Recombinants of *Bean common mosaic virus* (BCMV) and Genetic Determinants of BCMV Involved in Overcoming Resistance in Common Beans

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Abstract

Bean common mosaic virus (BCMV) exists as a complex of strains, which have been classified into seven pathogroups. These BCMV strains and strains of the closely related *Bean common mosaic necrosis virus* (BCMNV) can be distinguished based on their biological, molecular and serological properties. Seven resistance genes govern interactions of common beans (*Phaseolus vulgaris*) and BCMV/BCMNV strains: one dominant *I* gene, and six recessive genes, *bc-u*, *bc-1* and an allelic $bc\text{-}1^2$, *bc-2* and an allelic $bc\text{-}2^2$, and *bc-3*. Prior to this work, virus genetic determinants involved in pathogenicity interactions of BCMV with resistance genes were not identified or mapped. Here, we conducted a systematic study of biological and molecular properties of a group of field-collected and reference BCMV isolates in order to identify genetic determinants involved in interactions with three recessive genes, *bc-1*, *bc-2*, and $bc\text{-}2^2$, and the dominant *I* gene. Through comparative genomics of four closely related BCMV isolates from the RU1 strain group, and three other BCMV strains, US1, US10, and NY15P, putative genetic determinants interacting with recessive genes, *bc-1*, *bc-2*, and $bc\text{-}2^2$, as well as the dominant *I* gene in *P. vulgaris* were identified and mapped to the P1 and HC-Pro regions. Genome diversity found in this study indicated BCMV strains represented multiple recombinants, and the recombination in BCMV genome may or may not lead to pathogenicity changes. Due to wide spread of recombination in BCMV strains, partial sequencing is an unreliable means of BCMV strain classification.

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Chapter 1

Introduction

The common bean (*Phaseolus vulgaris* L.) is an economically important legume; as a crop it is widely grown all over the world. Both immature pods (green beans) and dry beans of common bean can be cooked and eaten as protein source food. In addition to being a source of fiber and protein, beans can also provide minerals (iron and zinc) and vitamins for human body, while dried pods and stems can be used as fodder (3, 26). According to FAO report, 23 million tonnes of dry beans and 20.7 million tonnes of green beans were produced in 2012 (5). Leading producers of common beans include countries in Asia, Europe, Oceania, and South and North America. Brazil and India are the main producers of dry beans while China produces the largest quantity of green beans (5, 9, 10). In the United States, dry edible beans and snap beans are grown on ca. 2 million acres in more than a dozen states with a farm-gate value averaging $850 million per year. The Pacific Northwest (Idaho, Oregon, and Washington) produces 130,000 acres of dry edible beans. Eighty-five percent of the seed industry for dry edible beans and snap beans is based in the Pacific Northwest. The 30,000 acres of seed production in the Pacific Northwest is for export as well as domestic use and is valued at $75 million per year (23). Thirty-five percent of the dry bean crop is exported, contributing favorably to U.S. trade balance.

Bean common mosaic virus (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) are closely related potyviruses in the family *Potyviridae* infecting a wide range of legume species (1). Both are important pathogens in common bean (*P. vulgaris* L.) that can cause severe economic losses in bean production. The BCMV genome is a single-stranded, positive-sense RNA of about 9.6-10 kb in length, with a viral encoded protein

(VPg) covalently attached to its 5' end and a 3' poly(A) tail (6). The viral genome encodes a single polyprotein, which is subsequently processed by virus-encoded proteases into ten protein products: P1 protease (multifunctional protein); the helper component/protease (HC-Pro, multifunctional protein); P3 protein; a 6-kDa peptide; the cylindrical inclusion protein (CI, RNA helicase); a second 6 kDa peptide; the VPg; the nuclear inclusion 'a' protein (NIa, a protease); the nuclear inclusion 'b' protein (NIb, RdRp) and the coat protein (CP, multifunction protein) (22). BCMV/BCMNV can be easily transmitted mechanically, and are aphid borne and seed borne viruses; when transmitted through seed, the efficiency can be up to 83%, which makes BCMV/BCMNV a worldwide problem in bean production (11, 19, 23). Control of BCMV/BCMNV mainly depends on seed certification and use of resistant cultivars.

BCMV exists as a complex of strains which can be distinguished into seven pathogroups (PGs) based on the interaction between virus strains and bean resistant genes. BCMV comprises strains from seven pathogroups while BCMNV strains all fell into pathogroup VI (7, 17). BCMV and BCMNV are also serologically distinct, BCMV exhibiting B-serotype while BCMNV displays A-serotype (18, 24, 25).

Seven resistant genes have been identified in common beans to confer resistance to BCMV/BCMNV: one dominant *I* gene and six recessive genes, including five strain specific genes, *bc-1* and an allelic *bc-1*^{2}, *bc-2* and an allelic *bc-2*^{2}, and *bc-3*, and one strain non-specific gene *bc-u*. The *bc-u* gene cannot function independently, however, the full functioning of other recessive resistant genes requires the presence of this gene (7). Due to complex interactions between the virus and bean resistance genes, wide range of symptoms can be induced in common beans. The symptoms displayed can vary depending on the virus

strains, bean cultivars and environment factors. When BCMV/BCMNV infect a susceptible cultivar, symptoms such as mosaic, blistering, yellowing and leaf deformation can be induced (7, 18, 19, 24); plants often show stunting and growth retardation which later result in reduction of quality and yield. The dominant *I* gene confers resistance to all strains of BCMV, when temperature stays below 30°C, and thus represents an efficient tool for bean protection from BCMV. However, when infecting a cultivar with the dominant *I* gene, a range of necrotic reactions including a distinct syndrome called "black root syndrome (BRS)" can be induced by BCMNV strains regardless of temperature; or by some BCMV necrotic strains at temperature above 30˚C (2, 4, 12, 13, 14, 15, 20, 21). Whole plant necrosis starting from the growing point is characteristic of BRS which can lead to a complete crop loss. In order to take advantage of the *I* gene without killing the plants, breeders have introduced recessive *bc* genes in the *I* gene containing cultivars. When bean cultivars carrying "protected" *I* gene are infected by BCMNV, only limited vein necrosis can be found, plants will survive and produce seed normally (8, 16).

The genetics of resistance to the BCMV/BCMNV complex in common bean is well characterized (7, 8), and all known resistance genes are widely used in bean breeding programs (7, 8, 16, 23). Nevertheless, from the virus perspective, the genome regions of BCMV and BCMNV involved in interactions with either the *I* gene, or recessive genes have not yet been identified, although the study of a natural BCMNV recombinant NL-3K suggested the P1 encoding genome region might be related to symptom expression in common beans (17, 24). Consequently, mechanisms of resistance to BCMV in common beans have yet to be elucidated. Characterization of the pathogenicity determinants in BCMV and BCMNV responsible for interactions with resistance genes in common beans is

currently hampered by two obstacles or knowledge gaps: 1) limited number of BCMV/BCMNV strains or isolates have been sequenced, and among these sequenced strains or isolates, few of them have been characterized with complete pathogenicity and molecular profiles, which makes it impossible to use these data for further analysis; 2) great genomic diversity found in known BCMV strains makes it difficult to screen good candidates which can link virus pathogenicity change with nucleotide sequence change.

In summer of 2011, symptomatic BCMV samples were found and collected by bean breeder James Myers (Oregon State University) in the Willamette Valley, OR. Two field isolates were sent to our lab for biological and molecular characterization. Both were found to belong to PG-VII and exhibit B-serotype. Initial limited sequencing of both genomes (partial HC-Pro, CI and 3' end) was found to be 98-99% identical to the known strain RU1. Interestingly, this original RU1 isolate was classified as a non-necrotic strain belonging to pathotype VI and was never found in the field. We immediately decided to continue sequencing these two field isolates as well as the control isolate US10, another strain exhibiting pathotype VII, to see if any pathogenicity determinant for pathotype VII can be identified. This initial characterization led to our attempts to identify and map putative genetic determinants of different BCMV pathogroups involved in the interactions between BCMV/BCMNV and common bean. The data obtained in this study is divided into three sections: in the first section, we report a systematic study of serological, biological and molecular properties of a group of novel BCMV field isolates and two reference BCMV strains from our laboratory virus collection. Comparison of the biology and molecular properties of these isolates, and other reported isolates with the same or differing pathogenicity on bean differentials, allowed us to localize putative genetic determinants of

pathogenicity interacting with the recessive *bc-1*(chapter 2). In the following section, using the same strategy, we also conducted the systematic study of a group of RU1-like BCMV strains and several reference BCMV strains (chapter 3 and 4) which helped us localize the putative genetic determinants of pathogenicity interacting with the *bc-2* gene (chapter 3) and the dominant *I* gene (chapter 4). In the end, the results of this study are summarized, and practical implications and future goal for following researchers are described (chapter 5).

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Chapter 2

Biological and molecular characterization of *Bean common mosaic virus* isolates from different pathogenicity groups

Abstract

Bean common mosaic virus (BCMV) exists as a complex of strains that can be distinguished biologically, on a set of bean differentials, into pathogenicity groups (PG) numbered I to VII. Up until now, these pathogenicity groups cannot be identified by laboratory methods, through molecular tools, due to the lack of understanding of genetic determinants linked to the strain pathogenicity. In order to establish molecular signatures characteristic of different pathogenicity groups of BCMV, we performed a complete biological and partial molecular study of 15 field BCMV isolates collected in Oregon, and also biological and molecular characterization of two control isolates from PG-I (US1) and PG-V (NY15P). All the field isolates were found to have B-serotype, with three of them belonging to PG-I, nine to PG-III, two to PG-IV, and one to PG-VI. An interesting syndrome was observed after inoculation of the four field isolates (all from PG-III) into some susceptible bean cultivars, including severe wilting and rapid, within 10-14 days, plant death; this syndrome was unrelated to the pathogenicity profile of BCMV isolates. Partial sequences of the HC-Pro region were determined for eight out of 15 field isolates, and no correlation was found between the PG assignment of a BCMV isolate and the HC-Pro sequence similarities. The complete genomes of US1 and NY15P isolates were sequenced and found closest to the BCMV strain NL1 (PG-I, 94% identity to US1 and 97% identity to NY15P). Sequence analysis revealed that strain US1 might be a product of recombination

between strains US10 and NY15P. The data obtained suggest that the search for the genetic determinants responsible for BCMV pathogenicity should continue through comparative genomics of BCMV isolates with known pathogenicities.

Introduction

The closely related potyviruses *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrotic virus* (BCMNV) are both economically important pathogens of common beans (*Phaseolus vulgaris* L.) (1). Both viruses are transmitted in the field by aphids in a non-persistent manner, but could also be efficiently transmitted through seed (8, 15, 17). This latter mode of transmission has facilitated the spread of both viruses anywhere beans are grown, and is the main factor responsible for BCMV and BCMNV importance in bean production world-wide. Management of BCMV and BCMNV relies on seed certification and use of resistant bean cultivars (see 17). The resistance to BCMV/BCMNV in common bean is conferred by seven resistance genes: one dominant *I* gene and six recessive genes *bc-u*, *bc-1*/$bc\text{-}1^2$, *bc-2*/$bc\text{-}2^2$, and *bc-3*, two of which have two alleles each (3). Based on their biological responses on bean differential hosts carrying different combinations of resistance genes, BCMV/BCMNV isolates are classified into seven pathogroups (PGs), numbered from I to VII. BCMNV isolates were found associated exclusively with PG-VI, while isolates of BCMV could be found in all seven pathogroups (3, 12). Both viruses are serologically distinct, with BCMNV displaying A-serotype, and BCMV displaying B-serotype (14, 18, 19).

At the moment, field isolates of BCMV and BCMNV can be easily distinguished by ELISA, using virus-specific serotyping (14, 19), however to determine the pathotype of an

isolate and assign it to the respective pathogroup, one needs to conduct a laborious and lengthy biological typing on bean differentials (3). Currently, only limited numbers of complete BCMV/BCMNV genome sequences are available in GenBank, and most of them were never tested on bean indicators to determine their complete biological profiles. If we want to replace this tedious biological characterization of BCMV isolates with rapid laboratory-based typing of BCMV to a pathogroup, through RT-PCR based methodology, we would need to map genomic polymorphisms associated with specific pathogroups of BCMV. Therefore, complete biological, serological and molecular characterization of reference BCMV/BCMNV strains and field isolates is needed.

Here, we present the complete biological and serological characterization of 15 field isolates collected in the Willamette Valley, OR, in the summer of 2013. Partial molecular characterization of several BCMV isolates was also performed. Comparison of the biological, serological and partial molecular properties of these field isolates with other known BCMV/BCMNV isolates in GenBank suggested that at least some of these isolates might have never been reported before. We also describe the full characterization of two reference isolates, US1 from PG-I, and NY15P from PG-V. The data obtained further extends our knowledge about the genome diversity of BCMV strains circulating in bean production areas.

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. BCMV isolates US1, NY15P, and BCMNV isolate

TN1 were provided by Drs. P. Berger and P. Shiel. Fifteen field isolates of BCMV, were collected in Corvallis, OR, from bean plants exhibiting typical mosaic symptoms in the summer of 2013. All virus isolates were propagated in the bean cultivar 'Dubbele Witte' using mechanical inoculation and then maintained under greenhouse conditions. BCMV isolates US1 and NY15P were purified as described previously (5).

Biological and serological characterization

The biological characterization of 15 field isolates on a set of bean differentials (3) was performed as described previously (5). Three reference strains, US1 (PG-I), NY15P (PG-V) and TN1 (PG-VI), were included in this analysis as controls. Eleven bean differential lines carrying different resistance gene combinations (Table 1) were inoculated with each isolate (one plant per cultivar due to space and time limitation), 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. In addition, at 4 weeks post-inoculation samples were collected for ELISA testing.

All BCMV and BCMNV isolates virus preparations, including 15 field isolates and 3 reference isolates, were collected from infected bean cv Dubbele Witte. Triple-antibody sandwich (TAS) ELISA tests using BCMV and BCMNV strain-specific, polyclonal antibodies from the laboratory collection were performed as described (5).

Cloning strategy, sequencing, and sequence analysis

For the control isolates US1 and NY15P, the whole-genome cloning strategy, sequencing, and sequence analysis were conducted as described previously (5). For the 15 field isolates, due to time and space limitations, only isolates 3915, 3918, 3921, 3982, 1755, 1574k, 1664k, 1759k isolates were subjected to a RT-PCR with degenerate primers targeting the HC-Pro region, which was amplified and sequenced. Isolates 3915 and 3918 were subjected to a RT-PCR with degenerate primers targeting the NIb-CP region which was amplified and sequenced. The RNA extraction and RT-PCR were performed according to the protocol described elsewhere (5). The amplified PCR products were purified using ExoSAP (Affymetrix) according to the manufacturer's instructions. The purified PCR products were then mixed with appropriate primers and submitted for sequencing by GENEWIZ (South Plainfield, NJ). All primers used in this study for RT-PCR amplifications and sequencing are listed in Table 2.

The whole genomes for BCMV isolates US1 and NY15P were amplified by RT-PCR using the same strategy described elsewhere (5; see Table 2); the overlapping PCR products were cloned into the AT-vector T-Easy and submitted for sequencing to GENEWIZ (South Plainfield, NJ) as described (5). The 5'-terminal sequences for both US1 and NY15P isolates were amplified using the RACE Kit (Roche), and subsequently cloned and sequenced the same way as the rest of the genome. The complete viral genome was assembled using SeqMan (DNASTAR, Madison, WI). The sequences for US1 and NY15P genomes will be deposited in the GenBank database. All sequences were initially analyzed using the BLASTn 2.2.17 (2) tool available at the National Center for Biotechnology Information (NCBI). Open reading frames (ORFs) were identified using the ORF Finder program

available at the National Center for Biotechnology Information (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) (13).

Results

Biological characterization

When screened on the eleven bean differential lines, most of the tested isolates induced typical mosaic, raised dark green blistering, leaf deformation, and growth retardation in susceptible bean varieties. Based on the pathogenicity profiles exhibited by these 15 field isolates on bean differential lines, three - 3915, 3918 and 3594 were classified as belonging to PG-I, nine - 1033, 2101, 2102, 3921, 3982, 1542k, 1574k, 1664k, and 1759k were classified as PG-III, two - 1748 and 3110 were classified as PG-IV, and one - 1755 was classified as belonging to PG-VI (see Table 1).

Unexpectedly, severe wilting and rapid plant death were recorded upon inoculation of four isolates, 1542k, 1574k, 1664k, and 1759k (all from PG-III) into bean cultivar Dubble Witte, RGLB, Sanilac, Amanda and IVT7233 (see Table 1 and Fig. 1, A and B). Letter "k" was added next to the isolate ID to designate its "killing" phenotype on cv Dubbele Witte. These severe wilting and plant death usually started at 7-10 days after inoculation (Fig. 1), and initially became visible on the lower inoculated leaf progressing upward to the growing point. As soon as the initial wilting showed up on the inoculated leaves, the symptom developed very fast, and usually in 1-2 days the whole plant showed severe wilting and then started to dry out and died in another 1-2 days. All four isolates,

1542k, 1574k, 1664k, and 1759k, could induce this syndrome in bean cvs Dubble Witte and Sanilac (Table 1). In contrast with this wilting syndrome starting on the lower, inoculated leaves and progressing upwards to the growing point, infection with the BCMNV isolate TN1 induced systemic necrosis only in cv. Jubila (Table 1), but in this case necrosis developed much later, at about 4 weeks post-inoculation. In addition, this systemic necrosis was expressed as mild necrotic spots on upper, non-inoculated leaves, and did not result in plant wilting and death up to the end of the experiment (8 weeks post-inoculation). The observed wilting syndrome was also quite distinct from the “black root syndrome (BRS)” characteristic of the infection of cv Jubila caused by the isolate RU1M (see 6), which developed as necrosis starting at the growing point and progressing downward to the lower leaves and eventually to the root system.

The symptomatic leaves from cultivars Dubble Witte and Sanilac inoculated with the four isolates, 1542k, 1574k, 1664k, and 1759k, were found positive for BCMV when tested by ELISA (Table 1 and Fig. 1). Isolates 1542k and 1574k could also induce the same syndrome in bean cvs Amanda and IVT7233, while 1664k isolate could induce the same wilting syndrome in bean cv RGLC. However, the symptomatic leaves from these three cultivars were found negative for BCMV when tested by ELISA (Table 1 and Fig. 1). Reference strains US1 (PG-I), NY15P (PG-V) and TN1 (PG-VI, BCMNV) displayed the expected virulence profiles with the differential lines based on their pathotypes (see Table 1). The presence of BCMV or BCMNV in inoculated bean plants was tested using TAS-ELISA (see Table 1), and the results always agreed with the symptom expression except that some cvs inoculated by killing isolates showed the severe wilting and plant death but were found to be negative in ELISA.

Serological characterization

The 15 field isolates were subjected to a serological characterization with TAS-ELISA side-by-side with reference strains US1 (B-serotype), NY15P (B-serotype), and TN1 (A-serotype), in order to determine serotypes for each of them. All the 15 field isolates and the control 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 (5). The anti-US10 antiserum reacted to all isolates tested, including all 15 field isolates, 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 suggested that all 15 field isolates displayed the B-serotype, characteristic of the control isolates US1 and NY15P (5).

Whole genome and partial genome cloning, sequencing, and sequence analysis

The small genome fragments from the HC-Pro regions of BCMV isolates 3915, 3918, 3921, 3982, 1574k, 1664k, 1759k, and 1755 were amplified using degenerate primers and sequenced using the approach described in materials and methods. The amplified segments were found to be around 700-nt long. Initially, the sequences of the eight isolates were compared to the known BCMV and BCMNV genomes using the BLAST 2.2.17 tool (2). The partial sequences determined for isolates 3915, 3918, 3982, 1664k, 1759k, and 1755 shared the closest similarity to the isolate NL1 (accession number AY112735; 90-97%

nucleotide identity), while partial sequences of isolates 3921 and 1574k shared the closest similarity to isolate MB (accession number KC832502; 95% nucleotide identity).

The partial sequences from HC-Pro region of BCMV isolates 3915, 3918 (all from PG-I), isolates 3921, 3982, 1574k, 1664k, and 1759k (all from PG-III), and isolate 1755 (from PG-VI), were aligned using CLUSTALX and further analysis was conducted with the RDP4 program package. Figure 2 shows the comparison of all sequences using the Neighbor-Joining algorithm analysis (see Fig. 2). Phylogenetic tree is labeled according to the pathogenicity grouping determined during biological characterization of the BCMV isolates (see Table 1 and Fig. 2). No compelling correlation could be seen between the phylogenies of the determined HC-Pro ca. 700-nt sequences and pathogenicity groupings. Based on the analysis of the genome sequence from partial HC-Pro of eight field isolates, all partially sequenced isolates could be classified into four groups. Isolates 3915 and 3918 were assigned to PG-I (see Table 1) and partial sequences of both isolates were 99% identical and shared 97% identities with the NL1 isolate (PG-I, accession number AY112735). Isolates 3921, 3982, 1574k, 1664k and 1759k all belonged to PG-III (Table 1), however, partial sequences of one clade including isolates 3921 and 1574k (99% identity between each other) were found to have 95% identities with the MB isolate (undetermined PG, accession number KC832502), while partial sequences of another clade including isolates 3982, 1664k and 1759k (>99% identity among each other) shared 92% identities with the NL1 isolate (PG-I, accession number AY112735). The HC-Pro sequences between these two clades with PG-III isolates were found to share 94% identities between each other. The partial sequence of isolate 1755 (PG-VI, see Table 1) was found to have 90% identity

with isolate NL1 (PG-I, accession numbers AY112735), which was the closest match found using the BLASTn analysis.

The partial sequences from the NIb-CP region of two BCMV isolates 3915 and 3918 were also amplifed with degenerate primers, cloned, and sequenced as described in materials and methods. Upon sequence assembly, the amplified segments were found to be around 750-nt long. Initially, the sequences of the two isolates were compared to the known BCMV and BCMNV genomes using the BLAST 2.2.17 tool (2), and found almost identical (99% identity) to each other and shared the closest similarity to isolate MS1 (undetermined PG, accession number EU761198; 90-91% nucleotide identity). This relatively low level of sequence identity indicated that both isolates represent a novel strain of BCMV.

The whole genomes of BCMV isolates US1 and NY15P were cloned and sequenced, using the approach described previously (5). Upon sequence assembly, US1 was found to be 10,051-nt long, excluding the poly (A). Based on conceptual translation, the BCMV US1 genome encoded a single polyprotein of 3,221 aa. NY15P was found to be 10,054-nt long, excluding the poly (A). Based on conceptual translation, the BCMV NY15P genome encoded a single polyprotein of 3,222 aa. Initially, the sequence of both isolates were compared to the known BCMV and BCMNV genomes using the BLAST 2.2.17 tool (2), and this revealed that the US1 and NY15P sequence 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, and NL1 (accession number AY112735) both from PG-I, together with NY15P (PG–V), were aligned using CLUSTALX and further analysis was conducted with the RDP4 program package. Figure 3 shows the comparison of all three

sequences using the manual distance plot analysis, with the full-length NY15P sequence as the reference (see Fig. 3). Based on the RDP4 analysis, the 5'- terminal sequences of isolates US1 and NL1, between nt 1-2090, shared more similarities to each other (98% identity) than to NY15p isolate (96% identity). The sequences of the downstream segment, between nt 2091-6850 (position in alignment) in the US1 and NY15P genomes were found to have close similarities to each other (97% identity), while NL1 was found to have 89% identities to the US1 and NY15P sequences in this area. The 3'-terminal genome segments between nt 6851-9330 (position in alignment), were found very similar among all three isolates (97-98% identity). In the 3'-terminal region, between nt 9,331-10,055 (position in alignment), sequences of US1 and NL1 analyzed were found to be 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 sections of the genome between nt 2091 to 6850 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 this similarity patterns (Fig. 3).

To see these possible recombination patterns in a more global context of known BCMV whole genome sequences, we compared the whole genome sequences of US1 and NY15P with the genomes of other BCMV isolates with known pathogenicity profiles, in addition to NL1 (isolates from PG-VI and PG-VII; 5, 6). This comparison conducted using the distance plot program from the RDP4 package is presented on Fig. 4. Interestingly, a large section of the US1 genome, from nt 2920 to 10055 (the 3'-terminus) was found common 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 6851 to 9331 was found common between isolates NL1 (PG-I), NY15P (PG-V), and US10 (PG-VII) (see Fig. 4). On the other hand, the two areas mentioned above, nt 1 to 2061 and 9331 to 10055 are the ones where the similarity between US1/NL1 and NY15P sequences is the lowest (Fig. 4), suggesting that pathogenicity determinants for PG-I and PG-V may be located in these two regions.

Discussion

Biological characterization on a set of bean indicators is currently the only reliable method of typing BCMV strains according to their pathogenicity (3, 4, 5, 12, 15). The genetic diversity of the BCMV isolates found in the field is high, and genetic determinants linked to specific pathogroups of BCMV have not yet been identified (5, 6). Although partial sequencing, for the capsid protein gene in particular, was often used in the past to characterize phylogeny and evolution of potyviruses, including BCMV, this approach was found problematic, especially for potyviruses with proven recombination history, like *Potato virus Y* (see 9, 10). In this work, a complete biological typing was conducted for 15 field isolates of BCMV collected in Oregon in 2013 on eleven bean cultivars carrying BCMV resistance genes (Table 1). Partial sequencing was performed for eight of these isolates, and phylogenies suggested no direct correlations between pathogroup assignment and specific clade placement in the sequenced region, HC-Pro cistron (Fig. 2). The most likely reason for this lack of correlations between phylogenies of HC-Pro and the pathogroup typing would be that HC-Pro is not involved in pathogenicity determinants for the PGs analyzed, *i.e.* PG-I, PG-III, and PG-V through –VII. The most striking was the distant placement of the two field isolates, 3921 and 1574k, relative to the other three field isolates, 1664k, 1759k, and 3982,

all five assigned to the same PG-III (see Fig. 2). Isolate 1755 was also found very distant from other PG-VI isolates which were grouped in the same lineage with at least three BCMV isolates from PG-VII. The low levels of sequence identities (below 91%) to the known strains of BCMV for some of these field isolates, *e.g.* 3915, 3918, 1755, suggest that they may represent novel strains of BCMV. Nevertheless, these partial sequences do not provide the information about the entire genome of BCMV isolates studied and possible recombination events in their genomes characteristic of other BCMV strains, like RU1 (5, 6).

Seven resistance genes govern interactions of BCMV and BCMNV with common beans, one dominant *I* gene, and six recessive genes, *bc-u*, *bc-1*, *bc-1*^{2}, *bc-2*, *bc-2*^{2}, and *bc-3*, and hence seven pathotypes are commonly distinguished among BCMV isolates, from PG-I through PG-VII (3). Specific genetic determinants of the BCMV genome responsible for these interactions have not yet been defined for most of these resistance genes. Identification of the genetic determinants involved in interactions with resistance genes is currently complicated because of the scarcity of full-length infectious clones of BCMV (7, 16), and wide spread of recombination between BCMV strains (see 5, 6). Nevertheless, using comparative genomics for several BCMV isolates from PG-VI and PG-VII, the genetic determinant of BCMV interacting with the *bc-2*^{2} gene between positions 1,287 to 1,976 of the BCMV genome were tentatively mapped (5, 6), while the genetic determinant of BCMV interacting with the dominant *I* gene was tentatively mapped between positions 513 to 1, 287 (6).

In this work, we continued our comparative genomic studies for BCMV strains from two other pathogenicity groups, PG-I (strain US1) and PG-V (NY15P). PG-I comprises BCMV isolates unable to overcome any of the recessive genes, *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 genes *bc-1* and *bc-2*, but not others. Interestingly, sequences determined for US1 (PG-I) and for NY15P (PG-V) were found relatively close to each other and to another BCMV isolate from PG-I, NL1 (see Figs 3 and 4). US1 was found to represent a recombinant between strains US10 and NY15P as the most likely parents (see Fig. 4). Based on the sequence analysis of NY15P, and six other complete genomes of BCMV isolates with defined pathogenicity (Fig. 4), the most likely genome areas involved in interactions with genes *bc-1* and *bc-2* may be located between positions 1 to 2,090 or positions 9,331 to 10,055.

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Table 2-1. Disease and ELISA reactions of bean differentials inoculated with BCMV strains[1)]

	Bean cultivar (*resistance genes*)										
Isolate ID	**Dubble Witte** (*none*)	**SGR** (*bc-u*)	**RGLC** (*bc-u,bc-1*)	**RGLB** (*bc-u,bc-1^2*)	**Sanilac** (*bc-u,bc-2*)	**UI35** (*bc-u,bc-1^2,bc-2^2*)	**IVT7214** (*bc-u,bc-2,bc-3*)	**Jubila** (*I,bc-1*)	**Amanda** (*I, bc-1^2*)	**US1006** (*I,bc-2^2*)	**IVT7233** (*I, bc-u, bc-1^2, bc-2^2*)
				PG-I							
3915	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
3918	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
3594	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
				PG-III							
3921	+/+	+/+	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
1033	+/+	+/+	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
2101	+/+	+/+	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
3102	+/+	+/+	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
3921	+/+	+/+	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
3982	+/+	+/+	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
1542k	WD/+	+/+	-/-	-/-	WD/+	-/-	-/-	-/-	WD/-	-/-	WD/-
1574k	WD/+	+/+	-/-	-/-	WD/+	-/-	-/-	-/-	WD/-	-/-	WD/-
1664k	WD/+	+/+	WD/-	-/-	WD/+	-/-	-/-	-/-	-/-	-/-	-/-
1759k	WD/+	+/+	-/-	-/-	WD/+	-/-	-/-	-/-	-/-	-/-	-/-
				PG-IV							
1748	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-
3110	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-
				PG-VI							
1755	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-
				Controls							

US1 (I)	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
NY15p(V)	+/+	+/+	+/+	-/-	+/+	-/-	-/-	-/-	-/-	-/-	-/-
TN1(VI)	+/+	+/+	+/+	+/+	+/+	-/-	nec/-	-/-	-/-	-/-	-/-

[1)] Disease reaction is shown first as a numerator followed by ELISA reaction as a denominator. One plant was inoculated for each BCMV isolate;

[2)] Numerator: + = symptoms on inoculated beans; - = no symptoms on inoculated beans; nec = systemic necrosic reaction on some uninoculated leaves; WD = "severe wilting and plant death".

[3)] Denominator: + designates ELISA signal (A_{405}) in an infected plant exceeding healthy control by 10-fold or more; - designates ELISA signal in an infected plant ca. equal to that of a healthy control.

Table 2-2. Primers used for cloning 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

[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.

Figure 2-1. Severe wilting and plant death induced upon inoculation of BCMV killing isolates. (A) Severe wilting and plant death induced upon inoculation of BCMV isolate 1542k and 1574k, in cv. Dubbele Witte, in comparison with the same isolates inducing typical mosaic reaction in cv. Stringless Green Refugee; 10 days post-inoculation. (B) Initial wilting symptom displayed on inoculated primary leave upon inoculation of BCMV isolate

1574k (PG-III), in cv. Dubbele Witte, in comparison with the non-killing isolate 2101 (PG-III) inducing typical stunting in cv. Dubbele Witte; 7 days post-inoculation.

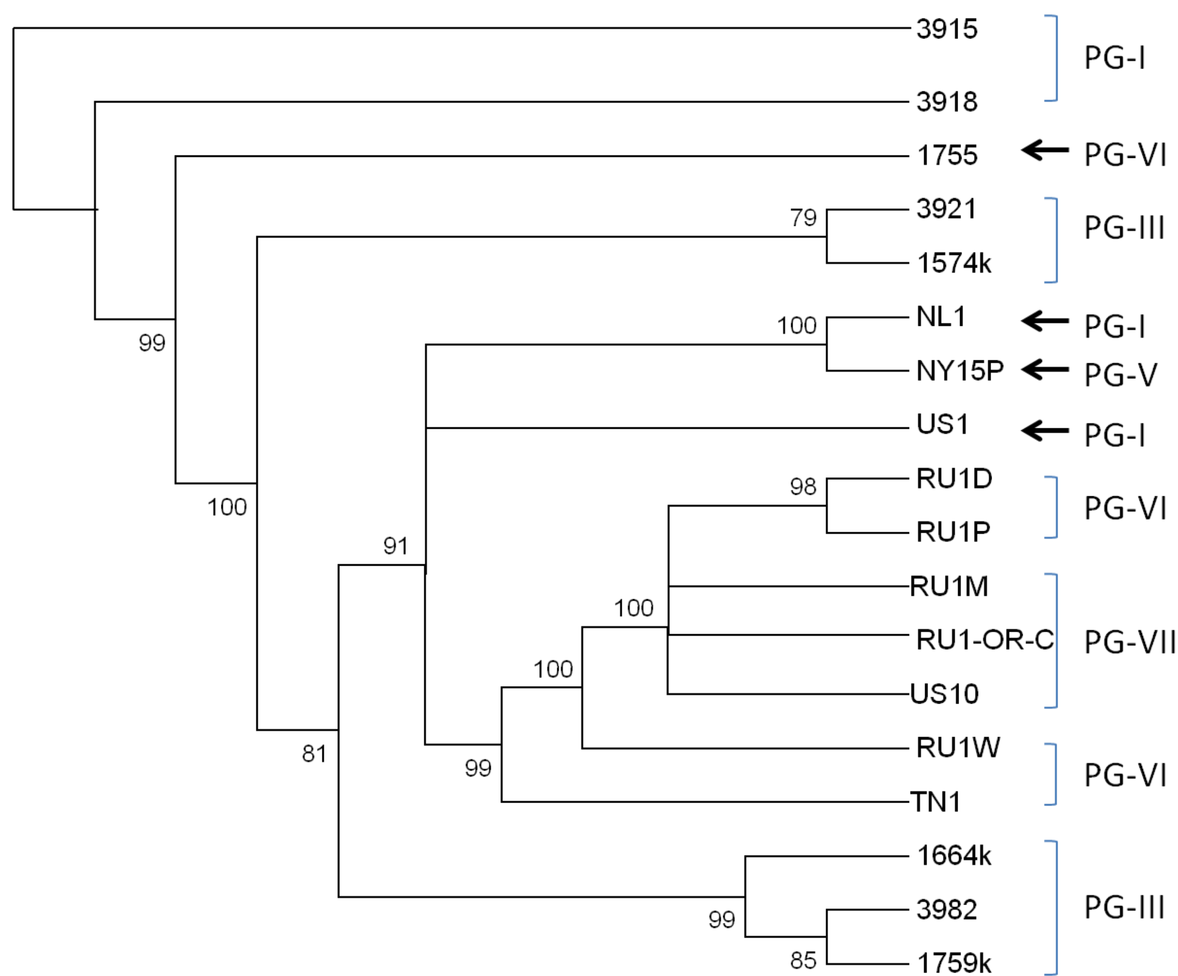

Figure 2-2. Phylogenetic tree created by Neighbor-Joining algorithm based on the aligned partial nucleotide sequences of HC-Pro of BCMV isolates 3915, 3918, 3921, 3982, 1574k, 1664k, 1759k, 1755, RU1M, RU1-OR, RU1P, RU1W, RU1D, US1, NY15P, NL1, US10 and BCMNV isolate TN1.

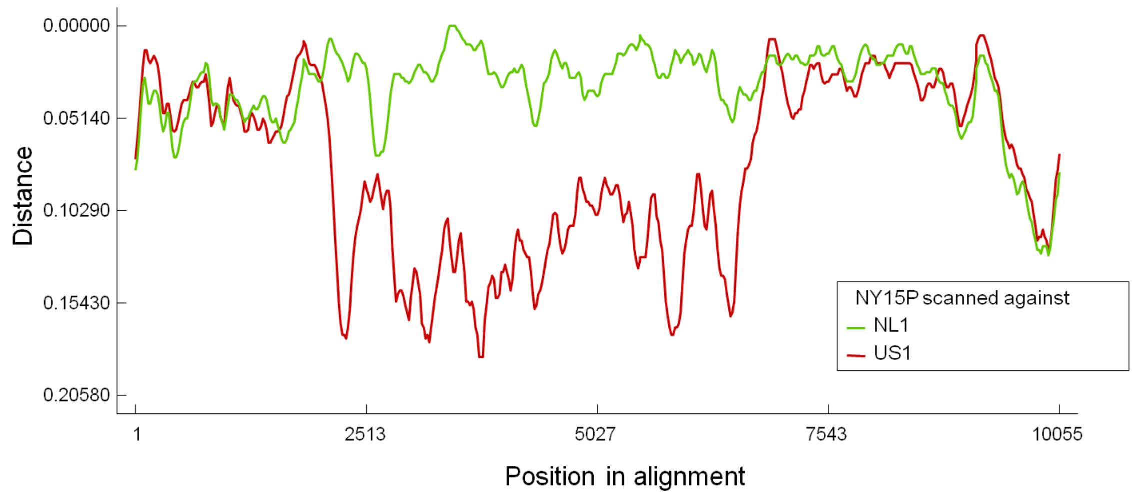

Figure 2-3. Manual distance plot based on the aligned full-length nucleotide sequences of BCMV isolates US1, NL1 and NY15P. NY15P (PG-V) was used as the reference strain. X axis represents nucleotide position in the alignment, Y axis represents relative distance from the reference sequence which is calculated using Kimura model (Kimura, 1980).

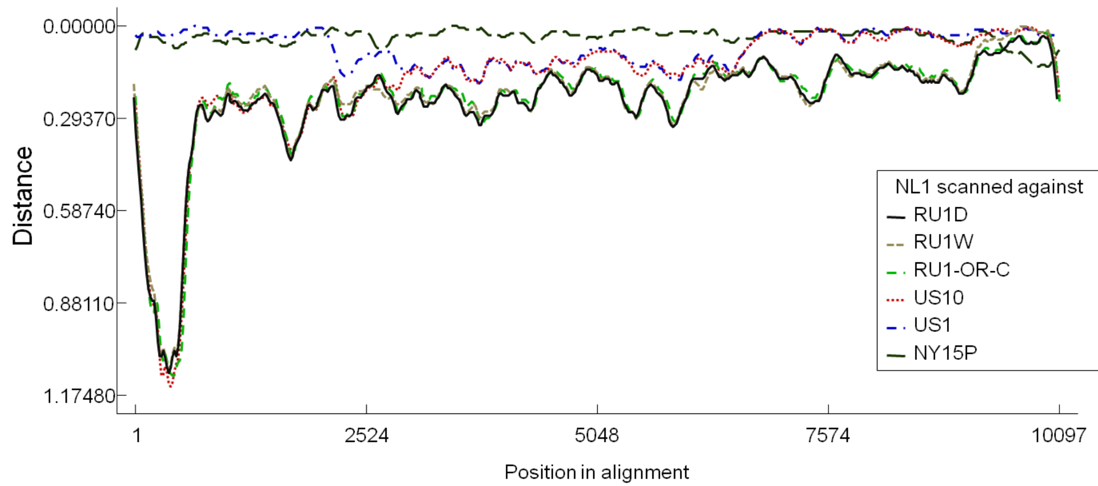

Figure 2-4. Manual distance plot based on the aligned full-length nucleotide sequences of BCMV isolates RU1-OR-C, RU1P, RU1D, US1, NY15P, NL1, and US10. NL1 (PG-I, accession numbers AY112735) was used as the reference strain. X axis represents nucleotide position in the alignment, Y axis represents relative distance from the reference sequence which is calculated using Kimura model (Kimura, 1980).

Chapter 3

Recombinants of *Bean common mosaic virus* (BCMV) and genetic determinants of BCMV involved in overcoming resistance in common beans

Abstract

Bean common mosaic virus (BCMV) exists as a complex of strains classified by reactions to resistance genes found in common bean (*Phaseolus vulgaris* L.); seven BCMV pathotypes have been distinguished so far, numbered I to VII. Virus genetic determinants involved in pathogenicity interactions with resistance genes have not yet been identified. Here, we describe the characterization of two novel field isolates of BCMV that helped to narrow down these genetic determinants interacting with specific *P. vulgaris* resistance factors. Based on a biological characterization on common bean differentials, both isolates were classified as belonging to pathotype VII, similar to a control isolate US10, and both isolates exhibited B-serotype. The whole genome was sequenced for both isolates and found to be 98-99% identical to the BCMV isolate RU1 (pathotype VI), and a single name was retained, BCMV RU1-OR. To identify a genetic determinant of BCMV linked to the BCMV pathotype VII, the whole genome was also sequenced for two control isolates, US10 and RU1P. Inspection of the nucleotide sequences for BCMV RU1-OR and US10 (both pathotype VII), and three closely related sequences of BCMV, RU1P, RU1D, and RU1W (all pathotype VI) revealed that RU1-OR originated through a series of recombination events between US10 and a yet unidentified BCMV parental genome, resulting in changes in virus pathology. The data obtained suggest that a fragment of the RU1-OR genome between positions 723-1,961 nt that is common to US10 and RU1-OR in the P1-HC-Pro

region of the BCMV genome may be responsible for the ability to overcome resistance in beans conferred by the *bc-2*^{2} gene. This is the first report of a virus genetic determinant responsible for overcoming a specific BCMV resistance gene in common bean.

Introduction

Bean common mosaic virus (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) belong to the genus *Potyvirus* in the family *Potyviridae* (1), and cause serious economic losses in common beans (*Phaseolus vulgaris* L.) (8, 33, 42). Both are typical potyviruses, transmitted by several aphid species in a non-persistent manner (33). BCMV and BCMNV are closely related, induce similar symptoms in beans, and exist as a complex of strains with multiple isolates which differ in their virulence on common bean cultivars. BCMV/BCMNV isolates are classified into seven pathotypes according to their reactions on 12 to 14 bean differentials with known combinations of resistance genes (5). All BCMV and BCMNV strains are seed-transmitted in beans, which is the main reason for the world-wide distribution and importance of this virus (33).

Five resistance genes govern interactions of BCMV and BCMNV isolates with common bean - one strain nonspecific dominant *I* gene, and four strain specific recessive genes, *bc-u*, *bc-1*, *bc-2*, and *bc-3* (5). Two of these recessive resistance genes have two resistance alleles each (*bc-1*/*bc-1*^{2} and *bc-2*/*bc-2*^{2}) for a total of seven genes or alleles that interact with the different pathotypes of BCMV/BCMNV. The strain specific resistance genes are unlinked, but *bc-u* is required for expression of resistance by the other *bc-* genes. The dominant *I* gene confers immunity or a hypersensitive response to all isolates of BCMV/BCMNV, i.e. no detectable virus replication occurs. If a BCMNV isolate is

inoculated into an *I*-gene carrying cultivar, a necrotic reaction occurs, regardless of the temperature, varying from limited vein necrosis on the inoculated leaf to a severe, whole plant necrosis, called “black root syndrome”. This reaction is called temperature insensitive necrosis (TIN). When such necrotic reaction occurs, no virus replication is detected in leaf tissues surrounding necrotic tissue, and no virus transmission through seed can be detected, resulting in a resistance reaction at the plant level. The necrotic reaction may be mitigated or prevented if the *I* gene is “protected” in the presence of one of the three recessive genes with various alleles, *bc-1*, $bc\text{-}1^2$, *bc-2*, $bc\text{-}2^2$ or *bc-3*. Whereas *bc-u* is necessary for expression of other strain-specific resistance genes, it is not required for protection of the *I* gene by the strain specific resistance genes. If a BCMV isolate is inoculated into an *I*-gene carrying cultivar, no necrotic reaction occurs if temperature stays below 30°C; however, above 30°C, necrotic reaction may occur, which may be very similar to the necrotic reaction caused by BCMNV isolates. This type of reaction is called temperature sensitive necrosis (TSN).

BCMNV isolates belong to pathotype VI, and all have A-serotype as opposed to serotype B in BCMV isolates (5, 31, 44, 50). Both BCMV and BCMNV isolates will induce mosaic, leaf deformations, blistering, and sometimes severe stunting in susceptible bean cultivars (5, 31, 33, 44). Not all pathotype VI isolates have serotype A and induce TIN; RU1 isolate was described as a BCMV isolate with pathotype VI and B-serotype inducing TSN (3, 22, 31). The genome regions of BCMV and BCMNV involved in interactions with either the *I* gene, or recessive genes are not yet mapped, although based on the analysis of NL-3K, a natural recombinant of BCMNV, the genome region in the P1 gene was found modulating symptom severity in common beans (22). Characterization of the pathogenicity determinants in BCMV and BCMNV responsible for interactions with resistance genes in common beans

is currently hampered by the diversity of isolates, and by the complexity of the biological assays, especially when recessive genes are studied. An additional complication is that in some cases nucleotide sequences determined for BCMV or BCMNV isolates were not complemented by concomitant biological experiments, and hence are difficult to interpret now in terms of pathogenicity determinants.

Here, we report a systematic study of biological and molecular properties of several BCMV isolates from a large virus collection maintained in Prosser, Washington. We also studied two unusual field isolates of BCMV that helped to initially map genetic determinants in the virus that interact with $bc\text{-}2^2$. Characterization of the biology and molecular properties of these isolates, and other isolates with the same or differing pathogenicity on bean differentials, allowed us to localize putative genetic determinants of pathogenicity interacting with the $bc\text{-}2^2$ gene.

Materials and methods

Virus sources, propagation, and purification

All reference BCMV and BCMNV isolates used in this work originated from the USDA-ARS Prosser, WA, collection. BCMV isolates US10, NY15P, and BCMNV isolate TN1 were provided by Drs. P. Berger and P. Shiel; BCMV isolate RU1P was provided by Dr. J. Crosslin. Two field isolates of BCMV, JMB and JMC, later named as RU1-OR-B and RU1-OR-C, were collected from the OSU Vegetable Research Farm, Corvallis, OR, (N44° 34' 28.91", W123° 14' 30.59") from bean plants exhibiting mild mosaic and stunting symptoms in the summer of 2011. The isolates appeared to be seed-borne in the line L192, a

brown-seeded dry bean with determinant growth habit most likely originally derived from interspecific hybridization work with *P. coccineus* conducted by H. Lamprecht (11,19,20).

All virus isolates were propagated under greenhouse conditions in the bean cultivar 'Dubbele Witte' using mechanical inoculation. Leaf tissue was harvested 14 to 21 days post-inoculation (dpi), and the virus was purified using a procedure described by Leiser and Richter (24) with some modifications. Briefly, 100 g of leaf tissue was homogenized in 100 ml cold extraction buffer (0.5 M potassium phosphate buffer, pH 7.5, containing 0.02 M sodium sulfite). Leaf homogenate was squeezed through cheesecloth and centrifuged at 6,000 rpm for 15 min. Triton X-100 was added to the supernatant to a final concentration of 3% (v/v). The mixture was stirred at 4°C for 30 min and centrifuged at 30,000 x g for 2 h. The pellet was resuspended in a resuspension buffer (0.5 M potassium phosphate buffer, pH 7.5) and then centrifuged at 15,000 x g for 15 min. The supernatant was collected and laid over a cushion of 20% (w/v) sucrose (in deionised water), followed by a high-speed centrifugation at 50,000 x g for 2 h. The final pellet was resuspended in the storage buffer (0.25 M potassium phosphate buffer, pH 7.5). The purified viruses were kept with the sodium azide preservative at 4°C for later use.

Antibody production, ELISA format and Western blots

Polyclonal antisera against TN1, US10, and NY15P were raised in rabbits and in guinea pigs following a series of 4 to 6 immunizations, with the first one in the presence of complete Freund's adjuvant, and all subsequent with the presence of incomplete Freund's adjuvant. The development of the virus-specific titer was monitored by indirect enzyme-linked immunosorbent assay (ELISA), with purified homologous BCMV or BCMNV isolate

captured on the ELISA plate. Two specific antisera were produced for each isolate, one in rabbit, and one in guinea pig.

Triple-antibody sandwich (TAS) ELISA tests were performed following the general protocol of Clark and Adams (4) with modifications described previously (17). Briefly, wells of Nunc MaxiSorp microtiter plates (Nunc, Rochester, NY) were coated with 100 µl of the rabbit antiserum at 1:10,000 dilution in 20 mM sodium carbonate buffer (pH 9.6). Plates were incubated with plant extracts for 16 to 20 h at 4°C, washed with phosphate-buffered saline plus Tween 20 (PBST), and an intermediate detecting antiserum from guinea pig at the appropriate concentration was applied to the wells. After incubation for 4 h at 37°C (or, alternatively, for 16 to 20 h at 4°C), plates were washed extensively with PBST, and goat anti-guinea pig (Sigma A-5062) IgG-conjugates with alkaline phosphatase at 1:30,000 dilution in PBST with 0.2% BSA were added, and the plates were incubated 4 h at 37°C (or 16 to 20 h at 4°C). After washing and adding *p*-nitrophenyl phosphate (Sigma) substrate, the color reaction was monitored by measuring absorbance at 405 nm using an ELISA reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA). BCMV/BCMNV-positive and -negative samples were included into each ELISA experiment as controls. Samples were defined as positive if the absorbance value exceeded the healthy controls three-fold.

For Western blots, purified virus preparations were mixed with the Laemmli Tris-SDS sample buffer, heated at 95°C for 4 min, and proteins were separated on 4 to 20% gradient polyacrylamide gels using Laemmli's Tris-SDS protocol (Bio-Rad, USA). Separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, USA) as described previously (17). The membranes were incubated for 2 h with the respective guinea pig polyclonal antiserum following incubation with alkaline phosphatase conjugated goat

anti-guinea pig IgG (Sigma A-5062). The immune complexes were revealed by incubating the membranes with BCIP/NBT substrate (Sigma) and the color reaction was stopped by washing them in water.

BCMV and BCMNV biological characterization on bean indicators

A set of bean differentials (5) representative of the various host groups (HG) were evaluated for reaction to infection with BCMV and BCMNV isolates. The differentials (Table 1) included cultivars Dubbele Witte (HG 0), Stringless Green Refugee (HG 1), Redlands Greenleaf C (HG 2), Redlands Greenleaf B (HG 3), Sanilac (HG 4), IVT-7214 (HG 7), Jubila (HG 9), Amanda (HG 10), US92-1006 (HG 11), and IVT-7233 (HG 11). All biological experiments were conducted under greenhouse conditions, following mechanical inoculation of bean indicator plants at the primary leaf stage. When infected plants were used as inocula, 0.2 g samples were ground in mortars with pestles with 1 ml Phosphate-Buffered-Saline (PBS, 4°C), plant sap was clarified by low-speed centrifugation (2,000 x *g*), and then used for the mechanical inoculation of bean leaves dusted with carborundum powder. Purified virus preparations of three reference strains NY15P (pathotype V), TN1 (pathotype VI), and US10 (pathotype VII) were included in this analysis as controls. Eleven bean differential lines carrying different resistant gene combinations (Table 1) were inoculated with each isolate in duplicates, and plants were placed in the greenhouse with standard summer-time growth conditions (16 hr photoperiod and daytime/nighttime temperatures of 25/20 °C). Symptoms were recorded at 4.5 and 7.5 weeks post-inoculation. In addition, at 4.5 weeks post-inoculation samples were collected for ELISA testing.

Cloning strategy, sequencing, and sequence analysis

The cloning strategy for all BCMV isolates was based on the initial amplification of three genome fragments through reverse transcriptase polymerase chain reaction (RT-PCR) using degenerate primers developed for conserved areas of potyvirus genomes, as described by Ha et al. (10). Briefly, three pairs of degenerate primers were used to amplify conserved areas in the HC-Pro, CI, and NIb-3'-end regions. Once these initial fragments were cloned and sequenced, the remaining gaps were filled through RT-PCR using specific primers based on the sequences determined (Fig. 1). All primers used in this study are listed in Table 2. The whole genome sequences for BCMV isolates have been deposited in the GenBank database under the following accession numbers: RU1-OR-B (KF919297), RU1-OR-C (KF919298), US10 (KF919299), and RU1-P (KF919300).

Virus RNA was extracted as described previously (14). Reverse transcription was performed using 6 µl of the total nucleic acid extract in a 50 µl reaction mixture that contained 5× first-strand buffer (Promega), 0.5mM of each dNTP, 0.15 µM N1T primer, 24 units RNase Out Ribonuclease Inhibitor (Promega), and 240 units of M-MLV reverse transcriptase (Promega). Before the reverse transcription reaction, 6 µl of RNA template was incubated at 70ºC for 5 min, then the reverse transcription mix was added. The profile used here consisted of incubation at 37ºC for 60 min and reverse transcriptase deactivation at 70ºC for 10 min. All PCR reactions, except the 5'-end fragment amplification, were accomplished by Takara LA Taq in a 50 µl reaction mixture that contained 10x LA buffer 5 µl, 0.2 mM dNTP, 0.2µM of each forward primer and reverse primer, 2.5 units LA Taq, 6 µl cDNA template. The PCR profile consisted of denaturing at 94ºC for 2 min, and 35 cycles of 94 ºC for 30 s, 55-65ºC for 30 s (depending on the melting temperature of primers used), 72

ºC for 0.5 to 4.5 min (depending on the fragment length amplified), followed by a final extension for 10 min at 72ºC. The 5' end of the viral genome was amplified using the 5' Rapid Amplification of cDNA Ends (5' RACE) Kit (Roche). The protocol followed the manufacturer's instructions. RNA extracted from purified virus prep was used as the template to synthesize the first-strand cDNA. The first-strand primer was 5'RACE rev1 and the PCR reverse primer was 5' RACE rev2 (see Table 2).

All PCR products were purified from low melting agarose gels and cloned into the plasmid vector pGEM-T Easy (Promega) as recommended by the manufacturer. Plasmid DNA was purified from transformed *E. coli* XL2-Blue Ultracompetent cells (Aligent) using alkaline lysis and clones containing inserts of expected size (based on primer positions) were identified by *EcoR* I digestion. Three independent clones for each RT-PCR fragment were sequenced in both directions by GENEWIZ, Inc. (South Plainfield, NJ) using the M13F (-21) and M13R primers for initial sequencing, and additional virus-specific primers for large fragments.

The sequences were initially analyzed using BLASTn 2.2.17 (2) tool available at the National Center for Biotechnology Information (NCBI). Complete viral genomes were assembled using SeqMan (DNASTAR, Madison, WI). Open reading frames (ORFs) were identified using the ORF Finder program available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). 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) (27). Further analysis for ClustalX-aligned sequences was performed by the program package RDP4.

Results

Biological characterization

When tested on the eleven bean differential lines, both the JMB and JMC isolates of BCMV RU1-OR showed identical disease reactions, producing typical mosaic, raised dark green blistering, and growth retardation in susceptible bean varieties. Presence of BCMV in inoculated plants was confirmed with TAS-ELISA (see Table 1), and it always matched the symptom expression. Reference strains NY15P (pathotype V), TN1 (pathotype VI), and US10 (pathotype VII) showed the expected susceptibility profiles with the differential lines based on their pathotypes. Both JMB and JMC isolates displayed susceptibility profiles identical to those of the pathotype VII reference strain, US10. No necrotic reactions were recorded in bean cultivars Jubila, Amanda, US 1006, and IVT 7233, harboring the *I* gene, upon inoculation with either JMB or JMC isolates. Plants deemed to be negative for virus replication by TAS-ELISA, showed no disease symptoms. Some Othello plants inoculated with either US10 or JMC virus preparations, did not show mosaic or score positive with ELISA until 7.5 weeks post-inoculation indicating late or very slow virus spread.

Serological characterization of BCMV and BCMNV isolates

Both JMB and JMC isolates were subjected to a serological characterization in TAS-ELISA and Western blots, side-by-side with reference strains US10, NY15P, and TN1, to determine their serotype. In these experiments, all BCMV and BCMNV isolates were captured on the ELISA plate using anti-TN1, anti-US10 or anti-NY15P rabbit antiserum, and were subsequently probed with different guinea pig detecting antibodies generated to specific isolates of BCMV/BCMNV mentioned above. Serological specificities of detecting

antibodies were also tested using Western blots, and were found to match the TAS-ELISA specificity. It should be noted that the titer of the antisera produced against all three viruses in either rabbits or guinea pigs was very high, and resulted in working dilutions for detecting antibodies to be in the range between 1:300,000 to 1:400,000, providing robust detection (and differentiation) tool for the BCMV strains studied. The serotyping data are summarized in Table 3. The anti-US10 guinea pig antiserum displayed universal specificity when used as detecting antibody in TAS-ELISA, binding all isolates tested, including JMB and JMC, and also the BCMNV isolate TN1. On the other hand, the anti-TN1 guinea pig antiserum showed considerable selectivity binding only the homologous isolate TN1 when used as the detecting antibody. The anti-NY15P guinea pig antiserum was able to bind the homologous isolate NY15P and one heterologous isolate US10. The data presented in Table 3 indicated that there was some cross-reactivity between different strains of BCMV and BCMNV in TAS-ELISA and in Western blots due to close relationship between BCMV and BCMNV. Nevertheless, JMB and JMC isolates displayed a clear serological distinction from the US10 isolate, despite identical pathotypes as determined on a set of bean differential cultivars (see Table 1).

Whole genome cloning, sequencing, and sequence analysis

The whole genomes of BCMV isolates JMB and JMC genome were cloned separately, using initially RT-PCR amplification of three conserved regions characteristic of potyviruses through the use of degenerate primers as described in Materials and Methods. The three initial clones were sequenced and, surprisingly, were found to be 98 to 99% identical to the known sequence of the BCMV strain RU1. Two large gaps between clones

BCMV-HP and BCMV-NIb were filled in via RT-PCR amplification using specific primers as listed in Table 2. The large 5'-terminal clone BCMV-mg1 was obtained through RT-PCR amplification using specific primers based on the RU1 sequence (BCMV isolate RU1-D, GenBank accession GQ219793). The very 5'-terminal sequences for both JMB and JMC were obtained through the use of the 5'-RACE technique as described in Materials and Methods. Upon sequence assembly, both JMB and JMC were found 9,984-nt long, excluding the poly (A). The two sequences were nearly identical, with only 23 nucleotide differences (ca. 99.8% nucleotide identity) resulting in 12 amino acid changes in the entire genome (Table 4); six of these amino acid changes were chemically conserved. Because of this high level of identity between the two sequences, a single name was retained, BCMV RU1-OR. Based on conceptual translation, the BCMV RU1-OR genome encoded a single polyprotein of 3,196 aa. The sequences of RU1-OR-B and RU1-OR-C were compared to the known BCMV and BCMNV genomes using BLAST tool, and the comparison revealed that both the RU1-OR-B and -C sequences shared the closest similarity to the sequence referred as RU1-D (accession number GQ219793; 98-99% nucleotide identity), except for a small fragment near the 5' terminus (see below).

Two other BCMV isolates, US10 and RU1P, were sequenced using the same approach. The full-length BCMV US10 genome was cloned, sequenced, and found to be 9,998-nt long. The BCMV US10 genome encoded a single polyprotein of 3,201 aa. A control RU1 isolate, RU1P (pathotype VI) was also cloned and sequenced. The whole genome of RU1P was found to be 10,003-nt long, coding for a single polyprotein of 3,202 aa. RU1P sequence was almost identical to RU1D with only 44-nt changes (ca. 99.6% identity) for the entire genome.

The whole genomes for RU1-OR, RU1P, RU1W, RU1D, and US10, were aligned using CLUSTALX program and subjected to a recombination analysis using the RDP4 package of programs. Figure 2 shows the comparison of all known RU1 whole genome sequences plus the sequence of US10 using the manual distance plot analysis, with the full-length BCMV RU1D sequence from the GenBank as the reference.

The RDP4 analysis demonstrated that the 5'-terminal fragment of RU1-OR genome (nt 1-722) was dissimilar from other RU1 sequences and also from the US10 sequence (Fig. 2). The downstream segment spanning nt 723 to 1,976 (position in alignment) was nearly identical (98%) for RU1-OR and US10 on one hand, and among RU1D, RU1P, and RU1W on the other hand (99% identity). The identity level between these two groups in the same region was 96%. A fragment of the RU1W genome between nt 1,977 to 2,720 was highly dissimilar from all other sequences analyzed (Fig. 2). A large section of the US10 genome, nt 2,721 to 9,114, displayed a more distant relationship to the RU1-OR and other RU1 sequences (Fig 2). In the 3'-terminal region, downstream of nt 9,120 (position in alignment), RU1D and RU1-OR on one hand, and RU1P, RU1W, and US10 on the other hand, formed two distinct similarity groups. These patterns of similarity and dissimilarities are presented in Fig. 3.

Discussion

Potyviruses are a large family of positive-strand RNA viruses well known for their propensity to rapid evolution through accumulation of mutations and extensive recombination (see 9). Recombination is thus one of the factors that allowed potyviruses to adapt to various hosts and different environments in order to survive and spread. The genetic

diversity of some potyviruses, like *Tobacco vein banding mosaic virus* (TVBMV; 52), *Zucchini yellow mosaic virus* (ZYMV; 23), *Turnip mosaic virus* (TuMV; 37,47,48), *Potato virus Y* (PVY; 14-15, 16,25), or *Soybean mosaic virus* (SMV; 40) has been well documented, and genetic determinants involved in interactions with resistance genes had been identified in *Wheat streak mosaic virus* (WSMV) (45), PVY (34,46,49), and in SMV (6,12,39). However, until recently, BCMV and BCMNV genetic diversity was not revealed to the same extent as in other potyviruses, despite an extremely well developed genetics of BCMV resistance in common beans (see 5,42). So far, only a few whole genomes for BCMV and BCMNV had been published (21,22,35,36). A natural recombinant of BCMNV, NL-3K, was described by Larsen et al. (21), carrying a small insert of the BCMV RU1 sequence near the 5'-terminus of the otherwise BCMNV genome; this recombinant was associated with more severe symptoms on certain bean cultivars (7,21,30,44).

RU1 is currently known as the only TSN isolate of BCMV assigned to pathogroup VI (21,31). The original RU1 strain was intercepted in mid-1980s in a dry bean entry imported from Russia (28,29,31). Since then it was confined to the lab, staying in the Prosser, WA, virus collection and was never reported from the field. Two complete genome sequences of RU1 were deposited in the GenBank database: RU1W sequence was published by Larsen et al. (21), and RU1D sequence was published by Naderpour et al. (36). Interestingly, both RU1D and RU1W sequences came from the same virus collection maintained in Prosser, WA. This indicates a substantial diversity in the original BCMV-RU1 strain intercepted in that dry bean shipment (31).

In this paper, we describe a novel BCMV isolate, RU1-OR, which was collected from L192 field grown beans in the Willamette Valley, Oregon. Hence, our RU1-OR

represents the first example of an RU1 strain found in the field in North America, and perhaps first in the entire world. Initial biological typing on a panel of bean indicators defined RU1-OR as a pathotype VII isolate (Table 1) capable of overcoming one of the most advanced resistance genes, *bc-2*^{2} (5, 42). Biologically, RU1-OR was found identical to the control isolate US10 displaying the same pathotype VII (Table 1). However, since serological properties between US10 and RU1-OR did not match (Table 3), the genomes of both US10 and RU1-OR were cloned, sequenced, and compared to each other and to other related sequences available in public databases. RU1-OR was found very close to the previously sequenced isolates RU1D and RU1W, which displayed a TSN pathotype VI (21,31,35,36). The sequence dissimilarities found between RU1-OR and US10 in the CP region (Fig. 2 and Fig. 3), especially in the 5'-terminal area of the cistron, may account for the serological distinction found between these two BCMV isolates (Table 3). It was demonstrated previously that the N-terminal one-third of potyvirus capsid proteins harbored most of the strain-specific epitopes (32,41). This apparent disconnect in serological differences between US10 and RU1-OR (Table 3), on one hand, and identical pathotype (Table 1), on the other hand, may suggest that the region of the BCMV genome responsible for the interactions with the recessive *bc-2* and *bc-2*^{2} genes lies outside of the CP gene.

Closely related molecular characteristics of RU1-OR and other RU1 isolates, and clearly different reactions towards *bc-2* and *bc-2*^{2} genes, make them perfect candidates to search for genetic determinant(s) responsible for the expression of BCMV pathotypes VI and VII in common beans. All RU1 isolates studied displayed the same symptoms on the first three indicator bean lines carrying recessive genes *bc-u*, *bc-1* or *bc-1*^{2}, Stringless Green Refugee (HG 1), Redlands Greenleaf C (HG 2), and Redlands Greenleaf B (HG 3), the only

difference observed was that RU1-OR could infect bean line carrying *bc-u* and *bc-1*^{2} or *bc-2*^{2} resistance genes instead of *bc-u* and *bc-2* for RU1P (Table 1). When inspecting the RU1-OR pathogenicity profile and our genome analysis, it appears that the region spanning the C-terminal half of P1 and the N-terminal part of HC-Pro in pathotype VII isolates may interact with the *bc-2*^{2} gene (Fig. 3). However, all the hypotheses on the locations of genetic determinants responsible for interactions with BCMV resistance genes in beans need to be tested using reverse genetic tools.

Many previous reports suggested that P1 and HC-Pro were both multifunctional proteins involved in important aspects of virus-host interactions in potyviruses (reviewed by 26,38). Klein et al. (18) found that an insertion mutant in the P1 region of *Tobacco vein mottling virus* was able to modulate disease symptoms. Recent reverse-genetics experiments demonstrated that the HC-Pro cistron of PVY is involved in triggering both *Nc* (34) and *Ny* (46) genes in potato and induction of necrosis. HC-Pro region was also demonstrated to be involved in host specificity and virulence of WSMV (43). Reverse-genetics also revealed the role of HC-Pro and P3 cistrons in overcoming resistance to *Soybean mosaic virus* in soybean (6, 51). NL-3K, the natural recombinant of BCMNV carrying a ca. 350-nt insert of the BCMV RU1 sequence in the P1 cistron, caused more severe symptoms on selected common bean lines (7, 21, 30, 44). A possible recombination event between *Blackeye cowpea mosaic virus* and BCMV found in the 5'-terminal region (5'-UTR and P1) in two isolates of BCMV from asparagus bean (*Vigna unguiculata* spp. *sesquipedalis* L.) was linked to symptom modulation in this host (53). The differences found in the P1 region among three strains of BYMV (BYMV, BYMV-MB4 and BYMV-S) were linked to the uncharacteristic symptom expression and host range changes (13). Our data showing that the P1-HC-Pro region of

BCMV may be involved in interactions with the *bc-2*^{2} gene are thus consistent with previous research conducted on other potyviruses.

The data presented here suggests that recombination between BCMV strains may be quite common, and BCMV strains US10 and RU1 represent multiple recombinants, with isolates sometimes changing their pathotypes. Of the sequences analyzed, US10 may be considered the most likely parental strain while other parents have yet to be identified (Fig. 3). With a limited number of whole genome sequences available for BCMV isolates at the moment, it is difficult so far to appreciate the entire spectrum of the BCMV genetic diversity and its role in BCMV pathogenicity.

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Table 3-1. Disease and enzyme-linked immunosorbent assay (ELISA) reactions of bean differentials inoculated with *Bean common mosaic virus* (BCMV) strains[1)]

Bean cultivar	Resistance genes	TN1 (VI)	US10 (VII)	JMB	JMC
Dubble Witte	none	+/+[2)]	+/+	+/+	+/+
SGR	*bc-u*	+/+	+/+	+/+	+/+
RGLC	*bc-u,bc-1*	+/+	+/+	+/+	+/+
RGLB	*bc-u,bc-1*^{2}	+/+	+/+	+/+	+/+
Sanilac	*bc-u,bc-2*	+/+	-/-	-/-	-/-
Othello	*bc-u,bc-1*^{2}*,bc-2*^{2}	-/-	+/+	+/+	+/+
IVT7214	*i,bc-u,bc-2,bc-3*	-/+	-/-	-/-	-/-
Jubila	*I,bc-1*	nec/NA	-/-	-/-	-/-
Amanda	*I, bc-1*^{2}	-/-	-/-	-/-	-/-
US1006	*I,bc-2*^{2}	-/-	-/-	-/-	-/-
IVT7233	*I, bc-u, bc-1*^{2}*, bc-2*^{2}	-/-	-/-	-/-	-/-

[1)] Disease reaction is shown first as a numerator followed by ELISA reaction as a denominator. Two plants were inoculated per each BCMV strain; results shown were the same for both plants inoculated with each virus preparation.

[2)] Numerator: + = symptoms on inoculated beans; - = no symptoms on inoculated beans; nec = whole plant necrosis reaction.

Denominator: + 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; NA designates “not applicable”, since the plant died before the test could be run.

Table 3-2. Primers used for cloning the whole *Bean common mosaic virus* (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	
RU1-OR specific primers		
RU1-OR mg1 For	CAACAACTTCTCGCAACCAACCAC	Specific primer to amplify the major gap 1
RU1-OR mg1 Rev	CCCTTTGCCCATTAGGATTCCTCC	Specific primer to amplify the major gap 1
RU1-OR mg2 For	GAGTCCAACTAAGAGACACC	Specific primer to amplify the major gap 2
RU1-OR mg2 Rev	CACGTCCTGGTGGTGTTGCTGATAC	Specific primer to amplify the major gap 2
RU1-OR mg3 For	GTATCAGCAACACCACCAGGACGTG	Specific primer to amplify the major gap 3
RU1-OR mg3 Rev	TCCGGTGAAGTGCCATTGTCAATAC	Specific primer to amplify the major gap 3
RU1-OR 5RACE Rev1	CTTAGCGCGCGCCATTCCTGC	1st strand primer for 5'RACE
RU1-OR 5RACE Rev2	GTGGCACAGTGATTGTTCC	PCR primer for 5'RACE
US10 specific primers		
US10 mg1 For	CAACAACTTCTCGCAACCAACCAC	Specific primer to amplify the major gap 1
US10 mg1 Rev	CTGTATTTGCTATAACCTTCCG	Specific primer to amplify the major gap 1
US10 mg2 For	CCATCCAGAAACCAGAAGTGC	Specific primer to amplify the major gap 2
US10 mg2 Rev	CAGCGAGAGGTCGTGTTGG	Specific primer to amplify the major gap 2
US10 mg3 For	GTCGAGATCAACACAATGGG	Specific primer to amplify the major gap 3

US10 mg3 Rev	CGCTCTTGTATGTCCTCATCGC	Specific primer to amplify the major gap 3
US10 5RACE Rev1	TGATACCCTCTCTCACCCC	1st strand primer for 5'RACE
RU1P specific primers		
RU1P mg1 For	CAACAACTTCTCGCAACCAACCAC	Specific primer to amplify the major gap 1
RU1P mg1 Rev	CCCTTTGCCCATTAGGATTCCTCC	Specific primer to amplify the major gap 1
RU1P mg2 For	GAGTCCAACTAAGAGACACC	Specific primer to amplify the major gap 2
RU1P mg2 Rev	CACGTCCTGGTGGTGTTGCTGATAC	Specific primer to amplify the major gap 2
RU1P mg3 For	GTATCAGCAACACCACCAGGACGTG	Specific primer to amplify the major gap 3
RU1P mg3 Rev	TCCGGTGAAGTGCCATTGTCAATAC	Specific primer to amplify the major gap 3
RU1P 5RACE Rev1	CACTTTGCCGATGTATTCCTTCTG	1st strand primer for 5'RACE
RU1P 5RACE Rev2	GTCTCCACTTCTATGAACTCACCAAC	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.

Table 3-3. 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 Strains				
Antibodies	TN1	NY15P	US10	JMB	JMC
Pre-immune	-	-	-	-	-
Anti-NY15p	-	+	+	-	-
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.

Table 3-4. Nucleotide and amino acid differences between RU1-OR-B and RU1-OR-C isolates of *Bean common mosaic virus* (BCMV).

Position of nucleotide in whole genome	Nucleotide change (RU1-OR-B/RU1-OR-C)	Amino acid change (RU1-OR-B/RU1-OR-C)
2726	A/C	-
2735	G/A	-
2744	G/A	-
3777	G/C	A/P
5799	G/A	V/I*
6287	G/A	-
6289	A/G	K/R
6301	A/G	K/R
6323	C/T	-
6360	C/G	H/D
6413	A/G	-
6463	C/T	P/L
6507	A/G	K/E
6515	A/G	-
6550	A/G	K/R
6571	A/G	N/S
7663	A/T	E/V
7964	G/A	-
8398	T/C	V/A
8876	C/T	-
9004	G/A	S/N
9041	C/T	-
9056	T/C	-

* Yellow shading designate chemically similar amino acids.

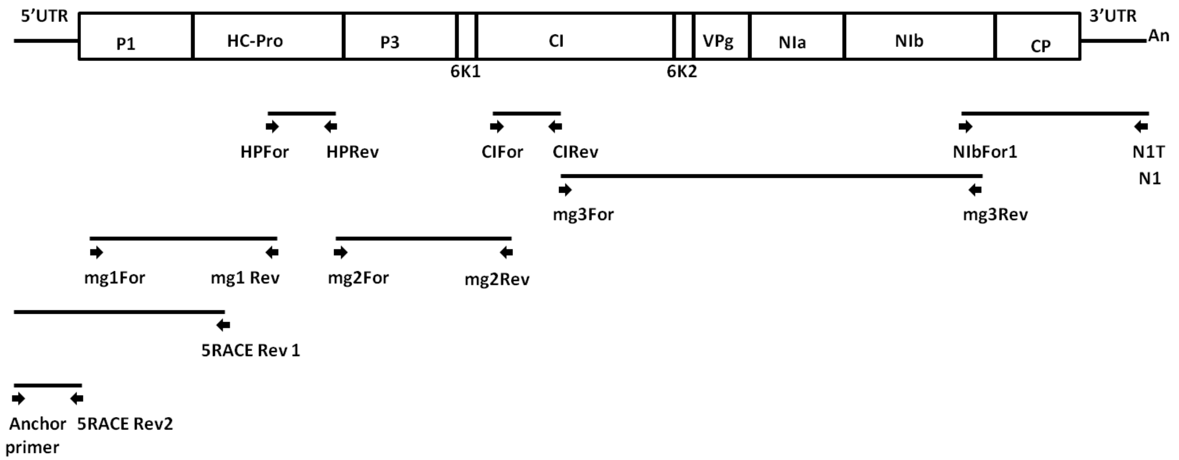

Figure 3-1. Diagram outlining the general cloning strategy for all *Bean common mosaic virus* (BCMV) genomes sequenced in this work. Relative positions of primers used to amplify and sequence the BCMV complete genome are also shown.

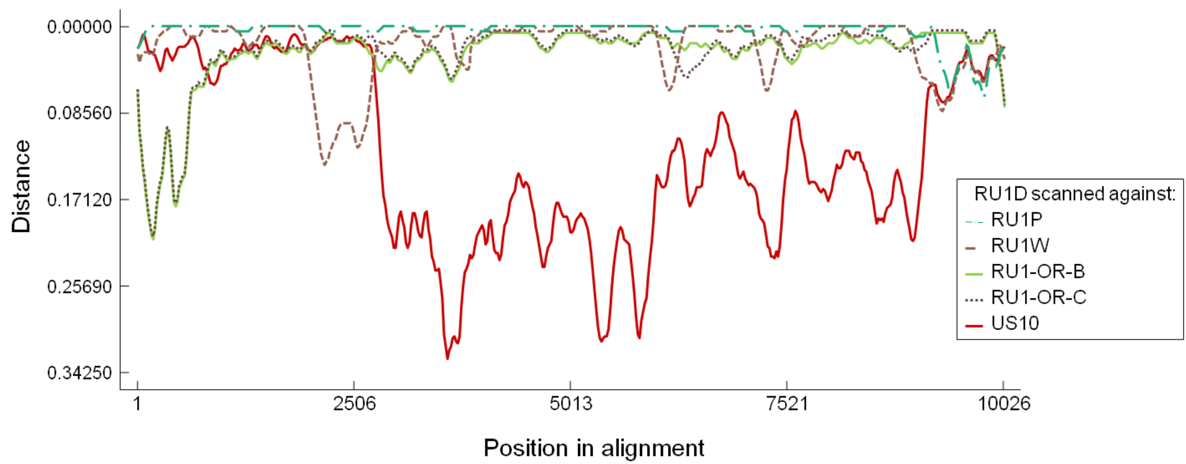

Figure 3-2. Manual distance plot based on aligned full-length nucleotide sequences of *Bean common mosaic virus* (BCMV) isolates. RU1D (GenBank accession GQ219793) was used as the reference strain. X-axis represents nucleotide position in the alignment, Y-axis represents relative distance from the RU1D sequence which is calculated using Kimura model (Kimura, 1980).

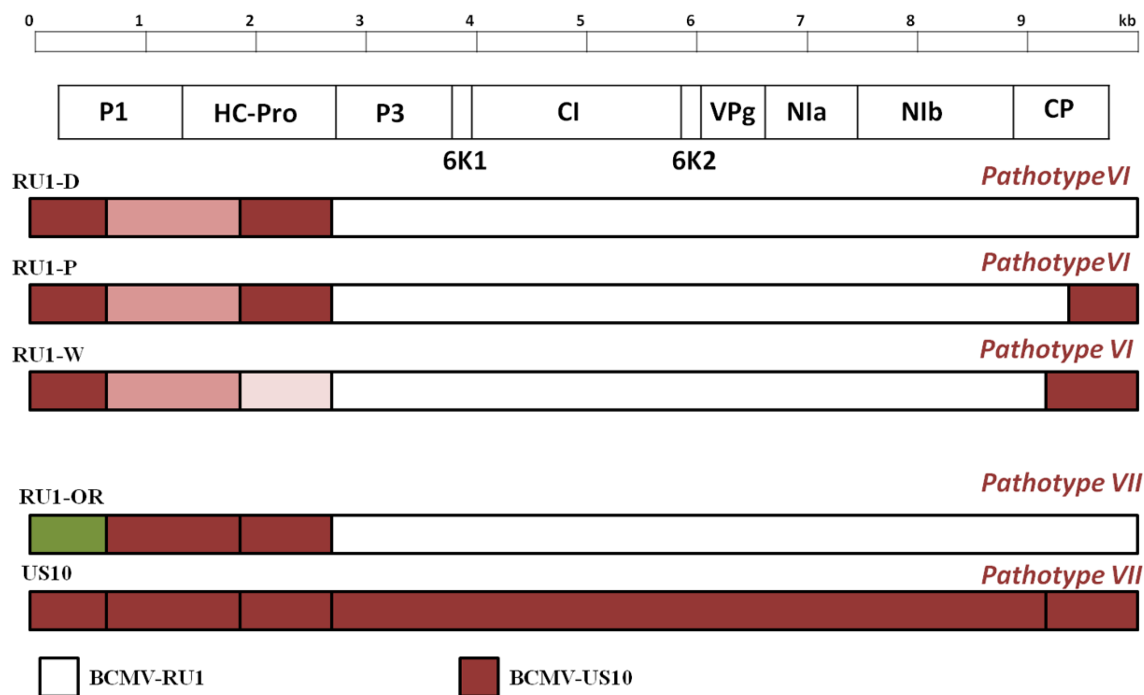

Figure 3-3. Schematic diagram showing putative *Bean common mosaic virus* (BCMV) recombination positions between US10 and an unknown BCMV parent. Putative recombinant junctions are shown as vertical lines. Shading of the different sections of the sequenced BCMV genomes reflects similarity/dissimilarity level as presented on Fig. 2: highly similar genome sections are shaded in the same color, while dissimilar sections are shaded in a different color.

Chapter 4

A recombinant of *Bean common mosaic virus* induces temperature insensitive necrosis in an *I* gene bearing line of common beans

Abstract

I gene is a single, dominant gene conferring resistance to all isolates of *Bean common mosaic virus* (BCMV) in common beans (*Phaseolus vulgaris* L.). However, the closely related *Bean common mosaic necrosis virus* (BCMNV) induces whole plant necrosis in *I*-bearing cultivars of common beans, and the presence of additional, recessive genes is required to prevent this severe whole plant necrotic reaction caused by BCMNV. All BCMNV isolates have so far been classified as having pathotype VI based on their interactions with the five BCMV resistance genes, and all have a distinct serotype A. Here, we describe a new isolate of BCMV, RU1M, capable of inducing whole plant necrosis in the presence of the *I* gene, that belongs to pathotype VII and exhibits B-serotype. Unlike other isolates of BCMV, RU1M was able to induce severe whole plant necrosis below 30°C in bean cultivar Jubila that carries the *I* gene and a protective recessive gene *bc-1*. The whole genome of RU1M was cloned and sequenced and determined to be 9,953-nt long excluding poly(A), coding for a single polyprotein of 3,186 aa. Most of the genome was found almost identical (>98%) to the BCMV isolate RU1-OR (also pathotype VII) that did not induce necrotic symptoms in Jubila. Inspection of the nucleotide sequences for BCMV isolates RU1-OR, RU1M and US10 (all pathotype VII), and three closely related sequences of BCMV isolates RU1P, RU1D, and RU1W (all pathotype VI) revealed that RU1M is a product of recombination between RU1-OR and a yet unknown potyvirus. A ca. 0.8-kb

fragment of an unknown origin in the RU1M genome may have led to its ability to induce necrosis regardless of temperature in beans carrying the *I* gene. This report is the first of a BCMV isolate inducing temperature-insensitive necrosis in the *I* gene containing bean cultivar.

Introduction

Bean common mosaic virus (BCMV) and *Bean common mosaic necrosis virus* (BCMNV) are closely related members of genus *Potyvirus* in the family *Potyviridae* (1). Both BCMV and BCMNV are important pathogens in common beans (*Phaseolus vulgaris* L.), both are transmitted by several aphid species in a non-persistent manner, and also through seed with very high efficiencies (14, 26, 31). Interactions of BCMV and BCMNV isolates with common bean are governed by five resistance genes, one dominant *I* gene, and four recessive genes, *bc-u*, *bc-1*, *bc-2*, and *bc-3* (8). Genes *bc-1* and *bc-2* have two alleles each ($bc\text{-}1/bc\text{-}1^2$ and $bc\text{-}2/bc\text{-}2^2$) for a total of seven genes or alleles that interact with the different strains of BCMV/BCMNV.

Resistance to BCMV was first discovered at the *I* locus in *P. vulgaris* L., cultivar ‘Corbett Refugee’ (29). Cultivars of beans (*P. vulgaris* L.) bearing the *I* gene display either no symptoms or necrosis ranging from local necrotic lesions on inoculated leaves to systemic vein necrosis and sometimes plant death following the challenge with BCMV or closely related BCMNV. Two types of necrotic responses could be induced: a) temperature insensitive necrosis (TIN) refers to systemic vein necrosis or whole plant necrosis (also called “black root syndrome” [BRS]) induced by all BCMNV isolates at all temperatures; and b) temperature sensitive necrosis (TSN) refers to a similar necrotic reaction induced by

some BCMV isolates only at temperatures above 30ºC (2, 5, 12, 15, 17, 18, 29, 30). Bean cultivars combining the *I* gene with recessive resistance genes could confer effective resistance to BCMV and BCMNV without inducing severe necrosis and plant death, resulting in the so-called "protected" *I* gene (9, 19).

BCMNV isolates can be easily distinguished not only biologically, as necrotic in *I*-gene bearing bean lines (8, 32), but also serologically, having a distinct A-serotype, whereas BCMV isolates all exhibit B-serotype (24, 25, 33). Sequence diversity among BCMNV strains was found very limited, with strains NL-3, NL-5, NL-8 and TN-1 exhibiting 97 to 99% genomic sequence identity (10, 21). A single natural recombinant NL-3K was reported having a typical BCMNV genome with a ca. 0.34-kb insert near the 5'-terminus coming from strain RU1 of BCMV (22, 32). This natural recombinant NL-3K displayed more severe symptoms on some bean indicators, but had the same pathotype VI as the parental isolate NL-3, and induced the necrotic reaction in the presence of the *I* gene like NL-3 (22, 32). The viral genetic determinants responsible for the *I*-gene mediated necrosis, or for exhibiting pathotype VI in beans have yet to be identified.

BCMV strains were found to be much more diverse than BCMNV isolates, displaying not only biological diversity with seven pathotypes (8, 9) but whole genome sequence diversity as well (11, 13, 22, 28). Recent sequence analysis of a series of whole genome sequences of BCMV isolates related to strain RU1 revealed that they often have recombinant genomes, and that recombinant events may result in biological changes shifting their pathotype specificity (11). The genome region spanning the P1 and HC-Pro cistrons was hypothesized to be involved in interactions with the $bc\text{-}2^2$ gene in common beans, thus

defining the pathotype VII of BCMV (11). Genetic determinants of BCMV responsible for interactions with other resistance genes have yet to be elucidated.

In this study, an unusual isolate of BCMV from the RU1 strain group, which we named RU1M, was characterized and found to induce severe whole plant necrosis regardless of temperature in bean cultivar Jubila that carries the *I* gene. Here, we describe a complete biological, serological, and molecular characterization of this RU1-like isolate. Comparison of the biological and molecular properties of RU1M with other isolates having the same or differing pathogenicity on bean differentials, allowed us to localize the putative genome region responsible for interaction with the *I* gene. Data presented here, together with previous studies, also allowed us to narrow down the putative genome region interacting with the recessive resistance gene $bc\text{-}2^2$.

Materials and methods

Virus sources, propagation, and purification

All reference BCMV and BCMNV isolates used in this work originated from the USDA-ARS Prosser, WA, collection. Control BCMV isolates RU1P and US10, and BCMNV isolate TN1 were provided by Drs. P. Berger and P. Shiel. The BCMV isolate designated by us as RU1M was originally obtained from Drs. P. Berger and P. Shiel, and this strain of BCMV was described and partially characterized previously (4). Isolate RU1-OR was originally collected by Dr. J. Myers in Oregon, and was described in details previously (11). All BCMV and BCMNV isolates were propagated on the bean cultivar "Dubbele Witte" and viruses were purified as described previously (11).

Biological and serological characterization

The biological characterization of RU1M on a set of bean differentials (Drijfhout, 1978) was completed as described previously (11). Two reference strains, TN1 (pathotype VI) and US10 (pathotype VII), were included in this analysis as controls. Eleven bean differential lines carrying different resistance gene combinations (Table 1) were inoculated with each isolate (three replicates), 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 4 weeks after inoculation. In addition, at 4 weeks post-inoculation samples were collected for ELISA testing.

RU1M virus preparations were purified from RU1M infected bean cv. Dubbele Witte according to the protocol described elsewhere (11); control virus preparations for isolates RU1-OR, RU1P, and US10 were from the laboratory collection and were described previously (11). Triple-antibody sandwich (TAS) ELISA tests using BCMV and BCMNV strain-specific, polyclonal antibodies from the laboratory collection were performed as described (11).

Cloning strategy, sequencing, and sequence analysis

The cloning strategy, sequencing and sequence analysis of BCMV isolates were all described previously (11). All primers used in this study for RT-PCR amplifications and sequencing are listed in Table 2. The whole genome sequence for BCMV isolates RU1M has been deposited in the GenBank database. The sequence was initially analyzed using the BLASTn 2.2.17 (3) tool available at the National Center for Biotechnology Information (NCBI). The complete viral genome was assembled using SeqMan (DNASTAR, Madison,

WI). Open reading frames (ORFs) were identified using the ORF Finder program available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). 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) (23).

Results

Biological characterization

When tested on the eleven bean differential lines, RU1M induced typical mosaic, raised dark green blistering, leaf deformation and growth retardation in susceptible bean varieties (Fig. 1, A and B, and Table 1). Based on the pathogenicity profile exhibited by the RU1M isolate on bean differential lines, it was classified as pathotype VII (Table 1); pathotype VII was previously found in one of the field isolates from the RU1 complex (11). Unexpectedly, systemic necrotic reactions were recorded upon inoculation of RU1M on bean cultivar Jubila (*I*, *bc-1*) visible as BRS or whole plant necrosis and plant death which started at 7-10 days after inoculation (Fig. 1, B and C). No necrotic reactions were observed in Amanda, US 1006, or IVT 7233, even at 6 weeks after inoculation with RU1M (Table 1). Upon inoculation with the TN1 isolate of BCMNV, systemic necrotic reactions were recorded in bean cultivar Jubila; however, in this case systemic vein necrosis was visible only on some upper, un-inoculated leaves, late in infection, and plants were alive and well even 6 weeks after inoculation (Fig. 2). By the time Jubila exhibited pronounced BRS induced by the RU1M infection, 2.5 weeks post-inoculation, systemic necrosis symptoms were not yet visible in Jubila plants inoculated with the BCMNV isolate TN1 (Fig.1, C).

These systemic necrotic symptoms became visible only later at around 4 weeks post-inoculation, and were displayed as modest vein necrotic spots on some upper non-inoculated leaves, which kept appearing through the entire experiment which was terminated at about 7 weeks post-inoculation (Fig. 2); by this time Jubila plants inoculated with RU1M were long dead. No systemic symptoms were observed in Amanda, US 1006, and IVT 7233 harboring the *I* gene upon inoculation with TN1. The presence of BCMV or BCMNV in inoculated plants was tested with TAS-ELISA (see Table 1), and the results always agreed with the symptom expression. Jubila plants infected with RU1M died at 10-14 days post-inoculation, providing no leaf tissue for ELISA tests; however, Jubila plants inoculated with TN1 were virus-negative as determined by ELISA at 4 weeks post-inoculation (Table 1). Reference strains TN1 (pathotype VI), and US10 (pathotype VII) showed the expected virulence profiles with the differential lines based on their pathotypes. Isolate RU1M displayed a virulence profile identical to that of the pathotype VII reference strain US10, and distinct from the control isolate TN1 (pathotype VI, Table 1). In summary, isolate RU1M exhibited pathotype VII on the bean differentials, however, it also displayed an ability to induce TIN in an *I* gene bearing cultivar Jubila (*I*, *bc-1*). The intensity, and time-course of this TIN was drastically different from that of the control isolate TN1 suggesting different types of interactions with the *I* gene between RU1M and TN1.

Serological characterization

The RU1M isolate was subjected to a serological characterization with TAS-ELISA side-by-side with reference strains US10 and TN1, to determine serotype. RU1M and control isolates were captured on the ELISA plates using either anti-TN1, anti-US10, anti-

RU1-OR or anti-NY15P rabbit antiserum, and were subsequently probed with the different detecting antibodies generated in guinea pig. The serotyping data are summarized in Table 3. The anti-US10 antiserum reacted to all isolates tested, including RU1M, and also the BCMNV isolate TN1, when used as detecting antibody. The anti-TN1 antiserum reacted only with the homologous isolate TN1 when used as detecting antibody. The anti-NY15P and anti-RU1-OR antisera were capable of binding BCMV isolates RU1M and US10, but not BCMNV isolate TN1. The data presented in Table 3 were consistent with data from a previous study (11), and RU1M displayed the same serological properties as US10. The serological typing of RU1M as B serotype was consistent with the preliminary characterization of this same isolate conducted previously by Berger et al. (4) who sequenced the 3'-terminal 1.2-kb of the BCMV RU1 genome and showed the RU1 capsid protein (CP) to group with other B serotype isolates of BCMV.

<u>Whole genome cloning, sequencing, and sequence analysis</u>

The whole genome of BCMV isolate RU1-M was cloned and sequenced, using the approach described previously (11). Upon sequence assembly, RU1-M was found to be 9,953-nt long, excluding the poly (A). Based on conceptual translation, the BCMV RU1-M genome encoded a single polyprotein of 3,186 aa. Initially, the sequence of RU1-M was compared to the known BCMV and BCMNV genomes using the BLAST 2.2.17 tool (3), and this revealed that the RU1-M sequence shared the closest similarity to the isolate RU1-OR (accession numbers KF919297 or KF919298; 98% nucleotide identity), except for a 0.8-kb fragment near the 5' terminus (see below).

The whole genomes for RU1-M, RU1-OR-B (KF919297), RU1-OR-C (KF919298) and US10 (KF919299) all having pathotype VII, together with RU1P (KF919300), RU1W (AY863025) and RU1D GQ219793) all having pathotype VI, were aligned using CLUSTALX and further analysis was conducted with the RDP4 program package. Figure 3 shows the comparison of all known RU1 whole genome sequences plus the sequence of US10 using the manual distance plot analysis, with the full-length BCMV RU1D sequence from GenBank as the reference (see Fig. 3).

Based on the RDP4 analysis, the very 5'- terminal sequences of isolates RU1M and RU1-OR, nt 1-512, were not very similar to each other (86% identity) or to all other isolates, RU1D, RU1W, RU1P, and US10 (84-85% identity, Fig. 3). On the other hand, the 5'-terminal genome segments were very similar between the latter four isolates (96% identity) (Fig. 3). Remarkably, RU1M was found to have an insert in its genome, between nt 513-1,287, with no close relationships to BCMV or BCMNV nucleotide sequences available in the GenBank database (Fig. 3). When this segment of the RU1M genome, between positions 513 to 1,287, was conceptually translated and the resulting amino acid sequence compared to the protein sequences available in the GenBank database using the BLASTP program, the closest matches were the P1 regions of polyproteins coded by isolates of *Cowpea aphid-borne mosaic virus* (CABMV; 50 to 58% identity) and isolates of BCMNV (35 to 36% identity). Downstream of this insert in RU1M, between nt 1,348-2,006 (position in alignment), sequences of all six isolates analyzed were found to show close similarities to each other (Fig. 3). Specifically, the three isolates belonging to pathotype VI, RU1W, RU1D, and RU1P exhibited >99% identity to each other, and the three isolates belonging to pathotype VII, RU1M, RU1-OR, and US10 exhibited more than 97% identity to each other,

while pathotypes VI and VII in this same region were 96% identical. In the 3'-terminal region, downstream of nt 9,129 (position in alignment), RU1D and RU1-OR (100% identity) on one hand, and RU1M, RU1P, RU1W, and US10 (96% identity) on the other hand, formed two distinct similarity groups (the two groups shared 93% identity between each other). These patterns of similarity and dissimilarities are presented as a schematic diagram in Fig. 3.

Discussion

Although the *I* gene is widely used in bean breeding programs (2, 5, 12, 15, 17, 18, 29, 30, 31), the exact mechanism conferring resistance to BCMV and of ensuing interactions between the *I* gene and BCMV or BCMNV is still unknown (6, 7). Experiments with isolated protoplasts from isogenic bean lines carrying different numbers of the *I* gene alleles demonstrated that even at low temperature (26°C), BCMV was still able to replicate in the *I* gene bearing genotypes, and hence necrosis seemed to occur when the virus was moving from the initially infected cell to a neighboring cell (6). Virus-encoded factors and virus genetic determinants involved in interactions between BCMV/BCMNV and the *I* gene in common beans have not been studied yet, and there are several factors that have precluded systematic research in this area. So far, there are no full-length infectious clones developed for any of the BCMNV isolates inducing TIN in the presence of the *I* gene. The two available full-length infectious copies were developed for the BCMV peanut stripe strain (13) and strain RU1 (27), which do not induce TIN (4, 13, 22, 25, 27). BCMNV isolates displayed remarkably low biological and molecular diversity belonging to the same

pathotype VI, and sharing sequence identity levels above 97% (21, 22) which also did not facilitate studies of BCMNV interactions with the *I* gene.

RU1 was the only known TSN inducing isolate assigned to the same pathogroup VI with BCMNV isolates (4, 22, 25). Given the low level of similarity between BCMV and BCMNV sequences (22), this meant that the genetic determinants responsible for the TIN induction and exhibiting pathotype VI in beans are distinct, and may reside in different parts of the virus genome. Recently, the RU1 strain was demonstrated to have a substantial biological and molecular diversity, comprising isolates with different recombinant structures and exhibiting pathotypes VI and VII (11). Comparative sequence analysis of several whole genomes for BCMV isolates from the RU1 complex suggested that the BCMV RU1 genome region between positions 723 to 1,961 may be a putative genetic determinant responsible for pathotype VII, interacting with the $bc\text{-}2^2$ gene (11). A similar comparative analysis for the BCMNV sequences was not possible because there was very little diversity within the BCMNV whole genomes sequenced to date (10, 21).

In this paper, we describe a novel BCMV isolate from the same RU1 complex, RU1M, which was found to have pathotype VII with serotype B, similar to the control isolate US10 (Table 1 and Table 3). But unlike US10 or other RU1-like isolates exhibiting pathotypes VI or VI, RU1M was capable of inducing TIN in an *I* gene bearing cultivar Jubila (*I*, *bc-1*). When challenged by RU1M, Jubila plants developed very quick systemic necrosis, or BRS, which caused plant death in only 10 days after inoculation. All four *I*-bearing cultivars tested, Jubila, Amanda, US 1006, and IVT 7233, harbored the *I* gene protected by four different recessive genes, and they were expected to exhibit complete resistance to all BCMV isolates. A mild to moderate systemic reaction was expected in cv.

Jubila (*I*, *bc-1*) in response to infection with a BCMNV isolate, clearly visible upon infection with isolate TN1 (Fig. 2). However, the response of Jubila plants to the RU1M infection was very different from the expectations either for BCMV or BCMNV infection, in fact the ensuing BRS was close to a rapid systemic reaction in bean cultivars with a non-protected *I* gene following inoculation with BCMNV. Nevertheless, the fact that the *I* gene was protected in our Jubila plants was confirmed following inoculation with the BCMNV isolate TN1 which produced only mild necrotic reaction in non-inoculated leaves very late post-inoculation (Fig. 2). To have a natural isolate from the RU1 complex capable of causing TIN in *I* gene bearing bean lines is very important since it may help to localize genetic determinants of the virus involved in interactions with the *I* gene through a comparative sequence analysis.

Indeed, when subjected to the whole genome analysis, RU1M was found very close to the previously sequenced isolate RU1-OR, which also displayed pathotype VII (11). Sequence identity level between RU1M and RU1-OR was found at or above 98% for the most of the genome with the exception of the 5'-terminal segment spanning the P1 and HC-Pro cistrons (Fig. 3 and 4). The most striking difference between RU1M on one hand, and RU1-OR and all other BCMV genomes on the other hand, was an apparent insert of approximately 770 nt, between nt 513 to 1,287 in the P1 encoding region (Fig. 3 and Fig. 4). This inserted nucleotide sequence had no close matches to any entry in the GenBank database, although it coded for a potyvirus protein product with CABMV and BCMNV identified as the closest matches found in GenBank. The identity levels in these cases, below 58% for CABMV and below 36% for BCMNV, were too low to suggest a recombination event between BCMV strains; most likely, this insert between positions 513 to 1,287 came

from an as yet unknown potyvirus. Given the otherwise similar recombinant structure of all RU1-like genomes (Fig. 4), we hypothesize that the presence of this insert in the genome of RU1M may be responsible for the unusual necrotic reaction, BRS, induced by this isolate in bean cultivar Jubila (see Fig. 1, Table 1). However, this hypothesis on the location of the genetic determinant responsible for interactions of BCMV with the *I* gene in common bean still needs to be tested using reverse genetics.

In addition to help in narrowing down the genetic determinant responsible for the interaction between BCMV and the *I* gene, isolate RU1M might also be helpful in narrowing down other genetic determinants of BCMV, i.e., responsible for interactions between BCMV and *bc-2* and *bc-2*^{2} genes in beans (see Fig. 4). Inspection of the RU1M pathogenicity profile and comparison to the genome structures of other RU1-like recombinants (11), suggests that the region coding the N-terminal part of HC-Pro cistron in the BCMV polyprotein, nt 1,287 to 1,976 may determine pathotype VII isolates, in other words this region may be responsible for interactions with the *bc-2*^{2} gene (Fig. 4). This 770-nt insertion in the RU1M genome thus narrowed down considerably the genome region of BCMV hypothesized to be involved in interactions with the *bc-2*^{2} gene, previously identified between positions 723 to 1,976 (11). However, just like in the case of the *I* gene, all the hypotheses on the locations of genetic determinants responsible for BCMV interactions with recessive BCMV resistance genes in beans need to be tested using reverse genetic tools.

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Table 4-1. Disease and ELISA reactions of bean differentials inoculated with BCMV strains[1)]

Bean cultivar	Resistance genes	TN1 (VI)	US10 (VII)	RU1M
Dubble Witte	none	+/+	+/+	+/+
SGR	*i,bc-u*	+/+	+/+	+/+
RGLC	*i,bc-u,bc-1*	+/+	+/+	+/+
RGLB	*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*	nec/-	-/-	BRS/NA
Amanda	*I, bc-1*^{2}	-/-	-/-	-/-
US1006	*I,bc-2*^{2}	-/-	-/-	-/-
IVT7233	*I, bc-u, bc-1*^{2}*, bc-2*^{2}	-/-	-/-	-/-

[1)] Disease reaction is shown first as a nominator followed by ELISA reaction as a denominator. Three plants were inoculated for each BCMV strain; results shown were the same for all three plants inoculated with each virus preparation.

[2)] Nominator: + = symptoms on inoculated beans; - = no symptoms on inoculated beans; nec = systemic necrotic reaction on some uninoculated leaves; BRS = “black root syndrome”.

[3)] Denominator: + designates ELISA signal (A_{405}) in an infected plant exceeding healthy control by 10-fold or more; - designates ELISA signal in an infected plant ca. equal to that

of a healthy control; NA designates “not applicable”, since the plant died before the test could be completed.

Table 4-2. Primers used for cloning 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	
RU1M specific primers		
RU1M mg1 For	CAACAACTTCTCGCAACCAACCAC	Specific primer to amplify the major gap 1
RU1M mg1 Rev	CCCTTTGCCCATTAGGATTCCTCC	Specific primer to amplify the major gap 1
RU1M mg2 For	GAGTCCAACTAAGAGACACC	Specific primer to amplify the major gap 2
RU1M mg2 Rev	CACGTCCTGGTGGTGTTGCTGATAC	Specific primer to amplify the major gap 2
RU1M mg3 For	GTATCAGCAACACCACCAGGACGTG	Specific primer to amplify the major gap 3
RU1M mg3 Rev	TCCGGTGAAGTGCCATTGTCAATAC	Specific primer to amplify the major gap 3

RU1M 5RACE Rev1	CCTGCGTCACTGATGAACCTTTC	1st strand primer for 5'RACE
RU1M 5RACE Rev2	CGTGCAGAAGTTGGTTTTGGATTCA	PCR primer for 5'RACE
	TGTAAATGCGCTCTTGTG	

1) All degenerate primers and 3' end primers are from Ha et al. (16)

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

Table 4-3. Serological characterization of BCMV isolates using strain-specific antibodies in TAS-ELISA[1].

	BCMV Strains		
Antibodies	TN1	US10	RU1M
Pre-immune	-	-	-
Anti-NY15P	-	+	+
Anti-US10	+	+	+
Anti-TN1	+	-	-
Anti-RU1-OR	-	+	+

[1] + designates ELISA signal (A_{405}) in an infected plant exceeding healthy control 10-fold or more; - designates ELISA signal in an infected plant ca. equal to that of a healthy control.

A

B
Jubila
Dubbele Witte

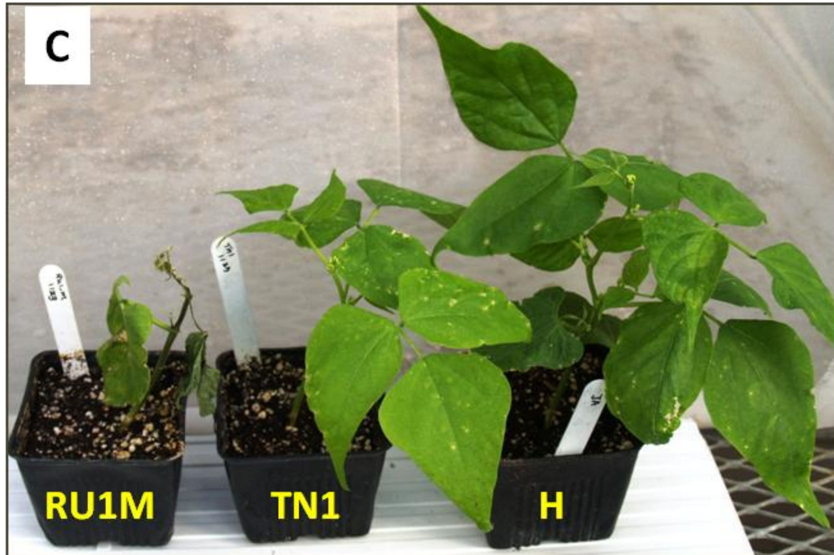

Figure 4-1. Foliar symptoms induced in common beans (*Phaseolus vulgaris* L.). (A) Bean plant infected with BCMV isolate RU1M, cv. Dubbele Witte, displaying severe mosaic, blistering, and leaf deformations 6 weeks post-inoculation. (B) Whole plant necrosis and plant death induced by RU1M (pointed by an arrow) in three plants of cv. Jubila (*I, bc-1*) 10 days post-inoculation; two control plants of cv. Dubbele Witte inoculated with RU1M at the same time are also pointed by an arrow. (C) Side-by-side comparison of Jubila (*I, bc-1*) plants inoculated with BCMV isolate RU1M and BCMNV isolate TN1 at 2.5 weeks post-inoculation; note the clear black root syndrome (BRS) or whole plant necrosis induced by RU1M and the absence of the BRS or any systemic necrotic reaction induced by TN1 by this time point; respective inoculum, RU1M or TN1, is labeled on each plant, (H) means healthy control.

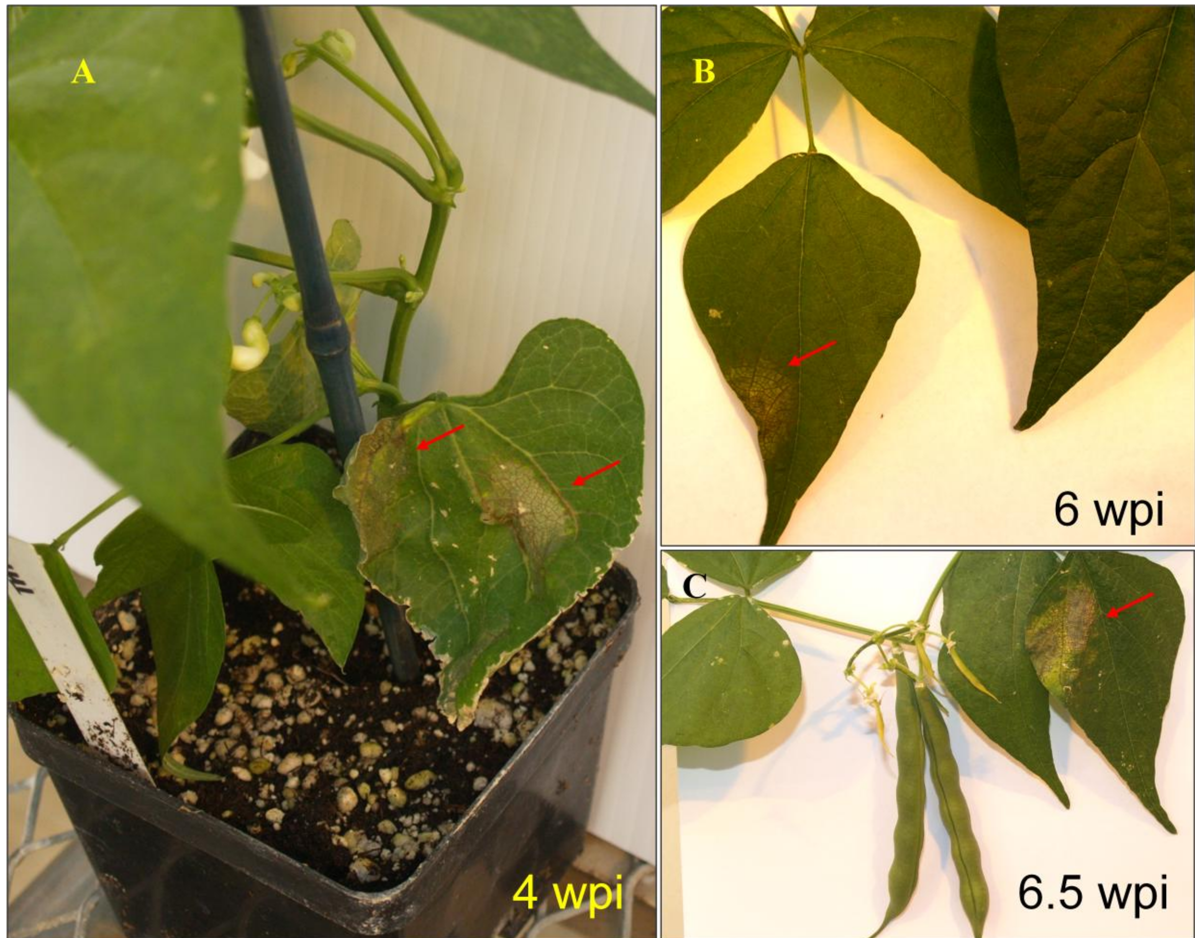

Figure 4-2. Systemic necrotic reaction induced by the control BCMNV isolate TN1 in cv. Jubila (*I, bc-1*) in upper, uninoculated leaves at various time points after inoculation: (A) 4 weeks post-inoculation (wpi); (B) 6 wpi; (C) 6.5 wpi. Note the absence of the whole plant necrosis or black root syndrome at any time point post-inoculation.

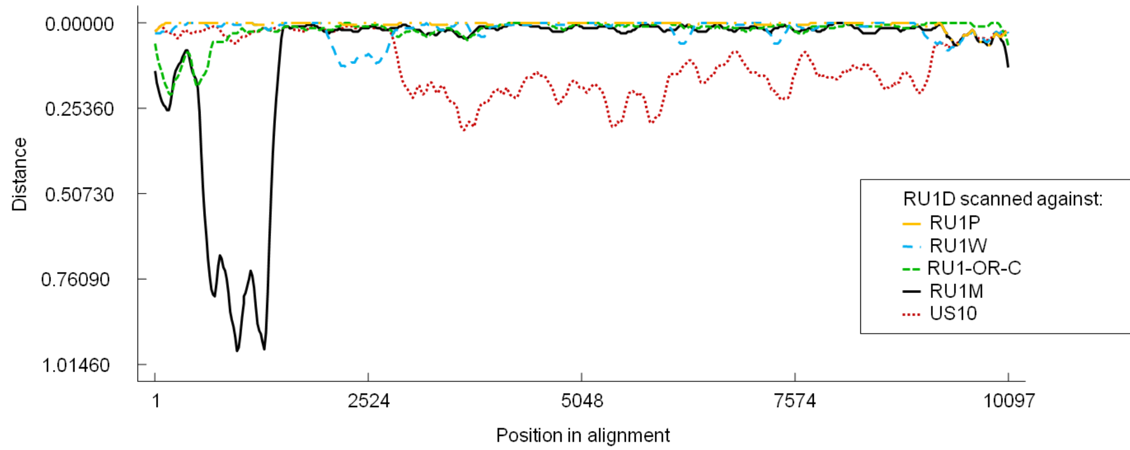

Figure 4-3. Manual distance plot based on the aligned full-length nucleotide sequences of BCMV isolates RU1M, RU1-OR, RU1P, RU1W, RU1D, and US10. RU1D (accession GQ219793) was used as the reference strain. X axis represents nucleotide position in the alignment, Y axis represents relative distance from the RU1D sequence which is calculated using Kimura model (20).

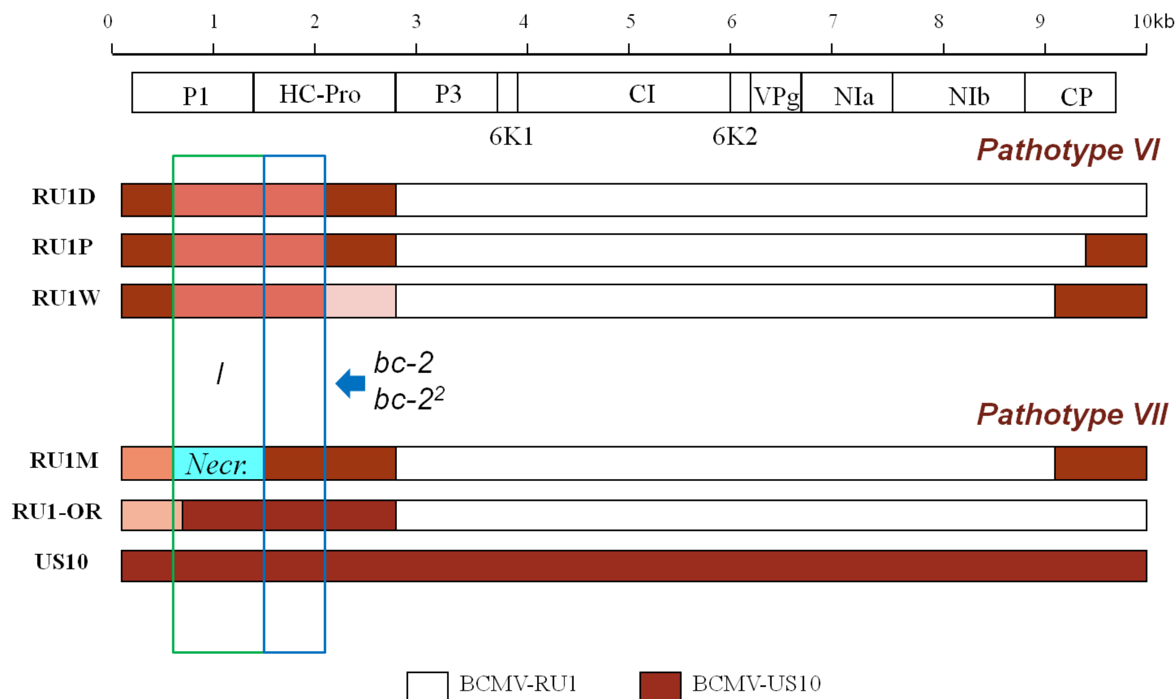

Figure 4-4. Schematic diagram showing putative BCMV recombination structures for *Bean common mosaic virus* (BCMV) isolates US10 and five isolates from the RU1 complex. Isolate US10 is selected as one of the most likely parents for all recombinants presented, the second parent is yet unknown. Putative recombinant junctions are shown as vertical lines. Shading of the different sections of the sequenced BCMV genomes reflects similarity/dissimilarity level as presented on Fig. 4-3: highly similar genome sections are shaded in the same color, while dissimilar sections are shaded in a different color. The position of the insert associated with the black root syndrome in the genome of isolate RU1-M is boxed and marked “Necr.” with a label indicating interaction with the *I* gene. Positions of the genome segments correlating with the pathotypes of the RU1 and US10 isolates are also boxed with a label indicating interactions with *bc-2* and/or *bc-2*^{2} genes.

Chapter 5

Summary

In the preceding chapters, several experiments designed to characterize a group of field and reference isolates of BCMV were described. Previously, pathogenicity of different BCMV strains was determined and differentiated on 12-14 bean indicators, and no molecular tools were available to address the BCMV pathogroups. Almost no data on linkage between genetic determinants of BCMV pathogenicity and molecular properties of the virus existed.

Two main accomplishments were achieved in this study. First, preliminary or tentative mapping of the genetic determinants of BCMV involved in interactions with the dominant *I* gene and the recessive genes *bc-1*, *bc-2*, and *bc-2*^{2} was proposed, located mainly in the P1 and HC-Pro cistrons. Second, genomes of several strains of BCMV, e.g. US1, NY15P, US10, and several strains we referred to as the RU1 strain group, were found to represent multiple recombinants exhibiting differences in their pathogenicity. Data obtained in this study will provide a foundation to study BCMV-bean interactions, both through comparative genomics and through the reverse genetics approach.

A serious limitation in a study of BCMV pathogenicity is the lack of an infectious clone for any of the described BCMV recombinants which would be necessary to test our hypotheses delineating putative genetic determinants of pathogenicity. Consequently, this would be the most obvious goal of any further research on BCMV pathogenicity in common beans. Once an infectious clone of BCMV is generated, we would be able to design chimeric genomes with the same or differing pathogenicity through genetic exchanges between BCMV strains from different pathogroups. These genetic swaps would be useful to prove

(or disprove) our hypotheses on positions of genetic determinants of BCMV responsible for overcoming resistance to the virus as well as identify and map novel genetic determinants of BCMV. We firmly believe that one other future direction of research on BCMV would be to continue comparative genomics of BCMV strains through systematic whole genome sequencing and pathogenicity studies of BCMV isolates. The data presented here suggests that recombination between BCMV strains may be quite common, and these recombination events may or may not lead to the virus pathogenicity changes. And last but not least, in the course of this work a large set of BCMV-specific antibodies was produced recognizing A and B serotypes of the virus, as well as a large number of specific primers distinguishing several distinct strains of BCMV. All these generated detection and differentiation tools will be of great value for applied plant pathologists involved in management of bean seed certification programs and in bean production.