A *Bean common mosaic virus* isolate exhibits a novel pathogenicity profile in common bean, overcoming the *bc-3* resistance allele coding for the mutated eIF4E translation initiation factor

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Abstract

Resistance against *Bean common mosaic virus* (BCMV) in *Phaseolus vulgaris* is governed by six recessive resistance alleles at four loci. One of these alleles, *bc-3*, is able to protect *P. vulgaris* against all BCMV strains and against other potyviruses; *bc-3* was identified as the *eIF4E* allele carrying mutated eukaryotic translation initiation factor gene. Here, we characterized a novel BCMV isolate 1755a that was able to overcome *bc-2* and *bc-3* alleles in common bean. Thus, it displayed a novel pattern of interactions with resistance genes in *P. vulgaris*, and was assigned to a new pathogroup, PG-VIII. The IVT7214 cultivar supporting the replication of BCMV-1755a was found to have the intact homozygous *bc-3* CAPS marker and corresponding mutations in the *eIF4E* allele that confer resistance to BCMV isolates from all other pathogroups as well as to other potyviruses. The VPg protein of 1755a had seven amino acid substitutions relative to VPgs of other BCMV isolates unable to overcome *bc-3*. The 1755a genome was found to be a recombinant between NL1, US1 (both PG-I), and a yet unknown BCMV strain. Analysis of the recombination patterns in the genomes of NL1 and US1 (PG-I), NY15P (PG-V), US10 and RU1-OR (PG-VII), and 1755a (PG-VIII), indicated that P1/HC-Pro cistrons of BCMV strains may interact with most resistance genes. This is the first report of a BCMV isolate able to overcome the *bc-3* resistance allele, suggesting that the virus has evolved mechanisms to overcome multiple resistance genes available in common bean.

Introduction

Bean common mosaic virus (BCMV) is an important pathogen of common bean (*Phaseolus vulgaris* L.). It is transmitted by aphids in a non-persistent manner, and can also be efficiently transmitted through seed (Morales and Bos, 1988; Flores-Estevez et al., 2003; Singh and Schwartz, 2010). The resistance to BCMV in common bean is governed by seven resistance alleles: one dominant *I*-allele and six recessive, *bc-u*, *bc-1*, *bc-1*^{2}, *bc-2*, *bc-2*^{2}, and *bc-3* alleles (Drijfhout, 1978; Kelly et al., 1995). Two recessive alleles, *bc-u* and *bc-3,* when present together even in the absence of the *I* allele, confer broad resistance against BCMV strains, and against other potyviruses, like *Bean common mosaic necrosis virus* (BCMNV) and *Clover yellow vein virus* (ClYVV) (Drijfhout, 1978; Drijfhout and Morales, 2005; Hart and Griffiths, 2013).

BCMV exists as a complex of strains that can be differentiated by their ability to overcome the individual recessive alleles or their combinations (Drijfhout, 1978; Drijfhout and Morales, 2005). Based on their biological responses on bean differential hosts carrying different combinations of these resistance genes, BCMV isolates are classified into seven pathogroups (PGs), numbered from I to VII (Drijfhout, 1978; Drijfhout and Morales, 2005; Larsen et al., 2005). BCMV is a member of the genus *Potyvirus*, family *Potyviridae* (Adams et al., 2011). It has a single-stranded, positive-sense RNA genome of ca. 10-kb excluding the 3'-terminal poly(A), with a covalently-linked VPg protein at the 5'-terminus (Morales and Bos, 1988; Adams et al., 2011). The BCMV genome encodes a single polyprotein which is cleaved co-translationally and post-translationally by three virus-specific proteases into 10 mature proteins (Adams et al., 2005). A single capsid protein (CP) encapsidates virus RNA forming filamentous particles of ca. 720-770 nm long and 12-15 nm wide (Morales and Bos, 1988). Recently, BCMV isolates were found to display substantial genome sequence diversity (Larsen et al., 2005;

Naderpour et al., 2009; Feng et al., 2014a,b; Martin et al., 2014). Specifically, several BCMV isolates related to the RU1 strain group revealed recombinant genomes, and these recombination events were hypothesized to result in biological changes shifting their pathotype specificity (Feng et al., 2014a,b). In particular, the genome region spanning the P1 and HC-Pro cistrons was hypothesized to be involved in interactions with the *bc-2*^{2} gene in common bean, thus defining the pathotype VII of BCMV (Feng et al., 2014a,b). However, specific mechanisms facilitating interactions with most of the resistance genes and genetic determinants of BCMV involved in these interactions are not known.

The only allele with a known mechanism of resistance to BCMV in common bean is *bc-3*, identified as the *eIF4E* allele carrying a mutated eukaryotic translation initiation factor gene (Naderpour et al., 2010). A cleaved amplified polymorphic sequence (CAPS) marker co-segregates with the *bc-3* allele; this polymorphism was due to a mutation in the *eIF4E* allele (Naderpour et al., 2010). The mutated *eIF4E* allele conferred resistance to BCMV and at least two other potyviruses, BCMNV and ClYVV (Naderpour et al., 2010; Hart and Griffiths, 2013). The *eIF4E* allele-mediated recessive resistance has been studied for several potyviruses infecting pepper, lettuce, potato, cereals, and cucurbits (Nicaise et al., 2003; Kang et al., 2005a; Abdul-Razzak et al., 2009; Borgstrom and Johansen, 2001; Bruun-Rasmussen et al., 2007; Charron et al., 2008; Moury et al., 2004; Kuhne et al., 2003; Kanyuka et al., 2004). Mutations in genes coding for translation initiation factors eIF4E and eIF(iso)4E resulted in multiple recessive resistance genes conferring effective resistance to potyviruses in both monocots and dicots (cf. Kang et al., 2005b; Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012).

In the common bean/BCMV pathosystem, *bc-3* conferred complete resistance to all strains and pathotypes of BCMV as well as to BCMNV and ClYVV, when present in a

homozygous state and in combination with the *bc-u* helper allele (Drijfhout, 1978; Drijfhout and Morales, 2005; Naderpour et al., 2010; Hart and Griffiths, 2013). Because of its ability to confer complete, strain non-specific resistance against BCMV and other legume-infecting potyviruses, the *bc-3* allele has been introduced by breeders into common bean germplasm to protect different market classes of dry and snap beans against BCMV (Drijfhout, 1978; Kelly et al., 1995; Singh and Schwartz, 2010; Hart and Griffiths, 2013).

Here, we describe the unique field isolate BCMV-1755a collected in the Willamette Valley, OR, in the summer of 2013. Comparison of the biological, serological, and molecular properties of this isolate with other known BCMV isolates suggested that, unlike any known BCMV strain, BCMV-1755a can overcome both the *bc-2* and *bc-3* alleles, and, thus, represents a new pathogroup, PG-VIII. The genome of BCMV-1755a as well as genomes of the two reference isolates, US1 from PG-I, and NY15P from PG-V, were found to be recombinant with extended sections of the genome having homologous sequences. This is the first report of a BCMV isolate able to overcome the *bc-3* resistance allele.

Materials and Methods

Virus sources and maintenance

All reference BCMV and BCMNV isolates used in this work originated from the USDA-ARS Prosser, WA, collection, and were described previously (Feng et al., 2014a,b). A field isolate of BCMV, named 1755a, was collected in Corvallis, OR, from the field-grown common bean accession 91-1755 exhibiting symptoms of mosaic and leaf deformation. This line is one of 59 accessions that were collected by Mike Dickson (Professor Emeritus, Cornell University) in China in 1991 and deposited in the USDA National Plant Germplasm System (NPGS) plant

introduction system in 1995 where it has been stored informally. These accessions were first grown in the field at Oregon State University in 2009. No virus symptoms were observed in 91-1755 at that time, but several other accessions exhibited classic BCMV mosaic mottle symptoms. When the collection was grown again in 2012, severe virus symptoms were observed in a number of lines, suggesting that the virus had spread during the initial grow out. After confirmation that isolate 1755a displayed BCMV-specific reactivity in ELISA, it was subjected to further characterization at the Plant Virology Laboratory at University of Idaho. All virus isolates were propagated in the bean cultivar 'Dubbele Witte' using mechanical inoculation and then maintained under greenhouse conditions. BCMV isolates US1, NY15P, and 1755a, and the BCMNV isolate TN1 were propagated and purified using the purification protocol described previously (Feng et al., 2014a).

Biological and serological characterization

The biological characterization of all three isolates on a set of bean differentials (Drijfhout, 1978) was performed as described previously (Feng et al., 2014a,b). This set of bean differentials represented the various host groups (HG) of common bean and included (Table 1) cultivars 'Dubbele Witte' (DW, HG 0), 'Stringless Green Refugee' (SGR, HG 1), 'Redlands Greenleaf C' (RGLC, HG 2), 'Redlands Greenleaf B' (RGLB, HG 3), 'Sanilac' (HG 4), UI 35 (HG 6), 'IVT7214' (HG 7), 'Jubila' (HG 9), 'Amanda' (HG 10), 'US1006' (HG 11), and 'IVT7233' (HG 11). Three reference isolates, BCMV-US1 (PG-I), BCMV-NY15P (PG-V), and BCMNV-TN1 (PG-VI), were included in this analysis as controls. All eleven bean differential lines carrying different resistance gene combinations (Table 1) were mechanically inoculated with each isolate (three plants per cultivar), and plants were placed in the greenhouse with

standard summer-time growth conditions (16-hr day photoperiod and daytime/nighttime temperatures of 25/20ºC). Symptoms were recorded at 4 weeks post-inoculation, and concomitantly, samples were collected for triple-antibody sandwich (TAS) ELISA testing as described elsewhere (Feng et al., 2014a). Experiments were repeated three times.

Cloning strategy, sequencing, and sequence analysis

For all three isolates, 1755a, US1, and NY15P, the whole-genome cloning strategy, sequencing, and sequence analysis were conducted as described previously (Feng et al., 2014a). Briefly, isolates were subjected to a RT-PCR amplification with degenerate primers targeting the HC-Pro, CI, and NIb-CP regions (Ha et al., 2008), which were amplified and sequenced after AT-cloning. These initial sequenced contigs were later linked through additional RT-PCR reactions using specific primers from the three contigs, followed by cloning and sequencing using primer-walking; the 5'-terminal sequence was determined through the use of the 5'-RACE approach as described previously (Feng et al., 2014a). The RNA extraction, RT-PCR, cloning and sequencing steps were performed according to the protocol described in Feng et al. (2014a). All primers used in this study for RT-PCR amplifications and sequencing are listed in Supplementary Table 1. The complete viral genomes were assembled using SeqMan (DNASTAR, Madison, WI). The sequences for NY15P, US1, and 1755a genomes have been deposited in the GenBank database and will appear under the accession numbers KT175568, KT175569, and KT175570, respectively. All sequences were initially analyzed using the BLASTn 2.2.17 (Altschul et al., 1997) tool available at the National Center for Biotechnology Information (NCBI). Open reading frames (ORFs) were identified using the ORF Finder program available at NCBI. Complete sequences of BCMV isolates were aligned using

ClustralX Ver. 2.0 (Conway Institute, UCD, Dublin). Further analysis was conducted with the Recombination Detection Program v.4.16 (RDP4) (Martin et al., 2005).

gDNA extraction, CAPS marker eIF4E-RsaI *analysis, and* eIF4E *sequencing*

gDNA was extracted from 0.1 g of young leaf tissue using the CTAB methodology as described in Naderpour et al. (2010). For CAPS analysis, a 541-bp fragment of *eIF4E* was amplified by PCR (Naderpour et al., 2010), using 1 µL of gDNA extract in a 25 µL reaction volume containing Taq-buffer (Genscript, Piscataway, NJ), 0.4m M of each of the oligonucleotide primers, 0.2 µM ENM-FWe and ENM-RVe primers (Naderpour et al., 2010) and 1.25 units of Taq DNA polymerase (Genscript). Amplification included 30 cycles: 20 s of denaturation at 94 °C, 20 s of annealing at 55°C and 35 s of elongation at 72°C in a thermal cycler (Mastercycler S-Pro, Eppendorf) after an initial denaturation at 95 °C for 2 min. Eight µL of PCR product were subsequently subjected to *Rsa*I (New England Biolabs, Ipswich, MA) digestion in a 20 µL reaction volume and analyzed by agarose gel electrophoresis.

The 541-bp fragments amplified from the gDNA extracted from bean cultivars 'SGR' and 'IVT'7214' were cloned into the T-Easy plasmid vector (Promega, Madison, WI). Three independent clones for each recombinant plasmid were selected and subjected to sequencing at the Genewiz laboratory (South Plainfield, NJ) from both ends of the cloned insert using plasmid-specific primers. The sequences were assembled using SeqMan (DNASTAR, Madison, WI) and analyzed using the BLASTn 2.2.17 (Altschul et al., 1997) tool available at the NCBI. Sequences of eIF4E were aligned using ClustralX Ver. 2.0 (Conway Institute, UCD, Dublin). The partial sequences of the *eIF4E* genes for 'SGR' and 'IVT7214' were deposited in the GenBank database under accession numbers KT175571 and KT175572, respectively.

Results

BCMV-1755a is serotype B isolate that overcomes bc-2 *and* bc-3 *alleles*

When screened on the eleven bean differentials, 1755a, US1, and NY15P, induced typical mosaic mottle, raised dark green blistering, leaf deformation, and often growth retardation in susceptible differentials. Based on the pathogenicity profiles exhibited on differentials of our two control isolates, US1 was classified as belonging to PG-I (as expected), while NY15P was classified as belonging to PG-V (as expected; see Table 1). Isolate 1755a, on the other hand, was able to infect cultivars 'Dubbele Witte', 'Stringless Green Refugee', 'Sanilac', and 'IVT7214' exhibiting a novel and unusual pathogenicity profile (Table 1). In particular, its ability to replicate in 'IVT7214' was quite unexpected. Symptoms induced by 1755a in 'IVT7214' were visible at 3-4 weeks post-inoculation as mild mosaic, mild leaf deformations, and slight downward leaf curling (Fig. 1a). Visual symptoms of BCMV infection were confirmed by laboratory diagnosis using BCMV-specific TAS-ELISA, with the OD_{405} signal for infected plants exceeding uninfected controls at least 10-fold (Fig. 1b). Isolate 1755a replicating in 'IVT7214' was also used as inoculum for re-inoculations of bean differentials and found to exhibit the same stable pathogenicity profile infecting cultivars 'Dubbele Witte', 'Stringless Green Refugee', 'Sanilac', and 'IVT7214'.

'IVT7214' carries the *bc-3* allele, in addition to *bc-u* and *bc-2* alleles, and hence is considered immune to all known BCMV strains (Drijfhout, 1978; Drijfhout and Morales, 2005; Hart and Griffiths, 2013). Based on this pathogenicity profile, it can be concluded that 1755a overcomes both *bc-2* and *bc-3* resistance alleles in *P. vulgaris* (Table 1), even in the presence of the *bc-u* helper allele, and hence represents a novel BCMV pathogroup, which we have

designated pathogroup VIII following the convention established for the BCMV pathotypes (Drijfhout, 1978; Drijfhout and Morales, 2005).

Isolate 1755a was subjected to a serological characterization with TAS-ELISA side-by-side with reference isolates US1 (B-serotype), NY15P (B-serotype), US10 (B-serotype), and TN1 (A-serotype), in order to determine its serotype. All four isolates were captured on the ELISA plate using either anti-TN1, or anti-US10 rabbit antiserum, and were subsequently probed with two different detecting antibodies as described previously (Feng et al., 2014a). The anti-US10 antiserum reacted to all BCMV isolates tested, and also, as expected, to the BCMNV isolate TN1, when used as the detecting antibody. On the other hand, the anti-TN1 antiserum reacted only with the homologous isolate TN1 when used as the detecting antibody. This serological reactivity profile (Supplementary Table 2) suggested that isolate 1755a displayed the B-serotype, also characteristic of the control isolates US1 and NY15P (Mink and Silbernagel, 1992; Vetten et al., 1992; Berger et al., 1997; Feng et al., 2014a,b).

IVT 7214 has the CAPS marker for bc-3*, and carries the mutated* eIF4E *allele associated with resistance to potyviruses*

In order to determine, if 'IVT7214' used in our experiments harbored the *bc-3* gene, we analyzed the presence of the *bc-3* specific CAPS marker as described by Naderpour et al. (2010). Two *P. vulgaris* genotypes were chosen for comparison: susceptible SGR, carrying only the *bc-u* allele, and 'IVT7214' carrying both *bc-u* and *bc-3* genes. For each cultivar, genomic DNA was used as a template for CAPS marker DNA amplification. Using the primer pair ENM-FWe/RVe, PCR fragments of 541-bp were amplified from both cultivars. Digestion with *Rsa*I of 'IVT7214' PCR products cleaved the 541-bp fragment into 381-bp and 160-bp fragments, as would be

expected in *bc-3* carrying, homozygous genotypes (Fig. 2a). The PCR products derived from SGR were not cleaved by *Rsa*I (Fig. 2a), as would be expected for a susceptible cultivar. To confirm if the mutations in the *eIF4E* gene involved in the disruption of the interactions between eIF4E and the VPg, were present in the 'IVT7214' *eIF4E* gene, these 541-bp PCR fragments were cloned and sequenced for both 'SGR' and 'IVT7214' cultivars. After the intron sequence removal, the partial coding sequence of a fragment derived from SGR (408-bp) was 99% identical to the published partial sequence of the *eIF4E* gene of 'IVT7214' (KC417369.1) available in GenBank, while the same partial coding sequence of a fragment amplified from 'IVT7214' (411-bp) was 100% identical to the same published sequence of the *eIF4E* gene of 'IVT7214' (KC417369.1).

These partial coding *eIF4E* gene sequences from both genotypes, 'SGR' and 'IVT7214', were aligned (Fig. 2b), and known mutations involved in expression of the eIF4E-mediated resistance to BCMV and other potyviruses (Naderpour et al., 2010; Hart and Griffiths, 2013) were analyzed. One-codon deletion and four codon differences leading to amino acid substitutions were found between 'SGR' and 'IVT7214' in this partial coding sequence: del/Thr (position 32), Asn/Lys (126), Phe/Tyr (161), Ala/Glu (194), and Asp/Gly (299). The nucleotide substitution at position 161 (T/A) introduced the *Rsa*I cleavage site into the 'IVT7214' sequence. Except for the deletion of an entire codon in the SGR sequence at nt 32, all other nucleotide changes observed in the *eIF4E* coding sequences were consistent with data published by Naderpour et al. (2010) and Hart and Griffiths (2013). Hence, it was confirmed that cultivar 'IVT7214' used in our host range tests had the intact CAPS marker for the *bc-3* gene, and carried the homozygous, mutated *eIF4E* allele associated with resistance to BCMV and other potyviruses.

BCMV-1755a has a recombinant genome carrying large sections homologous with isolates NY15P, NL1, and US1

Initially, small genome fragments from the HC-Pro, CI, and NIb/CP regions of BCMV isolate 1755a were amplified using RT-PCR with degenerate primers (Ha et al., 2008), and sequenced using the approach described by Feng et al. (2014a). Two of these three initial clones, CI and NIb/CP, were 98% identical to the known sequence of the BCMV strain NL1, while the HC-Pro clone sequence was only 92% identical to the NL1 sequence. Gaps between the three initial clones were filled in *via* RT-PCR amplification using specific primers as listed in supplementary Table 1. The very 5'-terminal sequences for isolate 1755a were obtained using 5'-RACE (Feng et al., 2014a). Upon sequence assembly, 1755a genome was found to be 10,064-nt long, excluding the poly (A) and, based on conceptual translation, encoded a single polyprotein of 3,222 aa. The 1755a sequence was compared to the known BCMV genomes which revealed that BCMV-1755a whole genome sequence shared the closest similarity to several sequences deposited in GenBank as NL1 strain (e.g. accession number AY112735; 94% nucleotide identity).

The whole genomes of BCMV isolates US1 and NY15P were cloned and sequenced, using the same approach (Feng et al., 2014a). Upon sequence assembly, the US1 genome was found to be 10,052-nt long, excluding the poly (A) and, based on conceptual translation, encoded a single polyprotein of 3,221 aa. NY15P genome was found to be 10,053-nt long, excluding the poly (A) and encoded a single polyprotein of 3,222 aa. Initially, the sequences of both isolates were compared to the known BCMV and BCMNV genomes, which revealed that the US1 and

NY15P sequences shared the closest similarity to the isolate NL1 [PG-I, accession number AY112735; 94% and 97% nucleotide identity to US1 (PG-I) and NY15P (PG-V), respectively].

The whole genomes for US1 (accession number KT175569), and NL1 (accession number AY112735) both from PG-I, together with NY15P (accession number KT175568, PG-V), were aligned using CLUSTALX and further analysis was conducted with the RDP4 program package (Table 2). Figure 3a shows the comparison of all three sequences using the manual distance plot analysis, with the full-length NY15P sequence as the reference (see Fig. 3a). Based on the RDP4 analysis, the 5'- terminal sequences of isolates US1 and NL1, between nt 1-2,124, shared more similarities to each other (98% identity) than to NY15p isolate (96% identity). The sequences downstream between nt 2,125-6,717 (position in alignment) in the US1 and NY15P genomes were quite similar (97% identity), while NL1 had 89% similarity to the US1 and NY15P sequences in this segment. The 3'-terminal genome segments between nt 6,718-9,381 (position in alignment), were very similar among all three isolates (97-98% identity). In the 3'-terminal region, between nt 9,382-10,055 (position in alignment), sequences of US1 and NL1 were similar to each other (98% identity) while NY15P shared only 91-92% identities with the two other sequences. It appears that genomes of isolates NL1 (PG-I) and NY15P (PG-V) on one hand, and isolate US1 (PG-I) on the other hand, carry long sequences between nt 2,125 to 6,717 that are quite different, while most of the other areas of the genomes for all three are much closer. This indicates a possible recombination event leading to these similarity patterns (Fig. 3a).

To view these possible recombination patterns in the more global context of known BCMV whole genome sequences, we compared the whole genome sequences of US1, NY15P, and 1755a with the genomes of other BCMV isolates with known pathogenicity profiles (Table

2), in addition to NL1 (isolates from PG-VII; Feng et al., 2014a,b). This comparison conducted using the distance plot program from the RDP4 package is presented in Fig. 3b. A large section of the US1 genome, from nt 2,833 to 10,055 (the 3'-terminus) was homologous between isolates US1 (PG-I) and US10 (PG-VII), suggesting that this large area is not involved in the pathogenicity determinants for either PG-I or PG-VII. A smaller section of the genome, between nt 6,718 to 9,381 was homologous between isolates NL1 (PG-I), NY15P (PG-V), and 1755a (PG-VIII) (Fig. 3b). On the other hand, the two areas mentioned above, nt 1 to 2,124 and 9,382 to 10,055 were the least similar between US1/NL1 and NY15P sequences (Fig. 3a), suggesting that pathogenicity determinants for PG-I and PG-V may be located in these two regions. Unexpectedly, the BCMV-1755a genome had a large section, nt 2,170 to 10,055, which was homologous to the corresponding section of the NL1 genome (97% identity), and a 5'-terminal section, nt 1 to 2,169 substantially different from all other BCMV genomes analyzed (Fig. 3b and Fig. 3c). These recombination patterns resulted in a complicated mosaic pattern presented on Fig. 3c, which suggests that the P1 and HC-Pro cistrons as largely responsible for interactions with multiple BCMV resistance genes in common bean.

The VPg protein of the BCMV-1755a isolate has only seven amino acid substitutions relative to other BCMV isolates unable to overcome bc-3

Since the VPg protein was implicated in interactions with the *bc-3* gene in common bean (Naderpour et al., 2010; Hart and Griffiths, 2013), sequences of several VPg proteins from BCMV strains unable to replicate in 'IVT7214' (NL1, US1, US10, RU1-OR, and NY15P) were aligned and compared to the VPg sequence of BCMV-1755a. The amino acid sequences of VPg proteins were identified based on the cleavage sites described for BCMV polyproteins by Wylie

and Jones (2011). The N-terminal cleavage site used was VTTQ/G, the C-terminal cleavage site used was VAVE/S (VGIE/S for RU1-OR). Only seven positions out of 190 amino acids were unique for the BCMV-1755a isolate overcoming the *bc-3* gene (Fig. 4), and distinct from other isolates unable to overcome the *bc-3* gene. Four out of these seven differences were located very close to the N-terminus of the VPg protein: position 2 (R/K), position 3 (N/K), position 5 (K/M), and position 8 (R/K). Two of these substitutions, at positions 3 and 5, resulted in chemically distinct amino acids that could change/disrupt the normal folding of a protein. The other three amino acid substitutions were scattered along the protein and located at position 55 (K/R), position 115 (T/K), and position 157 (S/A); only position 115 changed the chemical nature of the amino acid.

Discussion

The genetic diversity characteristic of potyviruses is driven by mutation and recombination (Roossinck, 2003; Nagy, 2008; Gibbs and Ohshima, 2010). This vast diversity allows potyviruses to adapt to new evolutionary niches, including their ability to overcome multiple resistance genes in different hosts (Gibbs and Ohshima, 2010; Karasev and Gray, 2013). One of the six recessive resistance alleles that govern interactions of BCMV isolates with common bean, *bc-3* was considered the best resistance gene conferring broad, strain non-specific resistance against all isolates of BCMV and against other potyviruses, e.g. BCMNV and ClYVV, in common bean (*P. vulgaris* L.) when present in a homozygous form with the *bc-u* helper allele (Drijfhout, 1978; Kelly et al., 1995; Hart and Griffiths, 2013), and was identified as the *eIF4E* allele coding for the eIF4E translation initiation factor (Naderpour et al., 2010; Hart and Griffiths, 2013). Isolate BCMV-1755a described here is the first BCMV isolate breaking *bc-3*

mediated resistance in common bean, its characterization allows the examination of possible BCMV genetic determinants interacting with the host *eIF4E* allele. Identification or narrowing down of the genetic determinants of BCMV interacting with all seven resistance alleles found in common bean is complicated at the moment due to the high genetic diversity of BCMV and relative scarcity of information about BCMV isolates from different pathogenicity groups; only some of them had been characterized (Larsen et al., 2005; Naderpour et al., 2010; Feng et al., 2014a,b; Martin et al., 2014).

Here, we continued our comparative genomic studies for BCMV strains from three different pathogenicity groups, PG-I (strain US1), PG-V (NY15P), and PG-VIII (1755a). PG-I comprises BCMV isolates unable to overcome any of the recessive alleles, *bc-1*, *bc-1*^{2}, *bc-2*, *bc-2*^{2}, or *bc-3* in the presence of the effector gene *bc-u*. PG-V comprises isolates that are able to overcome recessive alleles *bc-1* and *bc-2*, but not others. Interestingly, sequences determined for US1 (PG-I) and for NY15P (PG-V) were relatively similar to each other and to another BCMV isolate from PG-I, NL1 (see Figs 3a and 3b). US1 represented a recombinant between NL1 and another, yet unknown parent (see Fig. 3a). Based on the sequence analysis of NY15P, and six other complete genomes of BCMV isolates with defined pathogenicity (Fig. 3b), the most likely genome areas involved in interactions with genes *bc-1* and *bc-2* may be located between positions 1 to 2,124 or positions 9,382 to 10,055. The newly established PG-VIII comprises the 1755a isolate which is able to overcome *bc-2* and *bc-3* recessive alleles in the presence of the effector gene *bc-u*, but not others. The recombination analysis of 1755a (Figs. 3b and 3c) suggested that the unique sequence region between positions 1 to 2,169 may be responsible for the interactions with *bc-2* and/or *bc-3* alleles; the overlapping region, nt 1-2,124 was defined as a

possible determinant of the *bc-2* interaction based on the analysis of the NY15P sequence (see above).

The high level of genetic diversity characteristic of BCMV (Flasinski et al., 1996; Larsen et al., 2005; Feng, 2014a,b; Zhou et al., 2014; this work) that resulted in distinct evolutionary lineages comprising common bean, peanut, and soybean isolates of BCMV (Zhou et al., 2014) suggests successful adaptation and specialization of the virus to several leguminous hosts. In common bean, BCMV was recently found to undergo an extensive recombination related to the ability of BCMV recombinants to overcome multiple resistance genes incorporated into common bean cultivars (Feng et al., 2014a,b). Since molecular mechanisms involved in the expression of BCMV resistance in common bean are unknown for most of these resistance genes, with the *bc-3* allele being the only exception (Naderpour et al., 2010; Hart and Griffiths, 2013), recombinants may provide some clues as to where the molecular determinants of the resistance may reside in the BCMV genome (Feng et al., 2014a,b). Based on the molecular and biological analysis of three BCMV isolates, US1 (PG-I), NY15P (PG-V), and 1755a (PG-VIII), BCMV genome determinants interacting with genes *bc-1*, *bc-2*, and perhaps even *bc-3* may reside in the 5'-terminal region of the genome coding for the P1 and HC-Pro proteins, between positions 1 to 2,124 (Table 1; Fig. 3c), while a determinant involved in interactions with the *bc-1* gene may also reside close to the 3'-terminus of the genome, nt 9,382 to 10,055 (Table 1; Fig. 3c). Nevertheless, all these preliminary assignments will have to be confirmed through the reverse genetics experimentation.

Resistance breaking isolates have been described in several pathosystems that involve eIF4E/eIF(iso)4E recessive alleles and potyviruses (cf. Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012). Most of the examples of these resistance breaking potyvirus isolates had

mutations in the virus VPg cistron identified as responsible for the resistance-breaking phenotype (Abdul-Razzak et al., 2009; Borgstrom and Johansen, 2001; Bruun-Rasmussen et al., 2007; Charron et al., 2008; Moury et al., 2004; Kuhne et al., 2003; Kanyuka et al., 2005). Nevertheless, other potyvirus cistrons were also implicated in interactions with the *eIF4E/eIF(iso)4E* alleles, for instance the CI cistron in *Lettuce mosaic virus* isolates overcoming alleles $mo1^1$ and $mo1^2$ in lettuce (Abdul-Razzak et al., 2009), and the HC-Pro cistron in *Potato virus A* in both tobacco and potato (Ala-Poikela et al., 2011). The mechanism of the resistance breaking of the *eIF4E* allele was proposed to involve the disruption of interactions between the eIF4E translation initiation factor and the VPg protein of potyviruses (Charron et al., 2008; Roudet-Tavert et al., 2007; Yeam et al., 2007). It is interesting that most of the mutations in the eIF4E proteins that confer resistance to different potyviruses were located in the cap-binding cavity of the eIF4E translation initiation factor, likely affecting interactions between eIF4E and VPg (Monzingo et al., 2007; Yeam et al., 2007; Truniger and Aranda, 2009). Resistance breaking mutations in the VPg, on the other hand, were mostly found in the central region of the VPg thought to be directly interacting with the eIF4E protein (Roudet-Tavert et al., 2007). Of the seven amino acid substitutions found unique to the BCMV-1755a isolate VPg, four were clustered close to the N-terminus (Fig. 4). Precise identification of the residues in the BCMV-1755a VPg that might be involved in interactions with the mutated eIF4E protein will require reverse genetics and proteomics approaches.

From a practical perspective, finding of a BCMV isolate able to overcome both *bc-2* and *bc-3* alleles in common bean highlights the value of pursuing breeding material with multiple alleles conferring resistance to BCMV, even if perceived less effective, like *bc-1* or $bc\text{-}1^2$. Isolates of BCMV with pathogenicity profiles similar to 1755a (PG-VIII) should be included in

existing breeding programs as challenges, in search of other recessive genes conferring resistance to this novel pathotype of BCMV.

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539 **Table 1**. Disease and ELISA reactions of bean differentials inoculated with BCMV isolates.[1)]

Bean cultivar	Resistance genes	US1 (PG-I)	NY15P (PG-V)	1755a (PG-VIII)
'Dubbele Witte'	none	+/+	+/+	+/+
'Stringless Green Refugee'	*i, bc-u*	+/+	+/+	+/+
'Redlands Greenleaf C'	*i, bc-u, bc-1*	-/-	+/+	-/-
'Redlands Greenleaf B'	*i, bc-u, bc-1*^{2}	-/-	-/-	-/-
'Sanilac'	*i, bc-u, bc-2*	-/-	+/+	+/+
'UI35'	*i, bc-u, bc-1*^{2}*, bc-2*^{2}	-/-	-/-	-/-
'IVT7214'	*i, bc-u, bc-2, bc-3*	-/-	-/-	+/+
'Jubila'	*I, bc-1*	-/-	-/-	-/-
'Amanda'	*I, bc-1*^{2}	-/-	-/-	-/-
'US1006'	*I, bc-u, bc-2*^{2}	-/-	-/-	-/-
'IVT7233'	*I, bc-u, bc-1*^{2}*, bc-2*^{2}	-/-	-/-	-/-

540

541

542 [1)] Disease reaction is shown first as a numerator followed by ELISA reaction as a denominator.
543 Three plants were inoculated for each BCMV isolate per an experiment; numerator: + =
544 symptoms on inoculated beans; - = no symptoms on inoculated beans; denominator: + designates
545 ELISA signal (A_{405}) in an infected plant exceeding healthy control 10-fold or more; - designates
546 ELISA signal in an infected plant equal to that of a healthy control.

547

548 **Table 2**. Recombination patterns of select BCMV isolates detected by recombination-detecting
549 programs.

Isolate	Recombination breakpoint(s)*	*P*-value**
NL1	2124	3.95×10^{-27}
NL1	6717	3.95×10^{-27}
NY15P	2124	3.95×10^{-27}
NY15P	6717	3.95×10^{-27}
NY15P	9381	3.06×10^{-31}
US10	2832	1.87×10^{-167}
1755a	2169	4.39×10^{-80}
1755a	6680	4.83×10^{-10}

550 *Breakingpoints indicate positions in the alignment of all sequences.

551 **Greatest *P*-value among recombinants identified by the recombination-detecting programs

552 RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiScan, and 3Seq in RDP v4.16.

553

554

Figure legends

Fig. 1. (a) Mild mosaic, and leaf deformations induced upon inoculation of BCMV isolate 1755a in cv. 'IVT7214', 3 weeks post-inoculation. (b) An example of the triple-antibody sandwich (TAS)-ELISA assessment of BCMV-1755a infection in a set of bean differentials (see Table 1), 4 weeks post-inoculation. Three individual plants per cultivar were infected with isolate 1755a and one plant was left uninfected; for cultivar 'Dubbele Witte' (DW), two plants were inoculated in this experiment. Vertical bars represent averages of two duplicate samples for the same plant analyzed by TAS-ELISA in the same experiment. Samples were considered positive if the OD_{405} signal for the infected plant exceeded the one for an uninoculated plant 3-fold.

Fig. 2. Analysis of the *eIF4E* allele (*bc-3*) carrying mutated eukaryotic translation initiation factor gene in two common bean cultivars, 'Stringless Green Refugee' (SGR, permissive host for all isolates of BCMV including 1755a) and 'IVT7214' (non-permissive host for all BCMV isolates, but permissive for 1755a). (a) Agarose gel analysis of the 541-bp CAPS marker amplified and treated with *Rsa*I according to Naderpour et al. (2010). Two individual plants of each cultivar were tested and numbered underneath the gel; sizes of the restriction fragments and amplified PCR products are indicated with arrows, M – designates marker lanes. (b) Alignment of the *eIF4E* allele coding sequences, after removing the introns, for 'SGR' and 'IVT7214'. Nucleotide differences are highlighted in yellow, corresponding changes in amino acids encoded are given above the alignment. Position of the *Rsa*I restriction site in 'IVT7214' used for the CAPS marker is underlined.

Fig. 3. Recombination analysis of the two control BCMV isolates, US1 and NY15P and of the BCMV isolate 1755a, in comparison to the main BCMV strains.

(a) Manual distance plot based on the aligned full-length nucleotide sequences of BCMV isolates US1, NL1 and NY15P; NY15P (GenBank accession XXX) was used as the reference strain.

(b) Manual distance plot based on the aligned full-length nucleotide sequences of BCMV isolates US1, NL1, NY15P, 1755a, US10, and RU1-OR; NL1 (GenBank accession AY112735) was used as the reference strain. X axis represents nucleotide position in the alignment, Y axis represents relative distance from the reference sequence which is calculated using Kimura model (Kimura, 1980).

(c) Schematic diagram showing putative BCMV recombination structures for *Bean common mosaic virus* (BCMV) isolates 1755a, RU1-OR, US1, NL1, and NY15P. The diagram reflects distance plot data presented in Fig. 3. Similar shading of the rectangles indicates homologous sequences present in BCMV strains.

Fig. 4. Amino acid alignment of the VPg sequences for BCMV-1755a isolate, which is able to replicate in 'IVT7214', and several BCMV isolates unable to replicate in 'IVT7214', NL1, US1, US10, RU1-OR, and NY15P. Substitutions present only in BCMV-1755a are highlighted in yellow; substitutions that are not specific to BCMV-1755a are highlighted in pink.

3 wpi

Fig. 1a

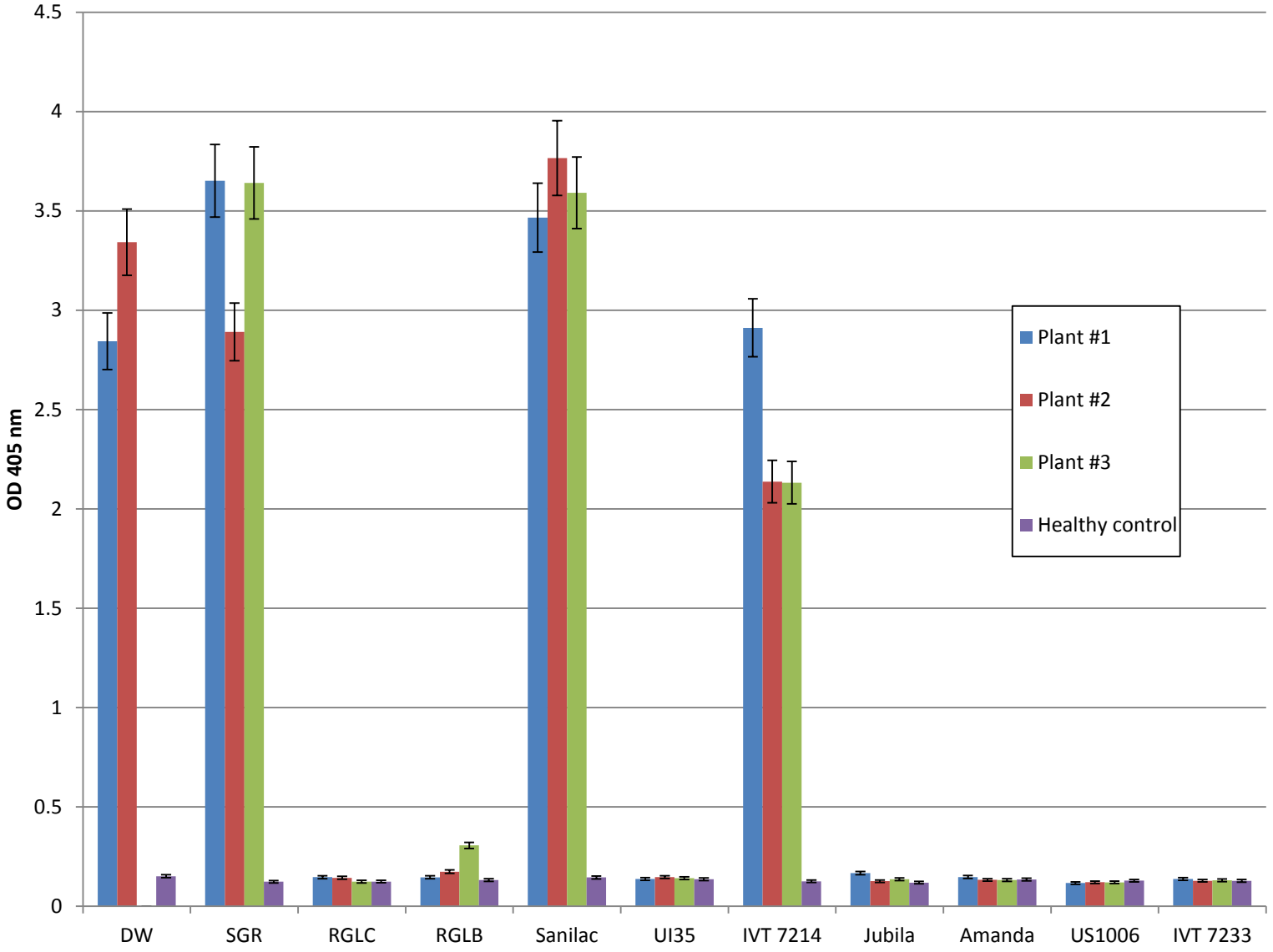
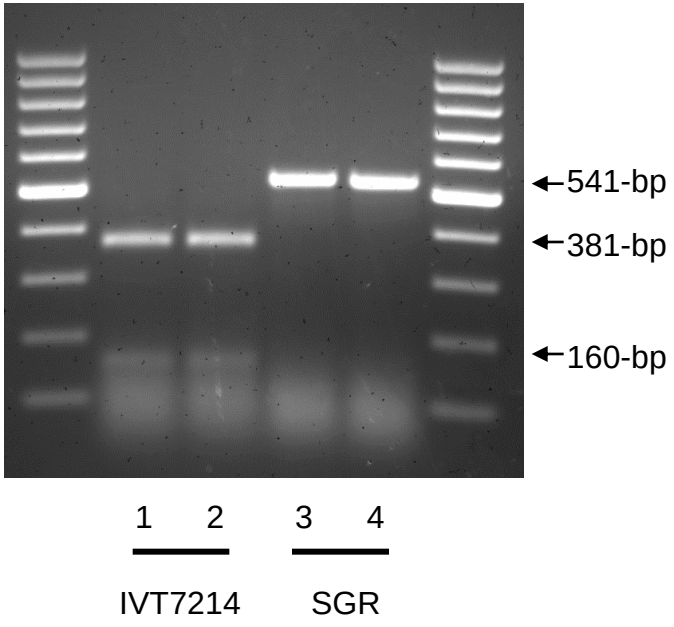


Fig. 1b

a



b

```
                                  del/Thr
                                  ↓
SGR      1   ACCGATGAGCAAAACCCTAGCAGGGTCGACA---ACGACGACGATCTCGAAGATGGGGAGATCCTCGAAGATGCCGACGACGCCGCCTCC  87
IVT7214  1   ********************************ACG********************************************************  90

                                  Asn/Lys                    Phe/Tyr
                                  ↓                          ↓
SGR     88   GCTGCCTCGAAGCCACCGTCCGCCTTCCTCCGCAACCCCCACCCTCTGGAGAATTCCTGGACCTTCTGGTTCGACAACCCTTCCGCCAAG 177
IVT7214 91   ***********************************A**********************************GTAC****************** 180
                                                                                    RsaI

                        Ala/Glu
                        ↓
SGR     178  TCCAAACAAGCCGCATGGGGCAGTTCCATCCGACCCATATACACTTTCTCCACCGTCGAAGAGTTTTGGAGCATTTACAATAACATTCAC 267
IVT7214 181  ************A************************************************************************** 270

                                  Asp/Gly
                                  ↓
SGR     268  CACCCGAGCAAGTTGGGTGTGGGGGCGGACTTTCACTGCTTCAAGCATAAGATTGAGCCCAGGAAAATGGACTATGACGTTCCAAAGGGG 357
IVT7214 271  ***************************G************************************************************ 360

SGR     358  GAAATCCGATACCAGTTGGTTG  408
IVT7214 361  **********************  411
```

Fig. 2.

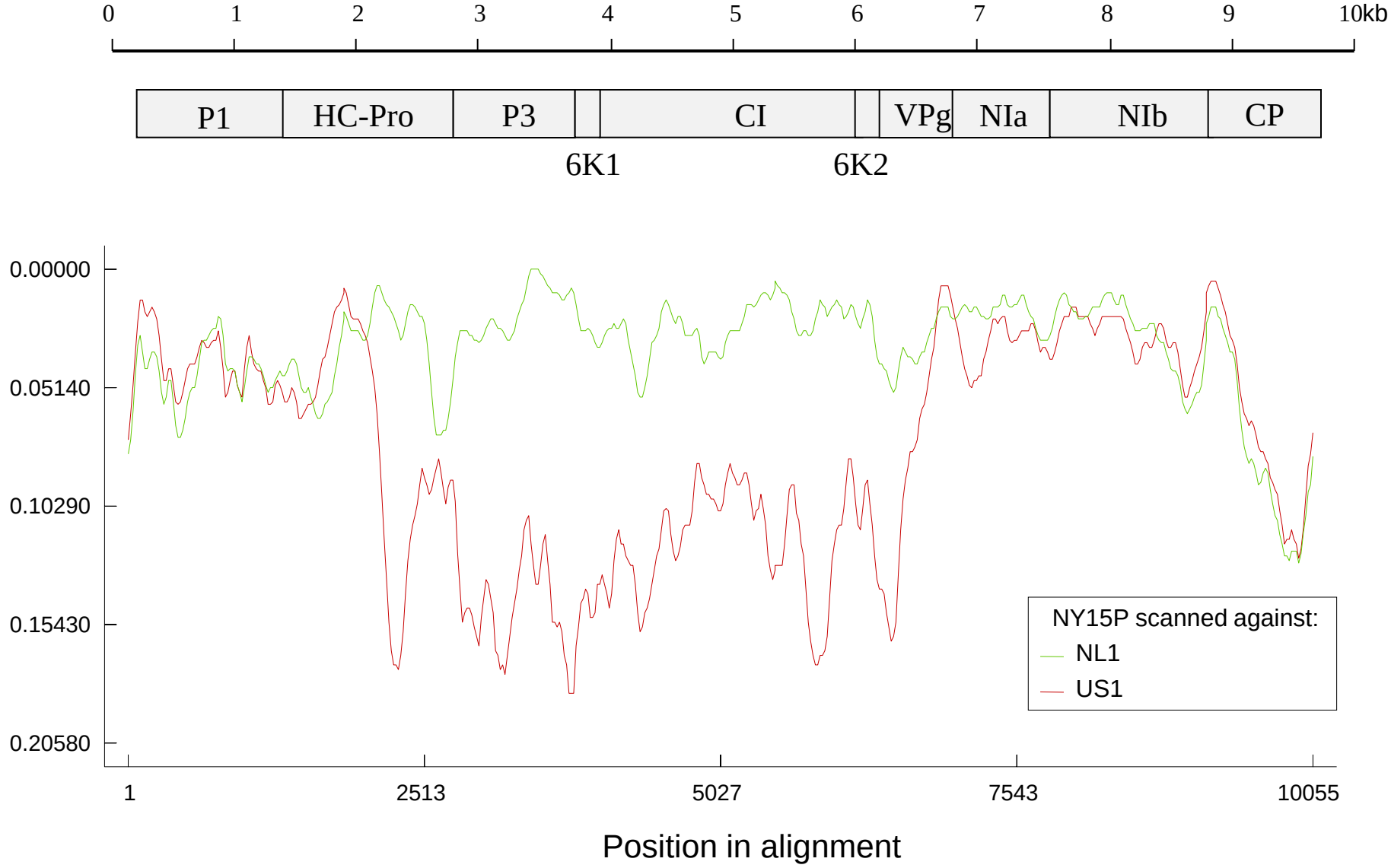


Fig. 3a

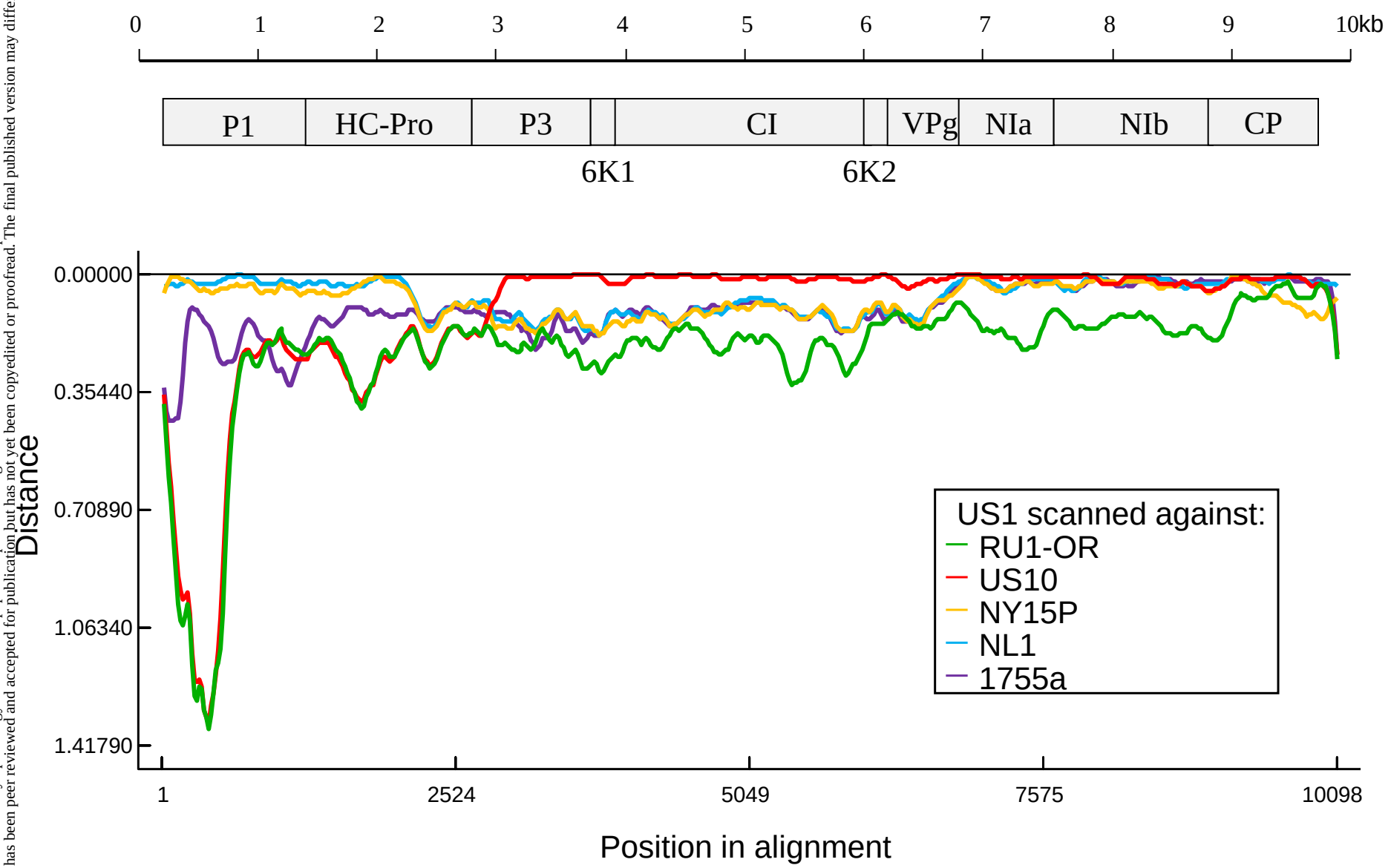


Fig. 3b

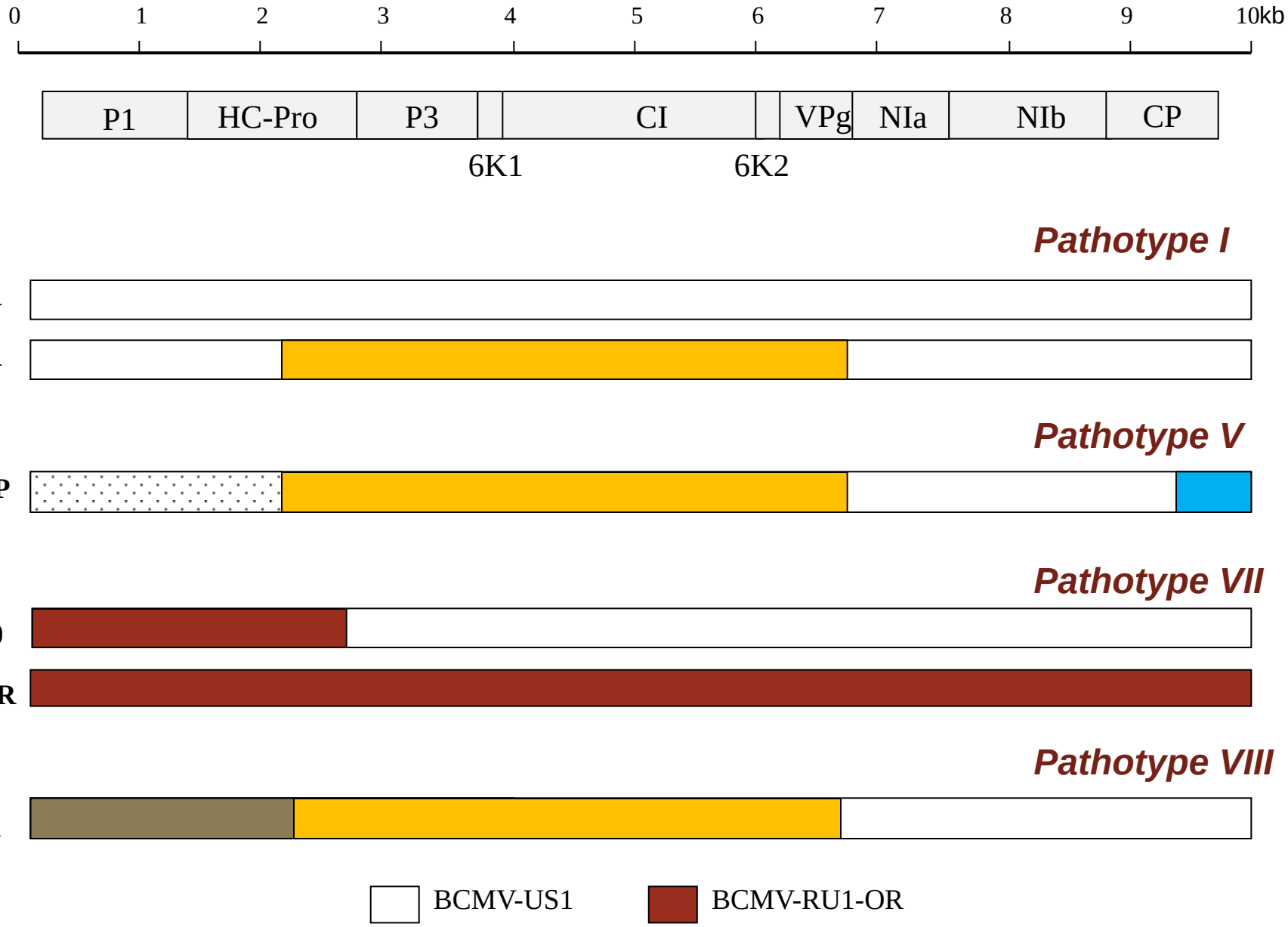


Fig. 3c.

```
                 |....| ....|....| ....|....| ....|....| ....|....| ....|....|
                 1          11         21         31         41         51
1755a            GRNRKL QRLKFRDAFD RKVGREVYAD DYTMEHTFGE AYTKKGKQKG STKTKGMGKK
NL1              GKKRML QKLKFRDAFD RKVGREVYAD DYTMEHTFGE AYTKKGKQKG STKTKGMGRK
US1              GKKRML QKLKFRDAFD RKVGREVYAD DYTMEHTFGE AYTKKGKQKG STKTKGMGRK
US10             GKKRML QKLKFRDAFD RKVGREVYAD DYTMEHTFGE AYTKKGKQKG STKTKGMGRK
RU1-OR           GKKRMM QKLKFRDAFD RKVGREVYAD EYTMEHTFGE AYTKKGKQKG STKTKGMGRK
NY15P            GKKRML QKLKFRDAFD RKVGREVYAD DYTMEHTFGE AYTKKGKQKG STKTKGMGRK

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     61         71         81         91        101        111
1755a            TRKFIHMYGV EPENYSMIRF VDPLTGATLD EGTRVDIRLV QEEFGEIRRQ KIDADELDTE
NL1              TRKFIHMYGV EPENYSMIRF VDPLTGATLD EGTRVDIRLV QEEFGEIRRQ KIDDNELDKE
US1              TRNFIHMYGV EPENYSMVRF VDPLTGATLD EGTRVDIRLV QEEFGEIRKE KINDGELDKE
US10             TRNFIHMYGV EPENYSMIRF VDPLTGATLD EGTRVDIRLV QEGFGEIRKQ KINDDELDKE
RU1-OR           TRNFIHMYGV EPENYSMIRF VDPLTGATLD EGTRVDIRLV QEEFGEIRKQ KIDEDELDKE
NY15P            TRNFIHMYGV EPENYSMIRF VDPLTGATLD EGTRVDIRLV QEEFGEIRRQ KIDNDELNKE

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    121        131        141        151        161        171
1755a            TVRNKPGIQA YFIGKNAEEA LKVDLTPHRP TLLCMNSNAI SGFPEREDEL RQTGLPVHIK
NL1              TVRNKPGIQA YFIGKNAEEA LKVDLTPHRP TLLCMNSNAI AGFPEREDEL RQTGLPVHIK
US1              TVRRNPGIQA YFVGKNAEEA LKVDLTPHRP TLLCMNSNAI AGFPEREDEL RQTGLPVRIK
US10             TVIRNPGIQA YFVGKNAEEA LKVDLTPHRP TLLFMNSNAI AGFPEREDEL RQTGLPVRIK
RU1-OR           TVRRNPGIQA YFIGKNAEEA LKVDLTPHRP TLLCMNSNAI AGFPEREDEL RQTGLPVRIK
NY15P            TVRNKPGIQA YFIGKNAEEA LKVDLTPHRP TLLCMNSNAI AGFPEREDEL RQTGLPVRIK

                 ....|....| ....
                    181
1755a            RSEVPEPNEE VAVE
NL1              RSEVPEPNEE VAVE
US1              RSEVPEPNEE VAVE
US10             RSEVPEPNEE VAVE
RU1-OR           RSEVPEPSEE VGIE
NY15P            RSEVPEPNEE VAVE
```

Fig. 4.

Supplementary Table 1. Primers used for cloning of the whole BCMV genome

Primer	Sequence(5′-3′)	Use
Degenerate primers[1]		
HPFor	TGYGAYAAYCARYTIGAYIIIAAYG	degenerate primer to amplify partial HC-Pro gene
HPRev	GAICCRWAIGARTCIAIIACRTG	degenerate primer to amplify partial HC-Pro gene
CIFor	GGIVVIGTIGGIWSIGGIAARTCIAC	degenerate primer to amplify partial CI gene
CIRev	ACICCRTTYTCDATDATRTTIGTIGC	degenerate primer to amplify partial CI gene
NIbFor	GGICARCCITCIACIGTIGT	degenerate primer to amplify partial NIb gene
3′ end[1]		
N1T	GACCACGCGTATCGATGTCGAC$(T)_{17}$V	generic 3′ end first strand primer
N1	GACCACGCGTATCGATGTCGAC	generic 3′ end PCR primer
5′ end[2]		
Oligo d(T) Anchor primer	GACCACGCGTATCGATGTCGAC$(T)_{16}$V	
US1 specific primers		
US1 mg1 For	GGAAAATCATCTGAAATGGC	Specific primer to amplify the major gap 1
US1 mg1 Rev	GAATGATATCCTCTCTCACCCC	Specific primer to amplify the major gap 1
US1 mg2 For	GCCACAGCAGTCTACATCC	Specific primer to amplify the major gap 2
US1 mg2 Rev	CCTTTCTTGGCCAAATGATG	Specific primer to amplify the major gap 2
US1 mg3 For	GTAGATGGGAGAACAATGC	Specific primer to amplify the major gap 3
US1 mg3 Rev	CCACCCCACCTTGTGACATGAATAAT	Specific primer to amplify the major gap 3
US1 5RACE Rev1	CACTTTGCCGATGTATTCCTTCTG	1st strand primer for 5′RACE
US1 5RACE Rev2	CAGTCTCCATACGCACATCCTGTTC	PCR primer for 5′RACE
NY15p specific primers		
NY15p mg1 For	GGAAAATCATCTGAAATGGC	Specific primer to amplify the

		major gap 1
NY15p mg1 Rev	GAATGATATCCTCTCTCACCCC	Specific primer to amplify the major gap 1
NY15p mg2 For	GCTACAGCGGTTTACATTC	Specific primer to amplify the major gap 2
NY15p mg2 Rev	CCCTTTCTTGGCTAAGTGATG	Specific primer to amplify the major gap 2
NY15p mg3 For	GTAGATGGGAGAACAATGC	Specific primer to amplify the major gap 3
NY15p mg3 Rev	CCACCCCACCTTGTGACATGAATAAT	Specific primer to amplify the major gap 3
NY15p 5RACE Rev1	GAATGATATCCTCTCTCACCCC	1st strand primer for 5'RACE
NY15p 5RACE Rev2	ATCGTGCTGAGCATCCTACAGTGAT	PCR primer for 5'RACE
1755a specific primers		
1755a mg1 For	GGAAAATCATCTGAAATGGC	Specific primer to amplify the major gap 1
1755a mg1 Rev	GAATGATATCCTCTCTCACCCC	Specific primer to amplify the major gap 1
1755a mg2 For	GACTTCACTAAGATGGTCAG	Specific primer to amplify the major gap 2
1755a mg2 Rev	CACGCATTCTGAGTGTGAC	Specific primer to amplify the major gap 2
1755a mg3 For	CAAATGCAGATATGATTCA	Specific primer to amplify the major gap 3
1755a mg3 Rev	GTATGTCCTCATCGCTCCATC	Specific primer to amplify the major gap 3
1755a 5RACE Rev1	GACTGTTGAGTGTGATTGAC	1st strand primer for 5'RACE
1755a 5RACE Rev2	GATGCTCTCCATAACTTGC	PCR primer for 5'RACE

[1)]All degenerate primers and 3' end primers are from Ha et al. (2008)

[2)]5' end anchor primer is from the 5'RACE Kit protocols.

Supplementary Table 2. Serological characterization of *Bean common mosaic virus* (BCMV) isolates using strain-specific antibodies in triple-antibody sandwich (TAS) enzyme-linked immunosorbent assay (ELISA)[1)].

	BCMV isolates				
Antibodies	TN1	US10	NY15P	US1	1755a
Pre-immune	-	-	-	-	-
Anti-US10	+	+	+	+	+
Anti-TN1	+	-	-	-	-

[1)] + designates ELISA signal (A_{405}) in an infected plant exceeding healthy control 10-fold or more; - designates ELISA signal in an infected plant equal to a healthy control; NT designates "not tested".