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Short communication

Movement protein of broad bean wilt virus 2 serves as a determinant of symptom severity in pepper

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ABSTRACT

Broad bean wilt virus 2 (BBWV2, genus *Fabavirus,* family *Secoviridae*) has a wide host range and infects many economically important crops. Various isolates of BBWV2 have been identified from diverse host plants, and their molecular and biological characteristics have been investigated. In our previous study, we demonstrated that BBWV2 RNA2 contains a symptom determinant(s) capable of enhancing symptom severity by utilizing infectious full-length cDNA clones of two distinct strains of BBWV2, pBBWV2-PAP1 (a severe strain) and pBBWV2-RP1 (a mild strain). In the present study, to identify the symptom determinant(s) of BBWV2, we exploited disease responses of pBBWV2-PAP1- and pBBWV2-RP1-derived chimeric viruses and amino acid substitution mutant viruses in *Nicotiana benthamiana* and pepper (*Capsicum annuum* Quarri) and demonstrated that the movement protein (MP) encoded in BBWV RNA2 is the determinant of disease symptom severity in both plants. A single amino acid substitution in the MP was sufficient for changing symptom severity of BBWV2. Our finding provides a role for the MP as a symptom determinant in BBWV2 and increases the understanding of the basis of molecular interactions between host plants and BBWV2.

The induction of disease responses in a plant infected with a virus is the result of complex interactions between host and viral factors. One well-characterized molecular interaction between plants and invading viruses is mediated by resistance (*R*) genes (Kang et al., 2005). *R*-gene-mediated resistance requires direct or indirect interactions between products of host *R* genes and avirulence (*Avr*) genes encoded by viruses (Bonas and Lahaye, 2002; Kang et al., 2005). Any protein component of a virus can function as the specific elicitor causing the symptomatic phenotype of disease (DeWit, 1997). Sometimes, the compatibility and strength of the interaction between host and viral factors regulates the severity of disease responses (Hajimorad and Hill, 2001; Kang et al., 2005; Seo et al., 2011). Many studies have shown that amino acid sequence variations in a viral protein were associated with different degrees of pathogenicity of distinct virus strains (Hajimorad et al., 2005; Seo et al., 2009; Szilassy et al., 1999).

Broad bean wilt virus 2 (BBWV2, genus *Fabavirus,* family *Secoviridae*) is a widespread viral pathogen that infects many economically important crops, including pepper, spinach, and sesame (Ferrer et al., 2011; Kwak et al., 2013a). The BBWV2 genome is composed of two single-stranded positive-sense RNA molecules, RNA1 and RNA2, which are encapsulated separately into icosahedral virions (Atsumi et al., 2013; Ferrer et al., 2011). BBWV2 RNA1 and RNA2, which are approximately 5960 and 3600 nucleotides (nt) in length, respectively, encode single large open reading frames (ORFs). The ORF encoded by BBWV2 RNA1 is translated into a single polyprotein precursor, which is cleaved to yield five mature proteins: protease cofactor (Co-Pro), NTP-binding motif (NTBM), VPg, protease (Pro), and RNA-dependent RNA polymerase (RdRp). The polyprotein precursor translated from the RNA2 ORF is processed to yield three mature proteins: movement protein (MP), large coat protein (LCP), and small coat protein (SCP) (Ferrer et al., 2011; Kwak et al., 2013a). Depending on the compatibility between virus strains and host plants, BBWV2 causes various symptoms, including mild mosaic, severe mosaic, mottling, vein clearing, wilting, and necrosis (Atsumi et al., 2013; Kwak et al., 2013a,b).

In our previous studies, we constructed infectious cDNA clones of two distinct BBWV2 strains, RP1 (a mild strain) and PAP1 (a severe strain), collected from pepper (Kwak et al., 2016). Virulence analysis of the pseudo-recombinants of the two strains demonstrated that a symptom severity determinant is located in BBWV2 RNA2. As shown in

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Region	Amino acid position on polyprotein precursor	pBBWV2-R2-RP1	pBBWV2-R2-PAP1
MP	49	Ile	Val
	59	Ala	Val
	165	Lys	Arg
	186	Ala	Val
	229	Ala	Val
	314	Asn	Asp
	390	Ser	Asn
	424	Lys	Arg
	426	Gly	Arg
	430	Ser	Pro
	433	Leu	Phe
	440	Ser	Gly
	444	Ile	Val
	446	Asn	Cys
LCP	483	Gly	Glu
	563	Gly	Glu
	645	Ile	Val
	656	His	Asn
	828	Asn	Ser
	838	Ala	Thr
	854	Asp	Asn
SCP	870	Ala	Val
	873	Glu	Lys
	941	Arg	Lys
	962	Thr	Ile
	988	Asn	Ser
	1026	Tyr	Glu
	1036	Asp	Gly

B

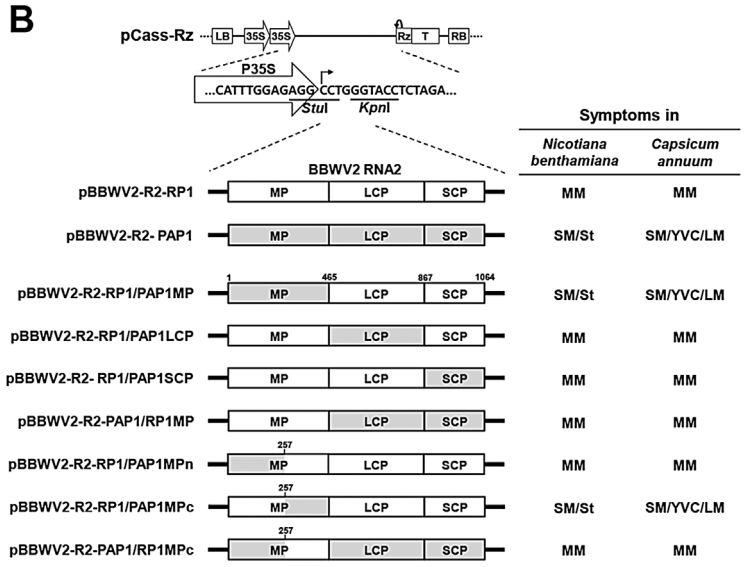

Fig. 1. (A) Amino acid differences between the viral proteins encoded in RNA2 of the BBWV2 strains RP1 and PAP1. (B) Schematic representation of chimeric RNA2 genomes of the BBWV2 strains RP1 and PAP1 and their symptoms observed in *Nicotiana benthamiana* and pepper (*Capsicum annuum* cv. Quarri) plants. Chimeric RNA2 constructs were generated by exchanging corresponding regions between pBBWV2-R2-RP1 and pBBWV2-R2-PAP1. Full-length cDNAs of RNA2 of BBWV2-RP1 and −PAP1 and their derivative chimeras were inserted between the *Stu*I and *Kpn*I sites in the pCassRz vector. The pCassRz vector contains, in sequential order, a T-DNA left border (LB), a double 35S promoter, multiple cloning sites (*Stu*I, *Kpn*I, *Xba*I, and *Bam*HI), a *cis*-cleaving ribozyme sequence (Rz), a 35S terminator (T), and a T-DNA right border (RB). pBBWV2-R2-RP1- and pBBWV2-R2-PAP1-derived regions are indicated by white and gray boxes, respectively. Symptom appearances were monitored visually for 4 weeks post-inoculation (MM, mild mosaic; SM, severe mosaic; St, stunting; YVC, yellowish vein clearing; LM, leaf malformation). BBWV2 infection was verified by RT-PCR using total RNA extracted from systemic leaves.

our previous study (Kwak et al., 2016), there are 28 amino acid differences between PAP1 and RP1 in RNA2, comprised of 14, 7, and 7 amino acid differences in the MP, LCP, and SCP, respectively (Fig. 1A). Thus, we hypothesized that one or more of these amino acid differences in RNA2 may be responsible for the symptomatic differences between the strains RP1 and PAP1.

In this study, we exploited disease responses of chimeric viruses of the strains RP1 and PAP1 and amino acid substitution mutant viruses in *Nicotiana benthamiana* and pepper (*Capsicum annuum* Quarri) to identify the symptom severity determinant in RNA2. Various chimeric BBWV2 RNA2 constructs were generated by a fusion-PCR-based method (Charlier et al., 2003) using overlapping primers for each junction (Fig. 1B and Table S1). The resulting full-length cDNAs of the chimeric RNA2 were inserted between the *Stu*I and *Kpn*I sites in the pCassRz vector (Kwak et al., 2016), a modified binary vector containing the cauliflower mosaic virus (CaMV) 35S promoter. The plasmids of the chimeric RNA2 constructs were transformed into *Agrobacterium tumefaciens* strain GV2260. Cells of *A. tumefaciens* harboring each chimeric RNA2 construct were grown as described previously (Kwak et al., 2016) and mixed in equal proportions with cells of *A. tumefaciens* harboring pBBWV2-PAP1-R1 for agroinfiltration (Kwak et al., 2016). Each mixture was infiltrated into the abaxial surface of the leaves of *N. benthamiana* and pepper plants.

The inoculated plants were grown in an insect-free growth chamber maintained at 26 °C with 16/8 h light/dark cycles. Symptom development was monitored for 4 weeks post-inoculation and BBWV2 infection was confirmed by RT-PCR using total RNA extracted from upper non-inoculated leaves (Kwak et al., 2013b). As shown previously (Kwak et al., 2016), pBBWV2-R2-RP1 caused very mild symptoms in *N. benthamiana* and pepper while pBBWV2-R2-PAP1 induced severe symptoms (Figs. 1 B and 2). Interestingly, pBBWV2-R2-RP1/PAP1MP, which is a pBBWV2-R2-RP1-derived chimera containing the PAP1 MP, induced severe symptoms similar to those caused by pBBWV2-R2-PAP1 in both *N. benthamiana* and pepper, while other pBBWV2-R2-RP1-derived chimeras containing either PAP1 LCP or SCP induced very mild mosaic symptoms (Figs. 1B and 2). Conversely, pBBWV2-R2-PAP1/RP1MP, which is a pBBWV2-R2-PAP1-derived chimera containing the RP1 MP, caused mild symptoms similar to those induced by pBBWV2-R2-RP1 (Figs. 1 B and 2). Together, these results suggest that the BBWV2 MP is the determinant of disease symptom severity in *N. benthamiana* and pepper.

To identify specific domains or amino acids in the MP associated with symptom severity, additional pBBWV2-R2-RP1-derived chimeras containing the N- or C-terminal half of the PAP MP were tested. As pBBWV2-R2-RP1/PAP1MPc but not pBBWV2-R2-RP1/PAP1MPc induced severe symptoms (Figs. 1B and 2), the results demonstrated that amino acid sequence differences in the C-terminal half of the BBWV2 MP are responsible for symptom severity. The C-terminal half of the PAP1 MP differs from that of the RP1 MP by nine amino acids (Fig. 3). To identify which of these 9 amino acid differences in the C-terminal half of MP affects symptom severity, single or multiple amino acid substitutions were introduced into pBBWV2-R2-RP1 and pBBWV2-R2-PAP1 by a fusion-PCR-based site-directed mutagenesis strategy using overlapping primers (Table S1) (Charlier et al., 2003). Briefly, the amino acid substitution mutants was constructed by introducing the amino acid residues of pBBWV2-R2-PAP1 into the pBBWV2-R2-RP1 background at the corresponding positions (Fig. 3). The virulence of the constructed BBWV2 amino acid substitution mutants was examined in *N. benthamiana* and pepper plants. RT-PCR detection of BBWV2 from upper non-inoculated leaves confirmed that the BBWV2 amino acid substitution mutants were replication competent (data not shown). Interestingly, pBBWV2-R2-RP1-MP$_{K424R}$, which contains a single amino acid substitution of Arg for Lys at position 424 in the MP, induced severe symptoms indistinguishable from those induced by pBBWV2-R2-PAP1 in both *N. benthamiana* and pepper plants (Figs. 2 and 3). In addition, introduction of the amino acid substitution of Lys for Arg at position 424 into pBBWV2-R2-PAP1 (the resultant clone was named pBBWV2-R2-PAP1-MP$_{R424K}$) resulted in a decrease in symptom severity (Figs. 2 and 3). Our results suggested that the amino acid residue at position 424 in the BBWV2 MP is associated with symptom severity alteration in *N. benthamiana* and pepper. To make sure that progeny viruses of the amino acid substitution mutants maintained the introduced mutations during replication, the nucleotide sequences of the MP C-terminus regions of the progeny viruses recovered by RT-PCR from the infected *N. benthamiana* plants were determined. The results showed that all introduced mutations were maintained in progenies