Technical advance

Agroinoculation of *Beet necrotic yellow vein virus* cDNA clones results in plant systemic infection and efficient *Polymyxa betae* transmission

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SUMMARY

Agroinoculation is a quick and easy method for the infection of plants with viruses. This method involves the infiltration of tissue with a suspension of *Agrobacterium tumefaciens* carrying binary plasmids harbouring full-length cDNA copies of viral genome components. When transferred into host cells, transcription of the cDNA produces RNA copies of the viral genome that initiate infection. We produced full-length cDNA corresponding to *Beet necrotic yellow vein virus* (BNYVV) RNAs and derived replicon vectors expressing viral and fluorescent proteins in pJL89 binary plasmid under the control of the *Cauliflower mosaic virus* 35S promoter. We infected *Nicotiana benthamiana* and *Beta macrocarpa* plants with BNYVV by leaf agroinfiltration of combinations of agrobacteria carrying full-length cDNA clones of BNYVV RNAs. We validated the ability of agroclones to reproduce a complete viral cycle, from replication to cell-to-cell and systemic movement and, finally, plant-to-plant transmission by its plasmodiophorid vector. We also showed successful root agroinfection of *B. vulgaris*, a new tool for the assay of resistance to rhizomania, the sugar beet disease caused by BNYVV.

Beet necrotic yellow vein virus (BNYVV) (Tamada and Baba, 1973), the leading infectious agent that affects sugar beet, is a member of the *Benyvirus* genus, together with *Beet soil-borne mosaic virus* (BSBMV), and both are transmitted through the soil from the plasmodiophorid *Polymyxa betae* (Gilmer and Ratti, 2012). BNYVV is the causal agent of rhizomania (Canova, 1959), a disease widespread in all the sugar beet-growing areas of Europe, Asia and America, which causes abnormal rootlet proliferation and losses of sugar yields.

The BNYVV genome consists of four plus-sense 5' capped and 3' polyadenylated RNAs (Tamada, 1999). The RNA-1 and RNA-2

carry 'house-keeping' genes involved in virus replication, assembly, cell-to-cell movement and suppression of post-transcriptional gene silencing (PTGS) (Chiba *et al.*, 2013; Dunoyer *et al.*, 2002; Tamada, 1999). The RNA-3 and RNA-4 play important roles in pathogenesis and vector transmission, respectively, for the efficient production of typical rhizomania symptoms, long-distance movement and vector propagation (Koenig *et al.*, 1991; Lemaire *et al.*, 1988; Peltier *et al.*, 2012). Sequence analysis of a number of isolates revealed the existence of two types of BNYVV: A type (found in most European countries, Iran, North America, China and Japan) and B type (present in France, Belgium, Germany, Sweden, the Netherlands, Iran, China, UK and Lithuania) (Lennfors *et al.*, 2000; Miyanishi *et al.*, 1999; Ratti *et al.*, 2005; Sohi and Maleki, 2004). Additional isolates, characterized by the presence of RNA-5 and typically more aggressive, were identified in France, Japan, China, Kazakhstan and the UK (Harju *et al.*, 2002; Koenig and Lennfors, 2000; Kruse *et al.*, 1994; Li *et al.*, 1998; Tamada *et al.*, 1989; Ward *et al.*, 2007).

The control of rhizomania disease relies on resistant sugar beet cultivars. Two proximal, but distinct, loci have been mapped on chromosome III of *Beta vulgaris*, represented by alleles *Rz1*, *Rz4* and *Rz5* and alleles *Rz2* and *Rz3* at the first and second locus, respectively (McGrann *et al.*, 2009). However, the identified loci confer only a partial resistance to rhizomania, reducing viral replication and titre, and new isolates of BNYVV able to cause significant yield penalties on resistant cultivars have recently evolved (McGrann *et al.*, 2009). New approaches to study virus–host–vector interactions therefore need to be explored in order to find alternative strategies to control the virus.

Up to now, reverse genetics for this virus has relied on full-length cDNA clones of all BNYVV RNAs and replicons based on RNA-3 and RNA-5 (D'Alonzo *et al.*, 2012; Quillet *et al.*, 1989; Ratti *et al.*, 2009) under the control of the bacteriophage T7 promoter for *in vitro* transcription. The use of these clones for *in vivo* experiments requires the performance of multiple, expensive and sensitive steps. In particular, clones containing a full-length DNA copy of viral RNA must be linearized and transcribed *in vitro* in order to

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obtain infectious transcripts that need to be mechanically inoculated onto a test plant, such as *Nicotiana benthamiana* or *Chenopodium quinoa*.

Full-length infectious clones of BNYVV RNA-3 and RNA-4, under the control of the *Cauliflower mosaic virus* 35S promoter, have been obtained by Koenig *et al.* (1991) and combined with BNYVV helper strain, containing RNA-1 and RNA-2 species, therefore skipping the *in vitro* transcription step for these two components. Field isolates must be used to investigate or assess Benyviruses–plant–vector interactions and rhizomania resistance (Bornemann and Varrelmann, 2011; Koenig *et al.*, 1991). These systems are expensive and time-consuming, and present serious limitations when applied to massive experiments.

A powerful alternative to these methods is agroinoculation (Grimsley *et al.*, 1986), a less expensive and more reproducible strategy for the infection of plants with transcripts obtained *in vivo*, leading to virus agroinfection. Agroinfection involves plant tissue infiltration with a suspension of a combination of *Agrobacterium tumefaciens* cell clones carrying binary plasmids, each harbouring a full-length cDNA copy of a viral genome component. A plant functional promoter and DNA copy of the RNA viral genome are transferred as T-DNA from *A. tumefaciens* into plant cells from which, after *in vivo* transcription, biologically active viral RNAs are generated and can initiate infection.

Agroinfection is extensively used for the study of insect-transmitted viruses that cannot be mechanically inoculated into test plants, a serious obstacle for the study of such pathogens. This is the case for *Turnip yellows virus* (TuYV) and *Beet mild yellowing virus* (BMV), both transmitted by aphids for which agroinfectious clones have been produced and successfully used for the infection of *N. benthamiana* plants (Leiser *et al.*, 1992; Stephan and Maiss, 2006), and for the whitefly-transmitted Geminivirus *Tomato yellow leaf curl virus* (Navot *et al.*, 1991) and the Crinivirus *Lettuce chlorosis virus* (Chen *et al.*, 2012).

Previous work has shown that two virus species affecting sugar beet and difficult or impossible to transmit through mechanical inoculation can overcome such a barrier through agroinfiltration: *Beet curly top virus* (BCTV), transmitted by leafhoppers (Bridson *et al.*, 1989), and the aphid-transmitted *Beet yellows virus* (BYV) for which mechanical transmission is inefficient (Chiba *et al.*, 2006; Polak and Klir, 1969; Russell, 1963).

Here, we describe the production of agroinfectious clones of BNYVV B-type RNAs able to infect *N. benthamiana* and *B. macrocarpa* plants and to reproduce a complete viral life cycle. Moreover, direct agroinoculation of sugar beet roots is possible and could be used to screen for rhizomania resistance in different cultivars.

Full-length cDNA copies of BNYVV RNA-1 to RNA-5 have been cloned previously under the T7 promoter giving rise to pB15, pB214, pB35, pB45 and pB55 clones, respectively (Link *et al.*, 2005; Quillet *et al.*, 1989; Ziegler-Graff *et al.*, 1988), as well as to

viral replicons Rep3GFP (Erhardt *et al.*, 2000), Rep5GFP (Schmidlin *et al.*, 2005), Rep3BNYVV-p26HA (Link *et al.*, 2005), Rep3BSBMV-p29HA and Rep5BNYVV-p25HA (A. Delbianco, unpublished data) expressing a tagged version of p29 and p25 usually expressed by BSBMV RNA-3 and BNYVV RNA-5, respectively. Expression vectors Rep3 and Rep5, containing minimal 5' and 3' untranslated regions (UTRs) of BNYVV RNA-3 and RNA-5, respectively, are replicated in the presence of BNYVV RNA-1 and RNA-2 and can be used to express foreign proteins (Bleykasten-Grosshans *et al.*, 1997; Schmidlin *et al.*, 2005).

Complete cDNA sequences of viral and replicon RNAs were introduced downstream of the *Cauliflower mosaic virus* double 35S promoter in binary vector pJL89 (Crivelli *et al.*, 2011; Lindbo, 2007).

The cDNAs of BNYVV RNA-2 (4612 nucleotides), RNA-3 (1773 nucleotides), RNA-4 (1467 nucleotides) and RNA-5 (1350 nucleotides) and replicons expressing different proteins were amplified from the available clones using Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies, Santa Clara, CA, USA) and ligated directly into the pJL89 vector digested with *StuI/SmaI*, and then introduced into *A. tumefaciens* cells (strain C58C1) by electroporation, leading to AgroBN-2, AgroBN-3, AgroBN-4, AgroBN-5, AgroRep3GFP, AgroRep3p26HA, AgroRep3p29HA, AgroRep5GFP and AgroRep5p25HA clones (Fig. 1a).

We could not perform cloning of the full-length cDNA amplicon of BNYVV RNA-1 (6746 nucleotides) with the same one-step approach: in fact, we removed from pB15 *SphI/SphI* (1995–3742 nucleotides) and *MluI/MluI* (4125–5396 nucleotides) fragments, obtaining the pB15-*SphI-MluI* clone (Fig. 1b). This cDNA sequence corresponding to partial BNYVV RNA-1 was amplified from pB15-*SphI-MluI* and introduced into the pJL89-*SphI* plasmid (pJL89 from which the *SphI* restriction site has been eliminated through Klenow treatment). The two fragments, previously removed from the BNYVV RNA-1 sequence, were introduced back into the afore-said vector in two steps to reconstitute the full-length RNA-1 sequence. All intermediate plasmids were cloned in *Escherichia coli* cells (strain MC1022) and the complete cDNA of RNA-1 in pJL89 (named AgroBN-1) was then introduced into *A. tumefaciens* cells (strain C58C1).

For leaf agroinfiltration, *A. tumefaciens* cells carrying clones of BNYVV RNA-1 to RNA-5 were grown overnight at 28 °C in 5 mL of Luria–Bertani (LB) medium supplemented with 100 µg/mL kanamycin and 50 µg/mL rifampicin. The bacteria were collected by centrifugation and resuspended in MA buffer (10 mM MgCl₂, 200 µM acetosyringone), adjusting the optical density at 600 nm (OD₆₀₀ nm) to 0.6 for *N. benthamiana* and 0.3 for *B. macrocarpa*. Each agroclone cell line was mixed in equal amounts with the others and left at room temperature for 3–4 h before leaf agroinfiltration of 3-week-old plants.

Local symptoms appeared 1 week after agroinfiltration as chlorotic spots, whereas systemic symptoms appeared 2 weeks later,

Fig. 1 (a) *Beet necrotic yellow vein virus* (BNYVV) constructs built and used in this study. Each clone has the same pJL89-derived backbone. Double *StuI*/*SmaI* (*) restriction digestion allows the insertion of blunt-end polymerase chain reaction (PCR) fragments between the double 35S promoter (2-35S) and the hepatitis delta virus ribozyme (Rz) followed by a Nos terminator. LB and RB represent the left and right borders of the T-DNA. Mtr, methyltransferase; Hel, helicase; Pro, protease; Pol, RNA polymerase; RT, readthrough; RTD, readthrough domain; star, suppressible UAG stop codon; CRP, cysteine-rich protein; GFP, green fluorescent protein; Ha, haemagglutinin tag. (b) Schematic representation of pB15 and pB15-*SphI*-*MluI* clones used for the production of AgroBN-1. Broken lines represent segments between restriction sites *SphI* and *MluI* removed from BNYVV RNA-1 clone (pB15) in order to facilitate cloning in the pJL89-*SphI* plasmid.

showing leaf distortion and providing evidence for the efficient long-distance movement of the virus within the plant (Fig. 2a,b). *Beta macrocarpa*-infiltrated leaves also displayed local lesions (Fig. 2c) and the systemic infection appeared to be particularly evident, as all leaves were completely distorted and the plant remained stunted, dying 1 month later (Fig. 2d, middle and right

plants). Both local and systemic symptoms were identical to those obtained when plants were infected with *in vitro* transcripts or wild-type BNYVV isolate (data not shown).

To ensure that the expression occurred efficiently in the infiltrated area, we used a replicon expressing the green fluorescent protein (GFP) only on viral replication (Link *et al.*, 2005). *Nicotiana*

Fig. 2 Symptom expression, particle detection and reporter gene expression obtained by agroinfiltration of *Agrobacterium tumefaciens* cells carrying AgroBN-1 to AgroBN-5 clones (a, b, c, d and f) or AgroBN-1 to AgroBN-4 and AgroRep5GFP clones (e and g). (a) Local symptoms in agroinfiltrated leaf of *Nicotiana benthamiana* at 7 days post-inoculation (dpi). (b) Symptoms in systemically infected leaf of *N. benthamiana* at 14 dpi. (c) Local chlorotic spots in leaf of *Beta macrocarpa* agroinfiltrated with AgroBN-1 to AgroBN-5 clones (left) and symptomless leaf of *B. macrocarpa* agroinfiltrated with empty pJL89 binary vector (right) at 7 dpi. (d) Systemic symptom development in *B. macrocarpa*: nonagroinfected plant on the left, infected plant in the middle, showing some distorted leaves (14 dpi), and infected plant with totally distorted leaves on the right (30 dpi). (e) Agroclone-mediated green fluorescent protein (GFP) expression in *N. benthamiana* cells. (f) Beet necrotic yellow vein virus (BNYVV) particles observed in *B. macrocarpa* roots at transmission electron microscopy (TEM) by immunosorbent electron microscopy (ISEM) (14 dpi). Bar represents 200 nm. (g) Agroclone-mediated GFP expression in agroinfected roots of *B. macrocarpa*, visualized by confocal microscopy (14 dpi).

benthamiana leaves agroinfected with AgroBN-1 to AgroBN-4 and AgroRep5GFP were observed using a Nikon E800 microscope (Nikon, Tokyo, Japan) equipped with a Nikon DXM1200 camera. Replicon-mediated GFP expression was observed in cells of the infiltrated area (Fig. 2e). To test for the completion of a full infection cycle, the leaves and roots of the inoculated plants were collected and used for transmission electronic microscopy (TEM) imaging employing the immunosorbent electron microscopy (ISEM) technique. Characteristic rod-shaped particles were visualized in all tissues, demonstrating that encapsidation had occurred and the virus was able to spread in the entire plant (Fig 2f).

To complete our analyses, total RNAs were extracted from leaves showing local symptoms (Fig. 3a) and further analysed by Northern blot to confirm the presence of BNYVV RNA-1 to RNA-5. All RNA species were detected using specific probes, as described previously (Link *et al.*, 2005; Schmidlin *et al.*, 2005), demonstrating the replication and systemic movement of all the viral RNAs. Similarly, expression of the coat protein (CP) and the proteins encoded by agroclones carrying Replicons 3 or 5 was shown by their respective immunodetection using specific

antibodies after Western blotting of total proteins from infiltrated or systemic tissues, as described by Link *et al.* (2005). BNYVV CP, haemagglutinin (HA)-tagged BNYVV p25 (and p26, not shown) and BSBMV p29 were detected using anti-HA antibody, whereas GFP was detected using a specific polyclonal antibody (Fig. 3b).

During the natural life cycle of *P. betae*, the zoospores infect the rootlets of the host plants, injecting their cytoplasmic content into the root cell and inducing plasmodium development. In this phase, the plasmodium acquires the virus from the infected plant and sequentially differentiates in a zoosporangium or in a cystosorus, leading to the production of secondary viruliferous zoospores able to infect new rootlets (Adams, 1991; Keskin, 1964).

To investigate the aptitude of 35S-driven RNAs to be transmitted by the plasmodiophorid vector, leaves of 3-week-old *B. macrocarpa* plants were infected with the agroclones of BNYVV RNA-1 to RNA-5 and the pots were successively infested with aviruliferous *P. betae* zoospores. The systemic infection arose 3 weeks after agroinfection and the presence of *P. betae* cystosori and BNYVV particles in the roots was confirmed by light microscopy and TEM

Fig. 3 Northern (a) and Western (b) blot analysis of *Nicotiana benthamiana* agroinoculated leaves: 1, AgroBN-1 + AgroBN-2 + AgroRep5p25Ha; 2, AgroBN-1 + AgroBN-2 + AgroRep3p29Ha; 3, AgroBN-1 + AgroBN-2 + AgroRep3GFP; 4, AgroBN-1 + AgroBN-2 + AgroRep5GFP. The equal loading of the Northern blot was visualized by ethidium bromide (EtBr) staining. Different RNAs were detected using complementary riboprobes. Immunodetection of virally expressed protein was performed on equally loaded gel as shown with membrane staining (MS).

observations (data not shown), respectively. Roots suspected to be infected by viruliferous *P. betae* cystosori were then collected, air dried and crumbled into pots carrying *B. vulgaris* seeds. After 2 weeks, viral particles were observed in the new seedling roots, and the root samples were analysed by reverse transcription-polymerase chain reaction (RT-PCR), Northern blot and Western blot (data not shown), demonstrating BNYVV transmission to new plants through the vector *P. betae*. Transmission occurred in all the 25 plants tested, leading to a 100% efficiency. BNYVV agroclones therefore represent a new tool to establish virus infection from cDNA, generating viral RNAs able to replicate, encapsidate, be acquired and transmitted by the vector to new host plants. Moreover, the behaviour of viral progeny was consistent with that observed using BNYVV wild-type isolate.

The capability of BNYVV agroclones to infect *B. vulgaris* roots was investigated in order to evaluate the possibility to develop a fast and reproducible rhizomania resistance test protocol, easily and economically applicable to a large number of plants. To date, resistance tests have been performed by growing sugar beet plants in soil infested by different BNYVV isolates. Alternatively, mechanically inoculated *C. quinoa* leaves, on which the virus is multiplied, can be used as a viral source to perform root inoculation in different experiments and resistance tests (Bornemann and Varrelmann, 2011; Koenig *et al.*, 1991; Tamada *et al.*, 1989). In our experiments, 10-day-old *B. vulgaris* plants were agroinoculated directly by vortexing the roots for 1 min in MA buffer containing carborundum (30 mg/mL) and a mixture of *A. tumefaciens* cells carrying AgroBN-1 to AgroBN-4 and AgroRep5GFP. Plants were successively planted in sterile sand and roots were analysed after 3 weeks. We demonstrated successful infection of *B. vulgaris* roots through BNYVV RNA and CP detection, viral particle observation (data not shown) and GFP expression visualization by confocal laser scanning microscopy with an LSM510 Zeiss laser scanning confocal microscope (Carl Zeiss Imaging Solutions GmbH, Göttingen, Germany) (Fig. 2g).

Using BNYVV agroclones, we were able to perform successful infection of *B. macrocarpa*, *B. vulgaris* and *N. benthamiana* hosts. Such a method represents an important achievement for studying BNYVV and opens up new possibilities for research studies. To our knowledge, this is the first time that agroinfection has been applied to a multipartite virus composed of five RNAs, although it has already been applied to bipartite viruses, such as *Lettuce infectious yellows virus* (Wang *et al.*, 2009) and *Lettuce chlorosis virus* (Chen *et al.*, 2012), and to the *Cucumber mosaic virus*, *Ourmia melon virus* and *Brome mosaic virus* with a tripartite genome (Crivelli *et al.*, 2011; Kwon and Rao, 2012; Yao *et al.*, 2011).

The agroinfectious clones described in this work represent a new tool for the verification of synergistic or antagonistic effects, as well as cross-protection, between BNYVV and other viruses affecting sugar beet, such as BSBMV. Previous studies have shown a high degree of reciprocal cross-protection in sugar beet mechanically inoculated with fresh sap of *C. quinoa* infected with BNYVV and BSBMV, but, when plants are grown in soil infested with viruliferous *P. betae* zoospores, it seems that BNYVV may suppress BSBMV, probably by competition for replicative or movement proteins inside the host cells (Mahmood and Rush, 1999; Rush, 2003; Wisler *et al.*, 2003). A major limitation of these methods is the difficulty in providing equal inoculum density and the changing environmental conditions, such as the soil temperature, which can influence vector efficiency (Rush, 2003). The production of BSBMV agroclones is in progress and, combined with BNYVV agroclones, will represent a new complementary tool to advance benyvirus interaction studies. Moreover, as the infection can be established with a precise viral inoculum density, synergistic or antagonistic effects could be tested directly starting from root infection.

Viral or foreign proteins can also be produced by these agroclones, when they are combined with the use of replicon vectors Rep3 or Rep5 (Bleykasten-Grosshans *et al.*, 1997; Schmidlin *et al.*, 2005), with the purpose to test their expression and/or their effects on the plant in the viral context. This approach represents an alternative method to perform experiments *in planta* aimed at studying interactions between different viruses, protein expression and virus–vector interaction or virus resistance. Finally, agroinfection of sugar beet roots represents an innovative test to assay rhizomania resistance of sugar beet cultivars in large-scale experiments characterized by homogeneity of viral infection pressure ensured by the precise quantification of *A. tumefaciens* cells. This represents a quick and economic way to infect plants, and therefore will widely enhance BNYVV research.

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