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RNA silencing machinery contributes to inability of BSBV to establish infection in *Nicotiana benthamiana*: evidence from characterization of agroinfectious clones of *Beet soil-borne virus*

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Abstract

Beet soil-borne virus (BSBV) is a sugar beet pomovirus frequently associated with *Beet necrotic yellow veins virus*, the causal agent of the rhizomania disease. BSBV has been detected in most of the major beet-growing regions worldwide, yet its impact on this crop remains unclear. With the aim to understand the life cycle of this virus and clarify its putative pathogenicity, agroinfectious clones have been engineered for each segment of its tripartite genome. The biological properties of these clones were then studied on different plant species. Local infection was obtained on agroinfiltrated leaves of *Beta macrocarpa*. On leaves of *Nicotiana benthamiana*, similar results were obtained, but only when heterologous viral suppressors of RNA silencing were co-expressed or in a transgenic line down regulated for both dicer-like protein 2 and 4. On sugar beet, local infection following agroinoculation was obtained on cotyledons, but not on other tested plant parts. Nevertheless, leaf symptoms were observed on this host via sap inoculation. Likewise, roots were efficiently mechanically infected, highlighting low frequency of root necrosis and constriction, and enabling the demonstration of transmission by the vector *Polymyxa betae*. Altogether, the entire viral cycle was reproduced, validating the constructed agroclones as efficient inoculation tools, paving the way for further studies on BSBV and its related pathosystem.

DATA SUMMARY

A section describing all supporting external data including the DOI(s) and/or accession numbers(s), and the associated URL.

INTRODUCTION

Beet soil-borne virus (BSBV) is a single-stranded, positive-sense ((+)ss) RNA virus that naturally infects sugar beet (*Beta vulgaris*), putatively using the plasmodiophorid *Polymyxa betae* (a protist parasite from the supergroup *Rhizaria*) as vector [1]. The BSBV genome is divided into three RNA segments (RNA 1–3) that are encapsidated into rod-shaped particles [2]. RNA 1 is 5.8 kb in length and encodes the replication proteins [3]. RNA 2 (3.5 kb) encodes the capsid protein (CP) and CP-readthrough (CP-RT) [4]. RNA 3 is the smallest RNA (3.0 kb) with three overlapping ORF encoding for the triple gene blocks proteins (TGBp 1, 2 and 3) that are required for efficient cell-to-cell movements [5]. Each genomic RNA is presumably 5′-capped [3] and harbours a tRNA-like structure at its 3′-terminus [3–5].

According to the current classification by the International Committee for Taxonomy of Viruses (ICTV), BSBV belongs to the genus *Pomovirus* within the family *Virgaviridae* [6]. Other recognized pomoviruses are the type member *Potato mop-top virus* (PMTV) [7], *Broad bean necrosis virus* (BBNV) [8], *Colombian potato soil-borne virus* (CPSbV) [9] as well as the other beet-infecting *Beet virus Q* (BVQ) [10]. Additionally, another pomo-like potato virus has been partially sequenced and tentatively named Soil-borne virus 2 (SbV2) [9]. All the aforementioned viruses share a similar genome organization, with the exception of PMTV encoding on its RNA 3 an additional cysteine-rich protein (CRP) of 8 kDa [11]. This CRP has been shown to be highly variable among

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Abbreviations: BSBV, beet soil-borne virus; CP, capsid protein; CRP, cystein-rich protein; DCL, dicer-like proteins; mRT-PCR, multiplex reverse transcription-polymerase chain reaction; PMTV, potato mop-top virus; RT-PCR, reverse transcription-polymerase chain reaction; VSR, viral suppressor of RNA silencing.

Seven supplementary figures are available with the online version of this article.

different isolates [12] and was demonstrated to act as a viral suppressor of RNA silencing (VSR) involved in virus accumulation [13], yet not required for long-distance movement [14].

The known host range of BSBV is quite narrow and limited to several plant species such as spinach (*Spinacia oleracea*), chenopodium (*Chenopodium quinoa*) and species from the genus *Beta* for which the most economically relevant member is sugar beet [1]. In crops of the latter, BSBV was initially identified in the UK from a field that had exhibited the 'Barney patch disorder' in the early 1980s [1]. Since then, the virus was shown to be widespread in Europe [15], Turkey [16], the Middle East [17, 18], Iran [19] and the USA [20]. In addition, it has been reported in China [21], Japan [22], and more recently in Morocco [23] and Brazil [24]. The virus has been found alone in roots of sugar beet but also in association with other soil-borne viruses transmitted by *P. betae* [15], in particular the benyvirus *Beet necrotic yellow vein virus* (BNYVV), the causal agent of the rhizomania disease [25]. However, unlike BNYVV, the real impact of BSBV on crop yield remains enigmatic. This virus has been regarded as a mild pathogen, and very few studies have focused on its potential economic significance [25]. Several field studies have nevertheless reported association of BSBV with plants showing symptoms [26, 27] and mechanical inoculations of beet resulted in significant reductions of taproot weight [28], advocating altogether for a possible deleterious effect on its host.

In order to decipher the life cycle of BSBV and its putative pathogenicity, we have previously constructed infectious cDNA clones for its three RNA segments based on the T7 *in vitro* transcription system [29]. Plant viruses can alternatively be studied via the construction of agroinfectious clones (or shortly 'agroclones'), i.e. viral cDNA cloned in binary vectors that are transformed into *Agrobacterium tumefaciens* [30]. Thanks to their bacterium-mediated inoculation, agroclones present several advantages in comparison with cDNA clones driven by T7 promoter such as their handling and the possibility of large-scale production [31]. As far as rhizomania-associated viruses are concerned, agroclones have already been produced for BNYVV and the other benyvirus *Beet soil-borne mosaic virus* (BSBMV) [32, 33], and were proven to enable fast and robust reverse genetic analyses [34, 35].

In this study, we developed similar agroclones for BSBV. Subsequently, we analysed their infectivity on *B. macrocarpa*, *N. benthamiana* and *B. vulgaris*. In addition, those clones were also efficiently used to demonstrate for the first time transmission of BSBV by *P. betae* zoospores derived from a single sporosorus.

METHODS

Plasmids and cloning

The full-length sequences of the three BSBV genomic cDNA were amplified by PCR with the primer pairs BSBV1F/1R, BSBV2F/1R and BSBV3F/3R (Table 1) and the Q5 polymerase (New England BioLabs) using T7-clones [29] as templates. In parallel, the pJL89 binary vector [36] was amplified with the primer pair pJL89F/R (Table 1) using the same enzyme. The insertion of BSBV cDNA inside pJL89 was performed using the Gibson Assembly kit (New England Biolabs) according to Blawid and Nagata [37]. The produced recombinant plasmids were used to transform *E. coli* DH5alpha competent cells by heat shock. Following a PCR-screening, bacterial colonies were selected and used to produce recombinant plasmids. After purification, the latter were verified for the presence of full-length viral sequences by restriction profile and sequencing. Verified plasmids were then transformed into *Agrobacterium tumefaciens* strain C58C1 by electroporation. The produced agroclones of BSBV 1/2/3 were then referred to as 'agroBS1/2/3', respectively.

The heterologous VSR P19 [38], HC-Pro [39] and 2b [40] have been previously cloned inside the pROK2 binary vector. Likewise, those plasmids were transformed into *A. tumefaciens* C58C1 by electroporation.

Agroinoculation

A. tumefaciens bacteria were grown at 28–30 °C in lysogeny broth (LB) liquid medium supplemented with kanamycin (100 mg l^{-1}) and rifampicin (50 mg l^{-1}). After overnight (O/N) growth, bacteria were pelleted by centrifugation (4000 ***g***, 5 min) and washed in MM buffer ($MgCl_2$ 100 mM, MES 100 mM, pH 5.6). Bacteria were then centrifuged again and resuspended in MM buffer supplemented with acetosyringone (100 µM). After 3–4 h incubation at room temperature (RT), the optical density (at 600 nm) of the bacterial suspensions were measured and adjusted to 0.4–0.5 for *N. benthamiana* or 0.2–0.3 for *B. macrocarpa* and *B. vulgaris*.

Bacterial infiltration in plant leaves was carried out using a 1 ml needleless syringe. The first pair of true leaves *B. macrocarpa* plants were infiltrated when the second pair appeared. Fully developed leaves of *N. benthamiana* plants were infiltrated at the five leaves stage. Agroinfection of sugar beet was tested either by infiltration of leaves/cotyledons or by vortexing the root in the presence of agroclones and carborundum as described previously [32].

Mechanical inoculation

Agroinfected leaves of *B. macrocarpa* or *N. benthamiana* were ground using a mortar and a pestle in cold phosphate buffer (KH_2PO_4 50 mM, pH 7.0, carborundum 30 g l^{-1}) at a ratio of 1:4 (leaf weight:buffer volume) to produce an infectious sap. This sap was used to perform mechanical inoculation by gently rubbing leaves of tested plants using latex gloves. Alternatively, sugar beet roots were mechanically inoculated using the same sap via the vortex method developed by Koenig and Stein for BNYVV [41].

Plant material

Plants were grown in twofold autoclaved soil in a grow chamber supplemented with 12/8 h light/darkness, at 20–22 °C. Experimentations with sugar beet were done using the cultivar Carma (Maribo). Seeds of this cultivar were first

uncoated using 10% (v/v) EtOH. Transgenic *N. benthamiana* lines down regulated for DCL (DCL1.13i, DCL2.11i, DCL3.10i, DCL4.9i, DCL2/4.5i) have been described before [42, 43].

Unless otherwise stated, for all experiments, at least three technical replicates were made consisting each time of at least four plants per treatments. Only representative symptoms are shown in the results section.

Western blot

Protein analysis was performed by Western blot as described by Crutzen *et al*. [29], with some modifications. Briefly, 100 mg of plant material was ground in liquid nitrogen and mixed with 300 µl of Laemmli buffer [44]. The mixture was then centrifuged at 13000 ***g***, for 15 min at 4 °C. One volume of supernatant was mixed with one volume of Laemmli buffer and heated for 3 min at 95 °C. Then 20 µl of this mix was used for SDS-PAGE analysis followed by transfer on a nitrocellulose membrane (Biorad). The membrane was then treated with TBS buffer (Tris-Base 50 mM, NaCl 40 mM) containing 0.1% (v/v) Tween 20 and 5% (w/v) powdered skimmed milk. After washing with TBS buffer, the membrane was serially treated with two antibodies diluted 1:10000 in TBS. The primary antibody was a rabbit anti-BSBV CP antibody (DSMZ). The secondary antibody was a goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma). After final washing, revelation was performed using a premixed BCIP-NBT solution (Sigma). Loading control was achieved using Coomassie (Serva) coloration of the gel.

Viral particles observation

In order to observe viral particles, the immunosorbent electron microscopy (ISEM) protocol developed by Roberts and Harrison for PMTV [45] was used, with some modifications. Briefly, 100 mg of leaf material was ground in 4 ml of cold Sörensen buffer (Na_2HPO_4 20 mM, KH_2PO_4 47 mM, pH 6.5). The mix was centrifuged at 1000 ***g***, for 1 min at 4 °C. Then, a drop of the supernatant was placed on Parafilm and kept at 4 °C. In parallel, carbon-coated 200 mesh-nickel grids (Agar scientific) were incubated with the aforementioned anti-BSBV antibody (diluted 1:1000) at 37 °C for 1 h, washed twice 15 min on drops of cold Sörensen buffer, and subsequently placed on the sample drop for 2 days at 4 °C. Then, grids were washed twice on drops of Sörensen buffer and visualized using the transmission electron microscope LEO 922.

Analysis of ssRNA

Detection of ssRNA was achieved by reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was first extracted from *ca*. 30 mg of plant material using Trizol reagent (Invitrogen) according to the manufacturer's indications. Then, samples were treated with the DNAse QI (Promega) in order to remove contaminant DNA. Subsequently, cDNAs were synthetized using the M-MLV reverse transcriptase (Promega) in combination with reverse primers BSBVfl14R, BSBV2(2)Rbis or BSBV3NBF (Table 1).

Table 1. Primers used in this study

Name	Sequence 5′–3′	Ref
pJL89F	GGGTCGGCATGGCATCTC	[37]
pJL89R	CCTCTCCAAATGAAATGAACTTCC	
BSBV1F	CATTTCATTTGGAGAGGGTATTAT TTTCTCAACGGTTATTTA	This study
BSBV1R	ATGCCATGCCGACCCGGGCTT GATTTCCCTCA	
BSBV2F	CATTTCATTTGGAGAGGGGTAA TTTTACTTCTTCGCC	
BSBV3F	CATTTCATTTGGAGAGGGGTATT TTTTTTTCTTGCCG	
BSBV3R	ATGCCATGCCGACCCGGGCT AGATTTCCCTCTA	
BSBVfl13F	TAAGGAAAAGACGGTAGATG	[29]
BSBVfl14R	TCAGTCATCGCAGACTTATC	
BSBV2(2)F	ATTAGGAAAGGAGGCGAGCACGAC	
BSBV2(2)Rbis	TTCAGGAACAGACTCAACGCGC	
BSBV3NBF	GCCCGGATTGTTCTTGGCAG	
BSBVIR3.4R	TAGGAACGACACTTAACCAC	
BNYVV2(1)for	ACATTTCTATCCTCCTCCAC	[15]
BNYVV2(1)rev	ACCCCAACAAACTCTCTAAC	
BSBV2for	CTTACGCTGTTCACTTTTATGCC	
BSBV2rev	GTCCGCA CTCTTTTCAACTGTTC	
BVQ1(1)for	GCTGGAGTATATCACCGATGAC	
BVQ1(1)rev	AAAATCTCGGATAGCATCCAAC	
PB4for	CAAACGCCTGAAATCATCTAAC	
PB4rev	GATGGCCCAATTCCTTACAC	

The cDNAs were then amplified by PCR using the GoTaq polymerase (Promega) in combination with corresponding primer pairs BSBVfl13F/BSBVfl14R, BSBV2(2)F/BSBV2(2) Rbis or BSBV3NBF/BSBVIR3.4R (Table 1).

Analysis of dsRNA

The dsRNA was extracted from infected leaves following a modified cellulose-based protocol [46]. Briefly, plant tissues were first cut into small pieces and dried on silica gel for at least 48 h at 4 °C. Then, 100 mg of the obtained dried material was ground in liquid nitrogen into a fine powder using a pestle and a mortar. Subsequently, the powder was mixed with 500 µl STE buffer (NaCl 100 mM, Tris 50 mM, EDTA 1 mM, pH 6.8), 100 µl bentonite 2% (w/v), 100 µl of SDS 10% (v/v) and 500 µl phenol. After vortexing for 1 min, the mix was centrifuged for 3 min at 8000 ***g***. Then 400 µl of the supernatant was mixed with 440 µl of STE buffer, 160 µl of EtOH and 100 mg of cellulose fibres (Sigma). After vortexing for 1 min, the mix was centrifuged as before. The supernatant was discarded. The cellulose was then washed twice with STE containing

EtOH (16%, v/v). Afterwards, the dsRNA was eluted from the cellulose by washing with 500 µl STE. Following ethanol precipitation, the obtained pellet was suspended in 35 µl of Depc-water. Removing ssRNA and DNA was achieved by using 1 U of S1 nuclease or DNase I (Thermo Fisher Scientific), respectively.

Transmission by *P. Betae*

In order to demonstrate transmission of the cloned BSBV isolate by zoospores of *P. betae*, roots of 7 to 10 day-old sugar beet were first mechanically inoculated using the vortex-based protocol already mentioned. Next, plants were grown in test tubes (20 mm in diameter, 12 mm in length) filled with twofold autoclaved coarse quartz and daily watered with modified half-strength Hoagland solution [47]. One week after the inoculation, 1 ml of zoospores suspension (10^4 zoosp. per ml) of the *P. betae* strain A26-41 [47] was inoculated around the crown part of beets. Zoospores were released in five-time diluted Hoagland solution from 6-week-old sugar beet plants grown in a soil-free automated hydroponic immersion system [47]. Inoculated plants were then grown for 1 month after which roots were screened for the presence of the virus and the protist by RNA extraction followed by a multiplex RT-PCR (mRT-PCR) developed for detection of *P. betae* and associated viruses [15]. Roots successfully infected with both the virus and the protist were dried under laminar flow for at least 2 days. Subsequently, dried roots were slightly ground in a mortar using a pestle in order to liberate resting spores from dried tissues. The obtained ground material was then used to infect new roots of beet plants. After 1 month of growth, roots were screened for the presence of BSBV and *P. betae* by mRT-PCR.

RESULTS

Agroinoculation of *B. macrocarpa*

Due to its susceptibility to both BSBV and *A. tumefaciens* [26], *B. macrocarpa* was chosen as the first plant species to test the agroinoculation procedure. Notably, infiltration of the combination agroBS1+2+3 in leaves of this plant lead to chlorotic symptoms, visible at 12–16 days post-infiltration (dpi), whereas leaves agroinfiltrated with the empty vector pJL89 remained symptomless, similar to non-treated leaves (Fig. 1a). In order to confirm viral replication, a dsRNA analysis was performed on infiltrated tissues. Three bands, presumably corresponding to dsRNA replicative intermediates of BSBV genomes were detected in the symptomatic leaves but not in the empty vector control (Fig. 1b). Those bands exhibit a size pattern similar to BSBV dsRNA found in naturally infected beets [48], and their dsRNA nature was proven by resistance to S1 nuclease and DNase I (Fig. S1). Following a Western blot analysis, one band with the size corresponding to BSBV CP (19 kDa) was obtained in symptomatic tissues but not in controls (Fig. 1c). In addition, typical tubular rod-shaped particles (*ca*. 50–300 nm in length) were visualized by ISEM (Fig. 1d), suggesting an efficient production of virions by the

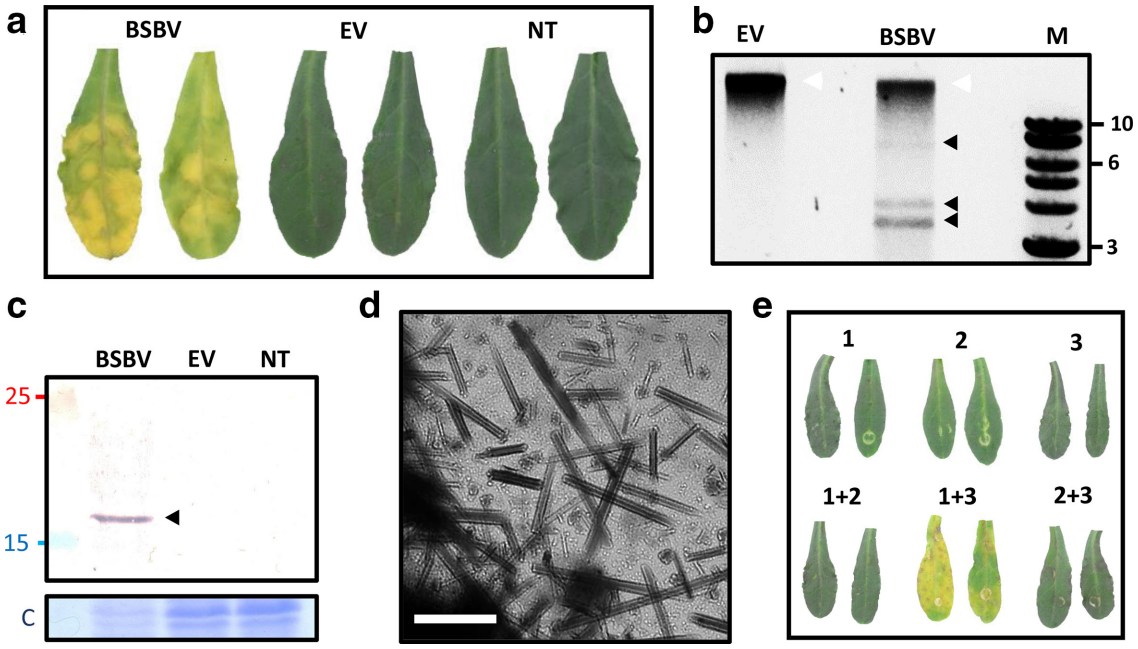

Fig. 1. BSBV agroinfection on *B. macrocarpa*. (a) leaves infiltrated with agroBS1+2+3 (BSBV), pJL89 empty vector (EV) or non-treated leaves (NT) at 13 dpi; (b) dsRNA analysis of leaves described in (a) M: Promega 1 kb ladder. Numbers to the right refer to chosen molecular weight expressed in kb. Plant DNA is visible for both sample (white arrow) while BSBV dsRNA (black arrow) are only detected in the BSBV sample; (c) Western blot analysis of leaves described in (a) BSBV CP in indicated by the black arrow. Numbers to the left refer to chosen molecular weight expressed in kDa. C: Coomassie loading control; (d) rod-shaped particles observed by ISEM. White bar=150 nm; (e) leaves infiltrated with individual or dual combinations of agroBS1/2/3, at 13 dpi.

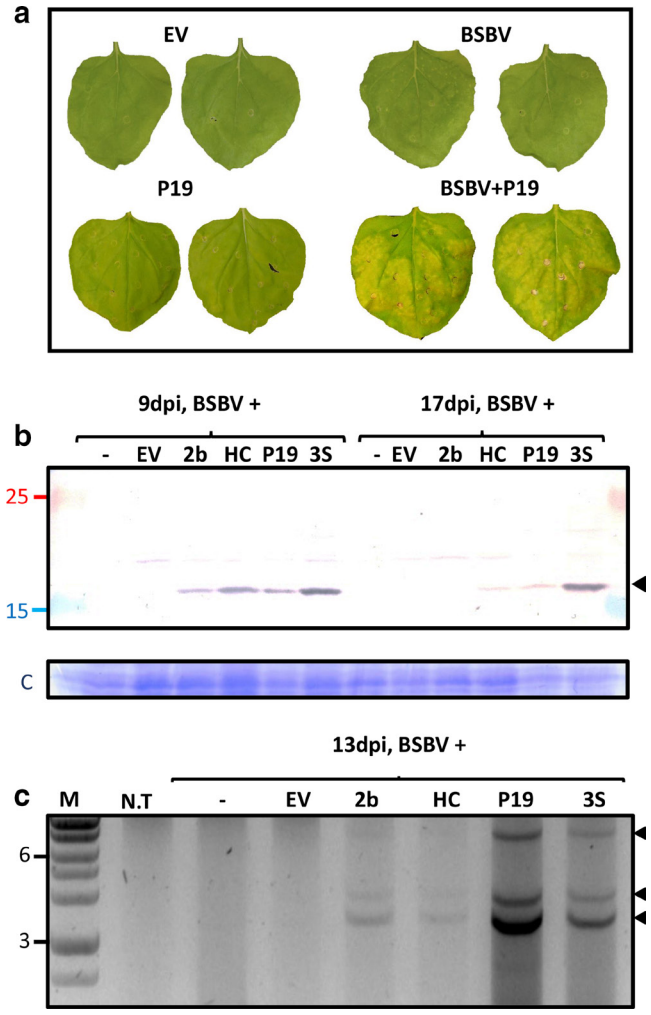

Fig. 2. BSBV agroinfection on *N. benthamiana* with co-expression of heterologous VSR. (a) Leaves agroinoculated with pROK2 empty vector (EV), agroBS1+2+3 (BSBV), pROK2 expressing P19 (p19) or BSBV and P19 in combination, at 9 dpi; (b) Western blot analysis of leaves agroinfiltrated with BSBV alone ('-') or in combination with empty vector pROK2 (EV) or expressing the VSR P19 (p19), HC-Pro (HC) or 2b (2b) alone or together (3S) at 9 and 17 dpi. BSBV CP in indicated by the black arrow. Numbers to the left refer to chosen molecular weight expressed in kDa. C: Coomassie loading control; (c) dsRNA analysis at 13 dpi for leaves as referred in (b) BSBV dsRNA are indicated by black arrows. M: Promega 1 kb ladder. Numbers to the left are molecular weight expressed in kb.

viral clones. Notably, some of the particles showed a tendency to associate together and form bundles, as already reported for previous studies on BSBV virions [2].

When each of the BSBV agroclones was infiltrated alone or in dual combinations, only the combination agroBS1+3 lead to chlorotic symptoms (Fig. 1e) and dsRNA detection (Fig. S2). This result was already observed for mechanical inoculation of *in vitro* transcribed BSBV RNA 1+3 on *Chenopodium quinoa* [29] and confirms the dispensability of RNA 2 for symptom induction and replication *in planta*.

Outside infiltrated leaves, no systemic symptom was observed and viral RNA was not detected by RT-PCR (data not shown), suggesting the inability of BSBV to move long-distance in this host as previously stated [1].

BSBV infection on *N. benthamiana* via expression of heterologous VSR

Following the results obtained on *B. macrocarpa*, agroinoculation was performed on *N. benthamiana*, a model plant in plant pathology that is host for other pomoviruses. In previous assays, using T7 clones, we were never able to detect BSBV infection on this species (data now shown). Likewise, when agroBS1+2+3 were infiltrated on leaves of this plant, no symptom was noticed (Fig. 2a, upper panel). In addition, Western blot and dsRNA analyses did not give signal of viral multiplication (Fig. 2b, c column '-').

Agroinoculation of virus is not always successful on *N. benthamiana*, as already described by other studies [49, 50]. Despite its susceptibility to many viruses [51], this plant species is not host for all of them thanks to, inter alia, its antiviral RNA silencing. The use of heterologous VSR has been shown to block this defence mechanism, allowing increased infection for some viruses such as *Citrus tristeza virus* [52] and yellow dwarf viruses [53]. Hypothesizing that antiviral RNA silencing might also be involved in the restriction of BSBV infection, heterologous VSR were tested in co-expression assays with BSBV agroclones. Interestingly, when P19 from *Tomato bushy stunt virus* was co-expressed, chlorotic lesions appeared at 9 dpi whereas no symptom occurs for the expression of P19 alone (Fig. 2a, lower panel). Similar symptoms were obtained for co-expression of agroBS1+2+3 with two other VSR : HC-Pro from *Potato virus* Y and 2b from *Cucumber mosaic virus* (Fig. S3).

A Western blot analysis revealed the detection of BSBV CP at 9 dpi when the different VSR were co-expressed individually or altogether (Fig. 2b). At 17 dpi, the CP signal was not detected for the co-expression of 2b, and was detected with reduced intensity for the co-expression with HC-Pro or P19. This result could be due to the decrease of transient expression level of individual VSR over time. Interestingly, the CP signal was relatively stronger for the combination of the three VSR together, at the two sampling time. This could be interpreted by the fact that the combination of VSR together generates a synergy, maintaining a higher suppression effect as suggested by other studies using combination of VSR [54, 55].

At 13 dpi, a dsRNA analysis showed the presence of BSBV replicative intermediates when VSR were co-infiltrated individually or in combination (Fig. 2c), confirming that viral replication occurs in the observed symptomatic tissues.

BSBV infection on *N. benthamiana* down regulated for DCL2 and DCL4

The results obtained from co-expression of heterologous VSR advocated that *N. benthamiana* can be host for BSBV when its antiviral silencing machinery is compromised. To confirm

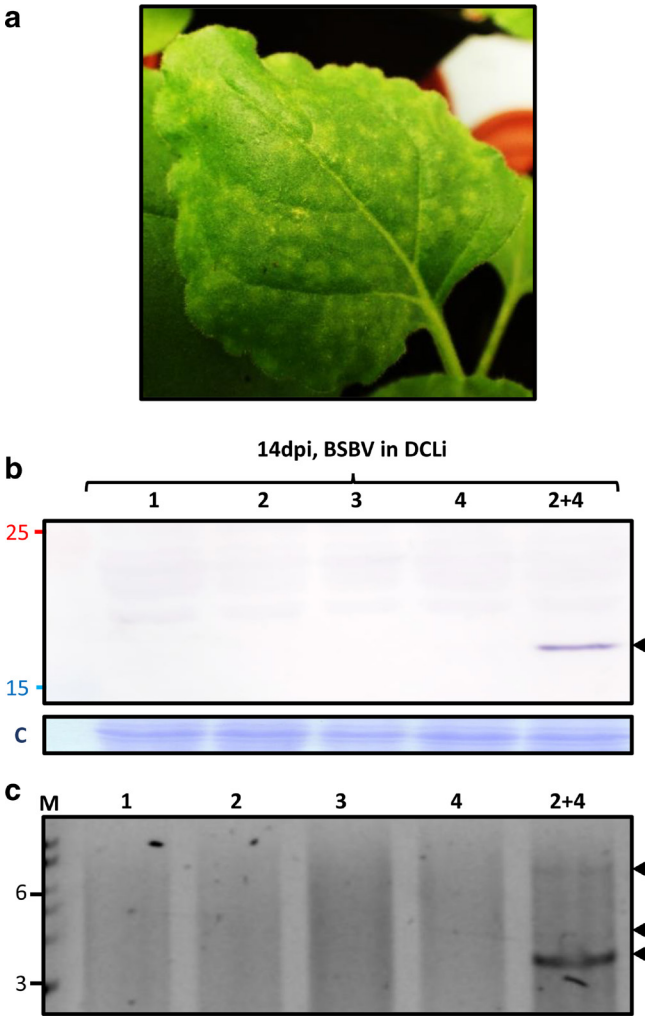

Fig. 3. BSBV agroinfection on transgenic *N. benthamiana* DCLi lines (a) local symptom of agroinfiltrated leaf of the DCL2/4.5i line at 7 dpi; (b) Western blot analysis of lines DCL1.13i (1), DCL2.11i (2), DCL3.10i (3), DCL4.9i (4), DCL2/4.5i (2+4) at 14 dpi. BSBV CP in indicated by the black arrow. Numbers to the left refer to chosen molecular weight expressed in kDa. C: Coomassie loading control; (c) dsRNA analysis for the same line at the same timing. BSBV dsRNA are indicated by black arrows. Numbers to the left are molecular weight expressed in kb.

this hypothesis, we used transgenic *N. benthamiana* lines down regulated for the expression of individual dicer-like proteins (DCL) 1–4, as well as one line down regulated for both DCL2 and 4. As part of the silencing mechanism, plant DCL are endonucleases that produce 21- to 24-nt small RNAs from longer RNA substrates [56]. Previously, down regulation of DCL in *N. benthamiana* was shown to allow overexpression of transient proteins [57] and increased viral infection [58].

Notably, the infiltration of agroBS1+2+3 in the DCLi lines lead to chlorotic symptoms that appeared at 9 dpi only for the line down regulated for both DCL2 and DCL4 (Fig. 3a). A Western blot analysis of infiltrated leaves at 14 dpi confirmed the detection of BSBV CP only in this line (Fig. 3b). Furthermore, the three BSBV dsRNA were also detected at the same time in this line but not in others (Fig. 3c), confirming efficient viral replication. This result is in agreement with studies that showed that down regulation of those two DCL in *N. benthamiana* was associated with increased viral titres for other (+)ss RNA viruses, while individual down regulation of DCL2 or four did not lead to such a result [43].

BSBV infection on sugar beet and vector transmission

Following efficient BSBV agroinfection of *B. macrocarpa* and *N. benthamiana*, the next investigated host was sugar beet. We focused on the cultivar Carma because it supported high levels of BSBV replication in roots when plants were grown in naturally infested soils (Fig. S4). However, unlike *B. macrocarpa* and *N. benthamiana*, direct agroinfection of leaves of this host turned out to be ineffective. Indeed, neither symptom nor detection of viral RNA or CP was obtained, even when heterologous VSR were co-infiltrated (data not shown). Likewise, agroinoculation of roots using the protocol developed for BNYVV agroclones [32] did not result into detectable infection, reflecting the same situation experienced for BSBMV agroclones [33]. The only reproducible agroinfection of sugar beet was obtained when cotyledons were infiltrated, as demonstrated by local yellowing symptoms visible at 14 dpi (Fig. 4a) in which the BSBV CP was detected (Fig. 4b).

As an alternative to agroinoculation, mechanical inoculation was performed to obtain a viral infection on sugar beet. To this end, due to their efficient agroinfection, BSBV-infected leaves of *B. macrocarpa* were used to produce a sap. The infectivity of this sap was first demonstrated on the indicator plant species *C. quinoa*. Indeed, on leaves of this host, mechanical inoculation of the sap produced typical chlorotic spots that appeared at 7 dpi and rapidly turned necrotic, whereas leaves inoculated with a mock sap (produced from healthy material) remained symptomless (Fig. 4c). In the symptomatic tissues, BSBV CP was readily detected by Western blot (Fig. 4d). Interestingly, similar results were obtained when using sap from *N. benthamiana* leaves agroinfiltrated with BSBV agroclones in combination with P19 (Fig. S5). When sap inoculation was tested on leaves of sugar beet, faint chlorotic patches and rings were visible at 20 dpi (Fig. 4e) and faded over time. Notably, in the symptomatic tissues, the three BSBV RNA were readily detected by RT-PCR (Fig. 4f) arguing for an efficient viral multiplication.

In natural infections, BSBV is likely transmitted by *P. betae* [1]. The exact mechanism of virus acquisition is currently unknown, but presumably occurs *in planta* when the protist plasmodium develop and feed upon root cytoplasmic content by pinocytosis [59]. BSBV virions are then internally carried inside *P. betae* resting spores. Under favourable conditions, resting spores produce zoospores that will allow BSBV infection of new roots. In order to demonstrate such transmission for the developed BSBV clones, sugar beet roots were first sap inoculated with BSBV via a vortex-mediated protocol. Then, the same roots were inoculated with aviruliferous *P. betae* zoospores. After 1 month, a

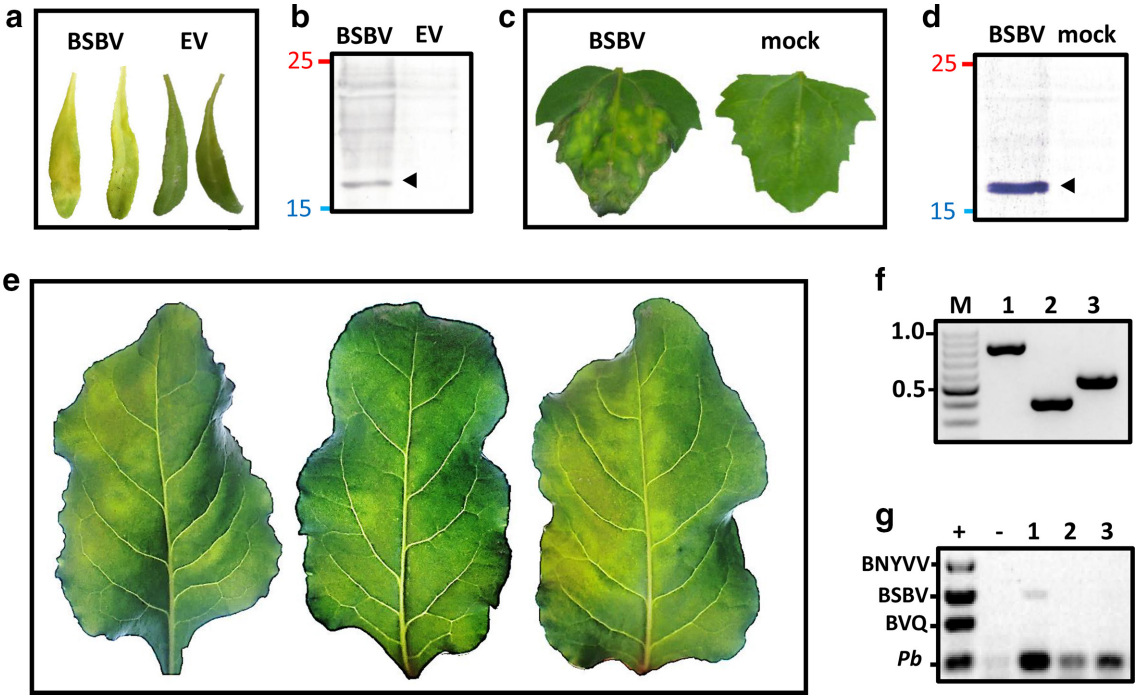

Fig. 4. Mechanical inoculation of BSBV-agroinfected material. (a) Beet cotyledons agroinfiltrated with agroBS1+2+3 (BSBV) or pJL89 empty vector (EV), at 14 dpi; (b) Western blot analysis for the cotyledons shown in A. Numbers to the left refer to chosen molecular weight expressed in kDa; (c) leaves of *C. quinoa* mechanically inoculated with the infectious (BSBV) sap or a healthy sap (mock), at 7 dpi; (d) Western blot analysis for leaves shown in C. (e) Local symptoms on leaves sugar beet (Carma), 20 dpi; (f) RT-PCR detection of BSBV RNA 1 (797 bp), 2 (400 bp) or 3 (531 bp) in leaves shown in E. M: Promega 100 bp. Numbers to the left refer to chosen molecular weight expressed in kb; (g) multiplex RT-PCR analysis of baiting plant of the transmission assay. Lane 1–3: samples from bait roots. Lane '–': root inoculated with aviruliferous *P. betae* . '+': Root naturally infected with BNYVV (545 bp), BSBV (399 bp), BVQ (292 bp) and *P. betae* (*Pb*, 170 bp).

mRT-PCR showed that all 36 doubly-treated roots gave positive signal for *P. betae* while nine roots were infected with BSBV as well (Fig. S6, yellow arrows). Two roots that gave strong signals for *P. betae* and BSBV (Fig. S6, white arrows) were subsequently desiccated and ground. Then, the obtained material (presumably containing viruliferous resting spores) was used to inoculate three new sugar beet rootlets. After 1 month, young parts of the three roots were positive for the protist and one root was positive for BSBV as well, confirming transmission of the virus (Fig. 4g). The same dried material kept for 15 weeks under laminar flow was used again for a repetition of the experiment. This time, three out of 12 inoculated roots gave positive signals for the virus by a similar analysis (Fig. S7).

In parallel to transmission, the vortex inoculation procedure was also used to analyse potential symptom development caused by BSBV on root tissues. To this end, a high number of beet roots were vortex-inoculated with the BSBV-infected sap or with a 'healthy' mock sap. Nine weeks after this procedure, six out of 70 BSBV-inoculated beets (8.6%) exhibited a visible taproot necrosis and constriction (Fig.S6A) whereas all 50 mock-inoculated plants remained symptomless. However, the majority of BSBV-inoculated plants were similar to mock, and no significative difference in root length was evidenced (Fig. S6b). A repetition of the experiment led to similar observations : four out of 48 (8.3%) BSBV-inoculated plants exhibited similar symptoms.

DISCUSSION

The results presented in this study argue that the developed BSBV agroclones are infectious. Direct demonstration of such infectivity was given on *B. macrocarpa* on which local yellowing was observed as well as accumulation of viral replicative dsRNA, CP and rod-shaped particles.

On *N. benthamiana*, inoculation with BSBV agroclones alone did not lead to viral infection. However, the co-expression of heterologous VSR allowed it, as demonstrated by symptom induction and detection of viral dsRNA and CP. Likewise, down regulation of both DCL2 and 4 also enabled viral infection, confirming the role of the antiviral silencing machinery as the factor limiting BSBV multiplication in this host. Establishing an infection of this species now opens up new possibilities in terms of experimental procedures given the numerous molecular tools available for this model organism. Indeed, *N. benthamiana* has been efficiently used to study plant viruses in general [60], and *a fortiori* soil-borne viruses and their underground infection

strategies [61]. The antiviral silencing is known to act differently in leaves and roots [62, 63], and further analysis of BSBV infection of the latter tissue seems relevant. In addition, *N. benthamiana* also represents a suitable platform for studying virus-virus interaction, as illustrated by recent studies demonstrating BNYVV-BSBMV competition [34]. Interestingly, in a preliminary test, we have found that BSBV can locally infect *N. benthamiana* when other rhizomania-associated viruses are co-inoculated (data not shown). We are currently investigating whether these results can be explained in light of a shared silencing suppression.

Despite several efforts to agroinoculate BSBV directly on sugar beet, no reproducible infection was evidenced on true leaves or roots. Indeed, only cotyledons developed local symptomatic infection following agroinoculation. Those results might reflect a tissue-specific recalcitrant response from this plant towards *A. tumefaciens*, as already pointed out by previous assays [64]. Yet, other studies have demonstrated efficient viral agroinfection of sugar beet [65–67], encouraging further research on adequate conditions allowing efficient agroinfection.

Local symptoms on true leaves of sugar beet were eventually obtained when this host was mechanically inoculated using an infectious sap produced from agroinfected plants. The observed mild fading symptoms were similar to those reported for inoculation of beet leaves with wild-type BSBV isolates [1].

The original isolate used to produce the agroclones was trapped from an Iranian soil sample associated with beets also co-infected with BNYVV and showing rhizomania symptoms. Our primary inoculation assays on root tissues resulted in root necrosis and constriction, but at low frequency. Nevertheless, to the best of our knowledge, this is the first time that symptoms are shown on sugar beet for an infectious clone of BSBV, also showing that maybe such a virus has been underestimated in the appraisal of the rhizomania syndrome. Similar inoculation procedure should be done on a large panel of cultivars in order to rule out the biological impact of BSBV on this plant species. The developed agroclones will be useful for this task. In parallel, further research is also needed in order to shed light on the conditions that favour BSBV infection in the field. For instance, the temperature seems to be a factor worth analysing since it has been shown to play a major role in the symptom expression of related viruses. Indeed, the other pomoviruses PMTV and BBNV induce symptoms only at low temperatures [68, 69] and similar observations were made for several cereal-infecting furoviruses [70, 71]. Interestingly, a preliminary study has already shown an increased accumulation of BSBV at 15–20 °C than at 20–25 °C [72], highlighting the importance of this parameter.

Like other pomoviruses, BSBV infection mainly occurs in the underground part of plant [9]. This soil-borne nature is conditioned by the life cycle of *P. betae* for which the role as vector has been demonstrated here. Yet, low transmission rates were observed for BSBV (1/3 and 1/4 of tested plants), contrasting with BNYVV and BSBMV for which higher rates are reported [33, 73]. Apart from different experimental conditions, the lower observed rates could be explained by the apparent lack of strong VSR in BSBV genome unlike BNYVV p31 and BSBMV p32 which were shown to greatly enhance *P. betae* transmission in similar experiments [74, 75]. Nonetheless, by establishing a system for the transmission of BSBV by *P. betae*, the natural infection process can now be reproduced and further investigated. Being obligate biotrophic root parasites, plasmodiophorids are challenging to handle [76]. Viral transmission studies have been consequently rare and developed only for a limited numbers of virus-plasmodiophorid vector combinations [77] but could help answer long lasting questions such as the role of the protist in disease development and whether it might actually be a host and not only a vector for the transmitted viruses [78].

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Author contributions
C. B., G. R., A. L., A. D. and M. M., designed the study; M. M., C. P. and A. D., performed experiments and analysed the data; M. M., wrote the manuscript; C. B., G. R., reviewed the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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