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Cross protection against the watermelon strain of *Papaya ringspot virus* through modification of viral RNA silencing suppressor

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ABSTRACT

Papaya ringspot virus watermelon strain (PRSV-W) causes huge economic losses to cucurbits production. Here, we constructed an infectious clone of PRSV-W, pCamPRSV-W, which can induce similar symptoms and accumulate to same levels as wild type virus in plants of *Cucurbita pepo*, *Cucumis melo*, *Citrullus lanatus* and *Cucumis sativus*. The green fluorescence protein gene *gfp* was cloned into pCamPRSV-W to produce pCamPRSV-W-GFP, which produced strong green fluorescence in systemic leaves of inoculated *Cucurbita pepo*, *Cucumis melo*, *Citrullus lanatus* and *Cucumis sativus* plants, indicating that pCamPRSV-W can be used to express foreign genes. Ten mutants of PRSV-W, obtained by site-directed mutagenesis in the RNA silencing suppressor helper-component proteinase encoding region, produced dramatically attenuated symptoms in plants of *Cucumis melo*. The *Cucumis melo* plants pre-infected with mutants K125D and G317 K showed effective protection against the challenge inoculation of wild type PRSV-W. The attenuated mutants generated in this study will be helpful for the eco-friendly control of PRSV-W.

1. Introduction

Cross-protection is a phenomenon that pre-infection with mild virus strains will protect the plants from the subsequent infections with more severe strains of the same or related viruses. This method has been successfully utilized for the control of many plant viruses, such as zucchini mosaic virus (ZYMV), tobacco mosaic virus (TMV), pepino mosaic virus (PepMV) and citrus tristeza virus (CTV) (Aguero et al., 2018; Lin et al., 2007; Müller and Costa, 1977; Rast, 1972). However, mild strains or attenuated mutants of plant viruses are scare, which has become a limiting factor for the application of cross protection. Site-directed mutagenesis in virus infectious clone is an effective way to solve this problem (Lin et al., 2007).

Mutations in viral RNA suppressors can attenuate the symptoms induced by plant viruses. For example, changes of amino acids in the 2b protein of cucumber mosaic virus (CMV) result in symptom attenuation and reduction in RNA silencing suppression activities (Dong et al., 2016; Nishiguchi and Kobayashi, 2011). Similarly, a substitution of cysteine (Cys) to tyrosine (Tyr) at the amino acid position 348 in the TMV 130-kDa protein produces an attenuated phenotype in infected plants and also a reduction in RNA silencing suppression activity of this protein (Nishiguchi and Kobayashi, 2011). Substitutions of amino acids in the helper-component proteinase (HC-Pro) of clover yellow vein virus (ClYVV) or ZYMV also result in symptom attenuation and reduction in RNA silencing suppression activities (Lin et al., 2007; Shiboleth et al., 2007).

Papaya ringspot virus (PRSV) is a widespread plant virus that causes severe losses to papaya and cucurbit production. PRSV belongs to the genus *Potyvirus* and is transmitted by aphids in a non-persistent manner. The genome of PRSV is single-stranded positive sense RNA and 10,300 nucleotides long. PRSV can be classified into two biotypes: the papaya type (PRSV-P) which infects both papaya and cucurbits, and the cucurbit type (PRSV-W) which infects cucurbits but not papaya (Gonsalves et al., 2010). PRSV-P is the most economically important virus affecting papaya cultivation. Crop losses due to PRSV-P typically range from 10% to 100% (Tripathi et al., 2008). PRSV-W is a major limiting factor in cucurbits production worldwide (Gonsalves et al., 2010). In this study, we construct an infectious clone of PRSV-W, modified it to an expression vector, screened ten attenuated mutants obtained by introducing site-directed mutations into the HC-Pro encoding region, and evaluated their efficiency of cross protection against PRSV-W in *Cucumis melo* plants.

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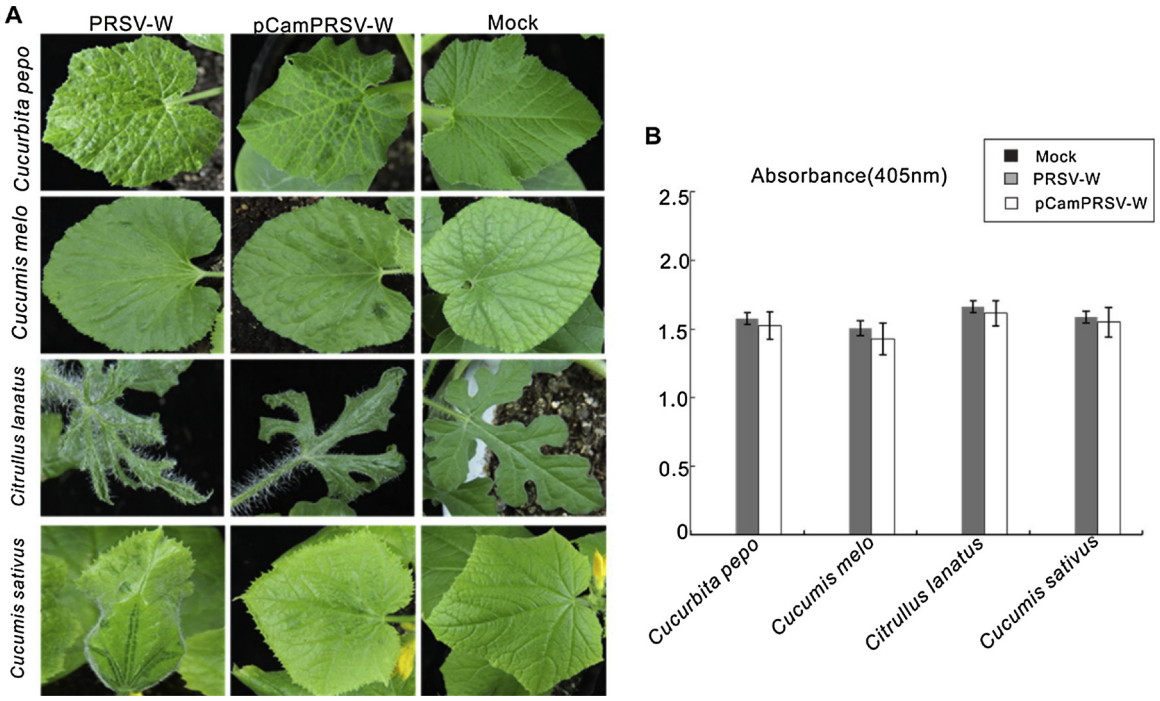

Fig. 1. Infectivity assay of PRSV-W in cucurbit plants.
A. Symptoms induced by wild type PRSV-W-SD and virus derived from pCamPRSV-W in systemically infected leaves of *Cucurbita pepo*, *Cucumis melo*, *Cucurbita pepo* and *Cucumis sativus* plants at 8 days post inoculation (dpi) or agro-infiltration (dpai). Mock plants were agro-infiltrated with an empty vector. B. The accumulation level of PRSV coat protein in systemically infected leaves determined by ELISA at 8 dpi.

2. Materials and methods

2.1. Plasmid construction

To construct an infectious clone of PRSV-W-SD, two fragments (1˜5396 bp and 5397˜10,337 bp) covering the full-length genomic sequence were PCR amplified from cDNA derived from total RNA of zucchini leaf showing mosaic symptom. The resulting PCR products were inserted into the binary vector pCambia0390 with CaMV 35S promoter by homologous recombination method (Li and Elledge, 2007). Site-directed mutagenesis was performed through PCR using specific primers as described previously (Liu and Naismith, 2008).

2.2. Plant growth and virus inoculation

Cucurbits plants were cultivated in a greenhouse at 25 ℃ with 16-h light and 8-h dark cycles. Plasmids pCamPRSV, pCamPRSV-W-GFP or its derivatives were introduced individually into *Agrobacterium* strain GV3101 by freezing-thawing method (Höfgen and Willmitzer, 1988). The transformed *Agrobacterium* cultures were grown overnight in Luria–Bertani culture medium containing 50 mg/mL kanamycin and 50 mg/mL rifampicin, followed by 3 h of incubation in an induction buffer [10 mM $MgCl_2$, 150 μM acetosyringone and 10 mM 2-(N-morpholino) ethanesulfonic acid (MES, pH = 5.6–6.3)] at room temperature. Individual *Agrobacterium* cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.5, and then infiltrated individually into cotyledons of cucurbits plants using needleless syringes.

2.3. Western blotting

Total proteins from the systemic leaves of *Cucumis melo* were extracted using an extraction buffer containing 25 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10% (v/v) glycerol, 2% (w/v) polyvinylpolypyrrolidone, 0.15% (v/v) Nonidet P-40, and protease inhibitor cocktail (Roche). The crude leaf extracts were centrifuged at 20,000 *g* for 15 min, and the supernatant was subject to 10% (w/v) sodium dodecylsulfatepolyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose membranes. The membranes were probed with antibody to PRSV CP (Huang et al., 2017). The detection signal was visualized using Pierce ECL western blotting substrates (Thermo Fisher Scientific, Rockford, IL, USA)

2.4. ELISA

As the method previously described (Gao et al., 2012), the cucurbit plant leaves were homogenized with 1:8 coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) in a tissue grinder. Aliquots of 100 μL protein crude extracts were added into a 96-well ELISA plate and incubated overnight at 4 ˚C. The plate was washed with PBST for 4 times. Antibodies against PRSV CP as a primary antibody (1:1000 dilution) were added into plates and incubated at 37 ˚C for 4 h. The plate was washed with PBST for 4 times. The mouse anti-rabbit IgG-conjugated alkaline phosphatase antibody (Sigma-Aldrich, Shanghai, China) as a secondary antibody was added into plates and incubated for 4 h at 37 ˚C. The plate was washed with PBST for 4 times. The p-Nitrophenyl phosphate (Sigma, 0.25 mg/mL) as a substrate for alkaline phosphatase (0.25 mg/mL) was added into plates and incubated at 37 ˚C. The plates were read by a multi-function microplate reader (BioTek Synergy™ Mx, Winooski, VT, USA). The original data was displayed in Fig. S1.

3. Results

3.1. Construction of infectious clone and expresion vector of PRSV-W

The full-length cDNA of PRSV-W genome was amplified from a zucchini sample collected from Jiyang, Shandong province, China (GenBank accession number: MF085000.1; Cheng et al., 2017) and ligated into a binary vector pCambia0390. The resultant infectious clone pCamPRSV-W was inoculated to plants of *Cucurbita pepo*, *Cucumis melo*,

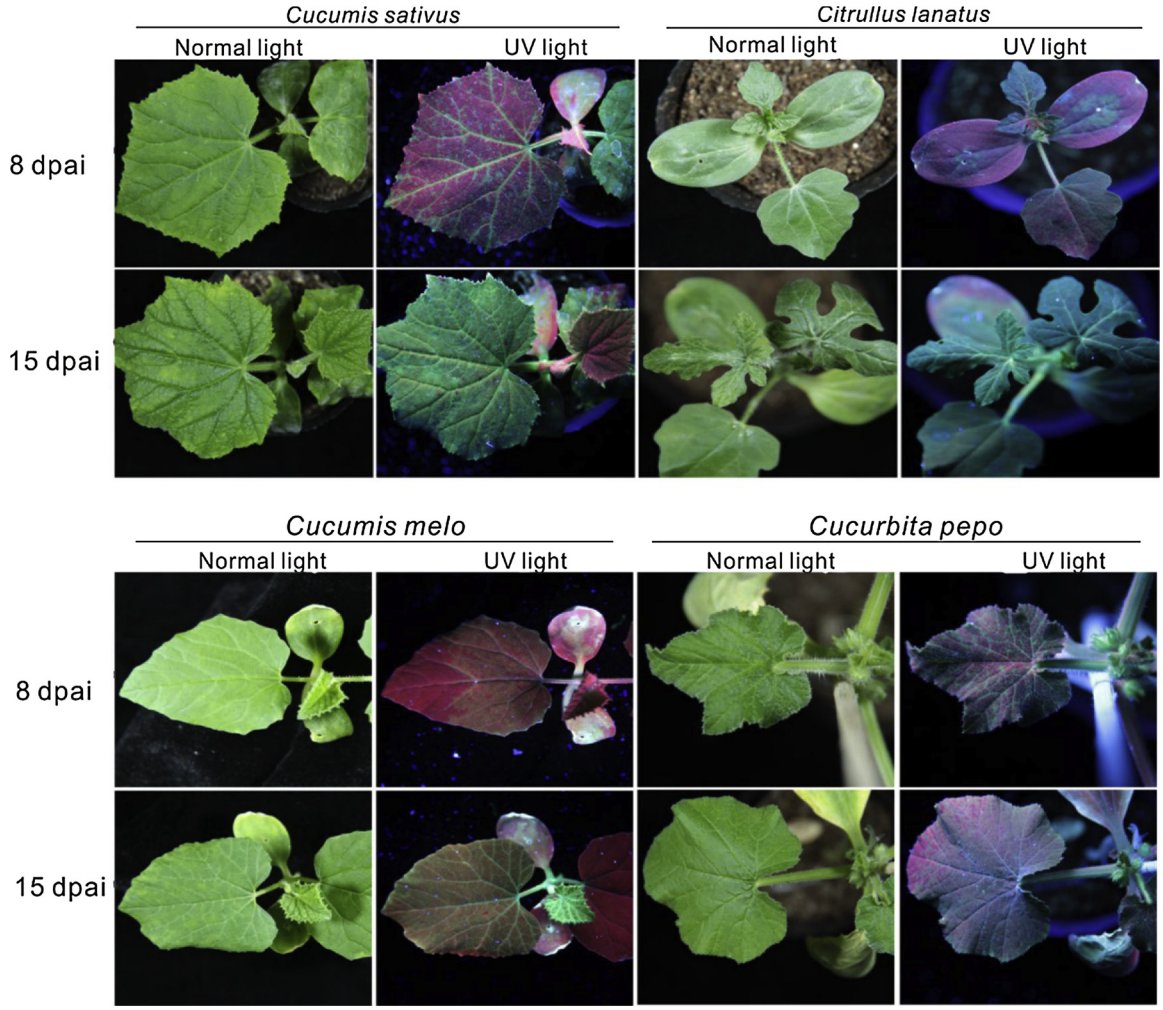

Fig. 2. Phenotypes of PRSV-W-GFP in plants of *Cucumis sativus*, *Citrullus lanatus*, *Cucumis melo* and *Cucurbit pepo* under normal light or UV illumination at 8 and 15 dpai.

Citrullus lanatus and *Cucumis sativus* by agro-infiltration. At eight days post agro-inoculation (dpai), distinct mosaic or chlorosis symptoms were observed in systemically infected leaves, which were similar to that on plants rub-inoculated with PRSV-W. No viral symptom was observed in mock plants agro-infiltrated with an empty vector (Fig. 1A). Results of Enzyme-linked immunosorbent assay (ELISA) showed that the viral coat protein accumulated to same levels in agro-infiltrated plants and rub-inoculated plants (Fig. 1B).

In order to evaluate the potential of using PRSV as an expression vector, a *gfp* gene was inserted into PRSV NIb- and CP-coding region to generate a GFP-expressing plasmid pCamPRSV-W-GFP (Fig. 2). In plants of *Cucumis sativus*, *Citrullus lanatus*p, *Cucumis melo* and *Cucurbita pepo* agro-infiltrated with the plasmid pCamPRSV-W-GFP, GFP fluorescence appeared in the leaf veins at eight dpai and expanded to the whole systemic leaves at 15 dpai (Fig. 2), indicating that pCamPRSV-W-GFP can effectively express foreign proteins like GFP.

3.2. Screening of PRSV attenuated mutants

Sequence alignment showed that there are several conserved motifs in potyviral HC-Pro and PRSV HC-Pro, such as a zinc-finger like motif $HXCX_8CX_{13}CX_4CX_2C$ in the N-terminus, an IDEKK motif in the middle region (Supplementary Figs. 1 and 2). Ten conserved amino acids distributed in different regions of HC-Pro were selected for further research (Fig. 3A). To obtain mild mutants of PRSV, site-directed mutations were introduced to the HC-Pro encoding region of pCamPRSV-W-GFP. The derived progeny mutant viruses will carry mutations in 10 conserved amino acids of HC-Pro. The mutant plasmids pgC26A, pgC57A, pgK125D, pgK126D, pgN137A, pgG317 K, pgP328A, pgN346A, pgL375A and pgV417A (the letter 'g' before a mutant name meant the plasmid carrying the *gfp* gene) were individually inoculated to the *Cucumis melo* plants. At 15 dpai, symptoms and GFP fluorescence induced by mutants were significantly attenuated than that induced by PRSV-W-GFP (Fig. 3A). As spontaneous mutations occur frequently during potyvirus replication in plants (Geng et al., 2015), we extracted the total RNAs of systemically mutants-infected *Cucumis melo* leaves at 15 dpai, and cloned the HC-Pro encoding region of the progeny virus amplified by reverse transcription polymerase chain reaction. The sequencing results showed that the introduced mutations were stable in the progeny virus of mutants (data not shown). Results of Western Blotting showed that the CP accumulation levels in mutants-infected *Cucumis melo* plants were significantly lower than that in *Cucumis melo* plants infected with PRSV-W-GFP (Fig. 3B).

3.3. Cross-protection effect of attenuated mutants in Cucumber melon plants

To evaluate the cross protection efficiency of these mutants, the same mutations were introduced to pCamPRSV which doesn't carry a *gfp* reporter gene. The mutant plasmids pC57A, pK125D, pG317 K and pP328A were agro-infiltrated to *Cucumis melo* plants individually. At 0, 3, 5, and 10 dpai, wild type PRSV-W-GFP was rub-inoculated to

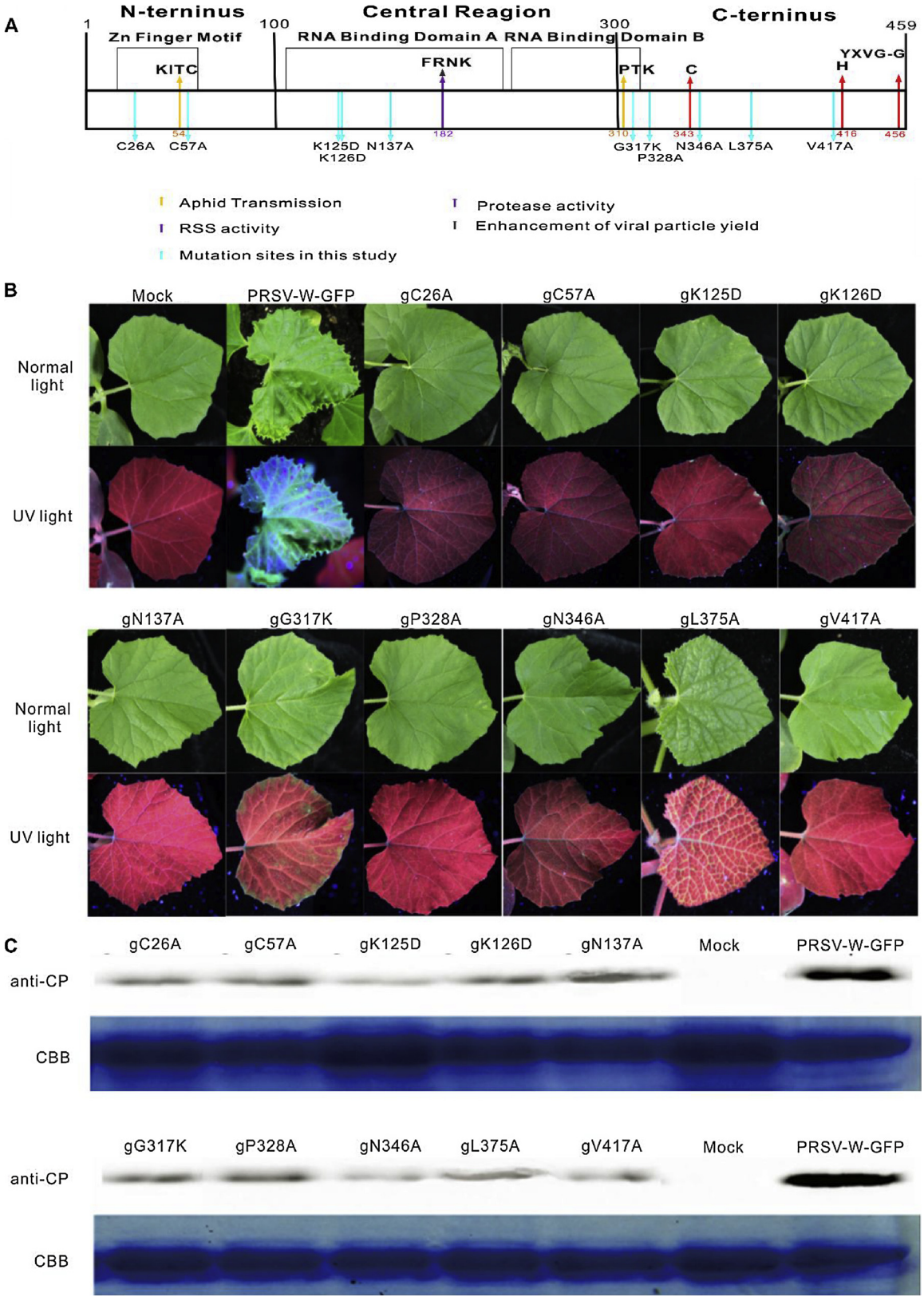

Fig. 3. Infectivity and accumulation levels of PRSV-W-GFP mutants in *Cucumis melo* plants.
A. Schematic representations of PRSV-W HC-Pro The location and function of domains, motifs key amino acids and mutation sites in this research were marked. B. Symptoms of wild-type and mutant PRSV-W-GFP in *Cucumis melo* plants at 15 dpai. C. The coat protein accumulation levels of wild-type and mutant PRSV-W-GFP in *Cucumis melo* plants at 15 dpai. Mock plants were agro-infiltrated with an empty vector.

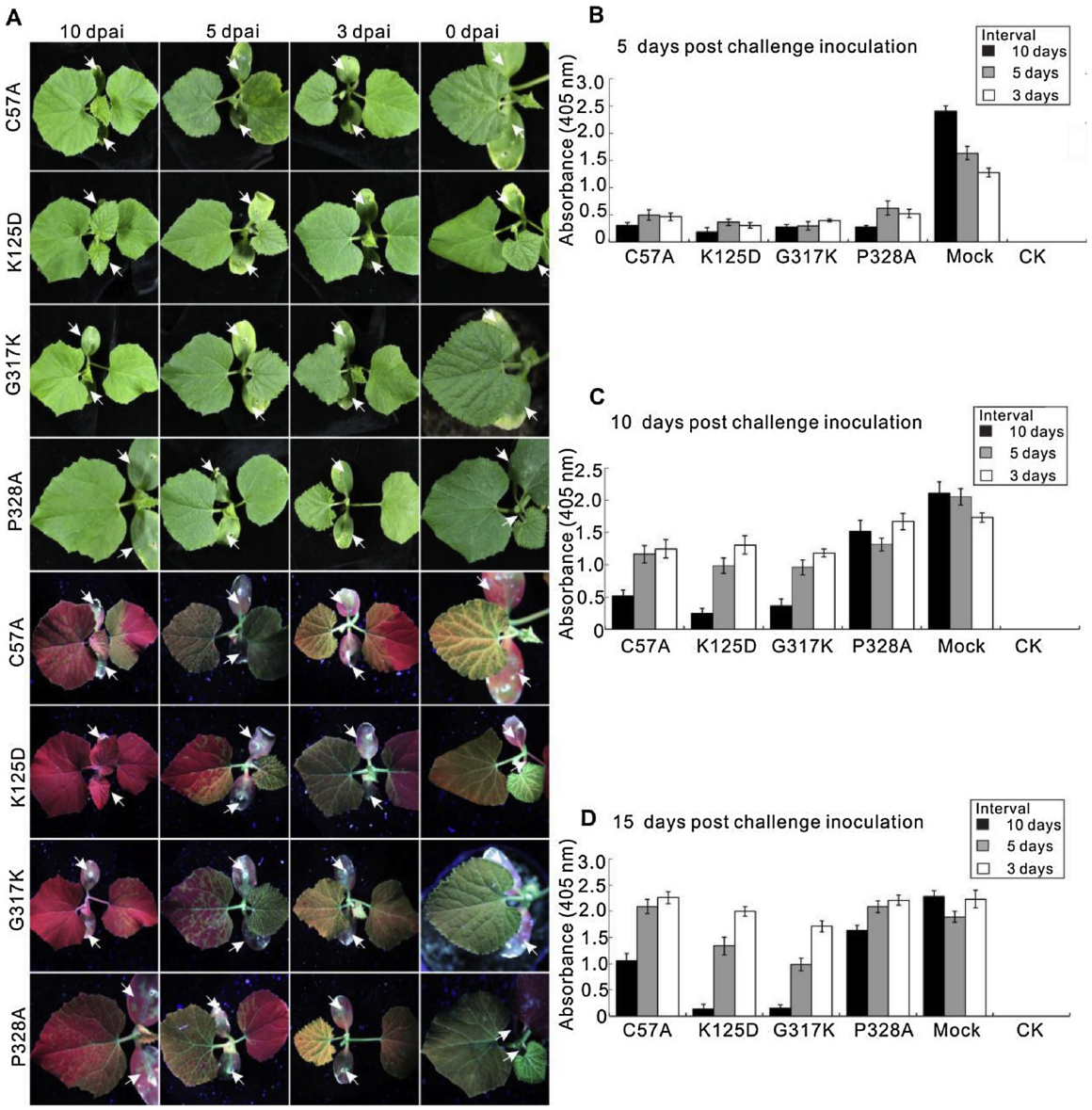

Fig. 4. Cross protection effect of four attenuated PRSV mutants.
A. Symptoms in *Cucumis melo* plants challenged with PRSV-W-GFP at 10 dpi with interval of 0, 3, 5 or 10 dpai. Arrows indicated the inoculated cotyledons. B, C and D. The coat protein accumulation levels of PRSV-W-GFP detected by ELISA at 5, 10 and 15 days post challenge inoculation.

systemic leaves of mutants-infected *Cucumis melo* plants. At ten days post inoculation (dpi), mosaic symptoms and strong GFP fluorescence appeared in non-protected control *Cucumis melo* plants and those *Cucumis melo* plants chanllenged at an interval of 3 days. With interval of 5 days, the protected *Cucumis melo* plants only displayed slight mosaic symptoms and week GFP fluorescence. When the interval was 10 days, no obvious virus symptom was observed in *Cucumis melo* plants; under UV light, only slight GFP fluorescence was observed in *Cucumis melo* plants pre-infected G317 K, no GFP fluorescence was seen in *Cucumis melo* plants protected with mild mutant K125D (Fig. 4A). The ELISA results at 5, 10 and 15 days post challenge inoculation were consistent with the symptoms and GFP fluorescence (Fig. 4B). All these results demonstrated that PRSV attenuated mutant C57A, K125D, G317 K, P328A can provide cross protection against PRSV-W when the interval was 5 days, while mutants K125D and G317 K show complete cross-protection effect on the challenge of wild type virus when the interval was 10 days.

4. Discussion

PRSV causes huge economic damage in papaya and cucurbit production. Here, we constructed a PRSV-W infectious clone based on an isolate from Shandong Province, China and then constructed a *gfp* reporter gene-expressing vector. Both the infectious clone pCamPRSV and vector pCamPRSV-W-GFP can be inoculated by agro-infiltration, which is a convenient, fast and low-cost method for PRSV-W inoculation. This infectious clone and *gfp* gene expression vector are powerful tools to dissect the molecular mechanism underlying the pathogenesis of PRSV-W. Yap et al. (2009) constructed a PRSV-W infectious clone and used it to inoculate zucchini seeds through bombardment. After 42 dpi, 69% of zucchini plants showed PRSV-W infection symptom. The new clone pCamPRSV-W can be inoculated to cotyledons by agro-infiltration and distinct symptoms appeared at 8 dpai.

In this paper, we selected the potyviral RNA silencing suppressor HC-Pro to perform site-direct mutagenesis. Differing from the previous studies on potyviruses (Lin et al., 2007), we were focusing on some new amino acid motifs in HC-Pro. The amino acid C57 belongs to the zinc-

finger like motif $HXCX_8CX_{13}CX_4CX_2C$ in the N-terminus of HC-Pro, which was reported to be responsible for self-interaction of HC-Pro (Guo et al., 1999). We speculated that the mutation of C57 to A impaired the self-interaction of HC-Pro and reduced virus titre. The amino acids G317 and P328 located in the C terminus of HC-Pro, while amino acid K125 is located in a conserved IDEKK motif in the middle region of HC-Pro. The function of these amino acids is unknown, but our results showed that these amino acids are important to the virulence of PRSV-W. The results of this paper will be insightful to screen mild strains of other potyviruses, which is the largest plant RNA virus group. The mutant K125D has the best cross protective effects among the attenuated mutants tested (Fig. 4). With interval of 10 days, K 125D showed cross protection efficiency of 100% against severe infection of PRSV-W-GFP. The conserved DAG triplet in potyviral CP and the KITC and PTK motifs of potyviral HC-Pro are responsible for the aphid transmission of potyviruses (Atreya et al., 1992, 1990; Gal-On et al., 1992; Granier et al., 1993; Harrison and Robinson, 1988). Mutations abolishing aphid transmissibility of PRSV-W will prevent the mild strains from being transmitted to non-target plants and thus increase bio-safety. In another experiment, the cross protection effects of a PRSV mutant could be maintained for 60 days in plants of *Cucurbit pepo* (data not shown). Furthermore, the infectious clone pCamPRSV can at least infect *Cucumis sativus*, *Citrullus lanatus*p, *Cucumis melo* and *Cucurbita pepo* plants. Therefore, the mild mutant K125D has great potential in PRSV-W control.

Taken together, we constructed a PRSV-W infectious clone that can infect plants of *Cucumis sativus*, *Citrullus lanatus*p, *Cucumis melo* and *Cucurbita pepo*, obtained ten mild strains among which K125D displayed the greatest potential in cross protection. Results of this study provide theoretical and practical guide for the control of PRSV-W and insightful hints to the control of other potyviruses via cross protection.

Conflict of interest

The authors declare that they have no conflict.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.03.016.

References

Aguero, J., Gomez-Aix, C., Sempere, R.N., Garcia-Villalba, J., Garcia-Nunez, J., Hernando, Y., Aranda, M.A., 2018. Stable and broad spectrum cross-protection against *Pepino mosaic virus* attained by mixed infection. Front. Plant Sci. 9, 1810.

Atreya, C.D., Raccah, B., Pirone, T.P., 1990. A point mutation in the coat protein abolishes aphid transmissibility of a potyvirus. Virology 178, 161–165.

Atreya, C.D., Atreya, P.L., Thornbury, D.W., Pirone, T.P., 1992. Site-directed mutations in the potyvirus HC-Pro gene affect helper component activity, virus accumulation, and symptom expression in infected tobacco plants. Virology 191, 106–111.

Cheng, D.J., Huang, X.D., Zhang, J.W., Tian, Y.P., Wang, X.Y., Li, X.D., 2017. First report of P*apaya ringspot virus* associated with a ringspot disease of Zucchini in Northern China. Plant Dis. 101, 847.

Dong, K., Wang, Y., Zhang, Z., Chai, L.X., Tong, X., Xu, J., Li, D., Wang, X.B., 2016. Two amino acids near the N-terminus of *Cucumber mosaic virus* 2b play critical roles in the suppression of RNA silencing and viral infectivity. Mol. Plant Pathol. 17, 173–183.

Gal-On, A., Antignus, Y., Rosner, A., Raccah, B., 1992. A zucchini yellow mosaic virus coat protein gene mutation restores aphid transmissibility but has no effect on multiplication. J. Gen. Virol. 73, 2183–2187.

Gao, R., Tian, Y.P., Wang, J., Yin, X., Li, X.D., Valkonen, J.P., 2012. Construction of an infectious cDNA clone and gene expression vector of *Tobacco vein banding mosaic virus* (genus *Potyvirus*). Virus Res. 169, 276–281.

Geng, C., Cong, Q.Q., Li, X.D., Mou, A.L., Gao, R., Liu, J.L., Tian, Y.P., 2015. Developmentally regulated plasma membrane protein of *Nicotiana benthamiana* contributes to potyvirus movement and transports to plasmodesmata via the early secretory pathway and the actomyosin system. Plant Physiol. 167, 394–410.

Gonsalves, D., Tripathi, S., Carr, J.B., Suzuki, J.Y., 2010. Papaya ringspot virus. Plant Health Instructor. Am. Phytopathol. Soc. https://doi.org/10.1094/PHI-I-2010-1004-01.

Granier, F., Durand-Tardif, M., Casse-Delbart, F., Lecoq, H., Robaglia, C., 1993. Mutations in zucchini yellow 2mosaic virus helper component protein associated with loss of aphid transmissibility. J. Gen. Virol. 74, 2737–2742.

Guo, D., Merits, A., Saarma, M., 1999. Self-association and mapping of interaction domains of helper component-proteinase of potato A potyvirus. J. Gen. Virol. 80 (Pt 5), 1127–1131.

Harrison, B.D., Robinson, D., 1988. Molecular variation in vector-borne plant viruses: epidemiological significance. Philos. Trans. R. Soc. Lond. B 321, 447–462.

Höfgen, R., Willmitzer, L., 1988. Storage of competent cells for Agrobacterium transformation. Nucleic Acids Res. 16, 9877.

Huang, X.D., Wang, Y., Tian, Y.P., Gu, Q.S., Li, X.D., 2017. Prokaryotic expression and antiserum preparation of *Papaya ringspot virus* -watermelon strain coat protein. Shandong Agric. Sci. 49, 86–89.

Li, M.Z., Elledge, S.J., 2007. Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. Nat. Methods 4, 251.

Lin, S.-S., Wu, H.-W., Jan, F.-J., Hou, R.F., Yeh, S.-D., 2007. Modifications of the helper component-protease of *Zucchini yellow mosaic virus* for generation of attenuated mutants for cross protection against severe infection. Phytopathology 97, 287–296.

Liu, H., Naismith, J.H., 2008. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. BMC Biotechnol. 8, 91.

Müller, G., Costa, A., 1977. Tristeza control in Brazil by preimmunization with mild strains. Proc. Int. Soc. Citric 868–872.

Nishiguchi, M., Kobayashi, K., 2011. Attenuated plant viruses: preventing virus diseases and understanding the molecular mechanism. J. Gen. Plant Pathol. 77, 221–229.

Rast, A.T.B., 1972. M II-16, an artificial symptomless mutant of tobacco mosaic virus for seedling inoculation of tomato crops. Netherlands J. Plant Pathol. 78, 110–112.

Shiboleth, Y.M., Haronsky, E., Leibman, D., Arazi, T., Wassenegger, M., Whitham, S.A., Gaba, V., Gal-On, A., 2007. The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is required for small RNA binding and mediates symptom development. J. Virol. 81, 13135–13148.

Tripathi, S., Suzuki, J.Y., Ferreira, S.A., Gonsalves, D., 2008. *Papaya ringspot virus*-P: characteristics, pathogenicity, sequence variability and control. Mol. Plant Pathol. 9, 269–280.

Yap, Y.K., Duangjit, J., Panyim, S., 2009. N-terminal of *Papaya ringspot virus type*-W (PRSV-W) helper component proteinase (HC-Pro) is essential for PRSV systemic infection in zucchini. Virus Genes 38, 461–467.