

Production of a *Beet chlorosis virus* full-length cDNA clone by means of Gibson assembly and analysis of biological properties

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Abstract

Beet chlorosis virus (genus *Polerovirus*, family *Luteoviridae*), which is persistently transmitted by the aphid *Myzus persicae*, is part of virus yellows in sugar beet and causes interveinal yellowing as well as significant yield loss in *Beta vulgaris*. To allow reverse genetic studies and replace vector transmission, an infectious cDNA clone under cauliflower mosaic virus 35S control in a binary vector for agrobacterium-mediated infection was constructed using Gibson assembly. Following agroinoculation, the BChV full-length clone was able to induce a systemic infection of the cultivated *B. vulgaris*. The engineered virus was successfully aphid-transmitted when acquired from infected *B. vulgaris* and displayed the same host plant spectrum as wild-type virus. This new polerovirus infectious clone is a valuable tool to identify the viral determinants involved in host range and study BChV protein function, and can be used to screen sugar beet for BChV resistance.

Yellowing of sugar beet (*Beta vulgaris*), or virus yellows, is a disease caused by different virus species that results in vein yellowing, leaf chlorosis and necrotic spots, and has the potential to significantly decrease root yield and sugar content [1–4]. Within this disease three species have been identified that belong to the genus *Polerovirus* (family *Luteoviridae*), i.e. *Beet western yellows virus* (BWYY), *Beet mild yellowing virus* (BMYV) and *Beet chlorosis virus* (BChV).

All beet-infecting poleroviruses are phloem-limited and aphid-transmitted preferentially by *Myzus persicae* in a circulative, non-propagative and persistent manner [5, 6]. To date, this disease can only be controlled by chemical vector control (i.e. neonicotinoids as a seed pellet), as no polerovirus resistance sources or genes have yet been described that could be exploited for resistant cultivar development [7].

BChV was described by Stevens in 1994 in the UK [8] and by Liu in the USA [9]. Based on its molecular, serological and biological traits, BChV was assigned as a distinct member of the genus *Polerovirus* in 2002 by the International Committee for the Taxonomy of Viruses (ICTV) [2, 4, 10]. BChV infection induces specific symptoms in the form of chlorotic and necrotic spots on older leaves, together with

vein yellowing. According to Stevens *et al.* [3], BChV infection can result in a sugar yield reduction of about 8–24 %, depending on the time-point of infection.

The host range of BChV includes *Beta* species, but also *Chenopodium capitatum*, *Spergula arvensis* and *Spinacia oleracea*. BMYV, on the other hand, can additionally infect *Capsella bursa-pastoris*, *Stellaria media*, *Senecio media* and *Montia perfoliata*, but not *C. capitatum* [2].

The genome of BChV consists of a single-stranded, positive sense RNA strand of 5776 nucleotides (AF352024.1), and possesses seven open reading frames (ORFs) [2, 4, 11]. The polerovirus ORFs at the 5′-proximal part of the virus genome – ORF0, -1 and -2 – can be translated directly by the viral genomic RNA, whereas ORF3a, -3, -4 and -5 are translated from a subgenomic RNA (sgRNA). The 5′-end of the genome is linked to a viral genome-linked protein (VPg), whereas the 3′-end does not contain a specific structure or poly(A)-tail [2, 5, 12]. Poleroviruses use a wide varieties of translation strategies to express their viral proteins.

The polerovirus P0 protein (nt 27–770, AF352024.1) is the viral silencing suppressor [13–15] but, intriguingly, according to Kozłowska-Makulska *et al.* [16], BChV-P0 does not exhibit such a function under the experimental conditions.

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Abbreviations: BChV, *Beet chlorosis virus*; BMYV, *Beet mild yellowing virus*; BrYV, *Brassica yellows virus*; BWYY, *Beet western yellows virus*; CABYV, *Cucurbit aphid-borne yellows virus*; CLRDV, *Cotton leafroll dwarf virus*; CP, coat protein; DAS-ELISA, double-antibody sandwich enzyme-linked immunosorbent assay; GA, Gibson assembly; HDV-rz, hepatitis delta virus ribozyme; ICTV, International Committee on Taxonomy of Viruses; PLRV, *Potato leafroll virus*; RTD, readthrough domain; TuYV, *Turnip yellows virus*.

The GenBank accession number for the genome sequence of *Beet chlorosis virus* is MH271171.

One supplementary table is available with the online version of this article.

Proteins P1 and P2, encoded by ORF1 (nt 163–2133) and ORF2 (nt: 1547–3404), are related to virus replication. P1 encodes the VPg [2, 5, 13]. P2, which possesses the RNA-dependent RNA polymerase (RdRp) motif, is expressed as a fusion protein with P1 by a –1 ribosomal frameshift at nt 1588 [5]. P3a protein is synthesized by translation from an alternative non-AUG start codon, recently described by Smirnova *et al.* [11]. This protein is involved in long-distance movement of *Turnip yellows virus* (TuYV).

The 22.6 kDa P3 protein, encoded by ORF3 (nt 3607–4215), is the major capsid protein (CP) [17]. By a translational ‘read-through’ mechanism of the ORF3 amber stop codon, the ORF5-encoded domain (4216–5610), referred to as the read-through domain (RTD), is expressed as a fusion protein with P3. The P3–P5 protein is not required for the assembly of the icosahedral virus particle, but is essential for aphid transmission and systemic spread [17]. The C-terminal half of the RTD might also play a role in the vector specificity and limitation of the virus to the phloem tissue [18, 19]. The ORF4 (nt 3638–4165) is expressed by a leaky scanning of the start codon of ORF3 and encodes P4, which has the characteristics of a host-specific viral movement protein [20, 21].

The natural vector-mediated mode of poleroviurs inoculation may be a drawback to analyse polerovirus host range, species-specific symptom development, polerovirus mixed infections or plant–polerovirus interactions. Developing full-length infectious cDNA clones of poleroviruses and agroinoculation circumvented the use of aphids to inoculate plants. Polerovirus infectious clones have been obtained for TuYV [22], BMYV [23, 24], *Potato leafroll virus* (PLRV) [25], *Cucurbit aphid-borne yellows virus* (CABYV) [26] and *Cotton leafroll dwarf virus* (CLRDV) [27], and from the proposed species *Brassica yellows virus* (BrYV) [28]. Reverse genetic analysis on the full-length clones is responsible for our existing knowledge on polerovirus gene function.

The construction of virus full-length cDNA clones by conventional cloning based on restriction enzymes and *in vitro* ligation is often inefficient and time-consuming. Simplification of the process is possible by either homologous recombination in yeast/*in vivo* [29, 30] or by using the isothermal one-step *in vitro* technique, Gibson assembly (GA), [31–36]. In this study, GA was used to produce an infectious cDNA clone of BChV. To ensure the exact processing of the viral RNA after *in vivo* transcription from T-DNA introduced into the plant cell, the viral sequence was introduced into the vector pDIVA (accession no. KX665539) downstream of the cauliflower mosaic virus 35S promoter and upstream the hepatitis delta virus ribozyme (HDV-rz) and the nopaline synthase terminator. The BChV construct was assayed for its ability to replicate, move systemically in different host plants and be aphid-transmissible.

The French BChV isolate 2a [2] (accession no. AF352024) produces typical symptoms when aphids are inoculated to field-grown beets, but greenhouse plants remain

symptomless. Total RNA was extracted from *B. vulgaris* plants systemically infected with BChV-2a using the RNeasy mini kit (Qiagen). As the complete BChV genome sequence initially determined by Hauser *et al.* [2] was lacking the genome extremities, the 3′- and 5′-terminal end sequences were identified by RACE-PCR, following the procedure of Frohman *et al.* [37] with minor modifications. Starting with total RNA, cDNA synthesis was performed using RevertAid H Minus transcriptase (Thermo Fisher Scientific) and the primer BChV-RACE-5_A (Table S1, available in the online version of this article) for the 5′ end and the primer BChV-RACE-3_A for the 3′ end negative strand. Subsequently, a tailing reaction was performed using terminal deoxynucleotidyl transferase (Promega) following the manufacturer’s instructions. The viral extremities were amplified from the tailed cDNA by PCR using Phusion DNA high-fidelity polymerase (New England BioLabs) and a primer complementary to the tailing nucleotide and one of BChV-RACE-5_B/BChV-RACE-3_B (Table S1). Within the extreme 5′ 100 nt two nucleotide changes were observed at positions G¹²-C¹² and C¹³-T¹³ in comparison to the previously published sequence. Within the extreme 3′ 100 nt of the RACE-PCR fragment six nucleotide changes were present, the deletion of C⁵⁷⁰⁵, the insertion of T⁵⁷⁵⁹ and T⁵⁷⁶⁵, and nucleotide exchanges at T⁵⁷⁵³-A⁵⁷⁵², 5762 A⁵⁷⁶²-T⁵⁷⁶⁸ and C⁵⁷⁶⁸-T⁵⁷⁶⁹. The new BChV consists of 5777 nt and is one nucleotide longer than the published sequence.

The primers for full-length cDNA synthesis and PCR were designed based on a newly assembled sequence based on both the original published sequence from Hauser *et al.* [2] and the new 3′ and 5′ extremities (MH271171). The viral cDNA synthesis from total RNA was performed using RevertAid H Minus transcriptase (Thermo Fisher Scientific) and the primers BChV-1_as, BChV-2_as and BChV-3_as (Table S1) to generate three cDNA fragments. Phusion DNA high-fidelity polymerase (New England BioLabs) was used for the amplification of the viral fragments and the binary vector. The pDIVA vector backbone was amplified with the primers HDV_s and 35S_as (Table S1). The viral fragments were generated with the primer pair BChV-1_s and BChV-1_as (Table S1) for the first fragment (nt 1–2000), and the primer pairs BChV-2_s and BChV-2_as and BChV-3_s and BChV-3_as (Table S1) for the second (nt 1953–3662) and the third fragments (nt 3616–5777), respectively. The viral fragments were generated to have a 50–60 nucleotide overlap. The 5′ viral genome fragment was supplemented with a 23 nucleotide overlap to the 35S end, whereas the 3′ viral fragment (nt 3616–5777) was supplemented with an 18 nucleotide overlap to the 5′-end of the HDV ribozyme. PCR products were purified from the agarosegel using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, GmbH and Co. KG). Cloning was performed by one-step isothermal GA, following the standard protocol [31]. Following visual estimation of the DNA concentration of the vector and insert after gel electrophoresis, 3 µl of each fragment and 1 µl of the linearized vector were used for assembly. Recombinant plasmids were transformed

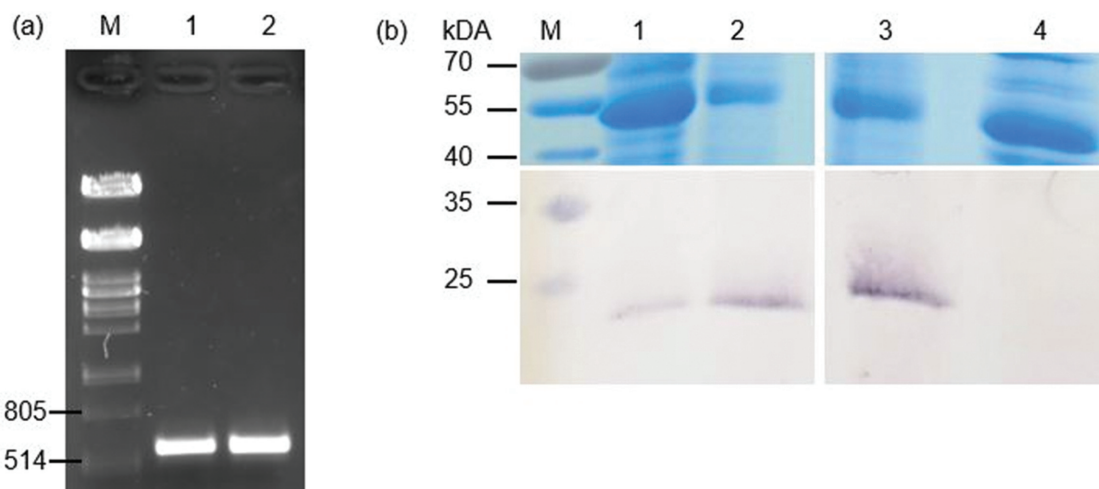


Fig. 1. Detection of BChV infection in inoculated plant material. (a) RT-PCR analysis of BChV infection in *N. benthamiana* leaves at 4 weeks post-inoculation. Lane M, lambda DNA *Pst*I digest marker; lane 1, BChV-2a-4 infected *N. benthamiana* leaf; lane 2, virus wild-type-infected *B. vulgaris* leaf. (b) Detection of the viral CP (22.6 kDa) by Western blot (bottom) and the protein loading control stained with Commassie blue (top) from BChV wild-type-infected and pDIVA-BChV-2a-4-infected *B. vulgaris* plants. M, PageRuler Prestained Protein Ladder, 10 to 180 kDa (Thermo Fisher Scientific); lane 1, virus wild-type-infected *B. vulgaris* young leaf; lane 2, virus wild-type-infected *B. vulgaris* old leaf; lane 3, BChV-2a_4 clone-infected *B. vulgaris* old leaf; lane 4, non-infected plant.

into *Escherichia coli* strain DH5 α to generate pDIVA-BChV-2a variants. Restriction enzyme analysis of the extracted plasmids was performed and 7 clones out of 64 (9%) showed the correct digestion profile. The full BChV genome, together with the plasmid/viral genome junction sites, were determined for one viral clone that was designated pDIVA-BChV-2a-4. The cloned BChV sequence showed 99% homology to the published sequence and no insertions or deletions were identified at the cDNA borders. All seven clones were transformed by electroporation into *Agrobacterium tumefaciens* strain C58C1 [38]. For agroinoculation, *A. tumefaciens* cells were grown overnight at 28 °C in 3 ml Luria Bertani medium (containing 50 $\mu\text{g ml}^{-1}$ kanamycin, 50 $\mu\text{g ml}^{-1}$ rifampicin and 5 $\mu\text{g ml}^{-1}$ tetracyclin). Cells were collected by centrifugation and suspended in inoculation buffer (10 mM MgSO₄, 10 mM MES and 10 μM acetosyringone) and adjusted to an OD₆₀₀ of 0.1 for the inoculation of *Nicotiana benthamiana* plants (three-to-four leaf stage) or an OD₆₀₀ of 0.5 for the inoculation of *B. vulgaris* plants (four-leaf stage) [36]. Each clone was inoculated to four *N. benthamiana* plants and three *B. vulgaris* plants by injecting the agrobacterium cell suspension into the mesophyll of three leaves per plant using a syringe without a needle [39]. After 6 weeks' growth in greenhouse conditions (14 h light, 24 °C/10 h dark, 18 °C) the infected plants displayed no obvious symptoms, similar to greenhouse plants aphid-inoculated with the wild-type virus. Virus infection was assayed by RT-PCR 4 weeks after the inoculation of *N. benthamiana* plants using the BChV-specific primers BChVP0_s and BChVP0_as (Table S1) on total RNA (DNase treated) extracted from newly emerged leaves of all inoculated plants. The expected PCR fragment of 580 bp

was observed in three out of seven samples prepared from inoculated *N. benthamiana*. (Fig. 1a). The infection of *B. vulgaris* by inoculation with the clone pDIVA-BChV-2a-4 was confirmed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using a polerovirus CP-specific antiserum (TYV-IgG, Loewe 07009S/500) (Table 1). Based on the ELISA OD values, it was observed that the viral clone pDIVA-BChV-2a-4 accumulated in non-inoculated leaves at a similar level to the wild-type virus (Table 1). DAS-ELISA confirmed the ability of the clone pDIVA-BChV-2a-4 to systemically colonize the natural host *B. vulgaris*. Although BChV systemic infection was detectable in *N. benthamiana* by RT-PCR following

Table 1. Detection of BChV by DAS-ELISA in *B. vulgaris* after pDIVA-BChV-2a-4 agroinoculation or aphid transmission of the wild-type virus

	No. plants infected/no. plants inoculated*	OD ₄₀₅ ±SD†
Wild-type	3/3	5.10±0.01
pDIVA-BChV-2a-4	4/5	5.31±0.35
Mock‡	0/2	0.00
Non-inoculated	0/2	0.00

*Number of plants infected/plants inoculated. Plants are considered to be infected when the absorption at 405 nm is above the mean value of the absorption of non-inoculated plants plus 3× the standard deviation (SD) of these values minus the sample buffer value.

†The mean value of absorbance at 405 nm 6 weeks after inoculation.

‡Mock, empty binary vector.

Table 2. Virus transmission efficacy for several host plant species when using wild-type-infected *B. vulgaris* plants and pDIVA-BChV-2a-4-agroinoculated *B. vulgaris* plants as the virus source

Systemic infection was assayed by DAS-ELISA 6 weeks after inoculation.

Plant species	BChV-2a	pDIVA-BChV-2a-4
	No. plants infected/no. plants inoculated*	No. plants infected/no. plants inoculated*
<i>Brassica napus</i> (Brassicaceae)	0/10	0/10
<i>Capsella bursa pastoris</i> (Brassicaceae)	0/10	0/10
<i>Montia perfoliata</i> (Portulacaceae)	0/10	0/10
<i>Nicotiana benthamiana</i> (Solanaceae)	0/10	0/10
<i>Nicotiana occidentalis</i> subsp. <i>Hesperis</i> (Solanaceae)	0/10	0/10
<i>Nicotiana clevelandii</i> (Solanaceae)	0/10	0/10
<i>Beta macrocarpa</i> (Amaranthaceae)	10/10	9/10
<i>Beta vulgaris</i> (Amaranthaceae)	10/10	10/10

*Number of plants infected/plants inoculated. Plants are considered to be infected when the absorption value at 405 nm is above the mean value of the absorption of non-inoculated plants plus 3× the standard deviation (sd) of these values minus the sample buffer value.

agroinoculation, further analysis is required to demonstrate the infectivity of the full-length clone in this plant species.

Western blot analysis was performed on protein extracts prepared from older, non-inoculated and newly developed leaves of *B. vulgaris* that were found to be positive by DAS-ELISA after aphid transmission with the wild-type virus or agroinoculation with the pDIVA-BChV-2a-4 clone. Protein extracts from a non-infected plant served as a control. Western blot was performed using the broad-spectrum monoclonal antibody (Mab) B-2-5 G4 (DSMZ AS-0049/1), which can detect the CP from several luteoviruses [40, 41]. As shown in Fig. 1(b), the BChV-CP of 22.6 kDa was clearly observed in one sample prepared from an old leaf of pDIVA-BChV-2a-inoculated *B. vulgaris*. This band was not reproducibly detected in all protein extracts, likely due to a lack of sensitivity of the technique or to a heterologous distribution of the virus in the plant.

For evaluation of the BChV-2a-4 host range, *B. vulgaris* plants inoculated with pDIVA-BChV-2a-4 were used as source for *M. persicae* virus acquisition. For the control, wild-type-infected *B. vulgaris* were used as a viral source. Aphids were placed in clip cages on each infected plant for 2 days. Then 10 aphids were transferred per test plant on 10 *Beta macrocarpa*, *N. benthamiana*, *Nicotiana hesperis*, *Nicotiana clevelandii*, *C. bursa-pastoris*, *Brassica napus* and *M. perfoliata* at the four-leaf stage. After 2 days of virus inoculation the aphids were killed by spraying with a systemic insecticide (Confidor, BayerAG; 0.035 %). DAS-ELISA (Table 2) was performed 6 weeks after inoculation on test plants grown under greenhouse conditions. Both wild-type and recombinant virus BChV-2a-4 displayed a similar host range, since they infected *B. vulgaris* and *B. macrocarpa*, whereas no infection was detectable in *B. napus*, *N. benthamiana*, *N. hesperis*, *N. clevelandii*, *C. bursa-pastoris* and *M. perfoliata* (Table 2). These results are in agreement with the known BChV host range [2]. Although it was possible to inoculate the model plant *N. benthamiana* via agroinoculation and confirm the

inoculation by RT-PCR in newly emerged leaves, aphid transmission of BChV was not successful for *N. benthamiana*. Whether the host plant spectrum of the virus might be increased by using agromediated transmission needs to be addressed in the future.

By using one-step cloning isothermal Gibson assembly we obtained another sugar beet-infecting polerovirus infectious clone that can be delivered to plants via agroinoculation. Up to now, only a full-length clone of BMV that is infectious in sugar beet has been developed [23, 24]. To obtain the BChV infectious clone, the exact 5′- and 3′-ends of the viral genome were obtained. The BChV recombinant virus displayed the same biological activity as the wild-type virus. Furthermore, the BChV full-length clone could be agroinoculated to sugar beet and the model plant *N. benthamiana*. Since BMV and BChV show a divergent host range, the construction of recombinant viruses between these two parental viruses is now conceivable to identify the viral determinants governing host specificity [2, 10, 23]. Moreover, the BChV infectious clone is a valuable tool to screen genetic resources for virus resistance. The identification of such resistance genes could circumvent the use of systemic insecticides that are widely sprayed to limit aphid populations and block virus dispersion. Finally, Boissinot *et al.* [42] recently introduced the enhanced green fluorescent protein (EGFP) into the TuYV genome and obtained the first fluorescent-labelled infectious clone displaying systemic infectivity. This opens up the possibility of constructing a fluorescent-labelled clone of *Beet chlorosis virus* for in-depth study of virus systemic movement in different plant species and in particular in the cultivated sugar beet.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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