Acquisition of Brassica Yellows Virus

Subjects: Plant Sciences
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Brassica yellows virus (BrYV) is a tentative species of the genus Polerovirus, which occurs widely, and mostly damages Brassicaceae plants in East Asia. Because BrYV cannot be transmitted mechanically, an insect-based transmission method is required for further virus research. Here, a reliable and unrestricted method is described, in which non-viruliferous aphids (Myzus persicae) acquired BrYV from transgenic Arabidopsis thaliana, harboring the full-length viral genome germinated from seeds and its frozen leaves. The aphids then transmitted the virus to healthy plants. There was no significant difference in acquisition rates between fresh and frozen infected leaves, although the transmission rate from frozen infected leaves was lower compared to fresh infected leaves. This simple novel method may be used to preserve viral inocula, evaluate host varietal resistance to BrYV, and investigate interactions among BrYV, aphids, and hosts.

Keywords: Brassica yellows virus ; Myzus persicae ; transgenic plants ; frozen BrYV-infected plants ; acquisition and transmission

1. Introduction

Brassica yellows virus (BrYV) is a tentative newly identified species in the genus of *Polerovirus*, and it is closely related to turnip yellows virus (TuYV) [1][2]. It is spread widely throughout China as well as in South Korea and Japan [3][4][5]. BrYV has a wide host range, although it mainly infects *Brassicaceae* crops, including cabbage (*Brassica oleracea* var. *capitata*), Chinese cabbage (*B. pekinensis*), cauliflower (*B. oleracea* var. *botrytis*), mustard (*B. juncea*), and turnip (*Raphanus sativus* var. *oleifera*), usually causing leaf malformations and yellowing in the field [3][2][4][5][6]. BrYV has at least three genotypes (BrYV-A, B, and C) as determined by sequence analyses, and full-length infectious cDNA clones of these three genotypes have been developed successfully [1][4][5][7][8]. The full-length amplicon of BrYV-C has been successfully transferred into *Arabidopsis thaliana*, resulting in two stable transgenic lines, 111 and 412, which exhibit severe symptoms, including dwarfism and purple leaves [9]. The BrYV-encoded P0 protein interacts with plant S-phase kinase associated protein 1, contributing to its stability, which allows it to evade autophagy and proteasomal degradation [10]. In addition, the P0 protein impairs the antiviral activity of *Nicotiana benthamiana* rubisco assembly factor 2 by altering its localization pattern to facilitate viral infection [11].

2. Analysis on Results

2.1. Viral Inocula Were Ready for Transmission

Two BrYV inocula were selected for aphid feeding and transmission. First, the seedlings of the seventh generation transgenic *A. thaliana* line 412 harboring the BrYV full-length cDNA clone under control of the ubiquitous 35S promoter and showing typical purple symptoms, were used as fresh BrYV inocula [9]. Then, the transgenic plants were kept at −20 °C as frozen BrYV inocula. Next, the full-length infectious BrYV clone was transferred to *A. tumefaciens* C58CI and inoculated into *A. thaliana*. At 14 days after inoculation, RT-PCR was used to detect BrYV in the inoculated and systemic leaves. The infection incidence in *A. thaliana* inoculated leaves by BrYV was 86.34%, while the incidence in systemic leaves was only 46.75% (**Table 1**). Therefore, transgenic *A. thaliana* line 412 and an *A. tumefaciens*-mediated BrYV infectious clone can be used as inoculation materials for aphid feeding and viral transmission.

Table 1. Efficiency of the *Agrobacterium tumefaciens*-mediated infiltration of BrYV into inoculated and systemic leaves.

Position	Efficiency (%)
Inoculated leaves (n = 58 plant)	**86.34**
Systemic leaves (n = 58 plant)	**46.75**

2.2. The Aphid Species Was Confirmed and a Non-Viruliferous Aphid Clonal Population Was Obtained

The aphids containing BrYV were identified as green peach aphids (*M. persicae*), and a non-viruliferous aphid clonal population Mp433-1 was obtained. DNA was extracted from a single aphid and universal primers (LEP-F and LEP-R) for the mitochondrial cytochrome oxidase gene (*COI*) were used for PCR amplification. The sequencing results were analyzed using the BLAST algorithm in NCBI, and the nucleotide sequence shared 99% identity with *M. persicae* strain YL. The cabbage *COI* (accession number: KM577343) indicated that the aphid species was green peach aphid (*M. persicae*). The aphid colony was screened to obtain virus-free and clonal aphids. A single aphid was identified for ovipositing and the progeny were subsequently reared on healthy turnip plants. Then, one nymph was transferred to healthy *A. thaliana*, and for approximately 5–10 generations, aphids were randomly selected and analyzed by RT-PCR to confirm the absence of BrYV. For another 5–10 generations, the offspring of these non-viruliferous aphids were classified as a clonal population, named *M. persicae* isolate Mp433-1. The selected aphid offspring from the single aphid were confirmed to be BrYV negative by RT-PCR with BrYV-specific primers (BrY4964F/BrY5635R).

2.3. Acquisition and Transmission of BrYV by the Aphids Were Available

Aphids were fed on the frozen leaves of the transgenic *A. thaliana* line 412 preserved for 180 d and 270 d, fresh leaves of line 412 and leaves of healthy *A. thaliana* for 2 days (60 aphids per treatment) and each experiment was repeated for three times. The mean numbers of living aphids were 35.33, 30.33, 55.33, and 51.33, respectively, and survival rates of 58.59%, 50.56%, 92.22%, and 85.56%, respectively (**Table 2**). Subsequently, 16 aphids from each treatment were selected randomly to independently inoculate 3–4-week-old healthy *A. thaliana*. At 2 days after inoculation, the presence of BrYV in the aphids was confirmed by RT-PCR. The mean survival rates of viruliferous aphids fed on line 412 and 180-d and 270-d frozen infected leaves were 93.75%, 87.5%, and 77.08%, respectively. Each experiment was performed three times (**Table 2**). There were no significant differences among the three treatments. Thus, both the fresh and frozen 412 leaves may be used for aphid BrYV acquisition, although the frozen leaves affected the aphid survival rate compared with the fresh leaves under the same experimental conditions.

Table 2. Percentages of viruliferous aphids and BrYV-infected plants as a result of aphids feeding on frozen or fresh infected leaves; each experiment was performed three times independently [1].

Treatment	Total No. of Aphids	No. of Surviving Aphids $p < 0.001$	Proportion of Viruliferous Aphids (n = 48) (%) $p = 0.0183$	Proportion of Infected Plants (n = 48) (%) $p = 0.0942$
Frozen for 180 d	**180 (60 each)**	**106**	**87.5**	**16.6**
Frozen for 270 d	**180 (60 each)**	**91**	**77.08**	**10.4**
Fresh infected leaves	**180 (60 each)**	**166**	**93.75**	**33.33**
Fresh healthy leaves	**180 (60 each)**	**154**	**-**	**-**

[1] Presence of BrYV assessed by RT-PCR.

At 14 days after inoculation, 16 inoculated *A. thaliana* were tested for BrYV infections using RT-PCR. A product of the expected size was amplified from infected plants but not from non-symptomatic plants (**Figure 1**a,b). The transmission rates to plants by aphids fed on line 412 and 180-d and 270-d frozen infected leaves were 33.33%, 16.67%, and 10.42%, respectively (**Table 2**). Thus, the aphids acquiring the virus from the BrYV-transgenic and frozen leaves transmitted the virus to healthy plants, producing symptoms that were identical to those of *A. tumefaciens*-mediated BrYV-infected and BrYV-transgenic plants.

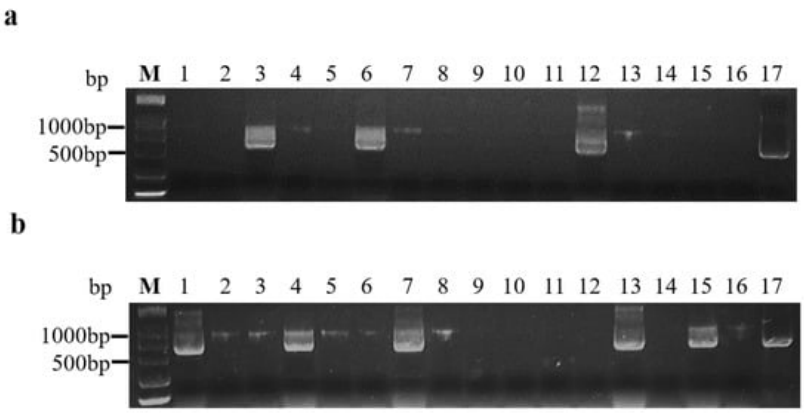

Figure 1. Agarose gel analysis of RT-PCR products using BrYV primers from *A. thaliana* plants inoculated by aphids. (**a**) Aphids that acquired the virus from frozen infected leaves; (**b**) aphids that acquired the virus from transgenic *A. thaliana* line 412 leaves. M: Marker (DCL 2000, Tsingke, Beijing, China); lines 1–16: aphids inoculated through *A. thaliana*; line 17: positive control.

2.4. The Greatest Transmission Efficiency of BrYV Was Determined by Assessing Minimal Aphid Numbers and Inoculation Times

To ensure the greatest transmission efficiency, inoculations with different numbers of viruliferous aphids were performed. The aphids fed on fresh 412 plants for 2 days, and then, 1, 2, 4, 6, and 10 aphids (second–third instar) were transferred independently to *A. thaliana* to act as inoculants. At 2 days after inoculation, the aphids were eliminated using an insecticide. At 14 days after inoculation, RT-PCR showed that the rates of infected *A. thaliana* produced by 1, 2, 4, 6, and 10 aphids were 30%, 80%, 90%, 100%, and 100%, respectively (**Table 3**). The experiments were repeated three times per group. The presence of six viruliferous aphids resulted in an infection efficiency of 100%. Typical symptoms were purple leaves on *A. thaliana* plants inoculated with six viruliferous aphids by 14 days post-inoculation, and western blotting revealed that the virus transmitted by aphids successfully infected *A. thaliana* (**Figure 2**a,b). The tested minimal inoculation times required for six viruliferous aphids to transmit the virus, to healthy *A. thaliana* plants were 6, 12, 24, and 48 h. At 14 days after inoculation, the RT-PCR results showed that the transmission rate for the six aphids with inoculation times of 6, 12, 24, and 48 h were 12.5%, 50%, 62.5%, and 100%, respectively (**Table 4**), indicating that six viruliferous aphids present for 2 days resulted in a 100% BrYV-infection rate for *A. thaliana* plants.

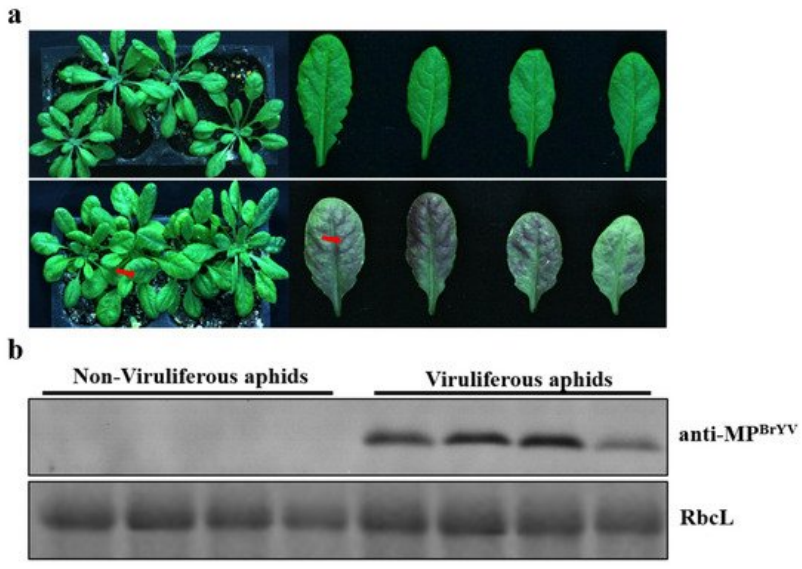

Figure 2. Symptoms and virus detection in *A. thaliana* inoculated with BrYV by aphids. (**a**) Typical symptoms of purple leaves on *A. thaliana* plants at 14 days post-inoculation by six viruliferous aphids. (**b**) Western blotting analyses of the accumulation of BrYV MP extracted from *A. thaliana* upper leaves.

Table 3. Viral transmission frequencies from different numbers of aphids.

Total Number of Insects	No. of Infected Plants (n = 30)	Proportion of Infected Plants (%)
1	9	30

Total Number of Insects	No. of Infected Plants (n = 30)	Proportion of Infected Plants (%)
2	24	80
4	27	90
6	30	100
10	30	100

Table 4. Minimal inoculation times for viral transmission by six aphids.

Inoculation Time (h)	Efficiency (%) (n = 16)
6	12.5
12	50
24	62.5
48	100

As previous report, BrYV could be detected in Mustard (*Brassica juncea* var. *tumida*) and Chinese cabbage (*Brassica campestris* L. ssp. *Chinensis*) [3][7]. To test whether aphids fed on fresh 412 could transmit BrYV to its natural host plants, six aphids fed on the transgenic *A. thaliana* line 412 were transferred to mustard and Chinese cabbage. At 3 days after inoculation, aphids were eliminated using an insecticide. At 14 days after inoculation, RT-PCR showed that the rates of infected Mustard and Chinese cabbage were 94.4% and 100% respectively (**Table 5**). The experiment was repeated three times per group, indicating that viruliferous aphids could successfully transmit BrYV to its natural host plants.

Table 5. BrYV transmitted to natural hosts-plants by aphids.

Host Plants	Total Number of Insects	Proportion of Infected Plants (%) n = 36
Mustard	6	94.4
Chinese cabbage	6	100

3. Current Insights

For plant virology research, virus preservation and utilization are very important [12][13]. Plant viruses in the *Polerovirus* (family *Luteoviridae*) cause emerging diseases that have serious economic consequences for many staple, vegetable, ornamental, and fruit crops, and the transmission by aphids is classified as persistent, circulative, and non-propagative [14][15][16]. Convenient tests to determine viral acquisition and transmission have not been available owing to the lack of viral inocula preservation. Therefore, it is important to explore a simple method for *Polerovirus* preservation and transmission. The technique presented here easily allows virus preservation and transmission by using transgenic plants and frozen infected leaves harboring the viral genome. The purpose of preservation is not only to maintain the infectivity of the virus, but also to eliminate mutations and contamination.

In this research, a very simple and feasible method was established using transgenic *A. thaliana* harboring the full-length BrYV genome and frozen infected leaves for aphid feeding and transmission. Previous research by Franco-Lara showed that PLRV genomic RNA transfers into common tobacco and potato, in which the viral genomic RNA and proteins undergo replication and translation. Furthermore, aphids (*M. persicae*) fed on the transgenic tobacco plants readily transmit PLRV to test plants, and the inoculation efficiency of five aphids present for 3 days was approximately 71% [17]. Boissinot extracted the virions from TuYV-infected plants, fed them to aphids, and then used 10 *M. persicae* (Sulzer) for 4 days for inoculations [18]. However, the method is technically demanding and requires viral purification equipment and instruments. In this study, six aphids (*M. persicae*) feeding in BrYV-transgenic *A. thaliana* for 2 days resulted in a transmission rate of up to 100% (**Table 3**), indicating that aphids can readily acquire the virus from transgenic *A. thaliana* plants. More conveniently, BrYV-transgenic plants may be germinated from transgenic *A. thaliana* seeds at any time for aphid feeding. The survival rate of aphids fed frozen diseased leaves was 58.89%, which was lower than that of aphids fed on the fresh infected leaves and fresh healthy leaves. Although the materials subjected to freezing conditions may affect aphid survival, there were no significant differences in the aphid viral acquisition rates (**Table 2**). The greatest survival rate for *M.*

persicae occurred after feeding on detached fresh infected leaves, perhaps because detached leaves infected with the virus had a lower water-loss rate. However, transmission rates for aphids fed on the frozen infected leaves were lower than those fed on the fresh infected leaves, indicating that viral acquisition form the frozen samples was difficult (**Table 2**). Zhou's previous work showed no significant difference in viral transmission between the SBPH fed on frozen or fresh infected rice leaves. The infected rice leaves had been preserved at −70 °C for 45 and 140 days [19]. Shikata et al. (1977) also showed that SBPH acquires viruses from 232-day frozen leaf tissues of RBSDV-infected rice leaves [20]. In this research, a single aphid acquired the virus from fresh infected leaves and 270-day frozen infected leaves, revealing a simple and feasible method for viral acquisition by aphids at any time, and the infected plants could be preserved at −20 °C for at least 270 days. This work may be further improved by feeding aphids on BrYV-infected leaves stored at −70 °C. *Agrobacterium tumefaciens*-mediated viral inoculation of *A. thaliana* and virus genomic transgenic plants may serve as a viral source for aphids that could successfully transmit it to its natural hosts. Because Polerovirus-infected plants are very difficult to preserve, this method overcomes the technical bottlenecks of viral transmission and preservation, and it can be used for screening resistant host varieties and for genetic analyses of a variety's resistance to BrYV.

This method provides a simple and reliable approach using transgenic *Arabidopsis* and frozen leaves harboring the full-length BrYV genome for viral acquisition and transmission by aphids. This novel method can be applied to the preservation of viral inocula, evaluation of host variety resistance, and biological research on interactions among BrYV, aphids, and hosts. It may also provide a foundation for establishing similar methods for research on other poleroviruses.

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