Short Communication

Biological properties of *Beet mild yellowing virus* derived from a full-length cDNA clone

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A German isolate of *Beet mild yellowing virus* (BMYV-IPP) was used for RT-PCR-based construction of the first infectious full-length cDNA clone of the virus ($BMYV_{fl}$). The complete genomic sequence was determined and displayed high similarity to the French isolate BMYV-2ITB. The host range of $BMYV_{fl}$ was examined by agroinoculation and aphid transmission. Both methods lead to systemic infections in *Beta vulgaris*, *Nicotiana benthamiana*, *N. clevelandii*, *N. hesperis*, *Capsella bursa-pastoris* and *Lamium purpureum*. Immunological investigation by tissue-print immunoassay (TPIA) of agroinoculated plant tissues revealed only local infections restricted to the agroinoculated mesophyll tissues in some plant species. In *Nicotiana glutinosa* and *N. edwardsonii*, BMYV was not found in either the agroinoculated tissue or distant tissues by TPIA. So far, $BMYV_{fl}$ agroinoculation did not extend or confine the BMYV host range known from aphid transmission experiments but it did describe new local hosts for BMYV.

The polerovirus *Beet mild yellowing virus* (BMYV) (family *Luteoviridae*) is an aphid-transmitted sugar-beet-infecting virus. BMYV, along with the closterovirus *Beet yellows virus* and the poleroviruses *Beet western yellows virus*-USA and *Beet chlorosis virus* (BChV), is the major cause of yellowing diseases in sugar beet (Smith, 2001; Stevens *et al.*, 2005). Like other poleroviruses, BMYV has a genome organization with six large open reading frames (ORFs) located on a single-stranded positive-sense RNA (5722 nt). The ORFs are arranged in a 5′ gene cluster (ORF0–2) and a 3′ gene cluster (ORF3–5), separated by a short intergenic region (Guilley *et al.*, 1995). ORFs 0–2 are believed to be expressed from viral genomic RNA by leaky scanning and ribosomal −1 frameshift strategies. ORF3–5 expression involves at least one subgenomic RNA (sgRNA) and includes leaky scanning and translational read-through of an amber stop codon. Despite the lack of direct evidence, the identical genome organization of poleroviruses allows the prediction of BMYV gene functions (for review see Mayo & Miller, 1999).

Like other poleroviruses, BMYV is limited to the vascular tissue of its hosts and mechanical inoculation is only possible in mixed infections with umbraviruses (Mayo *et al.*, 2000). Therefore, determination of the host range of BMYV requires the use of aphid vectors like *Myzus persicae* in transmission experiments. The classification of BMYV host plants is characterized by systemic viral movement from primary infected cells following aphid inoculation. However, local host plants are difficult to detect by this method. In local hosts, BMYV replication is possible in aphid-inoculated tissues, but systemic spread is inhibited by an incompatible virus–plant interaction.

We report here the construction of an infectious BMYV full-length cDNA clone ($BMYV_{fl}$) that was used to determine the host range of BMYV by agroinoculation and aphid transmission. For phloem-limited poleroviruses, agroinfection is a suitable alternative to infect plants without using aphids (Leiser *et al.*, 1992).

RNA of the German isolate BMYV-IPP ($BMYV_{wt}$) served as template in RT-PCR to amplify four cDNA fragments using specific oligonucleotides. These were based on the BMYV-2ITB sequence (GenBank accession no. X83110) provided by Guilley *et al.* (1995). Four cDNA fragments were assembled under control of an enhanced cauliflower mosaic virus 35S promoter (Töpfer *et al.*, 1987) and a hammerhead ribozyme sequence (Shintaku *et al.*, 1996) resulting in $pBMYV_{fl}$. For agroinoculation, $pBMYV_{fl}$ was inserted into a modified binary vector pBIN19 to generate $pBINBMYV_{fl}$ and subsequently transferred into *Agrobacterium tumefaciens* strains C58C1 and LBA4404 by electroporation. The infectious $BMYV_{fl}$ was sequenced (GenBank accession no. DQ132996) and BMYV-IPP revealed 96 % nucleotide sequence identity with the French isolate BMYV-2ITB.

In the first set of experiments, $pBINBMYV_{fl}$-recombinant *A. tumefaciens* strains LBA4404 or C58C1 were tested for their ability to agroinfect *Nicotiana benthamiana*. The recombinant *A. tumefaciens* cells were incubated in inoculation buffer (10 mM $MgSO_4$, 10 mM MES and 100 μM acetosyringone) and the suspension was injected into the abaxial

The GenBank/EMBL/DDBJ accession number for the sequence of the infectious full-length cDNA clone $BMYV_{fl}$ is DQ132996.

intercellular leaf space of three or four plant leaves, as described by English *et al.* (1997). It was possible in most cases to inoculate large patches of individual leaves. Both strains successfully delivered $BMYV_{fl}$. In all cases, systemic BMYV infections were detected in agroinoculated *N. benthamiana* by double-antibody sandwich (DAS)-ELISA 4 weeks after agroinoculation. Agglutination of bacterial cells caused problems when using LBA4404 for agroinoculation. As C58C1 demonstrated more uniform growth and less agglutination, this strain was used for all further experiments.

In a second series of experiments, the mean virus titre in both $BMYV_{fl}$-agroinfected and $BMYV_{wt}$ aphid-infected *N. benthamiana* plants was compared by DAS-ELISA. For $BMYV_{wt}$ aphid transmissions, 10 *Myzus persicae* aphids were transferred after a 48 h acquisition period on $BMYV_{wt}$-infected *Beta vulgaris* to each healthy *N. benthamiana* plant. Four weeks post-inoculation, the ELISA readings were similar [mean A_{415} for $BMYV_{fl}$-agroinfected, 0·66 ($n=18$); $BMYV_{wt}$ aphid-infected, 0·67 ($n=6$)] though, in both experiments, large differences were found between individual plants (A_{415} range for $BMYV_{fl}$ 0·25–1·8 and $BMYV_{wt}$ 0·26–1·08).

The $BMYV_{fl}$-agroinfected and $BMYV_{wt}$-infected test plants were examined for localization of BMYV capsid protein (CP) in stems, petioles and mesophyll cells by tissue-print immunoassay (TPIA) as described by Franco-Lara *et al.* (1999). The broad-range luteovirus monoclonal antibody 5G4 (AS-0227; DSMZ) was used to detect BMYV CP. A mouse anti-rabbit antibody conjugated to alkaline phosphatase was used as the second antibody. After the fast red/naphthol chromogenic reaction, purple-stained foci on the membranes indicated the presence of BMYV CP.

In all cases, BMYV was restricted to the vascular tissue in stems and petioles of *N. benthamiana*. More than 200 immunoprints were examined, but BMYV was never detected in epidermal cells of stems or petioles. This is consistent with the observation that BMYV replication and movement are limited to the vascular tissue. Almost all main vascular bundles in petioles and stems of $BMYV_{fl}$-agroinfected or $BMYV_{wt}$-infected *N. benthamiana* showed the presence of BMYV (Fig. 1a, b, e–g).

BMYV was detected in mesophyll leaf cells of *N. benthamiana* after removal of the lower epidermis and printing the sample onto nitrocellulose membranes. More than 83 000 stained or unstained mesophyll cells on TPIA from $BMYV_{fl}$- and $BMYV_{wt}$-infected plants, respectively, were counted using a digital video analysis system (LemnaTec Scanalyser). Irregularly distributed single infected mesophyll cells and also small clusters of up to five BMYV-infected cells were detected (Fig. 1i, j). The same irregular distribution of infected cells was observed in $BMYV_{fl}$-agroinfected and $BMYV_{wt}$-infected *N. benthamiana*. Some of these cells corresponded to tracks associated with vascular bundles, while others did not seem to have a vascular localization. For *Potato leafroll virus* (PLRV), Barker (1987) demonstrated for *Nicotiana clevelandii* and van den Heuvel *et al.* (1995) for potatoes that aphid-transmitted PLRV infects mesophyll cells by cell-to-cell movement from adjacent vascular tissue. In many plants, 'leaky' minor vein phloem cells are connected with the mesophyll via plasmodesmata of companion cells (Oparka & Turgeon, 1999). The route of infection in such cells is assumed to be the same in both $BMYV_{wt}$- and $BMYV_{fl}$-agroinfected plants. Consistent with previous observations, we demonstrated in this study that most of the mesophyll tissue in systemically infected plants was virus-free and the number of BMYV-infected cells was minor.

We agroinoculated different plant species from the Solanaceae, Chenopodiaceae, Brassicaceae and Lamiaceae with $BMYV_{fl}$ to investigate the host range of BMYV (Table 1). Additionally, some plant species were also used in aphid transmission experiments using *Myzus persicae* which had fed on $BMYV_{fl}$-agroinfected *N. benthamiana*. Systemic $BMYV_{fl}$ infections were tested by TPIA of petioles taken from stem positions acropetal of the inoculated leaves; systemic $BMYV_{fl}$ infections were found in *N. benthamiana*, *N. clevelandii*, *Nicotiana hesperis*, *B. vulgaris*, *Capsella bursa-pastoris* and *Lamium purpureum*.

In $BMYV_{fl}$-infected *N. benthamiana*, strong interveinal yellowing of leaves was observed. The intensity and the time of symptom appearance varied between individual plants, but symptoms were visible 6 weeks post-inoculation on all $BMYV_{fl}$-infected plants. In *N. clevelandii*, *L. purpureum* and *C. bursa-pastoris*, yellowing of older leaves was detected, whereas the latter plant species also showed leaf rolling of older leaves. In contrast, $BMYV_{fl}$-agroinfected *B. vulgaris* did not develop clear symptoms up to 8 weeks after agroinoculation. We did not detect any obvious difference in symptom development between test plants infected by aphid transmission or agroinfection.

In all tested BMYV hosts, systemic infections could be established, although the success of infection varied. Whereas 96 % of the agroinoculated *N. benthamiana* plants became infected, only 25 % of the *B. vulgaris* plants tested BMYV-positive. The reduced number of agroinfected *B. vulgaris* plants compared with $BMYV_{fl}$-infected beets after aphid transmission might reflect the difficulty of agroinoculating species of the family Chenopodiaceae. The agroinoculated patches in *B. vulgaris* were smaller in size and were often confined strictly to areas between leaf veins compared with those in *N. benthamiana*. In the latter plant species, it was possible to inoculate larger patches of leaf tissue easily. However, the determination of the BMYV-IPP host range by either $BMYV_{fl}$-agroinoculation or $BMYV_{fl}$ aphid transmission revealed no differences.

The ascertained host range mainly agrees with previous reports of systemic BMYV host plants as described by Russell (1965), Björling & Nilsson (1966), Graichen & Rabenstein (1996), Stevens *et al.* (1994), Mayo *et al.* (2000) and Hauser *et al.* (2002). However, divergent reports have appeared about species of the Solanaceae and

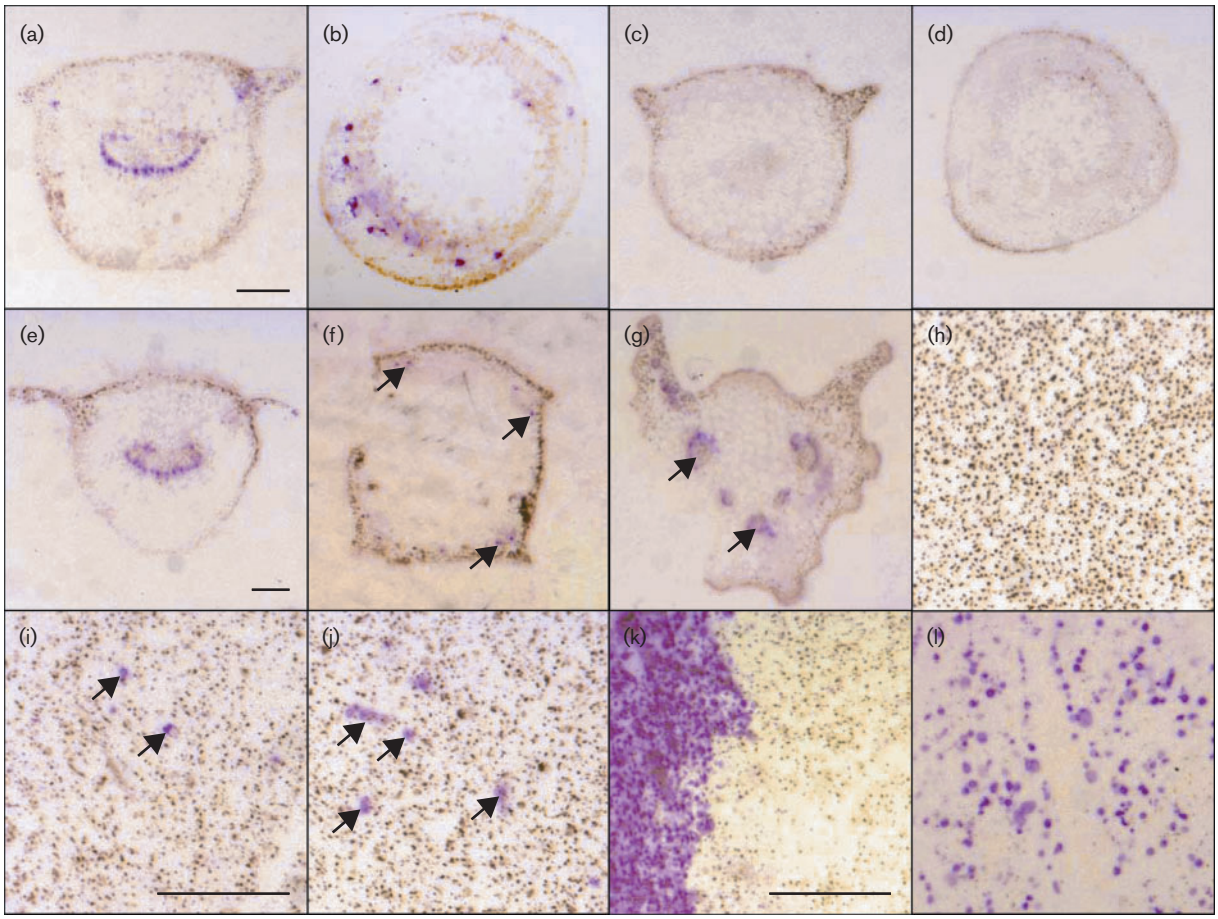

Fig. 1. TPIA of petioles (a, c, e, g), stems (b, d, f) and mesophyll tissue (h–l) after $BMYV_{fl}$ aphid transmission or $BMYV_{fl}$ agroinfection. (a–d) Petiole (a) and stem (b) prints of $BMYV_{fl}$-infected *N. benthamiana* after aphid transmission; virus-free controls are shown in (c) and (d). (e–g) Presence of BMYV CP in phloem bundles of systemically $BMYV_{fl}$-agroinfected *N. benthamiana* (e), *L. purpureum* (f) and *B. vulgaris* (g). (h–j) TPIA of mesophyll tissue of systemically infected *N. benthamiana* after $BMYV_{fl}$ aphid transmission (i) or agroinfection (j); a virus-free control is shown in (h). (k, l) $BMYV_{fl}$-agroinoculated mesophyll tissue of the systemic host *B. vulgaris* (k) and locally agroinfectable *N. tabacum* 'Xanthi-nc' (l). Red foci indicate the presence of BMYV CP and arrows show single infected mesophyll cells or clusters of such cells (i, j) or indicate the presence of BMYV CP in phloem bundles (f, g). Bars represent 1 mm.

Chenopodiaceae as BMYV hosts. Our results using $BMYV_{fl}$-agroinfection or aphid transmission of *N. benthamiana* and *N. clevelandii* agree with Mayo *et al.* (2000), describing these plant species as systemic BMYV hosts. In contrast, Russell (1965) and Graichen & Rabenstein (1996) did not detect BMYV infections in these plant species. Additionally, there are diverse reports about *Chenopodium capitatum* and *Chenopodium foliosum* as BMYV host plants. We obtained the same results as Russell (1965) and Hauser *et al.* (2002), showing that both plant species are local but not systemic BMYV hosts. This is in contrast to earlier work from Björling & Nilsson (1966) and Graichen & Rabenstein (1996), who reported that these two hosts could be infected systemically. These differences might be based on using different BMYV isolates or a BMYV/BChV mixed infection. BChV has been described recently (Hauser *et al.*, 2002); it infects *Chenopodium capitatum* and shows high CP sequence similarity to BMYV. Commonly used BMYV antisera detect both BMYV and BChV (Stevens *et al.*, 2005) and hence might have led to false-positive results.

Even if some plant species did not support a systemic $BMYV_{fl}$ infection, local virus replication restricted to the inoculated leaf tissues might be possible. It has been shown that aphids will occasionally probe on mesophyll and epidermal cells during feeding and allow poleroviruses to be transmitted and replicate in these tissues (Nurkiyanova *et al.*, 2000). Nevertheless, the number of aphid-inoculated cells will be small and difficult to detect. Therefore, the replication of $BMYV_{fl}$ in mesophyll cells of different plant species was examined by TPIA of agroinoculated leaves. All test plant species except for *Brassica napus* and *L. purpureum* were used for the analysis. Poleroviral CP is expressed from sgRNA; hence, the presence of BMYV CP in plant tissues

Table 1. BMYV host range using agroinoculation or aphid transmission

Systemic BMYV infection was determined by TPIA or DAS-ELISA 4 weeks after inoculation, whereas local agroinfection was determined by TPIA of agroinoculated mesophyll tissue. Numbers of BMYV-infected/numbers of inoculated test plants are given; NT, not tested. +, BMYV replication in inoculated mesophyll cells; −, no BMYV replication detectable by TPIA.

Plant species	Agroinfection		Aphid transmission
	Systemic	Local	
Solanaceae			
Nicotiana benthamiana	37/39	+	2/4
Nicotiana clevelandii	3/12	+	1/4
Nicotiana hesperis	4/5	+	3/4
Nicotiana tabacum cv. 'Xanthi-nc'	0/12	+	0/4
Nicotiana rustica	0/12	+	0/4
Nicotiana occidentalis	0/12	+	0/4
Nicotiana edwardsonii	0/17	−	0/4
Nicotiana glutinosa	0/17	−	0/4
Chenopodiaceae			
Beta vulgaris	3/14	+	4/4
Chenopodium capitatum	0/17	+	0/4
Chenopodium foliosum	0/12	+	NT
Brassicaceae			
Capsella bursa-pastoris	6/14	+	3/4
Brassica napus	0/11	NT	NT
Lamiaceae			
Lamium purpureum	4/9	NT	NT

indicates virus replication. A large number of $BMYV_{fl}$-agroinfected mesophyll cells was found in the inoculated tissues of systemic hosts like *N. benthamiana* and *B. vulgaris* (Fig. 1k). In the examined tissues, 50–90 % of the mesophyll cells contained BMYV CP. Additionally, no systemic infections were detected in *Nicotiana tabacum* cv. 'Xanthi-nc', *N. rustica*, *N. occidentalis*, *Chenopodium capitatum* or *Chenopodium foliosum*, but BMYV-infected mesophyll cells could be observed in the agroinoculated areas (Fig. 1l). A smaller number of infected mesophyll cells was seen in these agroinoculated areas than in systemically infected hosts. Furthermore, BMYV replication was restricted to the site of agroinoculation. Probably, $BMYV_{fl}$ agroinoculation also reached phloem-associated cells in these plant species but systemic spread was impeded. Carrington *et al.* (1996) suggested that compatible interactions of plant factors and viral movement functions are a prerequisite of successful viral infections. Additionally, plant RNA-silencing mechanisms are involved in non-host resistance (Vazquez Rovere *et al.*, 2002). Besides inefficient P0 suppressor activity, the absence of systemic BMYV infections is probably the consequence of inadequate movement functions.

Nicotiana edwardsonii and *N. glutinosa* were the only plant species that could not be locally agroinfected with $BMYV_{fl}$. *N. edwardsonii* is an interspecific hybrid derived from a cross between *N. clevelandii* and *N. glutinosa* (Christie, 1969). The BMYV incompatibilities could be therefore based on resistance genes derived from *N. glutinosa* or missing compatibility factors from *N. clevelandii*, as BMYV replicates and moves systemically in *N. clevelandii*. As *A. tumefaciens* also accepts *N. edwardsonii* (Kiernan *et al.*, 1989) or *N. glutinosa* (Tao & Zhou, 2004; Ueda *et al.*, 2004) as host plants, the failure to agroinfect these two species locally with $BMYV_{fl}$ might not reflect an overall incompatible bacterium–plant interaction.

It should be mentioned that the method of agroinfection itself might be unsuitable for specific virus–plant combinations. Mutterer *et al.* (1999) reported that agroinfections of *Lactuca sativa* and *N. benthamiana* with *Turnip yellows virus* (former BWYV-FL1) did not give systemic infections, but aphid transmission from *N. clevelandii* agroinfected with *Turnip yellows virus* to both plant species was successful. In our experiments, the host range determined by agroinfection and aphid transmission showed identical results, indicating that incompatible virus–plant interactions are not bypassed by artificial agroinfections. The smaller number of $BMYV_{fl}$-agroinfected mesophyll cells in local-host plant species could indicate that, even if acetosyringone is added, plant species differ in the extent of mesophyll cell wounding that is produced by the agroinoculation procedure. The number of agroinfected cells is definitely dependent on the efficiency by which *A. tumefaciens* is able to deliver the $BMYV_{fl}$ into mesophyll cells of different plant species.

In summary, all plant species in which a systemic BMYV infection after aphid transmission was verified were also successfully agroinfected with $BMYV_{fl}$. More interestingly,

agroinfection revealed that five plant species were merely locally infectable and two plant species turned out not to be agroinfectable with $BMYV_{fl}$. We described here the first $BMYV_{fl}$ cDNA clone that shows full biological activity. Its ability to agroinfect *N. benthamiana* efficiently provides a tool for further gene-function experiments. It is now possible to identify the genome regions which are involved in determining the BMYV host range. This could provide a deeper insight into the evolutionary relationship of the closely related poleroviruses *Beet western yellows virus*, BMYV and BChV.

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