A full-length infectious clone of beet soil-borne virus indicates the dispensability of the RNA-2 for virus survival in planta and symptom expression on Chenopodium quinoa leaves

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For a better understanding of the functionality and pathogenicity of beet soil-borne virus (BSBV), full-length cDNA clones have been constructed for the three genomic RNAs. With the aim of assessing their effectiveness and relative contribution to the virus housekeeping functions, transcripts were inoculated on Chenopodium quinoa and Beta macrocarpa leaves using five genome combinations. Both RNAs-1 (putative replicase) and -3 (putative movement proteins) proved to be essential for virus replication in planta and symptom production on C. quinoa, whereas RNA-2 (putative coat protein, CP, and a read-through domain, RT) was not. No symptoms were recorded on B. macrocarpa, but viral RNAs were detected. In both host plants, the 19 kDa CP was detected by Western blotting as well as a 115 kDa protein corresponding to the CP–RT.

Beet soil-borne virus (BSBV) is a pomovirus transmitted to Chenopodiaceae by the protist Polymyxa betae (Ivanović et al., 1983), which is also the vector of the aetiological agent of the rhizomania syndrome of sugar beet, beet necrotic yellow vein virus (BNYVV) (Tamada & Baba, 1973). Originally reported in Italy (Canova, 1959), rhizomania disease is now widespread in most countries where sugar beet is grown (McGrann et al., 2009) and BSBV is often found in beet infected with BNYVV (Meunier et al., 2003). However, the pathogenicity of BSBV and its contribution to the rhizomania syndrome remain unclear, with opinions still divided on this (Prillwitz & Schlösser, 1992; Kaufmann et al., 1993; Lindsten, 1993; Rush & Heidel, 1995).

The BSBV genome consists of three single-stranded RNAs of positive polarity, packaged into rod-shaped particles (Koenig et al., 1996, 1997; Koenig & Loss, 1997). RNA-1 (5.8 kb) encodes the putative viral replicase. The 19 kDa coat protein (CP) and a putative 85 kDa read-through (RT) domain are encoded by RNA-2 (3.5 kb). RNA-3 (3.0 kb) comprises three open reading frames (ORFs) encoding three putative proteins (48, 13 and 22 kDa) thought to be responsible for the viral cell-to-cell movement, resembling the well-known triple gene block proteins (TGBs) (Fig. 1).

In this study, the contribution of each RNA component to virus survival and symptom expression was investigated through the use of full-length cDNA clones on two host plants, Chenopodium quinoa and Beta macrocarpa. This last plant species was preferred to the natural host Beta vulgaris with a view of developing the basis for further molecular analysis of viral systemicity, following the example of previous studies on BNYVV (Lauber et al., 1998).

Full-length cDNA sequences were generated from an Iranian BSBV isolate (Nyshabour, Khorasan Razavi Province), which was trapped from infested soil in roots of B. vulgaris. After total RNA extraction from sugar beet roots using the SV total RNA isolation kit (Promega), the three genomic RNAs were reverse transcribed and amplified by PCR (RT-PCR) using expand reverse transcriptase and the expand long template PCR System (Roche). Primers matching the extremities of the three viral RNAs were designed following the only references known to date (Koenig et al., 1996, 1997; Koenig & Loss, 1997). Forward primers were flanked with a T7 promoter sequence (TAATACGACTCACTATAG) (Fig. 1). Ampli-
cons were gel purified using QIAquick gel extraction kit (Qiagen) and inserted into the pGEM-T vector (Promega). Ligation products were transferred into the *Escherichia coli* strain JM109. Full-length cDNA clones were then subcloned into pUC19 and maintained in the *E. coli* strain DH5α, to obtain the final constructs pUBS-1-wt (RNA-1), pUBS-2-wt (RNA-2) and pUBS-3-wt (RNA-3) (Fig. 1). A full-length cDNA clone of RNA-2 containing a frame shift mutation in the coding sequence for the putative CP–RT domain (clone pUBS-2-rtBsiWI, Fig. 1) was obtained by filling a BsiWI digestion (target site position 907–912) with DNA polymerase I Klenow fragment (Promega) followed by self-ligation with T4 DNA ligase (Roche).

The four full-length cDNA constructs in pUC19 were linearized with SpeI or Ncol (Fig. 1) before run-off transcription with the RiboMAX large-scale RNA production system - SP6 and T7 (Promega) in the presence of Cap analogue m7G(5')ppp(5')G (New England Biolabs). The transcripts were mechanically inoculated in a 50 mM KH2PO4 (pH 4.2) and 0.04% bentonite buffer on both *C. quinoa* and *B. macrocarpa* leaves. In vitro transcript combinations of RNA-1 (1), -2 (2), -2 carrying a frame shift mutation in the RT domain coding sequence (2*) and -3 (3) were tested as follows: 1+2+3, 1+2*+3, 1+2, 1+3 and 2+3. Inoculation buffer alone was applied for the negative control. *C. quinoa* and *B. macrocarpa* were exposed to light for 16 h per day at 15–20 °C and 20–25 °C for night and day, respectively. The experiment was carried out on one *C. quinoa* plant (three leaves inoculated) and two *B. macrocarpa* plants (two leaves inoculated per plant) for each condition and repeated twice to ensure reproducibility.

Symptomatic lesions appeared on all the *C. quinoa* leaves inoculated with BSBV full-length transcripts of RNAs 1+2+3, 1+2*+3 or 1+3 at 3 days post-inoculation (p.i.). Small necrotic spots, necrotic ringspots (data not shown) and chlorosis were clearly developed 7 days p.i. (Fig. 2). However, the infection of the *B. macrocarpa* plants with the same three combinations of BSBV full-length transcripts did not produce any symptoms on the inoculated leaves (data not shown). These observations indicated that the presence of the BSBV RNA-2 was therefore not necessary for symptom expression, which occurred only on *C. quinoa*.

*C. quinoa* - and *B. macrocarpa*-inoculated leaves were harvested at 7 and 10–17 days p.i., respectively. The RNAs were extracted from the whole leaves using the polysomes extraction protocol (Jupin et al., 1990) followed by phenol extraction and ethanol precipitation of the
RNAs. The viral RNAs were detected by Northern blot with random DNA probes synthesized with the Prime-a-Gene Labelling System (Promega) and labelled with $[^{32P}]dCTP$ (1.14 x 10$^{14}$ Bq mmol$^{-1}$; Perkin Elmer). The probes matched nucleotide positions 3510–4015 (NB-1), 2281–2711 (NB-2) and 1930–2330 (NB-3) of RNAs-1, -2 and -3, respectively (Fig. 1).

Genomic combinations 1+2*+3, 1+2+3 and 1+3 resulted in corresponding viral RNA amplification on both host plants (Fig. 3a, b, lanes 1, 2 and 4), whereas 1+2 and 2+3 did not (Fig. 3a, b, lanes 3 and 5). The BSBV RNAs -1 and -3 full-length transcripts were therefore required for the efficient viral replication in planta, in contrast with BSBV RNA-2 that appeared dispensable for the viral RNA replication. However, even if no viral RNAs were detected in plants inoculated with the combinations 1+2 and 2+3 (Fig. 3a, b, lanes 3 and 5), a potential undetected infection cannot be ruled out for the mix 1+2. Lacking the putative cell-to-cell movement proteins encoded by RNA-3, the RNAs-1 and -2 might indeed replicate in the first inoculated cells but couldn’t spread to the neighbouring ones (Hull, 2009). This will be further characterized using protoplast inoculation.

Fig. 2. C. quinoa leaves 7 days after inoculation with BSBV full-length transcript mixes 1+2*+3 (a), 1+2 (b), 1+2+3 (c), 2+3 (d) and 1+3 (e) and a negative control (f). Only genome arrangements 1+2*+3 (a), 1+2+3 (c) and 1+3 (e) provoked the appearance of symptoms on the inoculated leaves, with necrotic spots and ringspots (data not shown) and slight yellow chlorosis.

Fig. 3. Northern (a, b) and Western (c, d) blot analysis for the detection of BSBV RNAs and structural proteins in C. quinoa (a, c) and B. macrocarpa (b, d) leaves, 7 and 10–17 days p.i., respectively. Plant leaves were either inoculated with one of the five genome arrangements of BSBV full-length transcripts tested [combinations 1+2*+3 (lane 1), 1+2+3 (2), 1+2 (3), 1+3 (4) and 2+3 (5)] or mock-inoculated (6). The three BSBV genomic RNAs were detected in total RNA extracts using random DNA probes labelled with $[^{32P}]dCTP$ and diluted transcripts constituted positive controls (+C) (a, b). The 19 kDa CP and 115 kDa CP–RT proteins of BSBV were immuno-detected with primary polyclonal antibody directed against viral particles (c, d).
All three replicative combinations raised the infection rate by 100% in both host plants, except the 1+2*+3 which infected half of the inoculated Beta macrocarpa.

BSBV CP and CP–RT proteins were detected by Western blotting after SDS-PAGE separation of total protein extracts from symptomatic samples and whole asymptomatic leaves. Structural proteins were revealed using a polyclonal antibody directed against viral particles (DSMZ). The primary antibody was diluted 1:10 000 in TBS buffer containing 0.1% Tween 20, 5% powdered skimmed milk, and supplemented with total protein extracts from healthy Chenopodium quinoa or Beta macrocarpa. Anti-rabbit alkaline phosphatase-conjugated secondary IgG (Sigma–Aldrich) was diluted 1:10 000 in TBS-Tween–milk buffer and the blots were revealed with premixed BCIP–NBT solution (Sigma–Aldrich).

A 19 kDa protein was detected in both Chenopodium quinoa and Beta macrocarpa plants inoculated with the 1+2*+3 and 1+2+3 mixes of BSBV RNAs transcripts (Fig. 3c, d, lanes 1 and 2) but not when RNA-2 was omitted (combination 1+3; Fig. 3c, d, lane 4). Thus, the BSBV RNA-2 transcripts are indeed responsible for the expression of the 19 kDa protein in planta, which corresponds to the BSBV CP of the same predicted molecular mass that is encoded by the BSBV RNA-2 first ORF. Another protein with an estimated molecular mass of 115 kDa was also observed in the leaves of both plants inoculated with the genome combination 1+2+3 (Fig. 3c, d, lane 2) and not detected in the leaves inoculated with the BSBV RNA-2 transcripts mutated for the RT domain of the CP (genome combination 1+2*+3; Fig. 3c, d, lane 1). This product therefore corresponds to the predicted 104 kDa BSBV CP–RT protein. Transcript progeny analysis confirmed the stability of the frame shift mutation (data not shown).

According to the different genomic combinations of BSBV full-length transcripts inoculated, our study showed that both the putative replicase (RNA-1) and TGB proteins (RNA-3) of BSBV are essential and sufficient for the replication of such RNAs in the inoculated leaves of Chenopodium quinoa and Beta macrocarpa. In addition, the symptom expression on Chenopodium quinoa did not depend on the presence of the BSBV RNA-2. The BSBV CP and CP–RT encoded by the BSBV RNA-2 are therefore not required for viral infection in the plant hosts or for symptom production on Chenopodium quinoa leaves. The dispensability of the CP for viral replication in planta is a common phenomenon, shared by the closely related pomovirus potato mop-top virus (PMTV) on Nicotiana benthamiana and Nicotiana clevelandii (McGeachy & Barker, 2000; Savenkov et al., 2003), as well as other viruses such as the well-known tobamovirus tobacco mosaic virus (TMV) on Nicotiana tabacum (Siegel et al., 1962). However, whereas symptom production due to TMV does not depend on the presence of the CP, no symptoms are expressed when PMTV lacks the CP and CP–RT. Thus, despite their taxonomic proximity and a highly similar genome organization, the BSBV and PMTV pomoviruses retain their own particularities. Nevertheless, it should be borne in mind that PMTV is a potato virus and symptom expression could be host-dependent.

It is also worth noting that, as the primers used to amplify full-length cDNA sequences of BSBV were designed according to a previously published nucleotide sequence of a German isolate, the biological properties of the resulting full-length cDNA clone might have been modified. However, viral terminal sequences are mostly highly conserved for a virus species as they are involved in viral replication (Duggal et al., 1994; Koenig et al., 2000), and the BSBV full-length clone replicated efficiently.

Consistent with earlier findings reporting on foliar symptoms on Chenopodium spp. and occasional lesions on Beta spp. leaves when inoculating BSBV from the crude sap of infected plants (Henry et al., 1986), transcripts from the full-length clone of BSBV induced clear symptoms only on the Chenopodium quinoa leaves. Following the example of BSBV, the two first BNYVV RNAs, carrying the genetic material homologous to one of the three BSBV RNAs plus a cysteine-rich protein (p14) with PTGS suppressor activity (Bouzoubaa et al., 1986, 1987; Dunoyer et al., 2002), are also unable to induce a systematic symptomatic foliar reaction on Beta spp. However, lesions on Beta spp. leaves are clearly produced when at least BNYVV RNAs-1, -2 and -3 are present (Tamada et al., 1989; Rahim et al., 2007). Although BSBV and BNYVV both naturally infect sugar beet, BSBV lacks the genetic material necessary to induce clear symptoms on Beta macrocarpa leaves whereas BNYVV has this through RNAs-3 and -4.

The use of a polyclonal antibody directed against BSBV viral particles allowed two proteins expressed from BSBV RNA-2 transcripts to be detected. As expected, the Western blot analysis showed the production of the BSBV 19 kDa CP in both Chenopodium quinoa and Beta macrocarpa. However, whereas the expected molecular mass of the CP–RT is 104 kDa, a protein with an estimated molecular mass of 115 kDa corresponding to the CP–RT was identified in both host plants. It could be assumed that post-translational modifications of the CP–RT domain, such as phosphorylation or glycosylation events, are responsible for this 115 kDa observed molecular mass of CP–RT. Actually, post-translational modifications can reduce the protein’s electrophoretic mobility in SDS-PAGE and many viral structural proteins are described as glycosylated and/or phosphorylated (Hahn & Shepherd, 1980; Seddas & Boissinot, 2006; Akamatsu et al., 2007; Hafren & Makinen, 2008; Zayakina et al., 2008). Another hypothesis would involve the intrinsic properties of the BSBV CP–RT, knowing that highly hydrophilic domains containing many charged residues, such as arginine (Hu & Ghabrial, 1995), or the presence of transmembrane motifs (Rath et al., 2009) in proteins might influence their migration on SDS-PAGE. This is supported by the presence in the RT domain of the BSBV CP of two predicted transmembrane helices, as is also reported for the closely related beet virus Q (BVQ)
The rhizomania syndrome of sugar beet and its causal agent, BNYVV, have been widely studied since the 1970s because cropping losses, commonly about 50% and sometimes up to 80%, have been attributed to the disease (McGrann et al., 2009). However, the involvement of BVQ and BSBV in the disease, often associated with beet infected with BNYVV, has never been clearly established and no particular interest in understanding their specific functionality was followed. Our study describing the first infectious full-length cDNA clone available for a beet Pomovirus therefore provides a useful tool for further investigating the pathogenicity of BSBV in the complex rhizomania syndrome, as well as its replication and infection mechanisms, and the potential viral interactions with other beny- and pomoviruses on both susceptible and rhizomania-resistant beets.

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References


