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Biological properties and molecular characterization of beet chlorosis virus (BChV)

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Summary. Two distinct viruses belonging to the *Polerovirus* genus, in the family *Luteoviridae*, have been described as being able to induce mild yellowing on sugar beet: *Beet mild yellowing virus* (BMYV) and more recently, beet chlorosis virus (BChV). We have analysed biological properties and molecular organisation of two strains of BChV, one collected in England and the second from California. The biological data suggested that BChV displayed a narrower host range compared to BMYV and *Beet western yellows virus* lettuce isolate (BWYV). The complete genomic RNA sequence of the American isolate BChV-California and the European isolate BChV-2a showed a genetic organisation and expression typical of other *Polerovirus* members including 6 open reading frames (ORFs). Interspecific and intraspecific phylogenetic studies suggested that BChV arose by recombination events between a *Polerovirus*-like ancestor donating P0 and the replicase complex and either a BMYV or a BWYV progenitor providing the 3′ ORFs [3, 4 and 5]. The 5′- and 3′-parts of the BChV genome have evolved differently in the two continents, possibly due to different selection pressures to allow adaptation to the various environments, hosts and vectors. BChV is a distinct species of the *Polerovirus* genus.

Introduction

Viruses belonging to the *Luteoviridae* family [1] are phloem-limited with positive-strand RNA; the viruses are circulatively-persistently transmitted by several aphid species. There are three genera which compose the *Luteoviridae* family: *Luteovirus*, *Polerovirus*, and *Enamovirus* genera typified respectively by their type species *Barley yellow dwarf*-PAV (BYDV-PAV), *Potato leaf roll* (PLRV) and *Pea enation mosaic virus* RNA-1 (PEMV RNA-1).

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In 1994 Stevens et al. [20] identified a distinct strain of beet *Polerovirus* in Europe which did not react with monoclonal antibody BYDV-PAV-IL-1 and which did not infect *Capsella bursa-pastoris* or *Montia perfoliata*. At this time little was known about the molecular, serological and biological variability of beet poleroviruses; thus this strain was considered as a second serotype and biotype of BMYV. Simultaneously Liu et al. described a new yellowing disease of sugar beet in Colorado, Nebraska, Texas and California, induced by an aphid transmitted virus serologically related to BWYV [12].

Molecular variability studies of the beet poleroviruses [8] suggested that this new virus belonging to the *Luteoviridae* family could be a new species named beet chlorosis virus (BChV). Until now this species is not recognized by the International Committee for Taxonomy of Viruses (ICTV). The name of this virus proposed by Liu et al. is derived from symptoms such as interveinal yellowing, leaf chlorosis and necrotic lesions [12]. Two strains of this virus were analysed, one isolated from Europe as BChV-2a and the second one collected in North America as BChV-California or BChV-CR.

In 1999 Lewellen used BChV-California to inoculate sugar beet to determine the effects on yield and the occurrence of differential host-plant reactions [11]. Sugar yield losses were estimated between about 5–40%, and were as severe as those caused by BMYV.

In order to confirm that BChV represents a new polerovirus species we have analysed its host range and full-length genomic sequence. Thus we have sequenced the complete genomic RNA of an American isolate (BChV-CR) and a European isolate (BChV-2a). Sequence comparison of BChV with other *Luteoviridae* and between the two strains of BChV will provide new data on the origin and evolution of beet poleroviruses.

Materials and methods

Virus isolates

Two isolates of BChV were analysed: the first provided by Gail Wisler (USDA, Salinas, USA) collected from symptomatic sugar beets from California and called BChV-California or BChV-CR, the second was collected in East Anglia (England) and called BChV-2a. Both isolates were maintained on sugar beet (*Beta vulgaris* cv Trestel) by serial transmissions using 5 wingless *Myzus persicae* (clone NL). BWYV-FL1 lettuce isolate was maintained on *Physalis floridana*.

Virus transmission

A cloned population of *M. persicae* was reared on healthy pepper (*Capsicum annuum*), which is a plant species known to be immune to BMYV and BWYV. To inoculate test plants, aphids were given a 24 h acquisition access period (AAP) on infected source plants 8 weeks post inoculation. Ten aphids were transferred to each healthy test plant for an inoculation access period (IAP) of four days; the plants were then sprayed with an insecticide (4 ml/l Mevinphos). The inoculated plants were then grown in a growth chamber with 20 °C temperature, 16 h of daylight and a light intensity of 2520 Lux.

Triple antibody sandwich (TAS) ELISA

Infection of test plants was monitored 3 and 6 weeks later by TAS-ELISA using monoclonal antibody (MAb) MAFF 24 (raised against BMYV-1 isolate from UK), which is routinely used for detecting BMYV and BWYV, although it does not discriminate between them [19]. TAS-ELISA was performed as previously described [17, 19], with the following alterations. Leaves were ground in PBM buffer composed of phosphate buffered saline (PBS)-Tween-polyvinylpyrrolidone (PVP) with 0.1% dry milk at 1/5 w/v dilution. MAb MAFF 24 was diluted at 1 μg/ml in PBM and anti-rat alkaline phosphatase conjugate was used at 1/1000 dilution. Optical density (OD) readings at 405 nm were taken after 0.5, 1 and 2 h. Threshold values were deduced by calculating the mean of the OD values of 6 different healthy plants plus 3 times the standard deviation of these values.

Virus purification and genomic RNA extraction

Virus purification was performed on 300 g of sugar beet leaves collected and frozen in the −20 °C 8 weeks post inoculation, as described by Van den Heuvel [22]. Yields of 300 μg of purified virions from 1 kg of fresh material were achieved for both BChV isolates.

The PUREscript™ kit (GENTRA Systems Inc., USA) protocol was adapted for the extraction of viral genomic RNA. Six hundred μl of purified virus resuspended in sterile water, was incubated for 10 min at room temperature after the addition of 600 μl of cell lysis solution provided in the kit. Two hundred μl of protein-DNA precipitation solution (provided in the kit) was added and the mix was incubated for 10 min on ice followed by a 5 min 15000 rpm centrifugation. RNA was precipitated by adding 840 μl of isopropanol at room temperature to the supernatant and then centrifuged for 10 min at 15000 rpm and 4 °C. After washing the RNA with 70% ethanol, the pellet was resuspended in DEPC-treated water.

RT-PCR amplification and cloning

Complementary DNA (cDNA) was synthesised from purified RNA as described by Kotewicz [10] and amplified by PCR [18]. PCR reactions were carried out using a Hybaid Touchdown thermal cycler for 30 cycles. Primers used to obtain RT-PCR fragments are the following: o1: 5′-AAA CGC GTC GAC AAA AGA (A/T)(A/T)(A/C) (G/C)(A/C)G; o2: 5′-GCT CTA GAG GAT CC(T/C) TCC CA(A/G) TTN GGN GGN CC; 2aP1+ 5′-TTT GGC CCA CCC AAT TGG; 2a Zoll: 5′-CGC AGG GAA GTT AAT TCG GC; orf3+: 5′-GGC TTC GGS TGG CCC AAG TTC GG; rev3: 5′-CTC CAG TCA AAA CCR GAG CAA T; Frd3: 5′-ATT GCT CYG GTT TTG ACT GGA G; CP-: 5′-CCA GCT ATC GAT GAA GAA CCA TTG; Frd4: 5′-GAC GTT GCC AAG GAC CAA TT; rev 45: 5′-TCT CCC AGG TTG AGA CTG CC; 2ITB5+: 5′-GGC AGT CTC AAC CTG GGA GA; rev5: 5′-ACA CCG AAG TGC CGT AGG GA.

Purified RT-PCR products were cloned in pSK (+) Bluescript plasmid from PCR-Script Amp Cloning Kit™ (Stratagene) following the manufacturer's instructions. To clone the double stranded cDNA the reaction mix was precipitated and the pellet was resuspended in 5 μl, 18 Megohms, DNase and RNase treated water. Ligation was performed using solutions provided in the cloning kit and the ligation time was extended to overnight at 19 °C.

DNA sequencing and computer-assisted nucleotide and amino acid analysis

To obtain the complete genomic sequence of BChV-2a and BChV-California, 6 RT-PCR clones were constructed for each viruses (Fig. 1). The DNA fragments contained in recombinant clones 2a/1, 2a/2, 2a/3 and 2a/4 were synthesised by RT-PCR using primers designed to the BMYV-2ITB sequence [6].

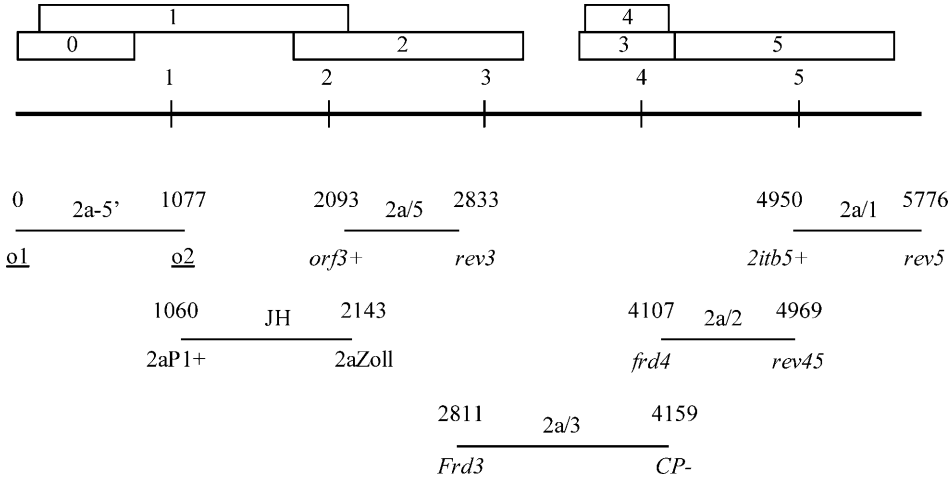

Fig. 1. Genetic map of the BChV-2a RNA. The major ORFs are represented by numbered rectangles. RT-PCR products are represented by thin lines, primer position and name are given at the extremities of the line. Primer designed with BMYV-2ITB sequences are represented in italics and degenerate primers provided by Guilley are underlined

The clones were then sequenced in both directions using the SequiTherm EXCEL II Long-Read DNA Sequencing Kit-LC (TEBU) with IRD41-labelled primers (M13 reverse and M13 forward). The sequences were analysed by LI-COR DNA sequencer 4000 L. Three clones for each cDNA were sequenced to ensure consistent and reliable sequence data.

Analyses of the amino acid sequences of BWYV, BMYV, PLRV and CABYV revealed the presence of a common peptide motif GPPNWE (located at nucleotide (nt) 1056, Fig. 2), mapping to a position approximately 1000 residues downstream of the 5′-terminus in the putative protein P1 encoded by ORF-1 [6]. On the assumption that this motif is conserved in the *Polerovirus* genus [6] we designed a degenerate antisense primer, o2, to prime reverse transcription. O1 is a degenerate primer designed to the first 15 residues of the 5′-terminus of BWYV, PLRV and CABYV. The 2a-5′ DNA fragment which corresponds to the first 1080 nucleotides in the 5′-termini of the genome, was synthesised by RT-PCR using the above primers kindly provided by H. Guilley (IBMP, France).

To make the junction between the clones 2a-5′ and 2a/4, we designed primer 2aP1+ based on the 2a-5′ sequence and primer 2aZoll designed from the 2a/4 sequence, which allowed the synthesis of a 1083 bp DNA fragment (clone JH, Fig. 1). The sequence of the 6 clones were aligned using Contig Manager (DNAsis package, Hitachi). ORF maps and Dot-plots were analysed using the GCG package [2].

Phylogenetic relationships between the beet poleroviruses and the *Luteoviridae* were observed using the PLATO analytical program (Partial Likelihoods Assessed Through Optimisation). The first complete genomic sequence of PLRV (X14600), *Soybean dwarf virus* (SbDV) (L24049), *Sugarcane yellow leaf virus* (ScYLV) (AF157029), BMYV-2ITB (X83110), BWYV-FL1 (X13062), BYDV-PAV (X07653), BYDV-MAV(D01213), PEMV-1 (L04573) and *Cucurbit aphid-borne yellows virus* (CABYV) (X76931) were aligned with the BChV sequence using the ClustalX program [21]. Phylogenetic tree and transition/transversion ratios were estimated using maximum likelihood by the Dnaml program from Phylip 3.572 package [3]. Recombination events were detected using PLATO analyses [5, 16]. The program PLATO utilises a sliding window of varying size (we used a minimum window length of $l = 50$) to find regions of an alignment which do not fit with a global (null) phylogenetic

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          _________
        1 ACAAAAGAATAGCAGGAGGACAGTGAATGAACTTTGAGATTTGCTTCAAAACGAACAGCG
c                             ORF-0  M  N  F  E  I  C  F  K  T  N  S  E

       61 AACTACTAGTGACGAGCGAAAGACACTTGCCACTAAAAGAGAGATCATTCATAATCGGAA
c            L  L  V  T  S  E  R  H  L  P  L  K  E  R  S  F  I  I  G  R

      121 GATTTCTCACGCAAATCCCTCAACTTTTGCACCATTTCAAATATGGACACCAAGTTGAAC
a                                                ORF-1  M  D  T  K  L  N
c            F  L  T  Q  I  P  Q  L  L  H  H  F  K  Y  G  H  Q  V  E  Q

      181 AGTTCTTACGCTCTATTCTTTTTCAGCTCCCTAATCTTATTTGGAGCTCTTGGGACCATG
a           S  S  Y  A  L  F  F  F  S  S  L  I  L  F  G  A  L  G  T  M
c            F  L  R  S  I  L  F  Q  L  P  N  L  I  W  S  S  W  D  H  G

      241 GCACCATCTTATATGGTGACAGGCCCTTTACACGCGCCGAAGATCTCCTCAGATTTTCCC
a           A  P  S  Y  M  V  T  G  P  L  H  A  P  K  I  S  S  D  F  P
c            T  I  L  Y  G  D  R  P  F  T  R  A  E  D  L  L  R  F  S  L

      301 TTACCACCGGATATTACCCAACTCCTACTAACACAGGACTCCGTGTGGGACTGCCCTCTT
a           L  P  P  D  I  T  Q  L  L  L  T  Q  D  S  V  W  D  C  P  L
c            T  T  G  Y  Y  P  T  P  T  N  T  G  L  R  V  G  L  P  S  S

      361 CAGAAAAAGCTGTCAGATTACAGCTACAAAGAACTTGTAATACACGTCTTGCAAAGAGGC
a           Q  K  K  L  S  D  Y  S  Y  K  E  L  V  I  H  V  L  Q  R  G
c            E  K  A  V  R  L  Q  L  Q  R  T  C  N  T  R  L  A  K  R  L

      421 TACAACGACACCCAGAAATACTTGCCACAACTAGCATCAATGGATTTCGGAAAGCACTGG
a           Y  N  D  T  Q  K  Y  L  P  Q  L  A  S  M  D  F  G  K  H  W
c            Q  R  H  P  E  I  L  A  T  T  S  I  N  G  F  R  K  A  L  G

      481 GATACCATCTCAGGGCAATTAACAGGATTGAATATCCTGGAAAGATTGATGTGGGGCCTC
a           D  T  I  S  G  Q  L  T  G  L  N  I  L  E  R  L  M  W  G  L
c            Y  H  L  R  A  I  N  R  I  E  Y  P  G  K  I  D  V  G  P  H

      541 ATACGGTTATGGGGTTATGTAATCTGGGTTGTCAGCTCATCCACGATGAGCTTCTTGATG
a           I  R  L  W  G  Y  V  I  W  V  V  S  S  S  T  M  S  F  L  M
c            T  V  M  G  L  C  N  L  G  C  Q  L  I  H  D  E  L  L  D  E

      601 AACAACTTGAGCCTGGCTATAATAGTGGCCTCATTGATGGCCTTAAGCGTGCTTATGGCA
a           N  N  L  S  L  A  I  I  V  A  S  L  M  A  L  S  V  L  M  A
c            Q  L  E  P  G  Y  N  S  G  L  I  D  G  L  K  R  A  Y  G  T

      661 CGGGCAGCTCAATTATTCTTCAAAATATTACAACTATGCCTACCTGTGTTGGCGGCAAGG
a           R  A  A  Q  L  F  F  K  I  L  Q  L  C  L  P  V  L  A  A  R
c            G  S  S  I  I  L  Q  N  I  T  T  M  P  T  C  V  G  G  K  D

      721 ATGGTGATGAGCGCGTTCACCACGATGAAGAGAGTTTGCATAGAGAGACCTAAATCCTAT
a           M  V  M  S  A  F  T  T  M  K  R  V  C  I  E  R  P  K  S  Y
c            G  D  E  R  V  H  H  D  E  E  S  L  H  R  E  T  *

      781 GTAAAAGAGTGTGCAGTCCGGGGGGTTCACTACATGGGCTGTACCAATGAAACCCCCAAAG
a           V  K  E  C  A  V  R  G  F  T  T  W  A  V  P  M  K  P  P  K

      841 AATTCGATTTTGCTCATAAGTCATGACGACGGTTCACACGCAGGTTATGCAACTTGCGTG
a           N  S  I  L  L  I  S  H  D  D  G  S  H  A  G  Y  A  T  C  V

      901 ACTTTACACGACAGACAATCAACCACCATTGGTTTGATAACCTGCTCGCATGCTCCCATC
a           T  L  H  D  R  Q  S  T  T  I  G  L  I  T  C  S  H  A  P  I
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Fig. 2 (*continued*)

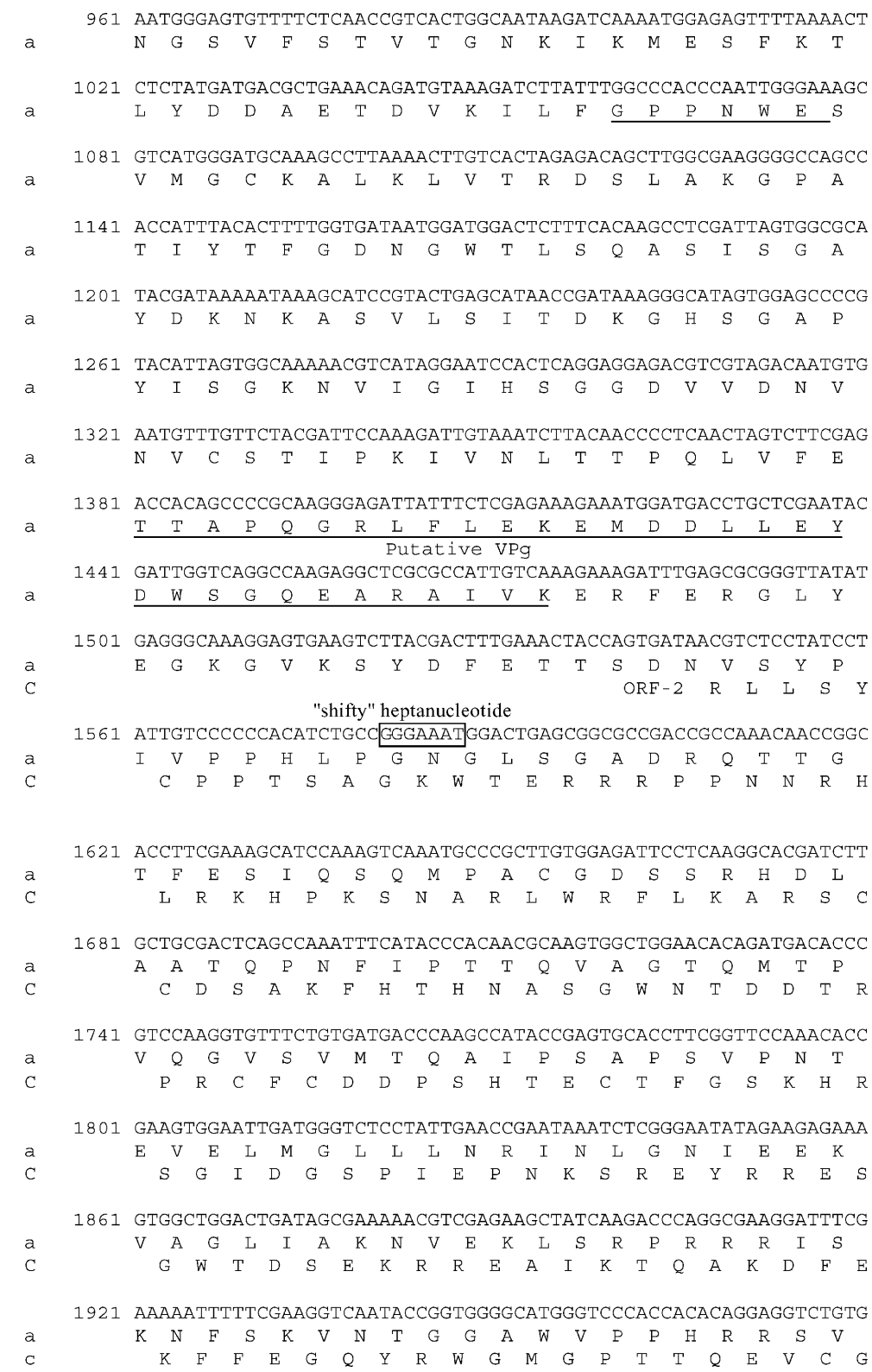

Fig. 2 (*continued*)

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    1981 GATTCGAAGAAGTCGGATCCCTCCCCAAGTTCTACCACTCTAAACAAAGGCTCAACTCAG
a         D  S  K  K  S  D  P  S  P  S  S  T  T  L  N  K  G  S  T  Q
c           F  E  E  V  G  S  L  P  K  F  Y  H  S  K  Q  R  L  N  S  E

    2041 AATACGGGGTTAGAGTGGCGGAAGAGTACAGTGAGCTCAAAGAGCTCACAGCGGGATTTG
a         N  T  G  L  E  W  R  K  S  T  V  S  S  K  S  S  Q  R  D  L
c           Y  G  V  R  V  A  E  E  Y  S  E  L  K  E  L  T  A  G  F  G

    2101 GGTGGCCCAAGTTCGGCGCCCAAGCCGAATTAACTTCCCTGCGACTGCAAGCTGACAGGT
a         G  G  P  S  S  A  P  K  P  N  *
c           W  P  K  F  G  A  Q  A  E  L  T  S  L  R  L  Q  A  D  R  W

    2161 GGCTCCAACGGATGGAGTCCGCACAAATACCCTCGACAGAAGGCGAGGAATCCGTGATAA
c           L  Q  R  M  E  S  A  Q  I  P  S  T  E  G  E  E  S  V  I  N

    2221 ACAGGCTAGTTGAAGCCTATAGTTGCGCGCGCACTAACGCACCAACATCAACCGCCGGCA
c           R  L  V  E  A  Y  S  C  A  R  T  N  A  P  T  S  T  A  G  N

    2281 ATTCCCTAACGTGGGAAGGTTTTATTGAAGACATCAAGGAAGCTGTAAGCTCCTTAGAAT
c           S  L  T  W  E  G  F  I  E  D  I  K  E  A  V  S  S  L  E  L

    2341 TAGACGCCGGTGTTGGGGTTCCGTACATCGCCTACGGGACTCGTACCCACAGAGACTGGG
c           D  A  G  V  G  V  P  Y  I  A  Y  G  T  R  T  H  R  D  W  V

    2401 TGTTCAACCAAGAGTTGCTACCTGTGTTGACTCGACTGACTTTCAACCGCCTACAGAAGA
c           F  N  Q  E  L  L  P  V  L  T  R  L  T  F  N  R  L  Q  K  M

    2461 TGTTGGAAGTCAACTCGGACGACTTGAATGCGGAACAACTGGTTCAACACGGACTTTGCG
c           L  E  V  N  S  D  D  L  N  A  E  Q  L  V  Q  H  G  L  C  D

    2521 ACCCGATAAGGGTATTTGTTAAGGGAGAACCCCACAAAGTCTCCAAGCTGGAAGAAGGTC
c           P  I  R  V  F  V  K  G  E  P  H  K  V  S  K  L  E  E  G  R

    2581 GCTACCGCCTTATAATGAGTGTATCCCTAGTGGATCAGTTGGTAGCCAGAGTTCTGTTCC
c           Y  R  L  I  M  S  V  S  L  V  D  Q  L  V  A  R  V  L  F  Q

    2461 AAAATCAGAACAAACGGGAAATAGAACTCTGGAGGGCAGTGCCCTCGAAGCCGGGATTTG
c           N  Q  N  K  R  E  I  E  L  W  R  A  V  P  S  K  P  G  F  G

    2701 GTTTATCAACCGATGACCAGATCGAAGACTTTGTTAAAGTCTTGGCTTCACAACTTGGTG
c           L  S  T  D  D  Q  I  E  D  F  V  K  V  L  A  S  Q  L  G  E

    2761 AGGAACCACAAGAAGTTTTCAATAACTGGTCCACCAAGTTGATACCAACAGATTGCTCTG
c           E  P  Q  E  V  F  N  N  W  S  T  K  L  I  P  T  D  C  S  G

    2821 GTTTTGACTGGAGTGTAGCGGACTGGATGCTCGAAGATGACATGGAAGTCCGCAATCGCT
c           F  D  W  S  V  A  D  W  M  L  E  D  D  M  E  V  R  N  R  L

    2881 TGACCAGAAACAACAACCACACAACAAAACGATTACGATCAGTGTGGTTAAAATGCATAA
c           T  R  N  N  N  N  H  T  T  K  R  L  R  S  V  W  L  K  C  I  S

    2941 GCAATTCAGTCTTATGCCTTTCTGATGGGTGCCTTCTTTCACAGAGAGTGCCTGGAGTTC
c           N  S  V  L  C  L  S  D  G  C  L  L  S  Q  R  V  P  G  V  Q

    3001 AGAAAAGTGGAAGTTACAATACTTCTTCTTCAAACTCCCGTATCAGAGTTATGGCTGCAT
c           K  S  G  S  Y  N  T  S  S  S  N  S  R  I  R  V  M  A  A  Y

    3061 ATCATTGCGGCGCTTCCTGGGCTATGGCGATGGGCGATGACGCCCTAGAATCTGTTGACT
c           H  C  G  A  S  W  A  M  A  M  G  D  D  A  L  E  S  V  D  S
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Fig. 2 (*continued*)

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    3121 CTAACCTAACAGAGTATAAAAAGTTGGGTTTCAAAGTCGAGGTAGCCAAACAACTGGAAT
c          N  L  T  E  Y  K  K  L  G  F  K  V  E  V  A  K  Q  L  E  F

    3181 TTTGCTCACATATCTTTAAGAATGAGCGCCTCGCATTACCGCTGAATGTAAAGAAGATGC
c          C  S  H  I  F  K  N  E  R  L  A  L  P  L  N  V  K  K  M  L

    3241 TTTACAAGCTAATTTACGGGTACAATCCTGATAGCGGTAACTTGGAAGCGATCAAGAATT
c          Y  K  L  I  Y  G  Y  N  P  D  S  G  N  L  E  A  I  K  N  Y

    3301 ACCTTGACGCTTGCCACTCGATCGTGAATGAAATTCGTCACGACGAGAGCTTGGTCCAGA
c          L  D  A  C  H  S  I  V  N  E  I  R  H  D  E  S  L  V  Q  K

    3361 AAATAATATCGTGGTTGGTCATTCCAGTCCAACCACAAAACTAATTCAGGAGGATCAAGC
c          I  I  S  W  L  V  I  P  V  Q  P  Q  N  *

    3421 ATACAAACAGCCGGGTAAACATCAGTTGCAAACACCGGAAGTTTTAGTCTGATTACACAA

    3481 CAAGCCAAAATAGACTTCAAATTTTTAGCAGGATTTTCAAGCGGTCTATGTCAGCAATAC

    3541 CCGTAACGGTAATTGGCTTGTATTTTGTCTACCTGAAAATCTCCCAACACGTAAGATCAA

    3601 TCGTTAATGAATACGGTCGTGGGTAGGAGAACAATCAATGGAAGAAGACGACCACGAAGG
a     ORF-3/5  M  N  T  V  V  G  R  R  T  I  N  G  R  R  R  P  R  R
b                                          ORF-4  M  E  E  D  D  H  E  G

    3661 CAAACACGACGCGCTCAGCGCAATCAGCCAGTGGTTGTGGTCCAAACCTCTCGGAGAACA
a          Q  T  R  R  A  Q  R  N  Q  P  V  V  V  V  Q  T  S  R  R  T
b           K  H  D  A  L  S  A  I  S  Q  W  L  W  S  K  P  L  G  E  H

    3721 CAACGCCGACCTAGACGACGACGAAGAGGTAATAACCGGGCAGGAAGAACTGTTTCTACC
a          Q  R  R  P  R  R  R  R  R  G  N  N  R  A  G  R  T  V  S  T
b           N  A  D  L  D  D  D  E  E  V  I  T  G  Q  E  E  L  F  L  P

    3781 AGAGGAACAGGTCAAAGCGAGACATTTGTTTTCTCGGAAGACAATCTCGCGGGAAGTTCC
a          R  G  T  G  Q  S  E  T  F  V  F  S  E  D  N  L  A  G  S  S
b           E  E  Q  V  K  A  R  H  L  F  S  R  K  T  I  S  R  E  V  P

    3841 AGCGGAGCAATCACGTTCGGGCCGAGTCTATCAGACTGCCCAGCATTCTCTAATGGAATG
a          S  G  A  I  T  F  G  P  S  L  S  D  C  P  A  F  S  N  G  M
b           A  E  Q  S  R  S  G  R  V  Y  Q  T  A  Q  H  S  L  M  E  C

    3901 CTCAAGGCCTACCATGAGTACAAAATCTCGATGGTCATTTTGGAGTTCGTCTCCGAAGCC
a          L  K  A  Y  H  E  Y  K  I  S  M  V  I  L  E  F  V  S  E  A
b           S  R  P  T  M  S  T  K  S  R  W  S  F  W  S  S  S  P  K  P

    3961 TCTTCCCAAAGTTCCGGTTCCATCGCTTACGAGCTGGACCCACACTGTAAACTCAACTCC
a          S  S  Q  S  S  G  S  I  A  Y  E  L  D  P  H  C  K  L  N  S
b           L  P  K  V  P  V  P  S  L  T  S  W  T  H  T  V  N  S  T  P

    4021 CTTTCCTCAACTATCAATAAGTTCGGGATCACGAAGCCCGGGAGGAGGGCGTTTACAGCG
a          L  S  S  T  I  N  K  F  G  I  T  K  P  G  R  R  A  F  T  A
b           F  P  Q  L  S  I  S  S  G  S  R  S  P  G  G  G  R  L  Q  R

    4081 TCTTACATCAACGGGACGGAATGGCATGACGTTGCCAAGGACCAATTCAGGATCCTCTAC
a          S  Y  I  N  G  T  E  W  H  D  V  A  K  D  Q  F  R  I  L  Y
b           L  T  S  T  G  R  N  G  M  T  L  P  R  T  N  S  G  S  S  T

    4141 AAAGGCAATGGTTCTTCATCGATAGCTGGTTCTTTCAGGATCACTATAAAGTGTCACTTC
a          K  G  N  G  S  S  S  I  A  G  S  F  R  I  T  I  K  C  H  F
b           K  A  M  V  L  H  R  *
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Fig. 2 (*continued*)

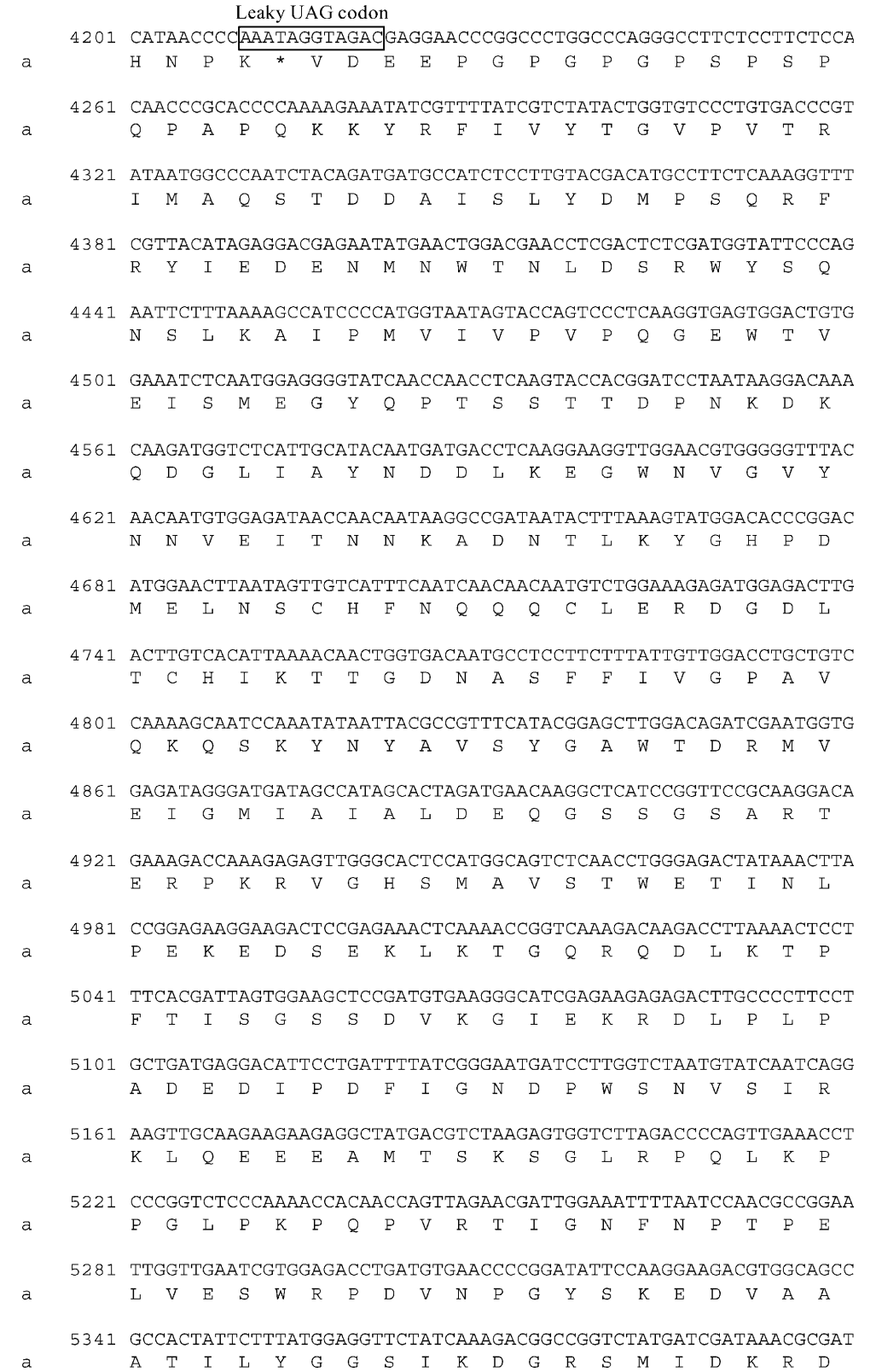

Fig. 2 (*continued*)

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     5401 AAAGCTGTGTTAGACGGCCGTAAGCATTGGGGTTCTTCCTTAGCTTCCTCCTTAACAGGA
a         K  A  V  L  D  G  R  K  H  W  G  S  S  L  A  S  S  L  T  G

     5461 GGAACGCTTAAGGCCTCTGCAAAGTCGGAGAAGCTTGCTAAACTCACCTCGCGTGAGAGG
a         G  T  L  K  A  S  A  K  S  E  K  L  A  K  L  T  S  R  E  R

     5521 GCGGAATTCGAACGAATTAAACGCCAGCAAGGCACCACACAAGCTTCAGAATACTTAGAA
a         A  E  F  E  R  I  K  R  Q  Q  G  T  T  Q  A  S  E  Y  L  E

     5581 TTTATTCTGAAGAGCATGAACCCCGACTAATGGTTTTGAAGGGATATAGCCTAACCCTTT
a         F  I  L  K  S  M  N  P  D  *

     5641 CTGGTCCTGATGAACCTGTCTAAATCATCACCGTCAAGCCCGTGACGTTAAACTAGGAAC

     5701 GACTCCCGAAAGGATAGGCACGAATGTTCCCCTTATTTAAAGGGTTATACAGTAGGATCC

     5761 TACGGCACTTCGGTGT 5776
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Fig. 2. Complete genomic nucleotide sequence of BChV-2a. The sequence is written as DNA. The conserved amino acid sequence in the N-terminal portion of ORF-1 gene product P2 used by Guilley et al. to design o2 primer is underlined. Perfectly conserved nucleotides in the 5′- and 3′-termini between BChV, BMYV and BWYV-FL1 are upperlined. Sequences needed for frameshift and readthrough are indicated. The BChV-2a nucleotide sequence has been assigned the accession number ***AF352024*** in the Genbank Data Library

hypothesis calculated *a priori* by Dnaml. The substitution model HKY85 [7] was specified to PLATO to calculate the likelihood of this null hypothesis for each site along the alignment. Those regions of the alignment which have the lowest average likelihood are then checked for significant departure from the null hypothesis using the Monte Carlo simulation. Significance indicates failure of the null model to explain the observed data, indicating the importance of recombination or selection events.

Results

Host range analysis

The host ranges of BMYV-2ITB, BWYV-FL1 and BChV-2a were tested on 10 plant species inoculated at the same age belonging to 5 botanical families; the results are shown in Table 1. This experiment was repeated twice and it was found that *M. persicae* transmitted the 3 virus species with a similar efficiency. Apart from *Chenopodium capitatum* and *Sinapis alba*, which was not tested, all plant species became infected when inoculated with BMYV-2ITB. Similar results were observed when plants were inoculated with BWYV-FL1 except that BWYV-FL1 infected only about 25% of inoculated sugar beet plants. The host range of BChV appears to be narrower than that of BMYV and BWYV with only 4 plant species out of 2 families becoming infected when inoculated with BChV. Additionally, 25 weed species were tested toward BChV, BMYV and BWYV. Only 2 plant species out of 25 became infected by BChV, whereas BWYV and BMYV were able to infect respectively 19 and 11 plant species out of 25 (data not shown). Conversely, BChV was the only beet polerovirus species able to infect *C. capitatum* as previously reported [8]. Two plant species appear to be

Table 1. The host range of three beet polerovirus species as determined by TAS-ELISA and transmission tests

Plant species with *family*	Infection rate and A405 values for plant inoculated with		
	BWYV-FL1	BMYV-2ITB	BChV-2a
Brassicaceae			
Capsella bursa-pastoris	**5/5** 2.14 (0.15)	**4/5** 1.35 (0.54)	0/5 0.17 (0.01)
Crambe abyssinica	**5/5** 2.35 (0.09)	**5/5** 1.27 (0.26)	0/5 0.18 (0.02)
Sinapis alba	**5/5** 1.83 (0.58)	nd	0/5 0.23 (0.1)
Caryophyllaceae			
Spergula arvensis	**5/5** 2.08 (0.44)	**5/5** 2.04 (0.46)	**4/5** 0.45 (0.13)
Stellaria media	**5/5** 2.14 (0.43)	**5/5** 0.64 (0.48)	0/5 0.16 (0.008)
Chenopodiaceae			
Beta vulgaris cv Trestel	**1/5** 2.01 (0.24)	**5/5** 2.34 (0.21)	**5/5** 2.41 (0.25)
Chenopodium capitatum	0/5 0.19 (0.02)	0/5 0.18 (0.03)	**5/5** 2.05 (0.58)
Spinacia oleracea	**5/5** 2.28 (0.31)	**5/5** 2.43 (0.02)	**5/5** 2.41 (0.04)
Asteraceae			
Senecio vulgaris	**5/5** 1.39 (0.46)	**5/5** 0.48 (0.11)	0/5 0.18 (0.01)
Portulacaceae			
Montia perfoliata	**5/5** 1.66 (0.36)	**5/5** 1.58 (0.39)	0/5 0.24 (0.08)

nd Not determined

Calculated threshold is: 0.293

Frequencies of infected plants are given in bold as well as positive absorbance values. ELISA absorbance means (OD 405 nm, after 2 h incubation) and standard deviation (between brackets) have been calculated on the positive OD values

a common host for the three beet polerovirus species; *Spergula arvensis* and *Spinacia oleracea*. This analysis reveals that BChV has a different host range than those of BMYV and BWYV.

Complete genomic sequence and genome organisation of BChV-2a and BChV-California

The complete nucleotide sequence (5776 nt) of BChV-2a genomic RNA is shown in Fig. 2. The sequences of the 5′ and 3′ extremities (within the untranslated region) were not determined experimentally by 5′ and 3′ RACE, and the terminal sequences of the 24 and 20 residues respectively have been assigned by oligonucleotides o1 and rev5. GenBank accession number of BChV-2a sequence is AF352024.

The complete genomic sequence of BChV-California was obtained by similar methods and a 5742 bp sequence was identified (GenBank accession number AF352025).

Analysis of the distribution of translation, initiation and termination codons revealed the presence of 6 long ORFs in the plus-strand (Fig. 1). No ORF of more than 300 nucleotides was present in the minus strand. The genetic organisation of the 6 ORFs is typical of other *Polerovirus* members. The first plus-strand ORF (ORF-0) begins with AUG (27–29) and terminates with UAA (771–773). The calculated molecular mass (M_r) of the corresponding putative protein P0 is 28259 Da. The second ORF (ORF-1) is in a different frame and begins with AUG (163–165) and terminates with UAA (2131–2133). The calculated M_r of the corresponding putative protein P1 is 71787 Da. The 5′-part of the third ORF (ORF-2) overlaps ORF-1 and is in the same reading frame as ORF-0. ORF-2 begins at position 1547 and finishes with UAA (3402–3404). The calculated M_r of the corresponding putative protein P2 is 70542 Da. Between the third and the fourth ORF there is a non-coding region (UTR) of 201 bp long (3405–3606). The fourth ORF (ORF-3) begins with AUG (3607–3609) and terminates with UAG (4213–4215) and has a coding capacity of M_r 22601 Da. The complete sequence of the fifth ORF (ORF-4) overlaps ORF-3 in a different reading frame. ORF-4 begins with AUG (3638–3640) and terminates with UAG (4163–4165) and has a coding capacity of M_r 19526 Da. The last ORF (ORF-5) is immediately adjacent to ORF-3 and is in the same reading frame. ORF-5 ends at UAA (5608–5610) and, if expressed, should encode a polypeptide of M_r 51637 Da. The 3′-noncoding region is 166 residues in length and displays great sequence homology with BMYV, BWYV and CABYV. The 5′-noncoding region of the RNA is very short (26 residues) and the first eight residues at the 5′ terminus (5′-ACAAAAGA) are identical with BMYV, BWYV and CABYV.

The P0 sequence did not display any significant homology with any other polerovirus P0 sequence, and currently no functions have been attributed to the P28 protein. However it has been proposed that it may be involved in determining the host range [13, 24]. Recently van der Wilk et al. showed that potato plants transformed with cDNA encoding P0 of PLRV expressed disease-like symptoms and suggested that P0 was involved in symptom expression [23]. At present there is no sufficient data to attribute any function to BChV P0; however, recent variability studies have shown strong relationships between the P0 sequence of beet poleroviruses and their host range [8].

Detailed analysis of the amino acid sequence of BChV ORF-1 and -2 reveals the presence of the consensus sequence motifs found in other *Luteoviridae*, indicating that P1 and P2 are involved in RNA replication [14]. In particular ORF-1 contains the putative active site residues for chymotrypsin-like protease while ORF-2 contains the well characterized “core” RNA polymerase sequence motifs present in the RNA dependent RNA polymerases of all plus-sense RNA viruses. The sequence of the putative genome linked protein (VPg) is mapped to position 1381–1476 (sequence underlined on Fig. 2). Except for BMYV [6] a “shifty” heptanucleotide just upstream of a pseudoknot has been found in the region which overlaps between ORF-1 and ORF-2 of the *Polerovirus* genome [4, 6]. A similar shifty heptanucleotide (1580-GGGAAAU) located upstream of a pseudoknot

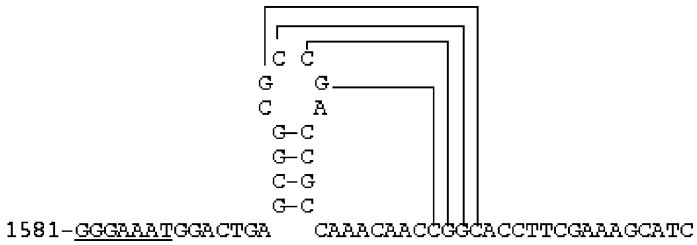

Fig. 3. Pseudoknot near the ORF-2/3 5′-terminus overlap of the BChV RNA. The "shifty" heptanucleotide thought to be involved in frameshifting of BChV RNA is underlined

(Fig. 3) has been observed for BChV and is probably involved in the expression of ORF2 as a P1-P2 fusion protein by –1 translation frameshift.

ORF-3 and ORF-4 sequences of BChV display strong homology with other beet poleroviruses, hence ORF-3 encodes for the viral coat protein. The function of P4 encoded by ORF-4 is not yet known. ORF-5 of *Luteoviridae* is expressed as a P3-P5 fusion protein (RT protein) by translational readthrough of the coat protein UAG termination codon. The sequence (AAAUAGGUAGAC 4210–4221) in the vicinity of the "leaky" UAG is identical for all *Luteoviridae* [14]. It is assumed that the RT protein is involved in the transmission of BChV by aphids as shown with BWYV.

Phylogenetic relationship between BChV and other Polerovirus *species*

Polerovirus interspecies comparisons have been done using 2 Dot-plot matrixes with BMYV-2ITB and BWYV-FL1 and BMYV-2ITB and BChV-2a, using a windows value of 21 and the stringency value of 15 to reduce background noise (data not shown). The dot matrix between BMYV-2ITB and BWYV-FL1 is similar to that performed earlier by Guilley et al. [6] and displays a close pattern when compared to the matrix between BMYV-2ITB and BChV-2a. We have observed that the 3′-proximal average 2000 residues of BChV-2a and BMYV-2ITB, the region encoding the viral structural proteins, are highly homologous. There is some homology between the central regions of the sequences which encode the domain of P2 containing the RNA-dependent RNA polymerase motifs common to all positive-strand RNA viruses. The 5′-proximal average 2000 residues display little similarity which is consistent with the P0 phylogenetic analyses [8]. The percentage identity between the individual putative proteins of BMYV-2ITB and BChV-2a reflecting the Dot-plot results are as follows: P0-20%, P1-28.4%, P2-56.7%, P3-91.6%, P4-86.9% and P5-85.7%.

However the dot matrix comparison between BChV-2a and BChV-California shows a different relationship (Fig. 4). Strong homology is observed in the 5′ part of genome, but within the 3′ region, less homology is seen. This intra-species and inter-species homology variation may be due to recombinations which can result in heterogeneous rates of homology along sequences. In order to understand the roles of recombination and selection in beet polerovirus diversity, the PLATO program was used to detect spatial variation in the phylogenetic relationships

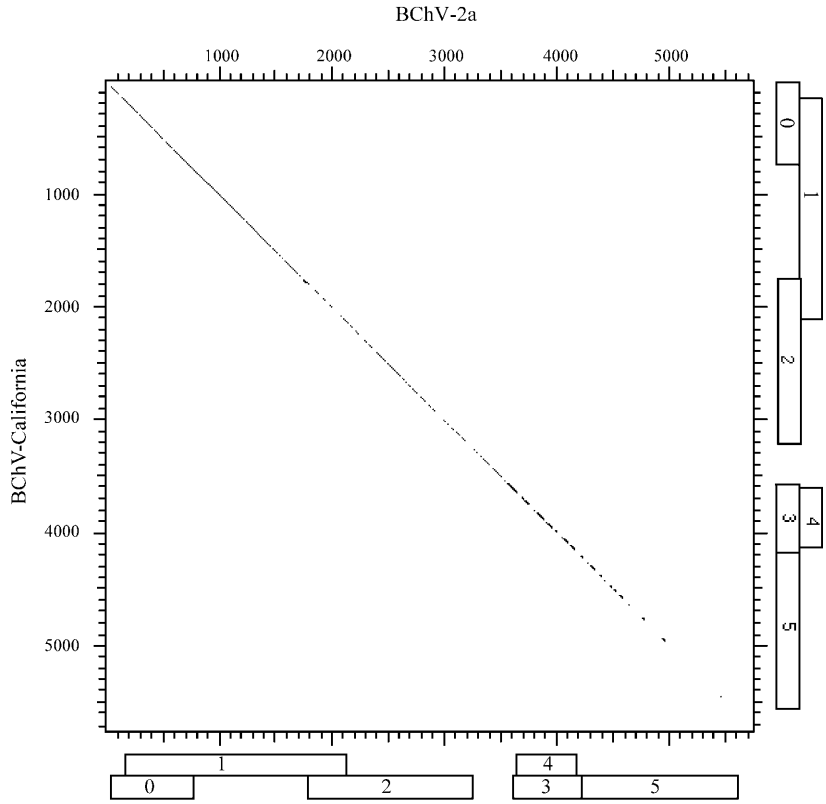

Fig. 4. Dot-plot matrix comparison between BChV-2a and BChV-California. Wordsize value 19 was used to reduce background noises

between beet poleroviruses and *Luteoviridae* [16]. It was observed that beet polerovirus genomes can be divided in two different parts with different phylogenetic lineages (Fig. 5). The first 3600 nucleotides have probably originated from different ancestors, which share little homology with other *Poleroviruses* and no significant homology with *Enamovirus* or *Luteoviruses*. Phylogenetic analyses have shown that BMYV and CABYV have related ancestor especially by comparing their P2, as proposed by Guilley et al. [6] and confirmed by the phylogenetic trees of Fig. 5. We observed the closest similarity of the putative VPg located on P1, downstream of the putative protease domain between BChV, CABYV, BMYV and an American sugar beet infecting isolate of BWYV. Beet polerovirus species share a common ancestor in the second part of the genome which starts near the initiation codon of ORF-3. In this part of the genome, sequences are well-conserved between species; BMYV, BChV, BWYV *Brassica spp* isolates and BWYV-FL1 share more than 85% of homology in their amino acid sequences (e.g. coat protein) [8]. Greater sequence variations are observed in the C-terminal of P5; this may be due to adaptation to different plant hosts or aphid vectors. Currently no data is available to predict what are the viral ancestors of these beet polerovirus species.

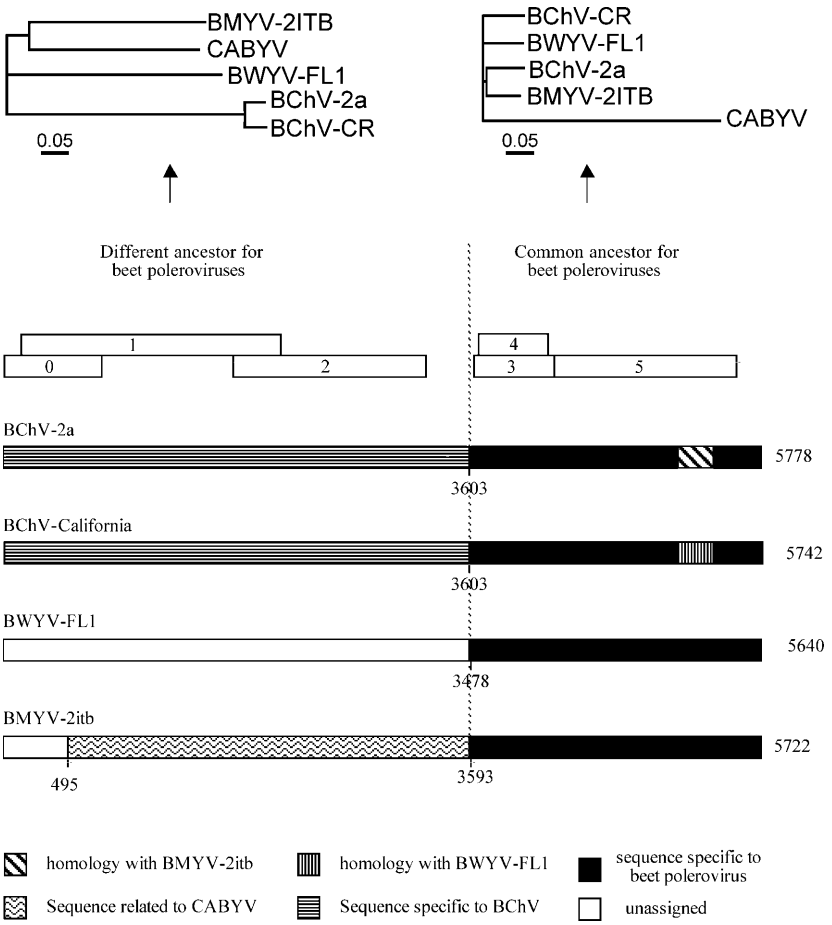

Fig. 5. PLATO analysis of *Luteoviridae* genomic sequence. The Beet polerovirus regions which differ by their phylogenetic relationships, are presented. Phylogenetic trees corresponding to the 5′ and 3′ halves of the polerovirus species used in this study have been obtained by ClustalW. Branch lengths are to the scale and the clusters corresponding to hypothetic ancestors are supported by significant boostrap values

Discussion

In these studies we demonstrated that BChV has a distinct and narrower host-range from BMYV and BWYV, suggesting that BChV has different weed hosts that can act as overwintering hosts and sources of infection. Most of the host range results (using 25 plant species) were clear-cut, except those obtained with *Capsella bursa-pastoris*. We showed that we were able to select at a low frequency (less than 5%), a BChV variant able to infect *C. bursa-pastoris* and still not reacting with Mab PAV-IL-1. This reflects the quasi-species structure of the viral population, being selected by the virus/host interactions for adaptation to new hosts. *C. bursa-pastoris* may accelerate the rate of BChV evolution by increasing the diversity of the viral population. This has important consequences in the epidemiology of virus yellows due to BChV and further

work is needed to identify the weed species harboring BChV and the role they play in the survival of the virus, and in maintaining BChV variants with selective advantages.

Recent molecular epidemiological studies have pointed out the increasing percentage of mixed infections between BMYV and BChV [9]. This phenomenon may have an influence either on recombination events creating variant viruses and isolates or on phenotypic mixing or genomic masking, potentially modifying the vector specificity or efficiency. Although our *M. persicae* clone (NL) transmitted the 3 virus species in our conditions, we do not know yet the vector efficiency and vector specificity of each aphid clone and species available in our aphid collection toward BChV. Transmission studies are required to complete the epidemiological features of this newly described beet polerovirus.

The molecular studies revealed that the term "beet polerovirus" does not only refer to polerovirus species that infect beet (which is obviously not the case for BWYV lettuce or rape isolates [8]), but corresponds to a group of poleroviruses which share a common ancestor in their 5′-part of genome. This feature concerning beet poleroviruses highlights the importance of recombination for the emergence of new *Luteoviridae* species or variants as outlined by Mayo and Miller [15] and by Moonan et al. [16] utilizing new phylogenetic and evolutionary tools (PLATO). These authors showed that RNA recombination events occurred between poleroviral and luteoviral ancestors to explain the origin of *sugarcane yellow leaf virus* (ScYLV), and that putative sites for such RNA recombinations could be associated with the known domains for the transcription of subgenomic RNAs. BChV probably appeared by recombination close to the internal UTR, between an unidentified polerovirus-like ancestor, which provided the 5′-part of the genome encoding P0 and the polymerase, and BMYV or BWYV lettuce or rape isolates which provided the 3′ half of the genome (ORF-3, -4 and -5). These recombination events might have been linked to adaptation to new hosts and vectors, as determined by new combinations of replicase, movement and readthrough proteins.

Different selection pressures allow the 5′-part and 3′-part of the genome to evolve differently. The 5′-part of beet polerovirus genome seems to be under strong selection pressure, maintaining the sequence integrity in order to conserve the fitness of replication and allow adaptation to hosts. The 3′-part of the genome is under divergent selection pressure to allow adaptation to hosts and vectors accessible in areas where the virus isolate is predominant.

The CP is an obvious target for pathogen-derived transgenic resistance because of the strong conservation among CP sequences of all beet polerovirus species. Transformed sugar beet expressing CP of BMYV could enable resistance against all beet polerovirus strains. However the propensity of beet poleroviruses to acquire new genes by recombination needs to be understood, in order to estimate the risk of emergence of new viral diseases by acquisition of beet polerovirus structural proteins generated from transgenic plants, by a non pathogenic strain of the virus, and/or by modifying the vector specificity and epidemiology of the disease.

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