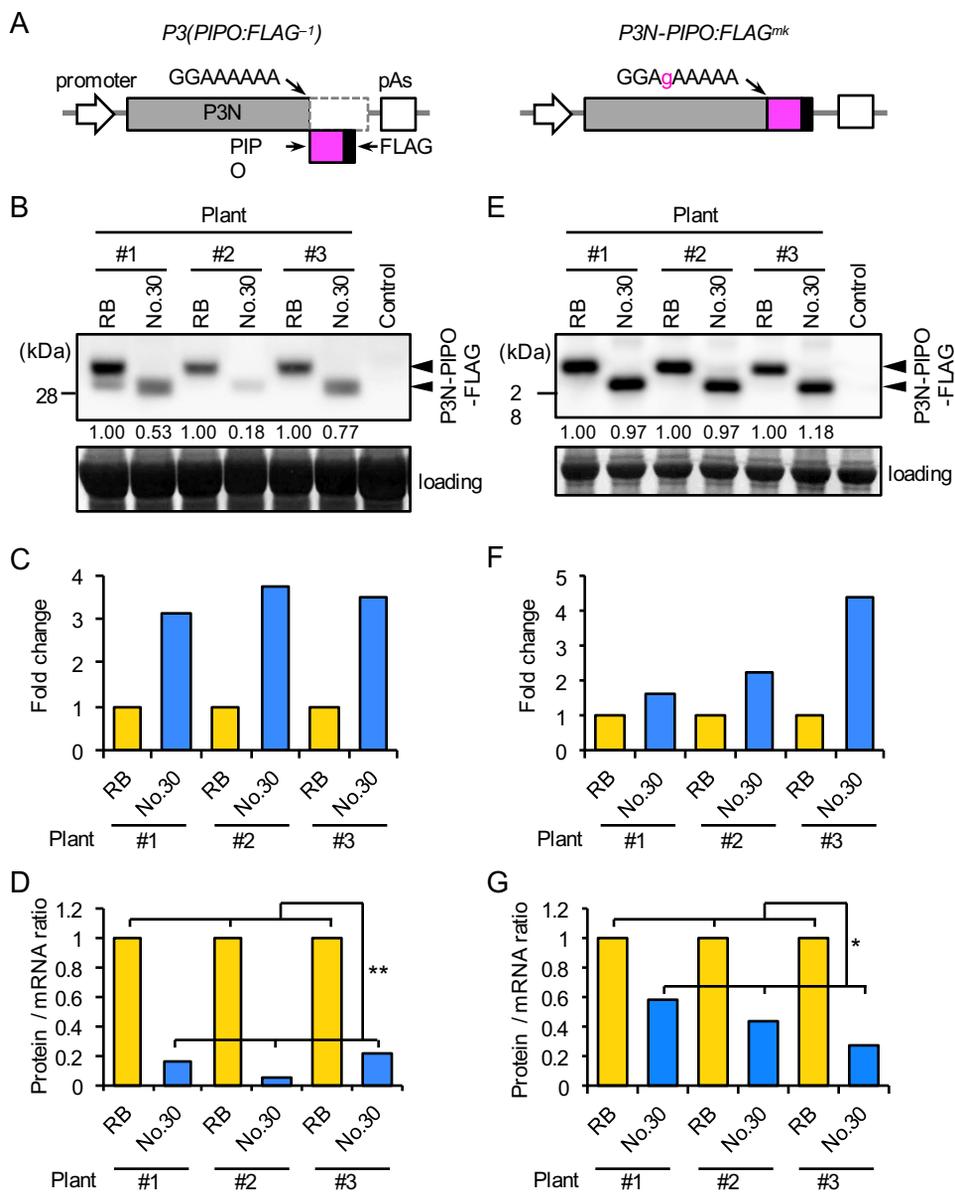


FIG 5

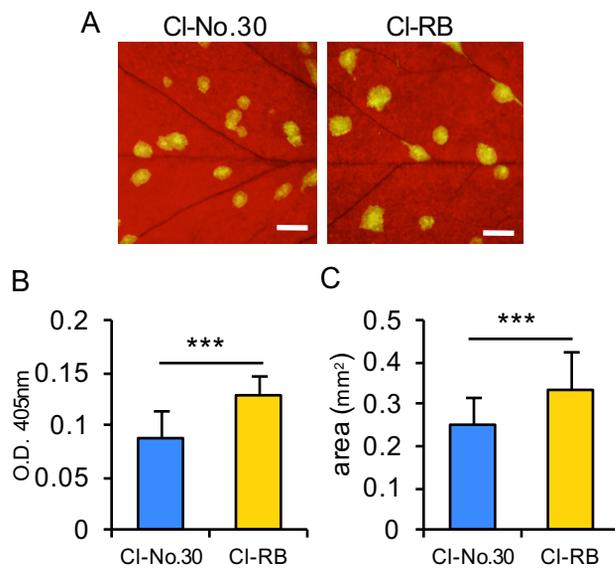


FIG 6

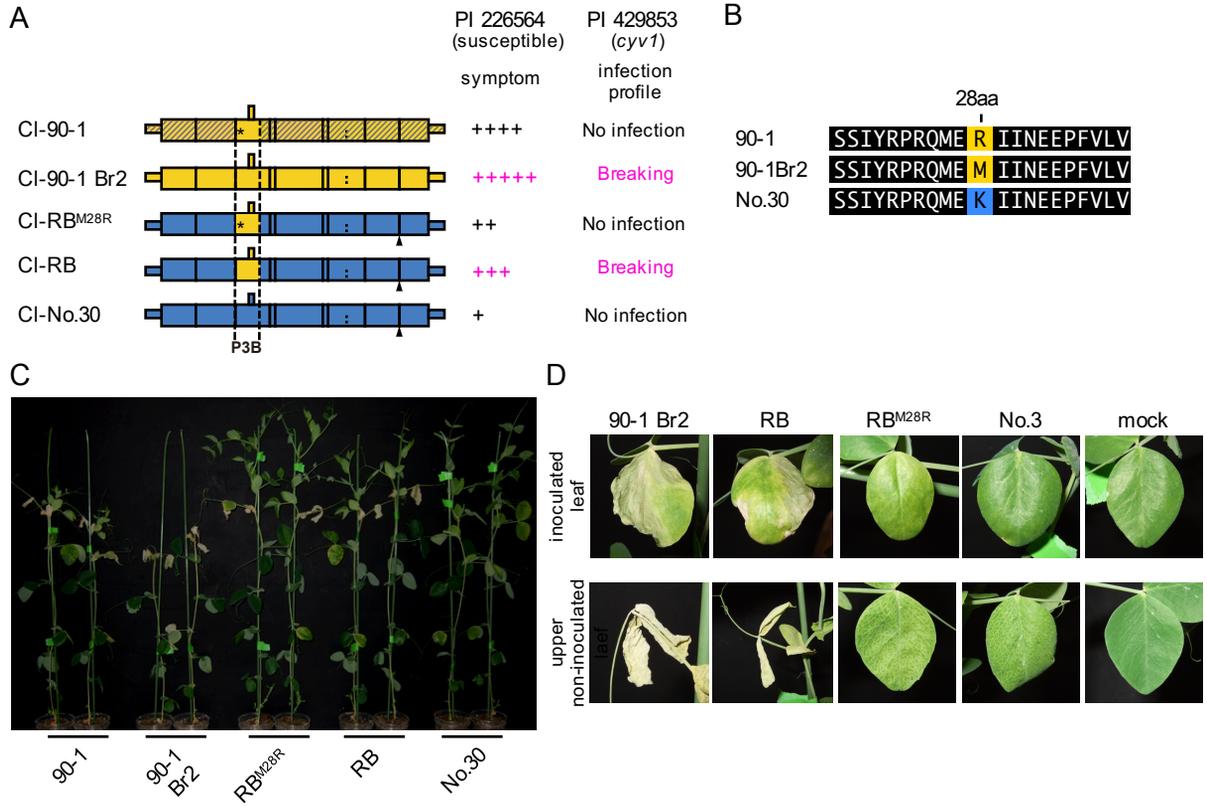


FIG 7

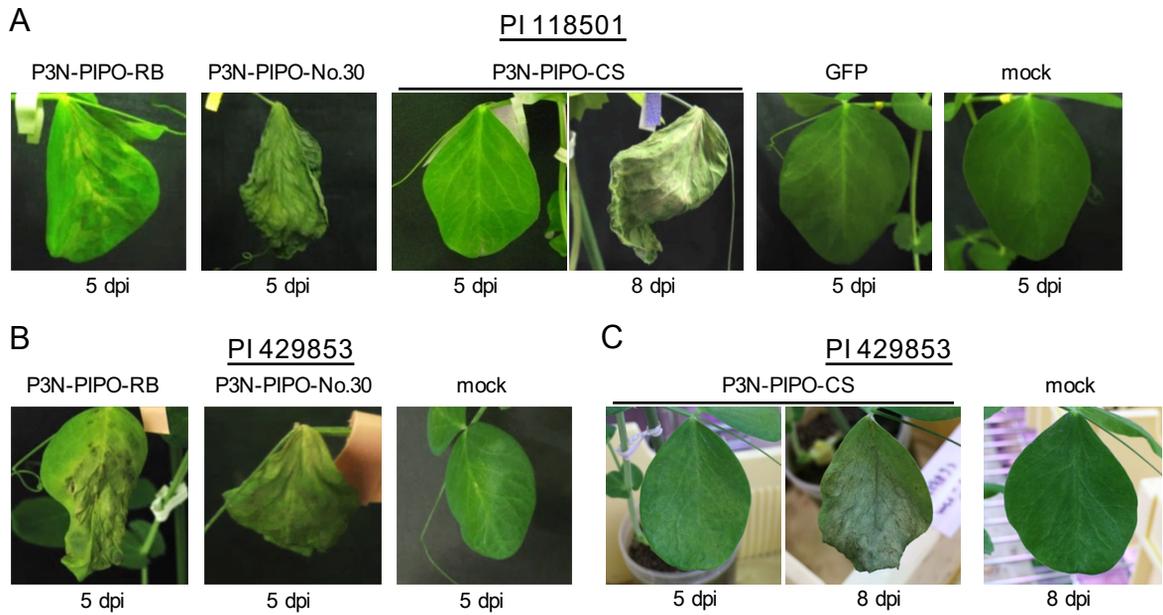


FIG 8

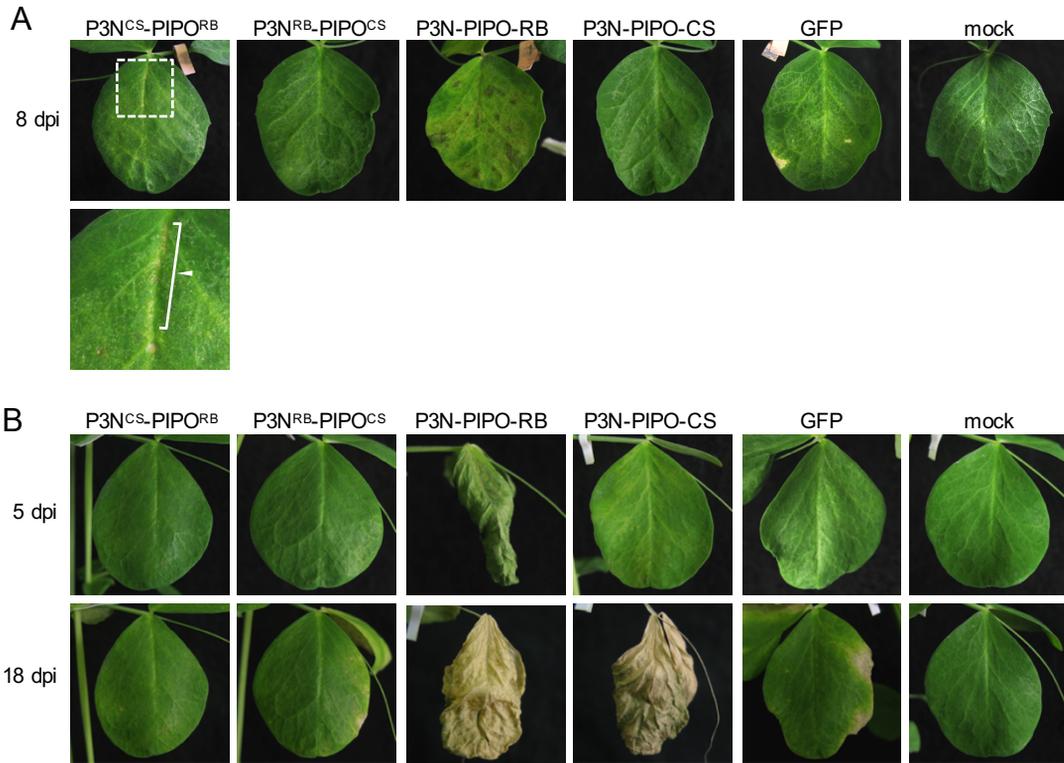


FIG 9

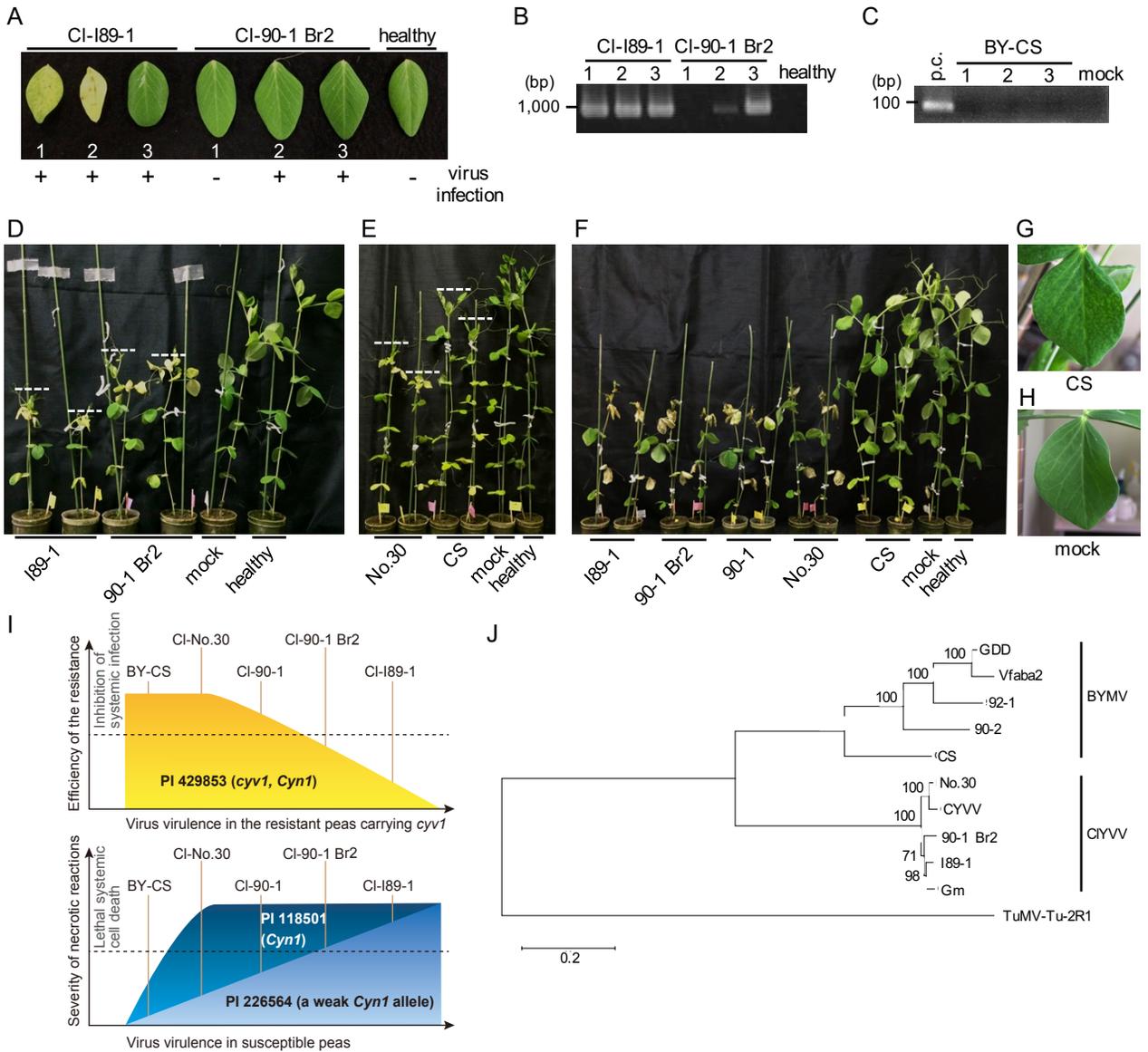


FIG 10

