Modifications of the Helper Component-Protease of *Zucchini yellow mosaic virus* for Generation of Attenuated Mutants for Cross Protection Against Severe Infection

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ABSTRACT

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A nonpathogenic mild strain is essential for control of plant viruses by cross protection. Three amino acid changes, Arg₁₈₀→Ile₁₈₀ (GA mutation), Phe₂₀₅→Leu₂₀₅ (GB mutation), and Glu₃₉₆→Asn₃₉₆ (GC mutation), of the conserved motifs of the helper component-protease (HC-Pro) of a severe strain TW-TN3 of *Zucchini yellow mosaic virus* (ZYMV), a member of the genus *Potyvirus*, were generated from an infectious cDNA clone that carried a green fluorescent protein reporter. The infectivity of individual mutants containing single, double, or triple mutations was assayed on local and systemic hosts. On *Chenopodium quinoa* plants, the GB mutant

induced necrotic lesions; the GA, GC, and GBC mutants induced chlorotic spots; and the GAB and GAC mutants induced local infection only visualized by fluorescence microscopy. On squash plants, the GA, GB, GC, and GBC mutants caused milder mosaic; the GAC mutant induced slight leaf mottling followed by recovering; and the GAB mutant did not induce conspicuous symptoms. Also, the GAC mutant, but not the GAB mutant, conferred complete cross protection against the parental virus carrying a mite allergen as a reporter. When tested on transgene-silenced transgenic squash, the ability of posttranscriptional gene silencing suppression of the mutated HC-Pro of GAC was not significantly affected. We concluded that the mutations of the HC-Pro of ZYMV reduce the degrees of pathogenicity on squash and also abolish the ability for eliciting the hypersensitive reaction on *C. quinoa*, and that the mutant GAC is a useful mild strain for cross protection.

Cross protection is the protection conferred on a host by infection with one strain of a virus that prevents infection by a closely related strain of that virus (26). This approach has been used for the control of viral diseases, such as *Tobacco mosaic virus* (TMV) (34), *Citrus tristeza virus* (CTV) (27), and *Papaya ringspot virus* (PRSV) (47). A naturally occurring mild strain of *Zucchini yellow mosaic virus* (ZYMV) obtained from France (21), designated ZYMV WK, has been characterized and used for control of ZYMV in cucurbits by cross protection under greenhouse and field conditions in south-eastern France (21), Taiwan (45), Hawaii (8), United Kingdom (44), Turkey (48), Israel (41), California (31), and Italy (10).

Cross protection is highly strain- or isolate-specific, and the specificity is probably determined by the genetic relatedness of the challenge viruses (28,29,33,39). In Taiwan, most ZYMV isolates are grouped in the same genotype and are different from ZYMV isolates reported from the rest of the world (23,24). When Wang et al. (45) tested the effectiveness of cross protection provided by the French mild strain ZYMV WK against four severe strains originating from France, Connecticut, Florida, and Taiwan in cucumber, melon, and squash, they found that ZYMV WK is more effective against the severe strain from France than the others. Therefore, the generation of an attenuated strain from a local prevalent severe strain is an essential step to avoid the problem of strain-specific cross protection at a particular area.

Studies using recombinant viruses between the mild strain of ZYMV WK and the severe strain ZYMV-NAT (12) followed by site-directed mutagenesis revealed that the amino acid substitution of Ile for Arg at amino acid position 180 of the helper component-protease (HC-Pro), in the conserved FRNK motif, is responsible for the attenuation of the recombinant ZYMV-AG that causes a dramatic symptom change from severe mosaic to mild mottling in squash plants (11). On the other hand, our previous recombination analyses between the severe Hawaii strain of (PRSV-HA) (46), and its nitrous acid-induced mild strain HA5-1 (47), which has been widely used for control of PRSV by cross protection (47), indicated that three amino acid changes in the P1 gene (Ile₃₀₉→Thr, Glu₃₅₁→Lys, and Lys₄₈₁→Gln) and the two amino acid changes (Phe₂₀₅→Leu and Asp₃₉₆→Asn) in the HC-Pro gene are critical for the attenuated symptoms induced by HA5-1 (7). Therefore, mutations in the HC-Pro gene apparently play important roles in the attenuation of potyviral pathogenicity.

The HC-Pro protein is the second gene product processed from the potyviral polyprotein by the *cis* cleavage of the P1 protein at the N terminus (35,42) and by autocatalytic protease activity of the HC-Pro protein at its C terminus (5). The HC-Pro gene mediates aphid transmission (32), exhibits viral cysteine-type protease activity (5), maintains genome amplification (18,20), is involved in cell-to-cell movement and long-distance virus transportation (9,36), enhances replication of an RNA virus that elicits a synergistic disease (40), suppresses virus-induced gene silencing (19), and inhibits miRNA-guided cleavage of target mRNAs (6). In addition, the HC-Pro gene has also been shown to be involved in alterations of symptom expression (2,7,9).

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In previous studies, infectious cDNA clones of a severe strain of ZYMV from Taiwan, TW-TN3, was constructed (25). That work provides a solid basis for molecular modification of the virus by mutagenesis of the HC-Pro gene in order to obtain mild strains for control of ZYMV by cross protection in Taiwan or elsewhere in Eastern Asia.

In this investigation, we created Taiwan mild strains of ZYMV for cross protection by mutating three conserved amino acids, Arg₁₈₀, Leu₂₀₅, and Glu₃₉₆, in the HC-Pro gene of a local severe strain of ZYMV, TW-TN3. A novel method to monitor the dynamic changes in accumulation of the protective strain and challenge strain, using two different reporters, during different stages of cross protection was also developed. The concentrations of the protective strains during different stages of the induction for cross protection were monitored. Furthermore, the gene-silencing suppression ability of the mutated strain on transgene-silenced transgenic squash plants was also investigated. Our results indicated that the mutant GAC containing two mutations at Arg₁₈₀ and Glu₃₉₆ is potentially a useful protective strain for control of ZYMV by cross protection, for it replicated in squash plants with high concentration, induced mild symptoms with recovering, and conferred squash plants complete protection against ZYMV severe strain of Taiwan.

MATERIALS AND METHODS

Multiple alignment of HC-Pro sequences of severe and mild strains of potyviruses. Amino acid sequences of HC-Pro proteins of severe and mild strains of potyviruses were compared to generate the amino acid changes on HC-Pro protein for attenuation. The severe strains of potyviruses compared included *Soybean mosaic virus* (SBMV; GenBank accession no. S42280), *Pepper mottle virus* (PepMoV; M96425), *Potato virus Y* (PVY; X12456), *Turnip mosaic virus* (TuMV; D10972), *Tobacco vein mottling virus* (TVMV; X04083), *Plum pox virus* (PPV; AJ243957), *Tobacco etch virus* (TEV; M15239), *Pea seed-borne mosaic virus* (PSBMV; D10930), and *Zucchini yellow mosaic virus* Taiwan strain (ZYMV TW-TN3, AF343979). The mild strains of potyviruses compared included PRSV HA5-1, a mild mutant of PRSV derived from the severe Hawaii strain (PRSV HA, S46722) (47), and ZYMV WK (X77756), a mild strain from France (21). Multiple amino acid sequences of HC-Pro proteins were aligned using the PILEUP program of the GCG package (version 10.0, 1999, Genetics Computer Group, Madison, WI).

Construction of mutations in the HC-Pro coding region using the in vivo infectious clone of ZYMV TW-TN3. To examine the involvement of the conserved amino acids in symptom severity, single, double, and triple mutations were conducted to change Arg₁₈₀, Phe₂₀₅, and Glu₃₉₆ in the HC-Pro coding region of TW-TN3 to Ile, Leu, and Asn, respectively, using the previously constructed p35SZYMVGFPHis3 that contains the full-length cDNA of TW-TN3 with a *Cauliflower mosaic virus* (CaMV) 35S promoter and a reporter green fluorescent protein (GFP) gene between the P1 and the HC-Pro genes (15).

The previously constructed p35Z5E2724GFP that contains a 2.7-kb fragment of the 5' terminal region of ZYMV TW-TN3 with the GFP gene inserted between the P1 and HC-Pro genes (15) was used as a mutagenesis shuttle vector. *Hind*III-digested fragment from p35Z5E2724 was inserted in pBluescript II SK(–) to create pSKHC1.6-3 that contained the entire HC-Pro coding region fragment (nucleotides 1120 to 2723). Site-directed mutagenesis was performed using the GeneEditor Kit (Promega, Madison, WI) with single-stranded DNA template of the pSKHC1.6-3 and three primers that introduced an amino acid change coupled with nucleotide changes that introduced a restriction enzyme site without creating additional amino acid changes. The primer PZHC1578M1, 5'-TGACTGGTGAAGAGGCCTTGAAGATG-TTCATAACAAGCGCTCT-3', changed the amino acid Arg₁₈₀

to Ile₁₈₀ (underlined) and two nucleotides to G and C to generate the unique *Sma*I restriction site (AGGCTT) (in bold) in which the corresponding amino acid remained the same. The primer PZHC1666M1, 5'-GGACAAGAATGGAAACCTGGTCTGGG-GGGAAAG-3', changed the amino acid Phe₂₀₅ to Leu₂₀₅ (underlined) and two nucleotides to C and one to G to generate the unique *Sac*II restriction site (CCTGG) (in bold). The primer PZHC2240M1, 5'-GGTGTATTCCATCCTAACACGCGTTG-TGCTGAATTACC-3', changed the amino acid Glu₃₉₆ to Asn₃₉₆ (underlined) and two nucleotides to G and T to generate the unique *Mlu*I restriction site (ACGCGT) (in bold). These mutated pSKHC1.6-3 clones were digested with *Hind*III and then introduced back to p35SZ5E2724. The plasmids containing the mutated HC-Pro region were double-digested by *Nhe*I/*Nde*I and the resulting fragments were used to replace the wild-type HC-Pro region of the p35SZYMVGFPHis3 to generate recombinant viruses.

Plant inoculation and host reactions. All mutated full-length cDNA clones were purified by alkaline lysis miniprep (38). Aliquots of 20 µl, containing 1 µg of DNA in sterilized water, were mechanically applied onto carborundum-dusted leaves of *Chenopodium quinoa* Willd. with a sterilized glass spatula. At 7 days postinoculation (dpi), single lesions were isolated and mechanically transferred to individual plants of the systemic host squash (*Cucurbita pepo* L. var. Zucchini). Inoculated plants were kept in a temperature-controlled greenhouse (23 to 28°C) for observation of symptom development.

Greenhouse-grown zucchini squash plants were inoculated at the cotyledon stage. ZYMV TW-TN3 (a Taiwan severe strain) (23), ZYMV-DerP5 (a ZYMV vector expressing Der p 5 protein) (15), and ZYMV WK mild strain (21) were used for comparison. Inocula were prepared from 0.5 g of leaves of infected squash with 2 ml of 0.01 M potassium phosphate buffer (pH 7.0) and mechanically applied onto two cotyledons dusted with carborundum.

GFP activity in local lesions monitored by fluorescence microscopy. Fluorescence in local lesions developed on *C. quinoa* plants after inoculation with different mutated viruses was monitored with a Nikon Labophot microscope equipped with a super-high-pressure mercury lamp as a source for blue light (Nikon HB 10101AF), an HFX-IIA epifluorescence attachment, and an FITC filter set XF23 (Omega Optical, Brattleboro, VT) containing a 488DF22 excitation filter, a 505DRLP02 dichroic filter, and a 535DF35 barrier filter. Photographs were taken with Kodak Ektachrome 100 ASA slide film. Images were scanned from slides with a Nikon scanner (LS 1000) and graphically arranged using Adobe Photoshop 7.0 software (Adobe Systems Inc., Mountainview, CA).

Enzyme-linked immunosorbent assay. A total of nine leaf disks (0.5 cm in diameter), punched from three different upper leaves (the first fully expanded leaf plus the next upper and the lower ones, three for each leaf), of each squash plant infected with individual viruses were taken at 5, 10, 15, 20, and 25 dpi. The disks from each plant were combined as one sample (with a repeat of three plants) and assayed by indirect enzyme-linked immunosorbent assay (ELISA) using the antiserum to ZYMV coat protein (CP) (22), Der p 5 (15), and GFP (15). Results were recorded by measuring absorbance at 405 nm with an ELISA reader (ELx800, Bio-Tek Instruments, Inc., Winooski, VT).

Confirmation of mutants from infected plants. To ascertain the presence of the mutation in the viral RNA, total mRNA was extracted from infected leaf tissues using the procedure described by Verwoerd et al. (43). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described by Bateson et al. (4). The N-terminal region of the HC-Pro gene of ZYMV was amplified using an antisense primer MZ2263, 5'-ATCGTG-TTTCAGGATGGAATACACC-3' (nucleotides 2239 to 2263, numbered from 5' end of the ZYMV TW-TN3 genome) and a sense primer PZ1328, 5'-ATTTCAAGGAGGGCTCTCAGTACG-

3' (nucleotides 1328 to 1351). The predicted amplified fragment covering the A and B mutation sites was digested by *Stu*I or *Sac*FI for verification. Another region covering the B and C mutation sites was amplified with an antisense primer MZ2339, 5'-GT-TAATGATCCATATGAATCAATGACATGC-3' (nucleotides 2310 to 2339) and a sense primer PZ1561, 5'-AGGAACCATG-CACCTGACTG-3' (nucleotides 1561 to 1582) and digested by *Sac*FI or *Mlu*I to confirm the mutations.

Cross-protection tests. The attenuated ZYMV mutants GAB (containing double mutations of A and B) and GAC (containing double mutations of A and C) did not cause conspicuous symptoms on squash. Their ability to protect the squash plants against challenge with the severe TW-TN3 or ZYMV-Derp5 strains was studied under temperature-controlled (25 to 28°C) greenhouse conditions.

Squash seedlings at the cotyledon stage were mechanically inoculated with ZYMV-GAC prepared from 0.5 g of infected squash leaves, using 2 ml of 0.01 M potassium phosphate buffer, pH 7.2. Since GAB mutant did not accumulate significantly in squash plants, the GAB inoculum for the protective inoculation was prepared from 0.5 g of the leaves of infected *C. quinoa* plants at 4 dpi. At 5, 10, and 15 days after the protective inoculation, the first fully expanded leaves of the plants were mechanically challenged with ZYMV TW-TN3 or ZYMV-Derp5, prepared from 0.5 g of infected squash leaves using 2 ml of 0.01 M potassium phosphate buffer (pH 7.2). Protection was evaluated by symptom development on the plants. The presence of the challenging virus was monitored by indirect ELISA at 15 days after the challenge inoculation as described above.

Analysis of GAC mutants for gene-silencing-suppression ability. The transgenic line *Squash mosaic virus* (SqMV)-127 carrying the CP gene of SqMV, a posttranscriptional gene

silencing (PTGS)-mediated silencer (16,30), was used to assay the gene-silencing-suppression ability of GAC mutant. The plants of the transgenic silencer were separately inoculated with TW-TN3 or GAC mutant. Three leaf disks from three leaves (the fully expanded leaf plus the upper and lower ones) of each virus-infected squash plant (a total of four plants) were taken at 7 dpi, and assayed by western blotting to monitor the expression of the silenced SqMV CP gene. The antiserum against SqMV virions (16) and the antiserum to ZYMV HC-Pro, which was prepared by rabbit immunization with ZYMV HC-Pro protein purified by Ni-NTA column from ZYMV-infected squash plants (17), were used for the immunoblot analysis at a 1:3,000 dilution.

RESULTS

Amino acid modifications of the conserved residues of TW-TN3 HC-Pro for symptom attenuation. Sequence comparison of the HC-Pro coding regions of the two mild viruses, ZYMV WK (12,21), PRSV HA5-1 (47), and 10 severe potyviruses are summarized in Figure 1A. The amino acid residues Arg₁₈₀, Phe₂₀₅, and Glu₃₉₆ or Asp₃₉₆ are conserved for all severe viruses. However, Ile₁₈₀ was found present for the mild strain ZYMV WK (11), and Leu₂₀₅ and Asn₃₉₆ present for the mild strain PRSV HA5-1 (7). Thus, the changes of Arg₁₈₀→Ile, Phe₂₀₅→Leu, or Asp₃₉₆→Asn in the three highly conserved residues of HC-Pro may result in attenuation of a potyvirus. Accordingly, site-directed mutagenesis was conducted to mutate these three amino acids of the HC-Pro of Taiwan severe strain ZYMV TW-TN3 and the mutants were assessed for symptom attenuation.

The Arg₁₈₀→Ile substitution was located at the FRNK motif of ZYMV TW-TN3 and the mutation was designated as site A.

Fig. 1. Amino acid differences within the helper component-protease (HC-Pro) gene among potyviruses and the schematic representations of the constructs of *Zucchini yellow mosaic virus* (ZYMV) infectious clones used for this investigation. **A**, Sequence alignment of three conserved motifs in the HC-Pro genes of two mild and 10 severe potyviruses. Black boxes represent amino acid identity and gray boxes indicate amino acids with similar properties. White boxes represent significant amino acid changes. An asterisk (*) indicates the critical amino acid differences in the HC-Pro of the mild strains with reference to the severe strain of ZYMV Taiwan strain (ZYMV TW-TN3, GenBank accession no. AF343979). *Papaya ringspot virus* (PRSV) HA5-1 (7), a mild mutant of PRSV derived from the severe Hawaii strain (PRSV HA, S46722); ZYMV WK (X77756), a mild strain from France (21); SBMV, *Soybean mosaic virus* (S42280); PepMoV, *Pepper mottle virus* (M96425); PVY, *Potato virus Y* (X12456); TuMV, *Turnip mosaic virus* (D10972); TVMV, *Tobacco vein mottling virus* (X04083); PPV, *Plum pox virus* (AJ243957); TEV, *Tobacco etch virus* (M15239); PSBMV, *Pea seed-borne mosaic virus* (D10930). **B**, Schematic representation of seven mutants of ZYMV constructed from the in vivo infectious clones (p35SZYMVGFPPhis3) of a Taiwan severe strain TW-TN3, which is driven by a *Cauliflower mosaic virus* (CaMV) 35S promoter and carries a green fluorescent protein (GFP) reporter gene (15). Three amino acids were selected for mutation: positions A, B, and C represent Arg₁₈₀, Phe₂₀₅, and Glu₃₉₆ of the HC-Pro gene of TW-TN3, respectively. An asterisk symbol (*) indicates the positions of amino acid changes by site-directed mutagenesis. Mutated amino acids are indicated below. The corresponding viruses derived from the wild and mutated infectious clones are indicated. **C**, The severe strain ZYMV-Derp5 carrying dust mite allergen Der p 5 was constructed from p35SZYMV-Derp5 infectious clone (15).

Moreover, Phe₂₀₅→Leu and Glu₃₉₆→Asn mutations were designated as sites B and C, respectively, because of their positions in the HC-Pro gene (Fig. 1B).

To examine the involvement of these amino acids in symptom development, individual cDNA fragments containing the mutated amino acids were used to replace the corresponding fragments in the infectious ZYMV TW-TN3 cDNA clone p35SZYMVGFPHis3 (15) that carried a GFP reporter and multiple cloning sites at the N-terminal region of the HC-Pro gene (Fig. 1B). A total of seven infectious clones containing single (A, B, and C), double (AB, BC, and AC), and triple (ABC) mutations were constructed (Fig. 1B).

Characterization of lesion formation by the mutant viruses on *C. quinoa* plants. All mutated clones were mechanically introduced to *C. quinoa* plants. The virus mutant GB derived from p35SZYMVGAB (Fig. 1B) induced local lesions on leaves of *C. quinoa* plants within 7 dpi similar to those produced by the wild-type virus TW-TN3 (Fig. 2A) (Table 1). The virus mutants GA, GC, and GBC, derived from p35SZYMVGA, p35SZYMVGC, and p35SZYMVGBC, respectively (Fig. 1B), caused chlorotic spots on leaves of *C. quinoa* plants, which were different from the necrotic lesions produced by TW-TN3 (Fig. 2A; Table 1). The virus mutants GAB, GAC, and GABC, derived from p35SZYMVGAB, p35SZYMVGAC, and p35SZYMVGABC, respectively (Fig. 1B), did not induce local lesions (Fig. 2A; Table 1).

The relationship between GFP expression and the formation of local lesions by virus mutants was monitored with a fluorescence microscope (Fig. 2A). High intensity of GFP expression was recorded in plants infected with ZYMV-GFP, the wild-type TW-TN3 carrying the GFP-derived form p35SZYMVGFPHis3 (Fig. 1B). On the contrary, the virus mutants GA, GB, GC, and GBC, expressed relatively lower levels of GFP, as reflected by the lower intensity of fluorescence. However, the GFP expression of the

GAB and GAC virus mutants was much lower than that of the other mutants and difficult to detect under fluorescence microscope. The GFP activity of GAB and GAC virus mutants on leaves of *C. quinoa* completely disappeared at 5 and 14 dpi, respectively (Fig. 2A). No GFP activity was detected on leaves of *C. quinoa* inoculated with the mutant GABC before 14 dpi.

Systemic symptoms of mutated viruses on squash. When local lesions induced by the virus mutants appeared on leaves of *C. quinoa* plants, they were transferred individually to squash by sap inoculation and systemic symptom development was monitored. Because GAB and GAC did not induce local lesions, the inoculated leaves were thoroughly examined by fluorescence microscopy at 4 dpi and spots with fluorescence were identified. The leaf tissue corresponding to the identified spots was cut and mechanically transferred to squash plants by sap inoculation similar to single lesion transfer. The GABC virus mutant could not be recovered from *C. quinoa* because this mutant did not form local lesions and no fluorescent spots were identified. The negative results of the GABC mutant were ascertained by repeated construction of p35SZYMVGABC and repeated introduction of the confirmed infectious clone to *C. quinoa* for an additional four times.

Symptoms induced by the six mutants on squash plants compared with those induced by the naturally occurring mild strain WK and the wild-type virus ZYMV-GFP are shown in Figure 2B. These mutants showed different levels of symptom severity that were divided into four types (Fig. 2B). Type I represented the severe symptoms induced by the wild-type virus ZYMV-GFP, with yellow mosaic, distortion and narrowing on leaves, and stunting on plant growth. Type II represented the symptoms induced by GA, GB, GC, and GBC mutants, with mild mosaic and slight distortion on leaves, but no stunting of growth. Type III

Fig. 2. Symptoms on the local lesion host *Chenopodium quinoa* and the systemic host squash (*Cucurbita pepo* var. Zucchini) induced by the wild type and the constructed mutants of Zucchini yellow mosaic virus (ZYMV). **A**, Local infection of *C. quinoa* plants after inoculation with the wild-type ZYMV-GFP (WT) or individual mutants. Virus accumulation in the infected tissue was reflected by green fluorescent protein (GFP) expression as monitored by fluorescence microscopy. Unless specified, leaf symptoms and fluorescence were photographed at 14 days postinoculation (dpi). Fluorescent spots induced by the GAB mutant were photographed at 5 dpi. The bar represents 10 μ m. **B**, Different symptoms developed on squash plants after inoculation with the wild type or individual ZYMV mutants. Type I: yellow mosaic with leaf distortion and narrowing induced by the wild-type ZYMV-GFP (WT). Type II: mild mosaic and slight distortion induced by GA, GB, GC, and GBC mutants. Type III: mild mottling induced by GAC mutant and the mild strain ZYMV WK (21). Type IV: no symptoms after inoculation with GAB mutant. H: buffer-inoculated control. The symptom types were indicated by I, II, III, and IV. The bar represents 5 cm.

represented infection induced by the GAC mutant and naturally occurring mild strain WK, with mild mottling on lower leaves, but recovered with symptomless upper leaves. Type IV represented no apparent phenotypic effects associated with inoculation by the GAB mutant.

Accumulation of CP and GFP of the mutants in infected squash plants. The titers of the mutants in infected squash plants were monitored at 5, 10, 15, 20, and 25 dpi by ELISA. In order to minimize the variation on each leaf of a squash, leaf disks from upper leaves of each infected squash plant were combined and ELISA readings were averaged from four plants.

Similar to the wild-type ZYMV-GFP, the GA, GB, GC, and GBC mutants continued to express observable GFP activity on squash and *C. quinoa* plants. ELISA readings revealed that the accumulations of the CP or GFP of virus mutants GA, GB, GC, and GBC were slightly less than that of the wild-type virus ZYMV-GFP on squash plant (Fig. 3A and B). Up to 5 dpi, the accumulation of CP and GFP of the mutant GAC gradually increased as did that of the other mutants. However, CP and GFP accumulations suddenly dropped to the background level at 10 dpi, and then increased slowly 10 to 25 dpi (Fig. 3A and B). The mild symptoms gradually became inconspicuous on new upper leaves between 9 and 14 dpi. After 14 dpi, some new upper leaves developed mild mottling and very weak GFP signal reappeared, but the shape of leaves and growth of plants were similar to those of uninfected plants. The transfer of the GAB mutant from *C. quinoa* to squash could only be conducted before 5 dpi, but no significant amount of the CP and GFP derived from the mutant GAB was detected from squash after transfer (Fig. 3A and B). The CP and GFP titers of GAB in squash plants were not significantly detected since the ELISA readings were less than two times the value of those of mock-inoculated controls (Fig. 3A and B). The GAB inoculation was repeated five times from *C. quinoa* to squash; however, the mutant was detected only from the inoculated cotyledons, but not the upper leaves, as reflected in weak positive reactions of western blotting and RT-PCR (data not shown). The results indicated the mutant GAB only existed in the inoculated leaves and lost the ability to infect squash plants systemically.

Confirmation of the mutations in HC-Pro gene. *Stu*I, *Scr*FI, and *Mlu*I restriction enzyme sites were introduced to detect the A,

B, and C mutation sites, respectively (Fig. 4A). Total RNAs of squash plants that had been individually infected with the six virus mutants were amplified by RT-PCR. The RT-PCR products obtained were analyzed by digestion with *Stu*I, *Scr*FI, or *Mlu*I, and directly by sequencing.

When the 936-bp product was digested with *Stu*I to check the A mutation site, two fragments (264 and 672 bp) were obtained specifically for the GA and GAC mutants (Fig. 4B). When the fragment was digested with *Scr*FI to check the B mutation site, two fragments (354 and 582 bp) were obtained specifically for the GB and GBC mutants (Fig. 4B). When the amplified fragment of 779 bp was digested, the expected smaller fragments were observed after digestion with *Scr*FI (658 bp) or *Mlu*I (697 bp) for the B or C mutations (Fig. 4C). The results of the restriction digestion analyses confirmed that the correct mutations were present in the viruses infecting squash plants. Furthermore, the RT-PCR products were also sequenced to verify that only one amino acid was changed in the corresponding HC-Pro regions for the single, double, and triple mutations.

Capacity of attenuated mutants to cross protect against severe ZYMV infection. The attenuated mutants, GAB and

TABLE 1. Symptom development and green fluorescent protein (GFP) expression on *Chenopodium quinoa* and zucchini squash plants caused by seven constructed mutants of *Zucchini yellow mosaic virus* (ZYMV), with single, double, or triple amino acids changes in the helper component-protease (HC-Pro) gene^a

Virus mutant ^b	Local lesions on <i>C. quinoa</i>	Zucchini squash		GFP expression in	
		Symptom	Type	<i>C. quinoa</i>	Zucchini squash
ZYMV-GFP	NL	Severe yellow mosaic	I	C, S	C
GA	CS	Mild mosaic	II	C, L	C
GB	NL	Mild mosaic	II	C, L	C
GC	CS	Mild mosaic	II	C, L	C
GAB	—	Symptomless	IV	T	T
GAC	—	Slightly mild mottling	III	T	T
GBC	CS	Mild mosaic	II	C, L	C
GABC	—	—	—	—	—

^a NL, necrotic lesions; CS, chlorotic spots; —, no local lesions; C, continued expression; S, strong expression; L, low expression; T, GFP transient expression; —, not noticed or detected.

^b ZYMV-GFP is a Taiwan severe strain TW-TN3 that carries the GFP reporter gene (15). GA, GB, GC, GAB, GAC, GBC, and GABC virus mutants contain different mutations of the HC-Pro gene of ZYMV. G indicates the virus mutant carrying the GFP reporter gene; A indicates Arg₁₈₀→Ile mutation; B indicates Phe₂₀₅→Leu mutation; and C indicates Glu₃₉₆→Asn mutation.

Fig. 3. Accumulation of the coat protein (CP) and green fluorescent protein (GFP) expressed by the six mutants of *Zucchini yellow mosaic virus* in squash plants as determined by indirect enzyme-linked immunosorbent assay. Each value of absorbance represents the average reading from three plants. **A**, CP accumulation determined by the antiserum to ZYMV CP (22). **B**, GFP accumulation determined by the antiserum to GFP (15).

GAC, and the naturally occurring mild strain WK (21) were evaluated for their capacity to protect zucchini squash plants from infection by a severe ZYMV strain. The severe virus ZYMV-Derp5 carrying group 5 allergen (Der p 5) gene of *Dermatophagoides pteronyssinus* constructed from TW-TN3 (15) was used as the challenge virus. Since the GAB mutant did not have significant titers on squash without conspicuous phenotype, the GAB inocula for the protective inoculation were from the leaf tissues of *C. quinoa* with GFP signal 4 days after inoculation with p35SZYMVGAB. The results of the plants challenged with the severe strain ZYMV-Derp5 at 5 days after the protective inoculation with GAB, GAC, or WK are shown in Figure 5A. At 15 days after the challenge inoculation, 100% protection (i.e., none of a total of 20 plants from five independent experiments for GAC showed severe symptoms) was observed for GAC-protected plants that showed mild mottling on the two or three leaves above the inoculated leaves, whereas no symptoms developed on the upper new leaves (Fig. 5A). Also, no severe symptoms were noticed on the WK-protected plants that only showed mild mottling on leaves, a characteristic of the mild strain (Fig. 5A). Using the Der p 5 antiserum to detect the challenge severe virus, the ELISA readings resulting from GAC-or WK-protected plants were similar to that of the mock control, indicating that the challenge virus did not accumulate in the protected plants (Fig. 5B). In addition, complete protection provided by GAC or WK was also observed when the challenge inoculation was applied 10 days (Fig. 5B) and 15 days (data not shown) after the protective inoculation. None of a total of 20 plants protected by each mild virus and challenged at 10 or 15 days from five independent experiments showed severe symptoms.

However, all plants first inoculated with the GAB mutant or buffer and then challenged with ZYMV-Derp5 5 days later showed severe symptoms on upper leaves (Fig. 5A) and a high concentration of Der p 5 protein was detected by ELISA (Fig. 5B). Moreover, the GAB mutant did not provide any protection when the protective intervals were increased to 10 (Fig. 5B) or 15 days (data not shown).

Time required for GAC mutant to provide protection. The GAC mutant provided complete cross protection at 5 dpi. ELISA results indicated that although the GAC titer dropped significantly

after 5 dpi, the accumulation of CP and GFP produced by the GAC mutant before 5 dpi increased similar to those resulting from inoculation with wild-type virus (Fig. 3). Therefore, the cross protection ability of GAC was further analyzed by challenge inoculations within 5 days after the protective inoculation.

Results of ZYMV-Derp5 challenge inoculation applied at 0, 2, 4, 6, or 10 days after inoculation with GAC are shown in Figure 5C and D. At 15 days after the challenge inoculation with ZYMV-Derp5, severe symptoms developed on plants protected with GAC for 0 day (co-inoculated), but no severe symptoms were observed for the plants protected for 6 and 10 days prior to inoculation with ZYMV-Derp5. However, the delay in development of mosaic symptoms was observed in plants protected for 2 or 4 days (Fig. 5C). The titers of challenge virus ZYMV-Derp5 were determined by ELISA using Der p 5 antiserum at 15 days after the challenge inoculation (Fig. 5D). The titer of ZYMV-Derp5 was similar to that of the nonprotected control when the challenge inoculation was applied at the same time as the protective inoculation, while it gradually decreased when the challenge inoculation was applied at 2 or 4 days after the protective inoculation. No significant ZYMV-Derp5 was detected when the protection period was increased up to 6 or 10 days (Fig. 5D). These results indicated that the GAC mutant provided incomplete cross protection if the plants were challenged at 2 or 4 days after the protective inoculation.

PTGS suppression ability of the GAC mutant. In order to understand whether the mutated HC-Pro of GAC suppresses PTGS, the restoration of transgene expression in a silenced transgenic squash line was analyzed by infection with GAC. The expression of the CP gene of SqMV was not detected from these transgenic plants after mock-inoculation (Fig. 6A, upper lane 3). When a transgenic plant was infected with the wild-type virus TW-TN3 at 7 dpi, SqMV CP was detected by the antiserum to SqMV CP (Fig. 6A, lane 1). When the GAC mutant was used for inoculation, SqMV CP was also detected on the inoculated leaf at 7 dpi (Fig. 6A, lane 2). In addition, the HC-Pro of TW-TN3 and GAC mutant also were detected by ZYMV HC-Pro antiserum (Fig. 6B, lanes 1 and 2, respectively). The results indicated that both HC-Pro proteins of the wild virus TW-TN3

Fig. 4. Verification of the mutations of the constructed mutants of *Zucchini yellow mosaic virus* (ZYMV) by reverse transcription-polymerase chain reaction (RT-PCR) and restriction enzyme digestions. The cDNA obtained by RT-PCR from total RNA of squash plants infected with ZYMV GA, GB, GC, GAC, GBC, and wild-type TW-TN3 was digested by *StuI*, *ScrFI*, and *MluI* to confirm the presence of the mutations at the specific sites. **A**, Schematic representation of the helper component-proteinase (HC-Pro) gene and the region amplified by RT-PCR. **B**, The cDNA fragment of 936 bp amplified by the PZ1329 and MZ2263 primers from the total RNA of infected squash plants was digested by *StuI* and *ScrFI* to confirm the A and B mutations. **C**, The cDNA fragment of 779 bp amplified by the PZ1562 and MZ2340 primers was digested by *ScrFI* and *MluI* to confirm the presence of the B and C mutations, respectively.

and the mutant GAC were expressed normally and had the capacity to suppress the PTGS of SqMV CP gene in the transgenic squash plants.

DISCUSSION

Three amino acid changes in conserved motifs of the HC-Pro of ZYMV TW-TN3 were designed from the consensus differences of the mild strains PRSV HA5-1 (7) and ZYMV WK (11) with 10 other severe wild-type potyviruses. Single substitutions of any of these three amino acids in the virus mutants GA, GB, and GC, respectively, resulted in reduced symptom severity in squash plants (i.e., from type I to type II). These three virus mutants caused milder mosaic symptoms on squash plants; however, stunting in growth of the infected plants was not observed. Single substitutions in these three amino acids apparently affected the virulence of the mutants and also significantly reduced the accumulation of GFP.

The GA mutant was designed according to the report that the FRNK motif affects symptom expression and that the engineered ZYMV AG containing a single mutation in the Arg residue induced mild symptoms in squash plants and protects against infection by a severe strain (11). In this investigation, we demonstrated that the GA mutant with the change Arg₁₈₀→Ile in the HC-Pro gene of TW-TN3 also caused a dramatic symptom change from severe to mild mosaic on squash. The Arg, Asn, and Lys residues in the FRNK motif are charged polar amino acids; therefore, the replacement of the basic Arg with the hydrophobic amino acid Ile may change the polarity of the motif and affect the interaction with a putative host factor or other viral products to cause symptom attenuation (11). Although the symptoms induced by the GA mutant were milder than that by the wild-type strain, the virus still caused rather prominent mottling symptoms that were quite different from the almost symptomless infection reported for ZYMV AG (11). Substitution of Arg₁₈₀→Ile in the FRNK motif of ZYMV AG dramatically affected symptom expression but did not affect virus accumulation (11). However, our ELISA data showed that the levels of accumulation of CP and GFP in GA-infected plants were slightly lower than those of the wild-type virus TW-TN3, depending on the time point for the virus (Fig. 3). These differences may result from the contribution of other genomic motifs of TW-TN3 to its pathogenicity.

The GBC mutant also induced type II symptoms, with mild mosaic and slight distortion on leaves of squash. However, the local lesions on *C. quinoa* were chlorotic spots that were different from the necrotic types produced by the GB mutant or wild-type virus and the GFP activity was lower than that associated with GA, GB, or GC infection. Substitution of Phe₂₀₅ with Leu does not change hydrophobicity, but the loss of an aromatic ring in an amino acid may interrupt its ability to interact with other amino acids through electron transfer. Furthermore, because Phe₂₀₅ is a conserved amino acid among potyviruses (Fig. 1A), and the GB mutant that has a single Phe₂₀₅→Leu substitution causes type II symptoms, it appears that Phe₂₀₅ plays a role in the pathogenicity of ZYMV TW-TN3. Our results also indicated that changes in Phe₂₀₅ and Glu₃₉₆ affect the local lesion phenotypes on *C. quinoa* plants. The mutation Glu₃₉₆→Asn replaces a highly acidic amino acid with an uncharged amino acid. This may be critical since the highly acidic amino acid, either Asp or Glu, are found at this position in most severe potyviruses compared (Fig. 1A).

With the exception of the GBC mutant, double substitution mutants decreased viral pathogenicity more dramatically than any of the single mutants. The GAC mutants showed much milder and recovering symptoms of type III on zucchini squash plants, coupled with low levels of GFP accumulation. Although the fluorescent spots on the leaf tissues of *C. quinoa* plants were

identified by microscope and the GAB mutant was then transferred to squash plants, but from the ELISA results we were not able to prove that the GAB mutant infected squash systemically. Only very low levels of the CP of GAB in inoculated leaves of squash were detected by RT-PCR and western blotting. Our results indicated that GAB is difficult to be transferred from *C. quinoa* to squash due to its low titer, or it may induce subliminal infection in the inoculated cotyledons of squash due to its inability to spread in squash tissues. It is also possible that the residue particles of the inoculum were detected. Therefore, we conclude that GAB cannot establish systemic infection in squash when it is transferred from *C. quinoa*. Our results imply that the changes at both A and B positions (Arg₄₉₀→Ile and Phe₅₁₅→Leu) affect virus replication or movement in squash plants. In addition, GFP activity in *C. quinoa* plants infected with the GAB and GAC mutants disappeared after 5 and 14 dpi, respectively. Therefore, the substitutions of Arg₁₈₀→Ile coupled with Phe₂₀₅→Leu or Glu₃₉₆→Asn are critical for the attenuated infection of TW-TN3. This is further reflected by the fact that the GABC mutant was not able to be recovered from inoculated *C. quinoa* leaves, probably due to not being infectious or only causing subliminal infection that was not detectable by the methods applied. Moreover, the GAB and GAC mutants produced no local lesions on *C. quinoa* plants. Thus, we conclude that Arg₁₈₀ together with Phe₂₀₅ or Glu₃₉₆ are also essential for the local lesion formation. Our results suggest that the HC-Pro gene is an important determinant for the induction of hypersensitive reaction on the local lesion host.

The HC-Pro is a viral suppressor of PTGS, a pathway that has evolved as a defensive mechanism against viral invasion (1,19). The ELISA curve of the GAC mutant showed that the GAC titer first increased and then dropped sharply within 10 days after inoculation (Fig. 3). Is the rapid decline in virus titer associated with the GAC mutation of the HC-Pro caused by a failure to suppress PTGS? Our western data showed that the GAC mutant still suppresses PTGS to a level that restores the expression of silenced SqMV CP gene in the transgenic squash plants, similar to the wild-type TW-TN3 (Fig. 6, upper lane 2). However, the results using the PTGS-inducing transgene in transgenic squash as a test model may not completely match with host reaction during different stages of virus infection, whether the dramatic decrease in the GAC titer is caused by other unknown aspects of the plant defensive reaction rather than the defect on the suppression of PTGS remains to be further investigated.

The cross protection reaction in squash plants by GAC was completely induced 5 days after the protective inoculation. Thus, we assume that the cross protection mechanism is triggered within 5 days after the protective inoculation and before this time the protection is incomplete, as reflected in the result that a delay in symptom expression was noticed when the challenge was applied 2 and 4 days after the protective inoculation with GAC. Furthermore, the ELISA readings of the challenge virus were inversely related with the increase in length of protection (Fig. 5D).

The explanation of the cross protection phenomenon provided by the mild strains WK, GAB, and GAC is summarized in Figure 7. The phenomenon can be separated into three stages: initiation, resistance, and maintenance. We suggest that the defensive reaction of squash plants is triggered when the mild strains of ZYMV start to establish infection in squash plants during the initiation stage that occurs within 5 days after the protective inoculation. After the plant defensive reaction is induced, the defective (mutated) HC-Pro proteins of the mild strains GAC and WK cannot antagonize the defensive reaction, resulting in a sharp decline of virus accumulation of the mild viruses, as reflected in the resistance stage that is from 5 to 10 days after the protective inoculation. However, the defective HC-Pro perhaps remains some ability to help mild strains to survive as low levels of virus accumulation still can be detected at this stage. Ten days after the protective inoculation, the virus replication rate is probably

Fig. 5. Cross protection of squash plants with mild strains of *Zucchini yellow mosaic virus* (ZYMV): GAC, GAB (both from this investigation) and WK (21), against the challenge inoculation with the severe strain ZYMV-Derp5 (15). **A**, GAB, a GAB-protected plant; GAC, a GAC-protected plant; Mock, a buffer-inoculated squash plant; and WK, a WK-protected plant. All plants were challenged with ZYMV-Derp5 5 days after the protective inoculation. The symptoms were photographed 15 days after the challenge inoculation. The bar represents 5 cm. **B**, Accumulation of Der p 5 protein in the challenged plants detected by indirect enzyme-linked immunosorbent assay (ELISA) at 5 or 10 days after the protective inoculation ($n = 3$; error bars indicate standard deviation [SD], the experiment was repeated three times). **C**, The symptoms on equivalent leaves photographed at 15 days after the challenge with severe ZYMV-Derp5. **D**, Accumulation of the challenge virus ZYMV-Derp5 monitored by indirect ELISA at 15 days after the challenge inoculation ($n = 3$; error bars indicate SD). The antiserum against the Der p 5 protein (15) was used and the ELISA readings at 405 nm were recorded 40 min after the addition of substrate. The intervals between the protective and the challenge inoculations are indicated. Plants infected with ZYMV-Derp5 or GAC were used as positive and negative controls, respectively.

balanced by the antagonistic ability of the host defensive reaction, and the mild strain virus survives in inoculated plants at low titers. The low titer of mild strains continues to stimulate the plants to maintain the defensive reaction and continue to provide a high degree of cross protection. This stage is therefore designated as the maintenance stage.

The initiation stage of cross protection is dependent on the virus accumulation of the protective strain and is considered similar to the initiation of virus-induced gene silencing (VIGS) by which the virus can induce PTGS in plants by carrying a sequence homologous to either a transgene or an endogenous nuclear gene (37). The cross protection of the host is not induced unless the mild strain virus starts replication and the virus titer reaches a certain threshold. The inability of the GAB mutant for cross protection is probably due to the fact that it is incapable of reaching the inducing threshold for cross protection. In the

initiation stage, the protection may result from competition for virus replication. The incomplete protection of GAC at the early stage, as shown in only the delay in the development of the severe symptoms, is probably a result of competition for replication factors that are already used by the protective virus. Although the major protection exhibited at the resistance and maintenance stages is considered to result from a plant defensive mechanism, the involvement of virus competition similar to that during the initiation stage cannot be excluded during the resistance and maintenance stages. At the maintenance stage, cross protection is virus-dependent and a low titer of the protective virus is needed. We suggest that low titers of virus may stimulate plants to continually synthesize host factors for maintaining the protection mechanism. On the contrary, the maintenance stage of VIGS is virus-independent, because this stage of VIGS is influenced by the continuous expression of a nuclear gene rather than the in-

Fig. 6. Analysis the ability of posttranscriptional gene silencing (PTGS) suppression of the GAC mutant. The wild-type virus *Zucchini yellow mosaic virus* (ZYMV) TW-TN3 and the GAC mutant were mechanically introduced to plants of a transgenic squash line SqMV-127 carrying the coat protein (CP) of *Squash mosaic virus* (SqMV), in which the transgene is posttranscriptionally silenced (16,30). The immunoblot analysis was conducted at 7 days postinoculation. Lanes 1 and 2, the extracts from the leaf tissue (0.01 g) of SqMV CP-transgenic squash plants that were inoculated with wild-type virus TW-TN3 and the GAC mutant, respectively. Lane 3, the extract from leaf tissue of a SqMV CP-transgenic squash plant inoculated with buffer. Lane 4, a SqMV-infected plant as control. **A**, Detection using the anti-SqMV CP serum. **B**, Detection with the anti-ZYMV helper component-proteinase serum. M, molecular size markers.

ducing virus (37). Interestingly, virus titers of WK, as reflected in CP, were similar to those for the GAC mutant, with a sudden drop close to the background values at 10 dpi and then a slow increase to a steady-state level that was maintained after 15 dpi (Fig. 6). Apparently the GAC and WK viruses induced a similar mechanism of cross protection, but at a different pace.

The GAC mutant was stable as reflected by the observation that no revertants with severe strain sequences were found after nine passages through squash plants after a period of 9 months (data not shown). In addition, the GAC mutant caused symptomless infection on plants of melon (*Cucumis melo* L. conomon group) and *Cucumis metuliferus* Naud. (Mey. Acc. 2459) on which ZYMV TN3 induces severe mosaic and stunting symptoms. When the reporter GFP was deleted from the GAC mutant, the resulting virus ZYMV AC behaves similar to the GAC mutant on these hosts and provides complete cross protection against TW-TN3 (data not shown), indicating that the attenuation and cross protection effectiveness are not influenced by the insertion of the markers, the GFP gene in GAC and the Der p 5 gene in TW-TN3. Since the mutant GAC induces a recovery type of symptoms that are milder than WK, the adverse effects due to the mild strain infection are less than WK. In this investigation, GAC provided similar degrees of protection against the Taiwan severe strain ZYMV TN3. Whether GAC would provide better degrees of protection against other Taiwan severe strains remains to be tested. However, we predict that ZYMV GAC can be widely used for control of ZYMV by cross protection in cucurbits in Taiwan and elsewhere in Eastern Asia since most of the ZYMV strains in these areas belong to the same genotype as GAC (21,24).

The conserved DAG triplet of potyviral CP genes (3,12,14) and the KITC motif of potyviral HC-Pro genes (2,13) are essential for aphid transmissibility. To minimize the adverse effects on other crops and environmental impacts, the DAG motif in the CP coding region and the KITC motif in the HC-Pro coding region are currently being mutated to abolish the aphid transmissibility of ZYMV GAC.

Cross protection is an important approach for control of plant viral diseases. The elucidation of the viral pathogenic genes opens a new and promising avenue to create mild strains that can be

Fig. 7. A model to explain the cross protection in squash plants provided by the constructed *Zucchini yellow mosaic virus* (ZYMV)-GAC and ZYMV-GAB mutants and the naturally collected mild strain ZYMV WK (21) against the severe strain ZYMV-Derp5 (15). The phenomenon of GAC- and WK-induced cross protection is separated into three stages: the initiation, resistance, and maintenance stages. The initiation stage reflects the time when the protection ability of squash plants against the severe strain is triggered at 0 to 5 days after the protective inoculation with the mutant. After the plant defensive reaction is fully induced, the mutated helper component-proteinase (HC-Pro) genes of the GAC mutant and the WK strain are not able to completely antagonize the host defensive reaction, resulting in a sharp drop of protective-virus accumulation and a build-up in protection against the severe infection. The time interval between 5 and 10 days after the protective inoculation reflects this phenomenon and is designated as the resistance stage. The maintenance stage reflects the period 10 days after the protective inoculation, at this time the mild strains GAC and WK are able to balance the host defensive reaction and the low level of accumulation of the mild viruses continues to stimulate the plants to induce high degrees of cross protection. The accumulation of the mutant GAB is not detected in the upper leaves at the initiation stage and thus is not able to trigger host defensive reaction that is essential for effective cross protection. The gray bars indicate the accumulation of Der p 5 protein at 15 days after the challenge inoculation with ZYMV-Derp5 at 0, 2, 4, 6, and 10 days after the protective inoculation.

used for control of different viruses. In this investigation, for the first time, we have demonstrated that two markers (GFP and Der p 5) can be used to monitor the accumulation and decline of the protective mild strain and the challenge severe strain of the same virus in cross protection. The unique system is useful for the study of the mechanism of cross protection. The two changes of the conserved amino acids of the HC-Pro are being applied to create new mild strains of other potyviruses for disease control by cross protection. In addition, the mild mutants GAC and GAB can also be used to further investigate the host factors involved in host responses to local lesion formation.

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