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Molecular Characterization and Variation of the *Broad bean wilt virus 2* Isolates Based on Analyses of Complete Genome Sequences

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(Received on March 27, 2013; Revised on May 26, 2013; Accepted on May 26, 2013)

The full-genome sequences of fourteen isolates of *Broad bean wilt virus 2* (BBWV2), collected from broad bean, pea, spinach, bell pepper and paprika plants in Korea during the years 2006–2012, were determined and analyzed comparatively along with fifteen previously reported BBWV2 genome sequences. Sequence analyses showed that RNA-1 and RNA-2 sequences of BBWV2 Korean isolates consisted of 5950–5956 and 3568–3604 nucleotides, respectively. Full-length genome sequence-based phylogenetic analyses revealed that the BBWV2 Korean isolates could be divided into three major groups comprising GS-I (isolates BB2 and RP7) along with isolate IP, GS-II (isolates BB5, P2, P3 and RP3) along with isolate B935, and GS-III including 16 BBWV2 Korean isolates. Interestingly, GS-III appears to be newly emerged and predominant in Korea. Recombination analyses identified two recombination events in the analyzed BBWV2 population: one in the RNA-1 of isolate K and another one in the RNA-2 of isolate XJ14-3. However, no recombination events were detected in the other 21 Korean isolates. On the other hand, out of 29 BBWV2 isolates, 16 isolates were found to be reassortants, of which each RNA segment (*i.e.* RNA1 and RNA2) was originated from different parental isolates. Our findings suggested that reassortment rather than recombination is a major evolutionary force in the genetic diversification of BBWV2 population in Korea.

Keywords : BBWV2, phylogenetic analyses, recombination, reassortment

Broad bean wilt virus (BBWV) is a destructive pathogen in many economically important horticultural and ornamental crops. BBWV is the type species of the genus *Fabavirus* in

the family *Secoviridae* (Taylor et al., 1972). It is transmitted by aphids, mostly *Aphis gossypii* and *Myzus persicae*, with infection rate of 60–90% in a nonpersistent manner and has a wide host range (Benner et al., 1985; Brunt et al., 1996). By serological and molecular studies, BBWV isolates are classified into two species, *Broad bean wilt virus 1* (BBWV1) and *Broad bean wilt virus 2* (BBWV2) (Kobayashi et al., 2003; Uyemoto et al., 1974). Although they show the similar genome structures and functions, the nucleotide (nt) sequence identity between the two BBWV species was limited (39%–67%). The genome is composed of two single stranded positive-sense RNA molecules, RNA-1 and RNA-2, that are encapsidated separately into icosahedral virions (Lisa et al., 1996). While BBWV1 has been reported mainly in Europe and North America, BBWV2 has a worldwide distribution. In Korea, BBWV1 has not been detected yet, but disease incidences caused by BBWV2 was reported firstly by Lee et al. (1979).

BBWV2 RNA-1 and RNA-2 are approximately 6 kb and 4 kb nucleotides (nt) in length, respectively, and each RNA segment contains a single open reading frame (ORF), and thus is translated into a single polyprotein precursor which is further processed by proteolytic cleavage to yield functional proteins. RNA-1 encodes five proteins including cofactor protease (Co-Pro), NTP-binding motif (NTBM), genome-linked viral protein (VPg), protease (Pro) and RNA-dependent RNA polymerase (RdRp) while RNA2 encodes three proteins including movement protein (MP), large coat protein (LCP), and small coat protein (SCP) (Goldbach et al., 1995). So far, the complete nt sequences of eight BBWV2 isolates (Ikegami et al., 1998; 2001; Kobayashi et al., 1999; Koh et al., 2001; Kuroda et al., 2000; Lee et al., 2000; Nakamura et al., 1998; Qi et al., 2000a; 2000b) and partial sequences of twenty-five BBWV2 isolates (Ferrer et al., 2011; Kobayashi et al., 1999; 2004; Kondo et al., 2005; Sui et al., 2009) from various host plants have been reported.

In Korea, natural infections of BBWV2 on red pepper

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(Lee et al., 2000), broad bean (unpublished), pea (Choi et al., 2001), spinach (Lee et al., 1979), perilla (Choi et al., 2001), lily (Chang et al., 1987), *Gentiana* spp. (Roh et al., 1998), gladiolus (Park, et al. 1998), and celery (Hahm, et al., 1998) have been reported. In present, disease caused by BBWV2 has increased gradually in pepper plants, i.e. red pepper (Cho et al., 2007), bell pepper, and paprika (Ryu et al., 2009). Nevertheless, molecular characterization of BBWV2 Korean isolates (except K isolate) has not been studied. Recently, we characterized seven BBWV2 isolates obtained from red pepper (Kwak et al., 2013). In this study, we determined complete genome sequences of fourteen BBWV2 isolates collected from various plants including broad bean, pea, spinach, bell pepper, and paprika around the Korean Peninsula during the years 2006–2012. We further analyzed molecular characteristics and genetic structure of BBWV2 Korean isolates in comparison with the previously reported isolates using a range of methods to understand their evolutionary relationships.

Materials and Methods

Isolates collection. Twenty-one BBWV2 isolates were collected from 6 natural plants in different areas in Korea

during the year 2006–2012 based on the symptoms on leaves and fruits. Out of 21 BBWV2 isolates, 3 isolates (BB2, BB5 and BB9) were collected from broad bean (*Vicia faba*), 2 isolates (P2 and P3) from pea (*Pisum*

Fig. 1. Geographical locations of the collected BBWV2 isolates in Korea.

Table 1. List of *Broad bean wilt virus 2* Korean isolates used in this study

	Plant isolated from	Isolates	Area collected	Year collected	NCBI accession No.	
					RNA-1	RNA-2
<i>Leguminosae</i>	<i>Vicia faba</i>	BB2	Namhae	2006	KC625492	KC625506
		BB5	Namhae	2006	KC625493	KC625507
		BB9	Namhae	2006	KC625494	KC625508
	<i>Pisum sativum</i>	P2	Jangheung	2007	KC625498	KC625512
		P3	Jangheung	2007	KC625499	KC625513
<i>Chenopodiaceae</i>	<i>Spinacia oleracea</i>	SP	Jangheung	2007	KC625505	KC625518
		SP1	Geomundo	2007	KC625503	KC625516
		SP2	Geomundo	2007	KC625504	KC625517
<i>Solanaceae</i>	<i>Capsicum annuum</i>	RP1	Cheongyang	2007	JX183221	JX183222
		RP2	Cheongyang	2007	JX183223	JX183224
		RP3	Yesan	2007	JX183225	JX183226
		RP4	Suwon	2007	JX183227	JX183228
		RP5	Yeongyang	2009	JX183229	JX183230
		RP6	Imsil	2010	JX183231	JX183232
		RP7	Suwon	2011	JX183233	JX183234
	<i>C. annuum</i> var. <i>gulosum</i> (Paprika)	PAP1	Gangjin	2010	KC625500	KC634010
		PAP2	Gangjin	2010	KC625501	KC625514
		PAP3	Gangjin	2011	KC625502	KC625515
	<i>C. annuum</i> var. <i>gulosum</i>	GP2	Hoengseong	2012	KC625495	KC625509
		GP4	Hoengseong	2012	KC625496	KC625510
		GP5	Hoengseong	2012	KC625497	KC625511

sativum), 3 isolates (SP, SP1 and SP2) from spinach (*Spinacia oleracea*), 7 isolates (RP1, RP2, RP3, RP4, RP5, RP6 and RP7) from red pepper (*Capsicum annuum* L.), 3 isolates (GP2, GP4 and GP5) from bell pepper (*C. annuum* var. *gulosum*), and 3 isolates (PAP1, PAP2 and PAP3) from paprika (*C. annuum* var. *gulosum*) (Table 1, Fig. 1). Virus isolates were inoculated to local lesion host, *Chenopodium quinoa*, reisolated from local lesions at least three times in succession, and propagated in *Nicotiana benthamiana*.

RT-PCR, cloning and sequencing. Total RNAs were extracted from infected leaf samples by easy-spin™ total RNA extraction kit (iNtRON, Korea) according to the manufacturer's instructions. RT-PCR was carried out two-steps procedure including RT using AMV reverse transcriptase (Promega, USA) and PCR using LA taq polymerase (TAKARA, Japan). Primer pairs for full-length nt sequence were designed group-specifically for RNA-1 and universally for RNA-2 (Table 2). End sequences of each

Table 2. Primer pairs for full genome sequencing of BBWV2 RNA-1 and RNA-2

Group	Primer	Sequence (5' → 3')	location	Size (nt)
I of RNA-1	BBWV2 1-1u	AAACAAACAGCTTTCGTTCCG	19-915	896
	BBWV2 1-1R	TTCCATGCATCGTGTGGCC		
	BBWV2 1-2F	ATTTCAGGCTCCATTAAAAAGAG	753-1701	948
	BBWV2 1-2R	TCTATCAACTTCTCCAAAGCC		
	BBWV2 1-3F	GAATGTGATTGCATGGTGGCT	1515-2400	885
	BBWV2 1-3R	CAAATGAAGTGGCTATGACCGT		
	BBWV2 1-4F	GTACACAAGGTTAAGGAGCA	2275-3203	928
	BBWV2 1-4R	CCAAGCACACTTGTGTTCTCA		
	BBWV2 1-3u	TGGGAG(A)TACAAGCAA(G)TGG C	2867-3888	1021
	BBWV2 1-5R	AAATGCCATTCTCTGGGG		
	BBWV2 1-6F	GAAGTGCCAGTTTTATGCCGT	3690-4626	936
	BBWV2 1-6R	TCATTCATGACACACTTGGGC		
	BBWV2 1-7F	GGAGGGATGTTTACCAGCT	4476-5252	776
	BBWV2 1-7R	CTATTGCGGAATTCCTTGGC		
	BBWV2 1-8F	TGAATCCAGATGGCACATGG	5078-5958	880
	BBWV2 1-8R	CCCTCACTACTGAAATTTACTTG		
II of RNA-1	BBWV2 1-1u	AAACAAACAGCTTTCGTTCCG	19-1173	1154
	BBWV2 1-1r	CTGAAATAGCTGCCATAGCAG		
	BBWV2 1-2f	TTTGCAGCGTGGATGCC	883-1981	1098
	BBWV2 1-2r	CTTGAGCCTGACCAAAAAGG		
	BBWV2 1-3f	GCTGATAGAGTTGCACAATTCAGT	1774-2850	1076
	BBWV2 1-3r	TACAAATACGCCCTTTGCCA		
	BBWV2 1-4f	GTGGAGCAGATGCAATTGAG	2603-3664	1061
	BBWV2 1-4r	GAAATACTTTGGGGAATTTCCC		
	BBWV2 1-5f	GATGTGGAACTTTGCCCAA	3495-4390	895
	BBWV2 1-5r	TTGCTCACTTCTCAAACCAAG		
	BBWV2 1-6f	CTTGATCGTGGTCCTGGTGA	4272-5355	1083
	BBWV2 1-6r	TCCATCTCAAATTTCTCAAGACTT		
	BBWV2 1-7f	TTGGAAATGGCAGAGGCAT	5239-5958	719
	BBWV2 1-7R	CCCTCACTACTGAAATTTACTTG		
Universal of RNA-2	BBWV2 2-1F	CAAACAGCTTTCGTTCCGAA	22-949	927
	BBWV2 2-1R	AAGCCATCTGCCACCAATTC		
	BBWV2 2-2F	GCCATGTCAGGTGCTGGA	801-1702	900
	BBWV2 2-1d	CGCATA(G)GTC(T)TCT(C)GTA(G)GCA(T)GA		
	BBWV2 2-2u	TACAA(T)TTG(A)CCTGGG(A)CCTAGC	1377-2496	1119
	BBWV2 2-2r	CATGTTCCCAACCTTTTCAATGT		
	BBWV2 2-3u	ATTT(C)TA(G)GATTTTACT(C)CTC(T)ATGAG	2348-3511	1163
	BBWV2 2-3d	AGGTCATGGAACCCATTTTAATGG		

Table 3. Database of the complete nucleotide sequences for BBWV isolates

Virus	Isolate	Host reported from	Country reported	NCBI accession No.	
				RNA1	RNA2
BBWV2	IP	<i>Capsicum annuum</i>	Japan	AB023484	AB018698
	ME	<i>Megaskepasma erythrochlamys</i>	Singapore	NC003003	NC003004
	IA	<i>Gentiana</i> spp.	Japan	AB051386	AB032403
	K	<i>Capsicum annuum</i>	Korea	AF144234	AF104335
	B935	<i>Vicia faba</i>	China	AF149425	AJ132844
	MB7	<i>Vicia faba</i>	Japan	AB013615	AB013616
	XJ14-3	<i>Lycopersicon esculentum</i> MILL	China	FN985164	HQ283389
	PatMMV ^a	<i>Pogostemon patchouli</i>	Japan	NC003975	NC003974
BBWV1	PV132	<i>Spinacia oleracea</i>	USA	AB084450	AB084451
	Ben	<i>Capsicum annuum</i>	Spain	AY781171	AY781172

^a Patchouli mild mosaic virus; an isolate of BBWV2

RNA segment were obtained with the 5' and 3' rapid amplification of cDNA ends (RACE) protocol (BM, Germany). cDNA clones containing the 5' end of the genomes were obtained using Xec primers (5'-AAAGAATTCCCCCCCC CCCCC-3'), and BBWV2 5'-RNA1 (5'-GCCATYTCAT TGGCATGGAAAAAGC-3') and BBWV2 5'-RNA2 (5'-GAAGGGTGCAACGATATATGCAAATTC-3') primers complementary to nt 384-360 and 424-395, respectively, of BBWV2 stain. In addition, cDNA clones containing the 3' end of the genomes were obtained using BBWV2 3'-RNA1 (5'-AGGCATATGAGAGAGGAGAG-3') and BBWV2 3'-RNA2 (5'-GAAATGGGGAATGTTTCATGAGTA-3') primers complementary to nt 5646-5665 and 3269-3291, respectively, and anchor primers (5'-GACCACGCGTATCGAT GTCGACTTTTTTTTTTTTTTTTTTV-3'). RT-PCR amplified DNA fragments were purified using MEGA quick-spin™ Kit (iNtRON, Korea) and cloned into pGEM-T easy vector (Promega, USA) according to the manufacturer's instructions followed by transformation into *E. coli* DH5α strain. Sequencing was done by a commercial company (Genotech, Korea). The resultant sequences were assembled using DNA Star v. 5.02 and have been submitted to GenBank database with accession numbers listed in Table 1.

Sequence and phylogenetic analyses. The complete nt sequences and the deduced amino acid sequences were aligned using Geneious method and ClustalW method in Geneious pro 5 and compared with the previously reported isolates, e.g. IP (Japan, Kobayashi et al., 1999) and ME (Singapore, Koh et al., 2001) isolates for Group I and IA (Japan, Kuroda et al., 2000), K (Korea, Lee et al., 2000), B935 (China, Qi et al., 2000a; 2000b), MB7 (Japan, Nakamura et al., 1998), XJ14-3 (China, unpublished) isolates and PatMMV (Japan, Ikegami et al., 1998; Ikegami et al., 2001) for Group II and with BBWV1 isolates, PV132 and Ben as

outgroup (Table 3). Phylogenetic analyses were performed employing maximum likelihood method implemented in the MEGA 5 (Tamura et al., 2011). All phylogeny tests were done by using the best substitution model for nucleotides/amino acids and 1,000 bootstrap replicates.

Recombination and reassortment analysis. Detection of recombination between 29 BBWV2 isolates was processed by RDP3 program (Recombination Detection Program, ver. 3). The previously saved mega format files which aligning sequences of 29 BBWV2 RNA-1 and RNA-2 segments were opened by RDP3. Then the detection of recombination among those segments, between all RNA-1 segments each, between all RNA-2 segments each and between RNA-1 and -2 each other. Used methods of recombination detections are RDP, GENECONV, BOOTSCAN, MaxChi, Chimaera, and SISCAN methods implemented in the RDP3 program. Parameter was set to default options following the manufacture's recommendation. Then recombination events were computed with highest acceptable P-Value set to 0.01.

Results

Genome characterization of BBWV2 Korean isolates.

To investigate the genetic diversity of BBWV2 Korean isolates, twenty-one BBWV2 isolates were collected from 6 natural plants (broad bean, pea, spinach, red pepper, bell pepper, and paprika) in 10 areas during the years 2006–2012: The full-genome sequences of 7 isolates from red pepper from our previous study (Kwak et al., 2013) along with full-genome sequences of 14 additional isolates determined in this study were summarized in Table 1. The representative symptoms include severe mosaic, vein clearing, stunting on the infected leaves and irregularly shaped coloring, chlorotic spot, and mosaic on the infected fruits

Fig. 2. Symptoms induced by BBWV2 on various host plants infected naturally in Korea. (A) severe mosaic, stunting and leaf curling on Broad bean, (B) mosaic on pea leaves, (C) mosaic on spinach leaves, (D) vein clearing and mosaic on red pepper leaves, (E) severe mosaic on leaves and irregularly shaped coloring on paprika fruits and (F) chlorotic spot and mosaic on bell pepper fruits.

(Fig. 2). The RNA-1 segment of BBWV2 Korean isolates consisted of 5950 to 5956 nt encoding 5 mature proteins including Co-pro (353 or 354 amino acids depending on subgroups), NTBM (582 amino acids), VPg (26 amino acids), Pro (209 amino acids), and RdRp (699 amino acids). The RNA-2 segment consisted of 3568 to 3604 nt encoding 3 mature proteins including MP (465 amino acids), LCP (402 amino acids), and SCP (197 amino acids). The sequences are available in the GenBank database with accession numbers listed in Table 1.

Phylogenetic relationship analysis. The complete nt and deduced amino acid sequences of 21 BBWV2 Korean isolates were analyzed by comparing with eight previously reported BBWV2 isolates (IP, IA, ME, MB7, K, PatMMV, B935, and XJ14-3). Two BBWV1 isolates (PV132 and Ben) were included as outgroup isolates in the phylogenetic analyses. Phylogenetic trees reconstructed using the complete nt sequences revealed that BBWV2 Korean isolates could be divided largely into two groups, Group I and Group II, in both RNA-1 and RNA-2 (Fig. 3). In addition, each group could be subgrouped according to the host plants and/or collected areas. Group I included two isolates (IP and ME) while Group II included six isolates (B935, IA, K, MB7, PatMMV and XJ114) for the RNA-1- and RNA-2-based phylogenetic trees (Fig. 3). For the RNA-1

segment of Korean isolates, four isolates (BB5, RP3, P2, and P3) were classified as Group II and the remains were classified as Group I. Meanwhile, the phylogenetic tree of the RNA-2 segment showed that two Korean isolates, BB2 and RP7, were classified as Group I and the remaining nineteen Korean isolates were classified as Group II. Furthermore, Group II of RNA-2 could be divided into 3 subgroups of II-a, II-b, and II-c. The nineteen Korean isolates, excluding BB2 and RP7, belonged to subgroup II-a along with the Chinese isolate B935.

The phylogenetic trees reconstructed based on the amino acid sequences of the mature proteins which are Co-Pro, NTBM, Pro, RdRp, LCP, and SCP, showed consistent grouping compared with the nucleotide sequence-based trees, but the trees of VPg and MP amino acids showed different patterns in grouping (Fig. 4). The BBWV2 VPg is composed of a short sequence of 26 amino acids and has little variation among isolates. Interestingly, the isolate K and Chinese isolate XJ14-3 were separately grouped in the trees reconstructed with the Co-Pro or MP amino acid sequences while these two isolates were most closely grouped in the RNA-2 nucleotide-based tree. The majority of RNA-1 genomes of the Korean isolates were grouped into Group I while their RNA-2 genomes were included in Group II. Thus, according to the group-combinations between RNA-1 and RNA-2 segments, BBWV2 could be

Fig. 3. Phylogenetic trees reconstructed using the complete nucleotide sequences of RNA-1 or RNA-2 of the BBWV2 isolates. The Maximum likelihood method implemented in MEGA 5 was used to reconstruct the phylogenetic trees.

designated as three genetic subpopulations of GS-I (both RNA-1 and RNA-2 genomes belong to Group I), GS-II (both RNA-1 and RNA-2 genomes belong to Group II) and GS-III (RNA-1 and RNA2 genomes belong to Group I and Group II, respectively). Based on this categorization, four isolates (BB2, RP7, IP, and ME) were classified to GS-I, nine isolates (BB5, P2, P3, RP3, B935, IA, MB7, PatMMV, K, and XJ14-3) to GS-II, and the other 15 Korean isolates (BB9, SP, SP1, SP2, RP1, RP2, RP4, RP5, RP6, PAP1, PAP2, PAP3, GP2, GP4, and GP5) to GS-III. The isolates belonging to GS-III have been detected newly and predominantly in Korea.

Sequence comparison. The nucleotide and amino acid sequence identities between BBWV2 isolates were analyzed. The results are summarized in Table 4 and Table 5. For the nt sequences of RNA-1, BB2, which belonged to Group I, showed 98.5% and 96.5% nt sequence identities with SP2 and IP, respectively, which belonged to the same group (Fig. 3). On the other hand, BB2 exhibited 78.7% and 78.2% nt sequence identities with BB5 and K, respectively, which belonged to Group II. In the mean time, BB2 showed 62–63% nt sequence identity with the BBWV1 isolates (Table 4). For the nt sequence of RNA-2 segment, BB2 showed 90.9% and 91.4% nt sequence identities with RP7 and IP,

respectively, while it exhibited 78–81% nt sequence identity with most of isolates in Group II (Table 5).

For the viral proteins encoded in RNA-1, the amino acid sequence identities between BB2 (as a representative of the Group I isolates) and the Group II isolates were 70–75% for Co-Pro, 90–92% for NTBM, 92% for VPg, 92–93% for Pro, and 91–92% for RdRp. The amino acid identity between two groups for Co-Pro was lower than those of the other proteins. Indeed, the amino acid alignment showed that the Co-Pro region contains high amino acid variations among isolates (Fig. 5). For the viral proteins encoded in RNA-2, the amino acid sequence identities between BB2 and the Group II isolates were 85–88% for MP, 85–94% for LCP, and 90–94% for SCP. In addition, the nt sequence identities between BB2 and the Group II isolates for the 5 non-coding region (NCR) and 3 NCR of RNA-2 were relatively lower (68–73% and 69–76%, respectively) than those for the 5 NCR and 3 NCR of RNA1 (74–87% and 85–89%, respectively).

Recombination and reassortment. Our phylogenetic analyses provided some clues for the possible recombination and reassortment events in the BBWV2 population: i) the inconsistent grouping of the isolate K and Chinese isolate XJ14-3 in the nucleotide or amino acid sequence-based

amine whether recombination and reassortment events have occurred in the BBWV2 population. To this end, the

Table 4. The nucleotide and amino acid sequence identities (%) between RNA-1 of the BBWV2 Korean isolate BB2 and RNA-1 of other BBWV isolates

Virus	Isolate	full genome (nt)	5' NCR (nt)	Co-Pro (aa)	NTBM (aa)	VPg (aa)	Pro (aa)	RdRp (aa)	3' NCR (nt)
BBWV2	BB5	78.7	86.8	73.4	91.1	92.3	93.3	91.2	87.2
	BB9	98.4	97.8	98.3	99.5	92.3	97.6	99.1	99.1
	P2	78.9	86.9	73.7	91.4	92.3	93.3	91.7	88.5
	P3	78.8	86.9	73.7	91.6	92.3	93.3	91.6	88.5
	SP	93.2	94.3	94.6	98.5	96.2	98.6	98.3	97.4
	SP1	98.4	97.8	98.3	99.0	100	100	99.3	98.3
	SP2	98.5	97.8	98.0	99.3	100	99.5	99.3	98.3
	RP1	95.8	97.4	95.8	98.8	100	99.0	98.7	99.1
	RP2	95.1	96.1	96.0	98.5	100	99.5	98.4	97.4
	RP3	78.9	86.9	73.7	91.2	92.3	93.3	91.7	89.4
	RP4	92.7	95.7	92.1	98.3	96.2	98.6	98.1	98.3
	RP5	95.9	96.1	96.6	99.3	100	99.0	99.0	96.6
	RP6	92.8	93.1	93.8	98.3	96.2	98.1	98.6	99.1
	RP7	94.7	96.1	95.2	99.0	100	99.5	98.1	96.6
	PAP1	93.1	95.7	94.1	98.6	96.2	98.1	98.0	98.0
	PAP2	96.6	96.5	97.2	99.1	100	100	98.9	99.1
	PAP3	93.1	94.3	95.5	98.8	96.2	98.1	97.9	98.3
	GP2	95.7	97.8	97.5	99.1	100	99.0	98.3	98.3
	GP4	95.7	97.8	97.5	99.1	100	99.0	98.4	98.3
	GP5	95.7	97.8	97.5	99.1	100	99.0	98.4	98.3
	IP	96.5	98.7	96.9	99.3	96.2	99.0	98.6	97.4
	ME	90.2	91.7	90.9	97.6	92.3	94.3	96.9	88.9
	IA	78.5	86.4	75.7	92.1	92.3	92.8	91.8	88.5
	MB7	79.5	86.9	74.0	92.1	92.3	92.3	91.7	86.0
	PatMMV	79.5	87.3	73.7	91.8	92.3	92.8	92.4	87.6
	B935	78.8	86.4	74.0	91.9	92.3	93.8	91.8	88.5
	XJ14-3	79.1	87.2	73.4	91.4	92.3	92.8	92.4	89.1
	K	78.2	74.4	70.4	90.7	92.3	93.3	91.0	85.7
BBWV1	Ben	63.2	65.7	36.8	67.2	57.7	58.9	68.0	55.4
	PV132	62.9	67.2	38.9	67.4	53.8	59.8	67.2	53.3

full-length nt sequences of RNA-1 and RNA-2 of 29 BBWV2 isolates were analyzed using RDP, GENECONV, BOOTSCAN, MaxChi, Chimaera, and SISCAN methods implemented in the RDP3 program with a highest acceptable P-Value set to 0.01. The analyses detected two recombination events in the analyzed BBWV population (Table 6). As expected from the phylogenetic analyses, one recombination event in RNA-1 genome was found to be occurred in the isolate K (Table 6). K was detected as a recombinant between XJ14-3 as a major parent and MB7 as a minor parent. In this recombination event, the genomic region (nt 286-2142) of XJ14-3 was replaced with the homologous region of MB7. In addition, the other recombination event was detected in the RNA-2 genome of XJ14-3, which was found to be a recombinant between K as a major

parent and IP as a minor parent. This result could explain why XJ14-3 and IP belonged to the same group in the MP amino acid-based phylogenetic tree. However, no recombination events were found in the BBWV2 Korean isolate population. Therefore, it is likely that genetic exchange by recombination is infrequent in natural populations of BBWV2.

On the other hand, out of 29 isolates, sixteen isolates were analyzed as reassortments (Table 7). This result is in agreement with the phylogenetic grouping by the group-combinations between RNA-1 and RNA-2 segments (i.e. three genetic subpopulations, GS-I, GS-II and GS-III). Four isolates (BB2, RP, IP, and ME), which belonged to GS-I, were reassortants having RNA-1 of the isolate SP (as a parental isolate of RNA-1) and RNA-2 of the isolate P2 (as

Table 5. The nucleotide and amino acid sequence identities (%) between RNA-2 of the BBWV2 Korean isolate BB2 and RNA-2 of other BBWV isolates

Virus	Isolate	full genome (nt)	5' NCR (nt)	MP (aa)	LCP (aa)	SCP (aa)	3' NCR (nt)
BBWV2	BB5	79.7	73.8	85.8	93.5	93.9	75.1
	BB9	79.5	73.2	86.2	93.3	94.4	74.9
	P2	79.8	72.7	86.7	91.8	93.4	75.4
	P3	79.9	72.3	86.7	93.8	93.9	75.4
	SP	79.9	72.7	86.9	94.0	93.9	75.4
	SP1	79.6	72.5	86.2	93.5	93.9	74.3
	SP2	79.6	72.5	85.8	93.8	93.9	75.4
	RP1	79.6	70.0	86.9	92.6	93.9	76.5
	RP2	79.3	70.4	86.2	93.3	93.9	76.5
	RP3	79.1	70.5	86.0	92.8	93.9	75.4
	RP4	79.3	71.7	86.9	93.5	93.9	74.3
	RP5	79.7	72.0	87.5	93.8	92.9	75.4
	RP6	79.8	71.7	86.5	94.5	93.4	73.7
	RP7	90.9	85.3	94.6	97.5	97.5	86.9
	PAP1	79.7	72.3	86.2	93.8	93.4	76.0
	PAP2	79.5	71.5	86.2	94.3	92.9	76.0
	PAP3	79.9	72.3	86.2	94.3	93.4	75.0
	GP2	79.3	71.2	86.7	94.3	93.4	76.0
	GP4	79.3	71.2	86.7	94.3	93.4	76.0
	GP5	79.2	71.2	86.7	94.0	93.4	75.4
	IP	91.4	88.6	95.7	98.0	97.5	86.8
	ME	87.4	78.8	92.3	93.8	93.4	66.3
	IA	78.6	73.3	84.1	93.8	90.9	74.0
	MB7	78.9	71.2	84.7	94.0	92.4	72.8
	PatMMV	78.9	71.6	86.2	85.1	91.9	73.5
	B935	79.1	72.0	86.1	93.8	91.9	75.4
	XJ14-3	81.6	70.7	92.0	93.3	92.9	71.4
	K	78.6	68.2	88.8	93.5	92.9	69.9
BBWV1	Ben	59.1	58.8	52.4	63.4	59.4	50.0
	PV132	58.4	62.4	53.1	63.7	57.9	51.4

Table 6. Recombination in the BBWV2 population detected by recombination detection programs

Genome	Recombination site	Recombinant	Parental isolates ^a	RDP3 (P-value) ^b
RNA1	nt 286-2142	K	XJ14-3 × MB7	R GBMCS (7.8×10^{-69})
RNA2	nt 484-1399	XJ14-3	K × IP	R GBMCS (3.3×10^{-33})

^a Parental isolates tested by recombination detection among analyzed isolates; Major parent × minor parent.

^b RDP3-implemented methods supporting the corresponding recombination sites; R (RDP), G (GENECONV), B (BOOTSCAN), M (MAXCHI), C (CHIMAERA), and S (SISCAN). The reported *P*-value in parentheses is the greatest *P*-value among the calculated *P*-values using RDP3-implemented methods and the corresponding method is shown boldface.

a parental isolate of RNA-2). Five isolates (BB5, P2, P3, RP3, and B935), which belonged to GS-II, were reassortments having RNA-1 of XJ14-3 and RNA-2 of SP as parental isolates. PatMMV was found to have MB7 RNA-1 and IA RNA-2 as parental isolates. Six isolates out of 15,

which belonged to GS-III, were determined as reassortants between the isolates in the same group GS-III. In conclusion, our results suggest strongly that reassortment is a major evolutionary force in the genetic diversification of BBWV2 population in Korea.

Table 7. Reassortment in the BBWV2 population detected by recombination detection programs

Reassortant ^a	Parental isolates ^b		RDP3 (P-value) ^c
	RNA1	RNA2	
BB2, RP7, IP, ME ^d	SP	Unknown (P2)	RGBMCS (3.2×10^{-247})
BB5, P2, P3, RP3, B935	XJ14-3	SP	RGBMCS (5.4×10^{-142})
PatMMV	MB7	IA	RBMC S (5.0×10^{-30})
SP, PAP1	RP6	PAP3	RGBMCS (9.1×10^{-21})
SP1, SP2 ^d	RP1	Unknown (RP2)	RMCS (6.4×10^{-12})
BB9 ^e	SP1	RP6	MC (2.4×10^{-18})
PAP2	BB9	SP	RGBMCS (2.1×10^{-27})

^aSome reassortants seemed to be ion programshe corresponding method is shown boldface. (Gd, ome reassortants seemed to be ion programshe corresponding me; e, reassortants seemed to be ion programshe c3 methods.

^be, reassortants seemed to be ion programshe c3 methodsing method is sho

^cRDP3-implemented methods supporting the corresponding reassortments; R (RDP), G (GENECONV), B (BOOTSCAN), M (MAXCHI), C (CHIMAERA), and S (SISCAN). The reported *P*-value in parentheses is the greatest *P*-value among the calculated *P*-values using RDP3-implemented methods and the corresponding method is shown boldface.

Discussion

Major sources of evolution of plant RNA viruses are known as recombination that occurs in either segmented or unsegmented viruses and reassortment that occurs only in viruses with segmented RNAs, as well as mutation that accumulates by error prone replication mechanism (Garcia-Arenal et al., 2001; Worobey et al., 1999). Recombination has been observed in many plant viruses, such as luteoviruses (Gibbs, 1995), bromoviruses (Allison et al., 1989), nepoviruses (Le Gall et al., 1995), cucumoviruses (Fraile et al., 1997), comoviruses (Zhang et al., 2007; Boulila, 2007), and potyviruses (Seo et al., 2009; Jonson et al., 2009). The potential involvement of recombination in evolution of BBWV2 was reported by Ferrer et al. (2011) based on phylogenetic analyses of partial sequences of BBWV2 isolates collected from various regions.

In this study, we sought to investigate the possible origin, diversity, and genetic structure of BBWV2 Korean population by analyzing full genome sequences of BBWV2 isolates. To this end, we have determined complete genome sequences of 21 BBWV2 isolates collected from various host plants and analyzed their genetic diversity by comparing with previously reported sequences of 8 BBWV2 isolates (Tables 1 and 2). Based on phylogenetic analyses, BBWV2 Korean isolates were divided into two groups for RNA-1 and RNA-2 segments, respectively (Fig. 3). Most of the Korean isolates, excluding BB2 and RP7, subgrouped into II-a together with B935. The Group II-a isolates could be further divided according to the collected areas rather than their host plants. For example, SP isolated from spinach in Jangheung had 99% nt sequence identity with P2 and P3 isolated from pea in Jangheung, while SP had 93% identity with SP1 and SP2 isolated from spinach in Geo-

mundo. Phylogenetic trees reconstructed using amino acid sequences of the mature proteins (excluding Co-Pro and MP) showed consistent grouping compared with the nt sequence-based trees (Fig. 4). Especially, isolates K and XJ14-3 showed high nt identities of 90–91% and amino acid identities of 97–100% in the same group, but they showed relatively low amino acid identities of 84% and 92% for amino acid sequences of Co-Pro and MP, respectively, and were grouped separately (Fig. 4).

One of the remarkable features of the BBWV2 Korean isolates compared to the previously reported isolates was group-combination between RNA-1 and RNA-2 segments. The isolates composed of Group I RNA-1 and Group II RNA-2 were predominant in Korea. Group-combination between RNA segments that belong to different groups could be explained by reassortment. To confirm the result of phylogenetic analyses, we investigated whether recombination and reassortment have occurred in BBWV2 population using the RDP3 program. Only two recombination events were detected in the analyzed BBWV2 population: One at Co-Pro and NTBM regions of RNA-1 of Korean isolate K and another one at MP region of RNA-2 of Chinese isolate XJ14-3 (Table 6). No recombination events were found in BBWV2 Korean isolates. The relatively high sequence variation in Co-Pro and MP regions suggests that these regions are under looser evolutionary constraints and vulnerable to mutation and recombination. In addition, our phylogenetic analyses indicate that the isolates collected in Korea during the years 2006–2012 might not be originated from the Korean isolate K reported in 2000.

Meanwhile, 16 isolates out of 29 were found to be reassortants (Table 7). This result is highly consistent with group-combination based subpopulation of GS-I, GS-II and GS-III. Four GS-I isolates (BB2, RP7, IP and ME) were

Fig. 5. Alignment of the amino acid sequences of Co-Pro of the BBWV2 isolates. Sequences were aligned by the geneious method. Same amino acid sequences are indicated with a ‘-’. Arrow indicates the amino acid position 118 on the polypeptide precursor.

reassortments having SP RNA-1 and P2 RNA-2, and five GS-II isolates (BB5, P2, P3, RP3 and B935) were reassortments having XJ14-3 RNA-1 and SP RNA-2. Six Korean isolates belonged to GS-III were determined as reassortments between the Korean isolates in the same group GS-III (Table 7). Japanese isolate PatMMV, which was detected as a reassortant, might be originated by reassortment between Japanese isolates (i.e. MB7 and IA). Collectively, our results revealed that BBWV2 Korean isolates have been evolved mainly by reassortment rather than recombination. In the case of *Bean pod mottle virus* (BPMV; genus *Comovirus*) having similar genome structures with BBWV2, reassortment events between distinct BPMV strains and recombination events at the putative helicase region of RNA-1 were reported (Zhang et al., 2007). In addition, Co-

Pro and the C-terminal domain of the putative helicase of BPMV were identified as symptom severity determinants (Gu et al., 2005). Since we also found high sequence variations in Co-Pro (Fig. 5), it is worthy to examine whether Co-Pro of BBWV2 is also a pathogenicity determinant in the BBWV2-pepper pathosystem. In the present study, we have analyzed the genetic diversity and structure of the BBWV2 population mainly collected from East Asia. Our findings revealed that reassortment occurred naturally in the BBWV2 population, and might play an important role in disease incidence occurred recently in Korea. Further analyses of genetic population structure of BBWV2 with more diverse full-genome sequences of worldwide isolates will be required for in-depth understanding of the evolutionary history of BBWV2.

Acknowledgements

This research was carried out with the support of “Cooperative Research Program for Agricultural Science & Technology Development (Project No. PJ00651901)” of the Rural Development Administration and iPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries: No. 110034-05-3-HD110), Ministry for Food, Agriculture, Forestry, and Fisheries, Republic of Korea.

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