Nucleotide Sequence-Homology-Independent Breakdown of Transgenic Resistance by More Virulent Virus Strains and a Potential Solution

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Controlling plant viruses by genetic engineering, including the globally important Papaya ringspot virus (PRSV), mainly involves coat protein (CP) gene mediated resistance via post-transcriptional gene silencing (PTGS). However, the breakdown of single- or double-virus resistance in CP-gene-transgenic papaya by more virulent PRSV strains has been noted in repeated field trials. Recombination analysis revealed that the gene silencing suppressor HC-Pro or CP of the virulent PRSV strain 5-19 is responsible for overcoming CP-transgenic resistance in a sequence-homology-independent manner. Transient expression assays using agro-infiltration in Nicotiana benthamiana plants indicated that 5-19 HC-Pro exhibits stronger PTGS suppression than the transgene donor strain. To disarm the suppressor from the virulent strain, transgenic papaya lines were generated carrying untranslatable 5-19 HC-Pro, which conferred complete resistance to 5-19 and other geographic PRSV strains. Our study suggested the potential risk of the emergence of more virulent virus strains, spurred by the deployment of CP-gene-transgenic crops, and provides a strategy to combat such strains.

The coat protein (CP)-mediated resistance strategy pioneered by Beachy and coworkers1,2 to control plant viruses through genetic engineering has been widely used to develop commercialised crops, including CP-transgenic squash and papaya3. Originally, CP-transgenic resistance was proposed to occur via protein-mediated interference with uncoating or by trapping the viral RNA, thereby inhibiting the replication of related invading viruses4. However, it was later found out that transgenic lines that do not accumulate transcript and protein from the transgene display even higher degrees of resistance, indicating that resistance occurs at the RNA level5. This RNA-mediated resistance is based on post-transcriptional gene silencing (PTGS), a host defence response to RNA6,7. Host RNA polymerase can target foreign RNA transcribed from a transgene and synthesise dsRNA that is recognised and cleaved by Dicer-like ribonucleases into 21-24 nt transgene-derived short-interfering RNAs (siRNAs)8,9. These siRNAs are loaded into ARGONAUTE1 (AGO1), a core protein of the RNA-induced silencing complex (RISC), which unwinds and retains the complementary siRNAs to guide the AGO endonuclease to target and cleave related RNAs in a sequence-homology-dependent manner10,11. Thus, PTGS-mediated transgenic resistance depends on the homology between a transgene and the corresponding viral genome12.

Because dsRNA intermediates are formed during the replication of plant RNA viruses, PTGS represent a major host response against invasion by RNA viruses. To counteract these host defences, plant viruses have evolved specialised proteins, such as the helper component-proteinase (HC-Pro) of potyvirus13 and the 2b protein of cucumovirus14, that suppress PTGS by compromising the components of the host gene-silencing pathway15.

Papaya (Carica papaya L.) is an important fruit crop in tropical and subtropical areas. The aphid-borne ss(+)RNA potyvirus Papaya ringspot virus (PRSV) is the main obstacle to large-scale commercial production.
of this fruit. The lack of naturally resistant sources for use in conventional breeding, as well as the ineffectiveness of control measures such as agricultural practices for repelling aphid vectors and cross-protection from mild PRSV strains, necessitate the generation of transgenic resistance. In Hawaii, Gonsalves and co-workers initiated the development of PRSV CP-transgenic papaya in the late 1980s. The CP-transgenic resistance of the resulting SunUp cultivar is triggered by PTGS and confers a high degree of resistance to PRSV Hawaii strain HA. In 1998, the SunUp and Rainbow papaya cultivars were deregulated and approved for commercial application in the United States, representing the first successful case of the cultivation of a transgenic fruit tree. However, the high genetic divergence of CP genes from other geographic PRSV strains limits the application of these two CP-transgenic cultivars in regions outside of Hawaii.

We previously used the CP coding sequence and contiguous 3′-untranslated region (CP-3′UTR) of a native PRSV strain, YK, to generate PTGS-based transgenic resistance using the papaya cultivar Tainung No. 2 in Taiwan. The resultant YK CP-3′UTR transgenic lines confer a high degree of broad-spectrum resistance to different geographic strains from Taiwan (YK), Hawaii (HA), Thailand (TH) and Mexico (MX). However, the infection of these YK-CP-3′UTR transgenic papaya lines by a different potyvirus Papaya leaf-distortion mosaic virus (PLDMV) during field trials necessitated the development of double-virus-resistant transgenic papaya lines that carry a chimeric untranslatable transgene comprising parts of the PLDMV CP and PRSV YK CP-3′UTR sequences. Nevertheless, during repeated field trials over 4 years, both PRSV single-virus- and PRSV + PLDMV double-virus-resistant lines were found to be susceptible to a more virulent strain, PRSV 5-19. The emergence of more virulent virus strains represents a serious threat to the application of CP-transgenic papaya cultivars resistant to either PRSV or PRSV + PLDMV.

The YK CP-3′UTR transgenic papaya lines exhibit broad-spectrum resistance to PRSV strains whose CPs share >89% nucleotide identity (nt) sequence identity with the transgene, including the different geographic strains TH (Thailand), HA (Hawaii) and MX (Mexico). However, PRSV 5-19 is able to overcome the single- or double-virus transgenic resistance despite the significantly higher nucleotide identity of its CP/3′UTR compared with YK (96/98)% nucleotide identity, implying that the resistance-breaking ability of PRSV 5-19 is not correlated with the level of sequence divergence between its CP-3′UTR and that of the transgene. Previously, Maki-Valkama reported that although potato lines containing the P7 gene of PVY (a common strain) are resistant to PVY, they are not resistant to PVY (a necrotic strain), despite complete sequence identity between the P7 genes of PVY and PVY. Such anomalous, homology-independent breakdown of PTGS-mediated transgenic resistance by a more virulent strain suggests the involvement of viral elements that suppress PTGS-mediated resistance and may shut off the RNA silencing mechanism.

In this study, the mechanism of the homology-independent breakdown of CP-transgenic resistance by virulent strain 5-19 was investigated by analysing the reactions of the PRSV single-virus- and PRSV + PLDMV double-virus-resistant lines to the transgene donor strain YK, the resistance-breaking strain 5-19, and their recombinants. The results of the recombinant analysis indicated that 5-19 HC-Pro, a PTGS suppressor, is responsible for the resistance-breaking ability of 5-19. Gene silencing suppression assays performed using transient expression in Nicotiana benthamiana verified that 5-19 HC-Pro exerts strong gene-silencing suppression than YK HC-Pro. Thus, the virulent strain 5-19 may have emerged in conventional plants and been inoculated into transgenic trees via aphid-mediated transmission. The stronger ability of this strain to suppress PTGS allows it to overcome CP-transgenic resistance in a sequence-homology-independent manner. To counter the threat of the 5-19 strain, transgenic papaya lines carrying an untranslatable 5-19 HC-Pro construct were developed. The resulting HC-Pro-transgenic papaya lines are resistant to the virulent strain 5-19, as well as to other geographic PRSV strains, suggesting that this approach may solve the problem of the emergence of more virulent strains from CP-transgenic lines.

**Results**

CP-transgenic resistance can be overcome in a nucleotide sequence-homology-independent manner. RNA viruses can be recombined at the cDNA level in the laboratory. Using an infectious, full-length *in vitro* YK cDNA clone as a backbone, which can generate a full-length, infectious YK RNA genome following *in vitro* transcription from a bacteriophage T3 promoter, the recombinant viruses YK/519HC and YK/519CP3U were generated by replacing the HC-Pro or/and CP-3′UTR regions of YK with the corresponding sequences from 5-19 (Fig. 1A).

A total of 30 plants, each from the PRSV single-virus-resistant lines 16-0-1, 17-1-0, 18-2-4 and 18-0-9 were mechanically inoculated with YK, 5-19 or the recombinant viruses under greenhouse conditions in four independent tests. Two months after inoculation, plants from lines 18-2-4 and 17-0-1 were completely resistant to YK infection, whereas 3 and 13% of the plants from lines 16-0-1 and 18-0-9 were infected with YK, respectively. The recombinant viruses YK/519HC and YK/519CP3U caused symptoms of severe leaf mosaic and distortion in non-transgenic (NT) papaya plants two weeks after inoculation. At two months post inoculation, YK/519HC and YK/519CP3U caused infection rates of 51% and 53%, respectively, in the four transgenic lines (Fig. 1B; Table 1). Similar symptoms of comparable severity were also caused by YK/519HC/CP3U in 56% of the transgenic plants, suggesting the absence of an additive effect in overcoming the resistance of CP-transgenic lines, even with the combined presence of both 5-19 HC-Pro and CP genes in the YK backbone (Fig. 1B; Table 1).

All three recombinants were also able to overcome the resistance of PRSV-PLDMV CP transgenic lines (Fig. 1C), with infection rates as high as 95–100% in the four tested lines 9-5, 10-4, 12-4, and 14-1 at eight weeks after inoculation (Table 2). Our results indicated that 5-19 HC-Pro enables recombinant YK/519HC, which possesses an identical CP-3′UTR to that of the transgene (100% nt identity), to overcome both single- and double-virus resistances in a sequence homology-independent manner. Because the resistance-breaking ability of 5-19 HC-Pro was not enhanced by the presence of the 5-19 CP-3′UTR in recombinant YK/519HC/CP3U (Table 1 and 2), we concluded that 5-19 HC-Pro plays a vital role in the breakdown of nucleotide sequence-homology-independent resistance.

Intriguingly, YK/519CP3U also significantly increased the percentage of breakdown, implying a possible contribution from CP-3′UTR. Because the nt identity of 5-19CP/3′UTR to that of YK is much higher than to the exotic strains HA, TH, and MX, which can not overcome single- and double-virus-transgenic resistance, the breakdown mediated by the recombinant YK/519CP3U is also probably not sequence-homology-dependent. However, we cannot exclude the possibility of homology-dependent breakdown mediated by the heterologous 5-19 CP and CP-RSV in recombinant YK/519CP3U because the two virus strains still show very little nucleotide divergence (95.9% identity for CP and 97.9% for 3′UTR).

**Breakdown of CP-transgenic resistance is due to suppression of the host defence reaction.** We investigated the ability of YK, 5-19 and the three recombinant viruses to suppress PTGS in double-virus resistant lines (10-4 and 14-1) carrying the chimeric construct (PLDMV CP-PRSV YK CP-3′UTR). In northern blots, the PLDMV and PRSV CP probes did not cross-hybridise with preparations from non-transgenic plants infected with the heterologous virus (Fig. 2A) due to the low degree of sequence homology (<60%). Thus, we chose the PLDMV CP probe to detect the chimeric transgenic transcripts to avoid
Figure 1 | Recombination analysis to study the ability of helper component protease (HC-Pro) and coat protein (CP) from the *Papaya ringspot virus* (PRSV) resistance-breaking strain 5-19 to overcome single-virus or double-virus resistance in CP-transgenic papaya lines. (A) Construction of recombinants carrying heterologous HC-Pro or/and CP from the resistance-breaking strain 5-19 using the *in vitro* infectious cDNA from the non-breaking strain YK as a backbone. Genomic regions of HC-Pro and CP (in yellow) amplified from 5-19 were used to replace one or both of the corresponding regions in YK to generate the recombinant viruses YK/519HC, YK/519CP3U and YK/519HCCP3U. (B) Resistance evaluation of the PRSV single-virus-resistant transgenic line 18-2-4 (carrying a YK CP-3’UTR transgene as indicated in the upper panel) against 5-19 and the recombinants. Non-transgenic (NT) plants of papaya cultivar Tainung No. 2 were used as a control. Symptoms were recorded 30 days post mechanical inoculation (dpi). (C) Resistance evaluation of the PRSV-PLDMV double-resistant-transgenic line 14-1 (carrying a chimeric construct comprised of parts of PLDMV CP and PRSV CP-3’UTR, as indicated above the panel) against YK, 5-19 and the recombinants. Symptoms were recorded 30 dpi.

Table 1 | Reactions of single-virus-resistant transgenic papaya lines carrying CP 3’UTR from *Papaya ringspot virus* (PRSV) YK after inoculation with PRSV YK, virulent strain 5-19, or individual recombinants carrying heterologous HC-Pro (YK/519HC), CP 3’UTR (YK/519CP3U), or both (YK/519HCCP3U) from 5-19.

| Virus | YK CP-3’UTR transgenic line | Mean | Non-transgenic Tainung No. 2 |
|-------|-----------------------------|------|-------------------------------|
|       | 18-2-4 | 17-0-1 | 16-0-1 | 18-0-9 |       |
| YK    | 0      | 0      | 3      | 13     | 4”     | 100   |
| 5-19  | 63     | 53     | 68     | 71     | 64”    | 100   |
| YK/519HC | 54     | 47     | 57     | 46     | 51”    | 100   |
| YK/519CP3U | 50     | 35     | 62     | 63     | 53”    | 100   |
| YK/519HCCP3U | 75     | 47     | 63     | 39     | 56”    | 100   |

Numbers indicate the percentages of plants showing symptoms two months after inoculation. Four independent tests, comprising a total of 30 plants from each line, were conducted for this assay. Adjacent mean values with the same superscript are not significantly different (P<0.05) based on Duncan’s multiple range test.
any possible interference from PRSV RNA. PTGS-mediated resistance to YK was clearly observed via the complete silencing of the chimeric transgenic transcript after inoculation with YK (Fig. 2B). However, the probe detected high levels of chimeric transgenic transcript in transgenic plants infected with 5-19 or the three recombinants (Fig. 2B). Our results indicated that 5-19 and the recombinants can infect and establish themselves in the transgenic lines by suppressing the PTGS-mediated degradation of the chimeric transgenic transcripts. This ability to suppress PTGS and compromise the degradation of the transgenic transcripts is apparently due to the different silencing suppression abilities of 5-19 and the YK recombinants. Once PTGS has been shut off, the chimeric transgenic transcripts and viral RNA are safeguarded from the host gene-silencing machinery.

Because the PLDMV CP probe did not cross-hybridise with the siRNA from PRSV-infected non-transgenic plants (Fig. 2A), the source of the detected siRNA must be the chimeric transgenic transcript. In these same infected transgenic plants, the corresponding 21-22 nt siRNAs were also observed to accumulate to higher levels than in YK-resistant transgenic plants, despite considerable accumulation of the chimeric transcript (Fig. 2C). Potyviral HC-Pro suppresses gene silencing by interfering with the RNA silencing machinery at multiple levels, including (i) reducing siRNA levels by incompletely inhibiting the processing of dsRNA by Dicer; (ii) preventing mRNA degradation by interfering with the function of the RNA-induced silencing complex (RISC) by inhibiting the 3'-methylation of siRNAs, which leads to enhanced oligo-uridylation and subsequent degradation of siRNA; and (iii) binding and trapping siRNAs to compromise signaling. Although the binding and trapping of siRNAs by HC-Pro in RDR6-dependent systems can inhibit the maintenance/amplification step and lower the total levels of signalling siRNAs, the sequestered siRNA may accumulate along with the preserved target RNA, as observed in the present study (Fig. 2B&C) and also reported elsewhere.

PRSV strain 5-19 has a stronger silencing suppressor HC-Pro than other strains. The gene-silencing-suppression capabilities of 5-19 in comparison with that of YK were compared via *Agrobacterium tumefaciens*-mediated transient expression of their respective HC-Pro constructs in the leaf tissues of *Nicotiana benthamiana* plants that were co-infiltrated with a translatable green fluorescent protein (GFP) construct (reporter) and a GFP hairpin construct (PTGS inducer), as described for *Tobacco etch virus* (TEV) P1/HC-Pro. UV irradiation of the *Agrobacterium*-infiltrated leaves 5 days after infiltration showed that the fluorescence intensity of 5-19 HC-Pro-infiltrated areas was 1.25-fold higher (mean of six repeats) than that of YK HC-Pro-infiltrated areas (Fig. 3A), indicating significantly stronger gene silencing suppression by 5-19 HC-Pro compared with YK HC-Pro (Fig. 3A). Since HC-Pro is the weapon of the virus to counteract the defensive RNA silencing from the host, if a virus mutant (strain) can resist RNA silencing, its titers would increase immediately and subsequently amplify the silencing suppression effect. This suggests that a slight difference would affect the consequence of the fight between the host and the invading virus dramatically. We strongly believe that a 25% increase in silencing suppression would significantly result in different biological phenotype as reflected by the results of this study, especially the virus recombination assays (Fig. 1). Similarly,
from diverse strains to which the YK CP-3’UTR-transgenic plants were resistant share 95-97% aa identity with 5-19 HC-Pro (Table S1). Potyviral HC-Pro is a multifunctional protein with different domains that fulfill its multiple roles38, and slight changes in these different functional domains may have a concerted effect on silencing suppression. Our results revealed that there was no correlation between particular sequence differences and the ability to break CP-transgenic resistance.

An approach for solving the problem of CP sequence-homology-independent transgenic resistance breakdown. Three transformation vectors, pBI-519HCF, pBI-519HCN and pBI-519HCC, carrying untranslatable full-length (nt 1-1371), N-terminal (nt 1-750) and C-terminal (nt 622-1371) 5-19 HC-Pro, respectively, were constructed. Each construct carries two engineered termination-codons, along with an inserted T nucleotide that causes frame shift, and is controlled by the CaMV 35S promoter and nos terminator (Fig. 4A). Somatic embryos derived from in vitro adventitious roots39 of papaya cultivar Sunrise were transformed using A. tumefaciens harbouring the individual vectors, and 60 transgenic lines were established (Table S2).

Micropropagated 5-19 HC-Pro transgenic plants were evaluated for their resistance to PRSV by mechanical inoculation with 5-19 or YK. Non-transgenic (NT) plants and plants from YK-CP-3’UTR transgenic line 18-2-440 were used as controls. Two weeks after inoculation in NT plants, 5-19 induced stunting and severe mosaic symptoms (Fig. 4B-a), which subsequently deteriorated into lethal wilting; mosaic symptoms were also observed in 18-2-4 plants (Fig. 4B-b). Thirty-one 5-19 HC-Pro transgenic lines were susceptible to 5-19 (Table S2; Fig. 4B-c), thirty of which were also susceptible to YK (Table S2), as defined by no delays in the development of symptoms. Of the twenty-nine 5-19-resistant transgenic lines, 10 were weakly resistant (WR) (Table S2), as defined by a delay in the development of symptoms, and 19 were highly resistant (HR) to 5-19 (Table S2; Fig. 4B-d), as defined by symptomless appearance and negative ELISA results 8 wk after inoculation. PRSV YK induced severe mosaic symptoms in NT plants (Fig. 4B-e). As reported earlier41, the plants from the 18-2-4 line were resistant to YK (Fig. 4B-f). The susceptible lines developed severe non-lethal vein-clearing and leaf-mosaic symptoms after inoculation with 5-19 or YK (Fig. 4B-c & g), whereas all 19 HR lines were also highly resistant to YK (Table S2; Fig. 4B-h).

A significantly higher percentage of HR lines (46.0%) was found among the full-length HC-Pro lines than among lines carrying the N-terminal 519HCN construct (29.0%) or the C-terminal 519HCC construct (21.4%) (Table S2). Southern hybridisation analysis of sixteen of lines that were highly resistant to both YK and 5-19 indicated the presence of 1-6 HC-Pro transgene inserts (Fig. S2). We also obtained 12 lines of 5-19 CP transgenic papaya, however, they did not show significant degrees of resistance to both PRSV 5-19 and YK strains (data not shown). Because high percentages of transgenic lines with different constructs of 5-19 HC-Pro (average 31.6%, Table S2) resistant to 5-19 were obtained, we did not proceed further experiments to obtain more 519-CP lines for comparison.

Four HR lines (F2-1-4, F2-7-1, F3-2-2, and N11-1) with single insertions of the untranslatable 5-19 HC-Pro construct were further analysed for their resistance to different geographical PRSV strains. Line F2-7-1 was susceptible to the exotic PRSV strains HA, TH and MX. Lines F2-1-4 and N11-1 showed delayed symptoms to HA and TH but were susceptible to MX (Table S3). However, line F3-2-2 showed complete resistance to all the exotic strains tested and was thus regarded as an extremely HR line.

Of the nine HR lines and three S lines (three HR and one S lines for each full-length, N-half, and C-half untranslatable HC-Pro) analysed by northern hybridisation, five HR lines (F2-7-1, N11-1, C9-4-1, C9-5-3, C10-13) accumulated significantly lower levels of transgenic transcripts than the S lines. However, transgenic transcripts were not
detectable in the other four HR lines (Fig. 4C). In addition, siRNAs were detected in all the nine HR lines but not in any of the S lines (Fig. 4C). The highest level of siRNA was detected in the single-insert full-length HC-Pro transgenic line F3-2-2 that conferred broad-spectrum resistance to 5-19 and all other PRSV strains (Table S3). Our results demonstrated that the transgenic resistance to the
virulent virus 5-19 and the other PRSV strains is based on the targeting of the gene-silencing suppressor HC-Pro by PTGS. However, HC-Pro resistance is not merely dependent on sequence identity but also on its function as a suppressor.

Segregation analysis of the nptII gene in R1 progeny. The segregation ratios of the nptII gene in the R1 progeny from the 2 HR lines (F2-1-4 and F3-2-2) were 3:1, as calculated by the ratios of gminating seedling resistant or sensitive on the selection medium containing kanamycin (Table S4), verifying that the nptII gene was integrated into the chromosome at a single locus, as revealed by Southern blotting (Fig. S2). When these kanamycin-resistant plants were mechanically challenged with PRSV 5-19, all of them were resistant to viral infection. Thus, our results further indicated that the transgenic resistance to 5-19 is linked with kanamycin resistance and undergoes nuclear inheritance as a dominant trait.

Discussion

The severity of viruses can be increased by a few mutations in gene silencing suppressors. As demonstrated by the present investigation, such mutant viruses can overcome PTGS-mediated transgenic resistance independent of transgene sequence homology. Although transgenic papaya lines grown in Hawaii are not resistant to PRSV strains from other geographic areas, no breakdown in the transgenic resistance to PRSV strains indigenous to Hawaii has been observed. The geographical isolation of Hawaii restricts the diversity of PRSV strains and contributes to the stable and continuous effectiveness of PRSV CP-transgenic papaya for more than a decade. However, the emergence of more virulent virus strains through the selection mechanism described in this study is expected to reveal in prevails where more diverse PRSV strains exist.

Influenced by European trends, the government of Taiwan has yet to approve any of the transgenic crops cultivated on the island. Nevertheless, field tests of CP-transgenic papaya lines under isolated conditions have been strongly supported by the government over the past two decades. During more than 10 years of regulated field trials, the complexity of the viruses that infect CP-transgenic papaya lines has helped advance the development of single-virus, double-virus, and virulent virus (this study) resistance to PRSV, PRSV/PLDMV, and virulent PRSV strains, respectively. The lethal wilting type PRSV 5-19 with a stronger HC-Pro described herein infects the YK-CP-3’UTR-transgenic papaya plants as a non-lethal mosaic virus. Here, we demonstrate that the virulent strain 5-19 exhibits a strong ability to suppress gene silencing and overcome CP-transgenic resistance, which allows it to establish in CP-transgenic plants and cause non-lethal mosaic symptoms. The emergence and accumulation of such virulent strains in CP-transgenic plants poses a serious risk that may completely wipe out CP-transgenic and non-transgenic papaya plantations and also threatens other cucurbitaceous crops that can be infected by PRSV. Our results further indicate that the breakdown of CP-transgenic resistance by virulent strains, such as PRSV 5-19, is independent of the genetic diversity of PRSV CPs. Thus, regardless of investigations into the diversity of CPs to develop CP-transgenic resistance against different PRSV strains present in a particular region, CP-transgenic plants could still facilitate the emergence of PRSV mutants or variants with stronger RNA silencing suppression from amongst the natural virus population. This complexity emphasises the importance of monitoring the possible emergence of more virulent virus strains during the cultivation of CP-transgenic crops.

Our study also demonstrated that untranslatable constructs targeting the gene silencing suppressor HC-Pro can disarm viral protections against host defences and minimise the potential emergence of more virulent virus strains that can overcome CP-transgenic resistance. Currently, we are pyramiding single-virus, double-virus, and virulent virus transgenic resistances in papaya to develop a hybrid variety with broad-spectrum resistance for global application and reduced risk for the emergence of more virulent virus strains during cultivation.

Methods

Virus isolates. Papaya ringspot virus (PRSV) isolates YK40, 5-19, HA41, TH and MX1, and Papaya leaf-distorting mosaic virus (PLDMV) isolate P-TW-WF22 were used in this study. PRSV YK, a typical mosaic type strain collected from southern Taiwan, was previously characterised and its genome sequenced. PRSV 5-19 and PLDMV P-TW-WF22, both severely infecting transgenic papaya (Carica papaya L. cultivar Tainung No. 2) lines carrying the PRSV YK CP coding region along with the contiguous PRSV 5-19-UTR, were collected from central Taiwan during field trials. The geographically distant PRSV strains HA41, TH and MX1, which originate from Hawaii, Thailand and Mexico, respectively, were used to evaluate the broad-spectrum resistance of the transgenic papaya lines. All virus isolates were maintained in papaya (Carica papaya L. cv. Tainung No. 2) plants.

Construction of viral recombinants between PRSV YK and 5-19. The in vitro infectious PRSV YK cDNA clone pT3PYKFK1, driven by the bacteriophage T3 promoter, was constructed in our previous study. A cDNA fragment corresponding to the HC-Pro gene of PRSV 5-19 was amplified by RT-PCR from total RNA isolated from 5-19-infected non-transgenic papaya plants (Ultraspect M) using primers based on the PRSV 5-19 HC-Pro sequence (Table S1), and cloned into a PCR-TOPO vector (Invitrogen, San Diego, CA, USA) to generate p519(905-1008)/TA. The C-terminus part, along with the contiguous 3’-UTR, was amplified using the primers 5’-CTAGATATGTCCTTG-3’ and oligo-5’TGACAT-NT5 (5’-ATTGTGCTCGCCGCGCTTGTTC-3’), NotI underlined, and cloned into a PCR-TOPO vector to generate p519(905-1008)-TA. The Apal-NotI restriction fragment from p519(905-1008)/TA and the PstI-NotI restriction fragment from p519(1095-1100)/TA were ligated and cloned together into pBluescriptIIIMK+ SK+ to generate p519CP/SK+ carrying the complete CP coding region and contiguous 3’-UTR (CP-3’-UTR) of PRSV 5-19. The in vitro infectious PRSV YK recombinant cDNA clone pT3YK/SK+CPU, in which the PRSV YK CP coding region was replaced with that from PRSV 5-19, was constructed by replacing the Apal-NotI restriction fragment of pT3PYKFK1 with that from p519CP/SK+ (Fig. 1A). Another in vitro infectious PRSV YK recombinant cDNA clone, pT3YK/S19HCCP3U, in which both PRSV YK HC-Pro and CP-3’-UTR were replaced with the corresponding regions from PRSV 5-19, was also constructed (Fig. 1A).

Confirmation of recombinant viruses in infected plants. NotI-linearised constructs for the wild-type and recombinant viruses were transcribed in vitro, and the products were mechanically introduced into papaya seedlings (cv. Tainung No. 2) as described previously. The inoculated plants were maintained in a temperature-controlled (25 ± 3 °C) greenhouse to observe symptom development. Appropriate cDNAs amplified by RT-PCR using RNA extracted from virus-infected papaya plants were analysed by restriction-enzyme digestion and sequencing to confirm the identities of the recombinant viruses. cDNA containing the HC-Pro coding region was amplified using the primers H1K164/MK339, and analysed by XhoI digestion to specifically release the fragment corresponding to the 5’-19 HC-Pro gene. cDNA containing the CP coding region along with the 3’-UTR was amplified by RT-PCR using the YK905/YK10326 primer pair and then sequenced to verify its origin from PRSV 5-19.

Infectivity of recombinant viruses in transgenic papaya lines. Infectivity assays were performed with (i) the single-virus-resistant YK CP-3’UTR transgenic papaya lines 18-2-4, 18-0-9, 17-0-1 and 16-0-1, which carry a transgene corresponding to the PRSV YK CP coding sequence with the contiguous 3’-UTR (CP-3’-UTR), and (ii) the double-virus-resistant PY16 transgenic papaya lines 10-4, 12-4 and 14-1, which carry a chimeric transgene comprising parts of both PLDMV CP and PRSV YK CP-3’UTR. Both the single-virus-resistance to PRSV1 and the double-virus-resistance to PLDMV and PRSV1 are based on post transcriptional gene silencing (PTGS).

All transgenic lines were evaluated against the recombinant viruses YK/519HC, YK/519CP3U and YK/519HCCP3U (Fig. 1A), which were derived from the pT3YK/519HC, pT3YK/519CP3U and pT3YK/519HCCP3U constructs, respectively, by mechanical inoculation, with YK and 519 serving as controls. Inocula of the challenge viruses were prepared by extracting leaf tissues from virus-infected papaya plants
Hybridization was carried out in ULTRAHyb-Oligo solution (Ambion Inc., Austin, TX), and signals were detected by autoradiography. The Dynazyme® PreStain Marker (BioDynamics Laboratory Inc., Tokyo, Japan) for small RNA was used as the molecular weight standard. The mRNA/siRNA signals were quantified by Kodak 1D image analysis software (Eastman Kodak, Rochester, NY).

Cloning and sequencing of PRSV TH and MX HC-Pro coding sequences. Total RNA was isolated from papaya leaves infected with PRSV TH or PRSV MX 3 weeks after inoculation. The primer MYK3234 (5'-GTAAAAGTACGCTGTCGTGACATC-3'), which corresponds to nucleotide positions 3211-3235 in the PRSV YK RNA sequence, was used for first-strand cDNA synthesis by reverse transcription (RT).

The forward primer PPYK-TH (5'-ACAGGATGACGACATATTGATGAC-3') and the reverse primer PRSV-TH (5'-CTTGTGGAGAATATGCTTTGTCGT-3'), which corresponds to nucleotide positions 1607-1626 in the PRSV YK RNA sequence, was used with the reverse primer MYK3234 to PCR amplify the HC-Pro gene from each virus. RT-PCR-amplified PRSV TH and MX HC-Pro fragments were cloned into pCR-TOPO (Invitrogen, San Diego, CA, USA) and sequenced.

The nucleotide sequences of HC-Pro from PRSV TH and MX were compared with those from 5-19, YK3 and HA using PR/GENE 6.85 software (IntelliGenetics, Inc., Mountain View, CA, USA). Multiple sequence alignments were constructed using the PILEUP program of the GCG package, version 9.0 (Genetics Computer Group, Madison, WI, USA).

Construction of untranslatable PRSV 5-19 HC-Pro constructs. To construct untranslatable PRSV 5-19 HC-Pro coding sequence in binary vector, the forward primer 519HCSclop (5'-GCAGAATACCATGTTACGTCGTGACATC-3') and the reverse primer PRSV-519 (5'-TCTTGTGGAGAATATGCTTTGTCGT-3') were used to amplify the C-terminal fragment (750 bp) of the PRSV 5-19 HC-Pro gene. The amplified fragment was digested with XbaI/SacI and ligated into XbaI/SacI-digested pBI121 to generate the untranslatable construct pBI-519HCSclop.

In addition, the primers 519HSCOa (5'-GACAGAAATACCATGTTACGTCGTGACATC-3') and 519HSCOa (5'-TCTTGTGGAGAATATGCTTTGTCGT-3') were used to amplify the C-terminal fragment (750 bp) of the PRSV 5-19 HC-Pro gene. The amplified fragment was digested with XbaI/SacI and ligated into XbaI/SacI-digested pBI121 to generate the untranslatable construct pBI-519HSCOa.

Generation of HC-Pro transgenic papaya lines. Papaya transformation and regeneration were carried out as described previously4, with the required modifications. Embryogenic tissues derived from the adventitious roots of the PRSV-susceptible papaya cultivar Sunrise wounded in liquid-phase by carborundum were transferred to the Agrobacterium tumefaciens carrying pBI-519, pBI-519HC, pBI-519HCSaCB or pBI-519HCSaC, respectively, and then injected into the lower side of leaves using a syringe without a needle, and the infiltrated plants were kept in a temperature-controlled (25 ± 3 °C) greenhouse. The empty vector pBACa was used as a negative control. GFP fluorescence was monitored at 5 dpi using a handheld UV light-800AP (UVG, CA, Upland, USA) and photographed using a digital camera (D7000, Nikon, Tokyo, Japan) with a Nikon P series filter (Cokin, 84 mm, 524 nm, R-31). The fluorescence intensity and the variation of mean fluorescence intensity was performed (n = 6). The GFP fluorescence signals were quantified by Kodak 1D image analysis software (Eastman Kodak, Rochester, NY). The density of GFP fluorescence in each line was arbitrary set to 1.0 to compare the intensity in each line. The same half leaves infiltrated with 5-19 HC-Pro. The transient expression of recombinant proteins was monitored at 5 dpi via chemiluminescent western blotting (Amersham, Bucks, U.K.) using 5-19 HC-Pro antiserum (prepared as described above), GFP antiserum49, or WSMoV NSs MAb48 as the primary antibody and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Amersham, Bucks, U.K.) or horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham, Bucks, U.K.) as the secondary antibody.

The protein signals were quantified by Kodak 1D image analysis software (Eastman Kodak, Rochester, NY).

Evaluation of PRSV HC-Pro transgenic lines for resistance to 5-19 and YK. The viral resistance of the putative HC-Pro transgenic lines was evaluated by mechanical inoculation with PRSV 5-19 or PRSV YK using sixty micropropagated R0 plants (5 plants/line) at approximately the 15-cm stage. The first two fully expanded leaves were dusted with 600-mesh carborundum and rubbed with 200 μl of inoculum that had been prepared from papaya leaf tissues 3 weeks after inoculation with PRSV 5-19 or PRSV YK (1:20 w/v in 0.01 M potassium phosphate buffer, pH 7.0). Non-transgenic papaya plants (cv. Sunrise) and YK-CP3'-UTR transgenic papaya plants line 18-2-4 were used as controls. Plants were kept in a temperature-controlled greenhouse (25 ± 3 °C), and symptom development was monitored for 7 weeks.

Southern hybridisation. The DNeasy Plant Mini kit (QIAGEN Inc., Valencia, CA, USA) was used to extract genomic DNA from HC-Pro transgenic papaya plants. Thirty micrograms of genomic DNA was digested with Asel (two Asel sites are located at nt 4764 and 4823 in pBI121, prior to the 35S promoter) and the electrophoretically resolved profile (0.8% agarose gel) was transfected to a Gene Screen Plus nylon
membrane (Dupont Co., Boston, MA, USA). The Primer-It II random primer labelling kit (Stratagene, La Jolla, CA, USA) was used to prepare the 2'-3'-dATP-labeled HC-Pro probe from 25 ng of DNA (1371 bp) amplified from pBl-S19HC by using the primer pair S19HCStApA/S19HCSacB. Southern hybridisation and autoradiography were performed as described previously[20].

Resistance of HC-Pro lines to different geographic PRSV strains. Plants from four highly resistant transgenic lines that contained a single transgene insert, as identified by Southern blotting, were tested against geographically distinct PRSV strains from Hawaii (HA)[21], Thailand (TH)[1], and Mexico (MX)[11] by mechanical inoculation, as described above.

Analysis of transgene segregation in R1 progeny. R0 plants from two highly resistant 5-19 HC-Pro transgenic papaya lines, F2-1-4 and F3-2-2, were self-crossed under greenhouse conditions, and the R1 premature seeds were collected at 90 days after pollination to analyse the inheritance of the transgene. To examine the pattern of inheritance of the nphl gene, the R0, premature seeds of F3-2-2 were cultured on MSNB medium containing 100 mg/l kanamycin for two weeks, until plantlets developed. The kanamycin-resistant R1 progeny were further established and challenged by mechanical inoculation with PRSV 5-19, as described above, to ascertain the inheritance of the transgenic resistance.

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Author contributions
Y.J.K, B.J.Y, and S.D.Y designed the experiments. Y.J.K, B.J.Y, K.C.C, C.H.H, H.J.B, C.F.Y, C.H.H, and C.P.C performed the experiments. Y.J.K, B.J.Y, J.A.J.R, and S.D.Y wrote the manuscript. All authors reviewed the manuscript.

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