Highly efficient inoculation method of apple viruses to apple seedlings

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Abstract

Virus inoculation to original plants is an important step in research for many reasons. For example, it is used to satisfy Koch's postulates, to test resistance to viruses in breeding programs, and to analyze gene function by virus vectors etc. However, it is generally difficult to inoculate viruses to woody fruit trees like apple, and an efficient inoculation method has not been developed thus far. In this study, we showed that a biolistic inoculation of total RNAs from infected tissues or virus RNAs resulted in a high infection rate in apple seedlings. Total RNAs extracted from *Chenopodium quinoa* leaves infected with *Apple latent spherical virus* (ALSV) or *Apple chlorotic leaf spot virus* (ACLSV) and ALSV- RNAs from purified virus were biolistically inoculated to the cotyledons of apple seedlings by a Helios Gene Gun system (BIO-RAD) or a PDS-1000/He Particle Delivery System (BIO-RAD). Analysis of true leaves 2-4 weeks after inoculation by Northern blot hybridization, RT-PCR, or ELISA showed that 36 out of 38 plants (95%) inoculated with total RNAs from ALSV-infected tissues, 39 out of 41 plants (95%) inoculated with ALSV-RNAs, and 6 out of 7 plants (86%) inoculated with total RNAs from ACLSV-infected tissues were infected with each virus. Thus, the biolistic inoculation of total RNAs from infected tissues or virus RNAs from purified virus to apple seedlings is found to be an efficient inoculation method of apple viruses. We think that the method can be applied to other virus-fruit tree combinations.

Keywords: ALSV, ACLSV, biolistic inoculation, apple, cotyledon

Introduction

Virus inoculation to an original plant is an important step to establish the viral etiology of a disease, to test resistance to viruses in a breeding program, and to analyze gene function by virus vectors etc. In woody fruit trees like apple, it is generally difficult to inoculate viruses by conventional inoculation methods, and an efficient inoculation method has not been developed thus far.

Recently, we reported that *Apple latent spherical virus* (ALSV)-based vectors could be used for reliable and effective VIGS and expression of foreign proteins in a broad range of plants (Li et al. 2004; Igarashi et al. 2009). However, the infection efficiency of ALSV inoculation to apple trees by conventional methods had been poor (Ito et al. 1992; Li et al. 2004). Thus, it is necessary to establish an effective inoculation method to analyze gene function of apple by using the ALSV vectors. In this study, we have established a highly efficient inoculation method of apple viruses to apple seedlings by biolistic inoculation.

Materials and methods

Viruses: ALSV and ACLSV (P205) were used in this study (Li et al. 2000, 2004; Sato et al., 1993).

Apple plants: Apple seeds were germinated at 4°C, sown in soil, and grown in a growth chamber (25°C, 16:8 light:dark photoperiod). Germinated apple seeds before being sown in soil were also used for biolistic inoculation.

Inocula: For rubbing and stem slashing inoculation, ALSV was purified from infected *C. quinoa* leaves by homogenizing with 0.1M Tris buffer (pH7.8), clarification by bentonite, precipitation with PEG6000, and sucrose density gradient centrifugation as described previously (Li et al. 2000).

For biolistic inoculation by particle bombardment, total RNAs were extracted from ALSV- or ACLSV- infected *C. quinoa* leaves by Tri reagent (Sigma) or Tripure isolation reagent (Roche Applied science) as recommended by the instruction manuals. The RNA samples were re-extracted by phenol/chloroform and then precipitated with ethanol. ALSV-RNAs were extracted from purified virus preparations by Tripure isolation reagent (Roche Applied science) as recommended by the instruction manual or by phenol/chloroform and then precipitated with ethanol.

Plant inoculation: For rub inoculation, the opened and expanded cotyledons of apple seedlings were dusted with 600 mesh carborundum and then rubbed with purified virus (1-6 μg/μl). For stem slashing, apple seedlings were grown to the 11-20 true leaf stages, and their stems were inoculated by cross-cutting with blades dipped in purified virus (1-6 μg/μl). Biolistic inoculation was performed by using a Helios Gene Gun system (BIO-RAD) or a PDS-1000/He Particle Delivery System (BIO-RAD). Microcarriers (gold particles) coated with total or virus RNAs were prepared as described previously (Yamagishi et al., 2006) with some modifications. For inoculation of total RNAs by a Helios Gene Gun system, microcarriers were prepared in the same amounts as those for total RNAs and gold particles which were approximately 5.0 μg and 0.3 mg per shot. For inoculation of virus RNAs by a Helios Gene Gun system and a PDS-1000/He Particle Delivery System, the amounts of virus RNAs and gold particles were approximately 3.0 μg and 0.4mg (0.6 μm in diameter) per shot, respectively.

The cotyledons of germinated seeds (Fig.1A) were bombarded with gold particles coated with total or virus RNAs at a pressure of 250 to 320 psi (Helios Gene Gun system) or 1100 psi (PDS-1000/He Particle Delivery System) using helium gas. Cotyledons of the apple seedling were bombarded with 3 to 4 shots (Helios Gene Gun system) or 2 shots (PDS-1000/He Particle Delivery System) per cotyledon. After particle bombardment, germinated seeds were sprayed with water and placed on a KIMWIPE (Crecia) soaked with water to retain humidity in the petri dish. The petri dish was then covered and placed at 4°C in the dark for 1-2 days. Then, the petri dish was wrapped with paper to lower light intensity, and placed into a growth chamber (25°C, 16:8 light:dark photoperiod) for 1day. The next day, the paper was removed and the apple seedlings were grown under the same conditions. When the apple seedlings grew up to the cover of the petri dish, the cover was removed from the petri dish. Then, the petri dish was placed into a large plastic case containing a paper towel soaked with water. When the second true leaf developed, the seedlings were planted in soil and acclimatized to the open air. In another procedure, apple seedlings were grown to the 2-19 true leaf stages (Fig.1B), and total RNAs were bombarded onto the leaves with 1-3 shots per leaf.

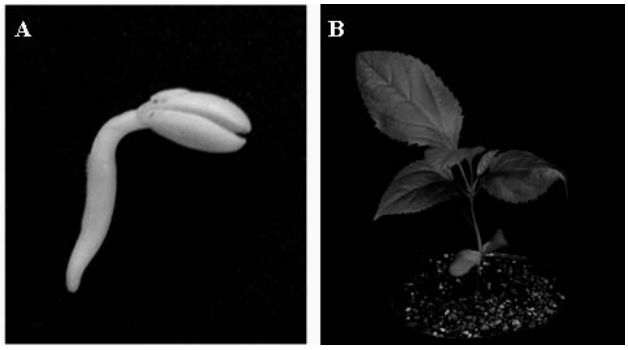

Fig. 1 Plant materials for biolistic inoculation. A, A germinated apple seed in which the seed coat was removed. Total RNAs or virus RNAs were bombarded to the cotyledons of this stage. B, An apple seedling at true leaf stage. Total RNAs from infected tissues were bombarded to the true leaves.

Analyses of inoculated plants: The infection efficiency was determined by Northern blot analysis, ELISA, and RT-PCR 2-4 weeks after inoculation as follows: total RNAs from inoculated apple seedlings were prepared according to Gasic et al. (2004) and Northern blot analysis was conducted as described previously (Yamagishi et al. 2009). For RT-PCR, first-strand cDNA was synthesized from total RNA using an oligo (dT) primer and Superscript reverse transcriptase (Promega). PCR amplification was performed using the following primer pairs: R2ALS1363+ (5'-GCGAGGCACTCCTTA -3'; homologous to positions 1362-1376 of ALSV RNA2) and R2ALS1551- (5'-GCAAGGTGGTCGTGA -3'; complementary to positions 1524-1510 of ALSV RNA2). The ELISA procedure used was the direct method of the conventional double antibody sandwich.

Results and discussion

The infection efficiency of viruses to apple seedlings by three inoculation methods is shown in Table 1. By rubbing and stem slashing inoculation, no virus infection was found. Although ALSV was previously shown to infect apples by rubbing and stem slashing inoculations (Ito et al., 1992; Li et al., 2004), these inoculation methods had a low level of efficiency. On the other hand, when total RNAs and virus RNAs were inoculated to the cotyledons of germinated seeds by particle bombardment, 36 out of 38 plants (95%) inoculated with total RNAs and 39 out of 41 plants (95%) inoculated with virus RNAs were found to be infected with ALSV. Similarly, 6 out of 7 plants (86%) were infected with ACLSV by the same methods. Our results showed that biolistic inoculation with RNAs resulted in high infection efficiency. In particular, it is thought that biolistic inoculation by using a Helios Gene Gun system with total RNAs from infected tissues is an attractive inoculation method, because total RNAs from infected tissue were sufficient for highly efficient inoculation. Furthermore, ALSV could be detected from the first true leaf which was developed just above the bombarded cotyledons (Fig. 2).

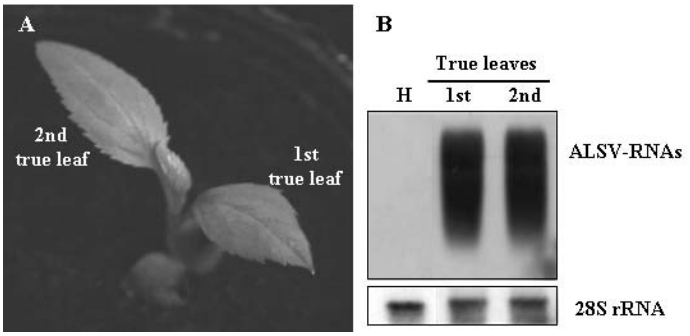

Fig. 2 Systemic infection of ALSV at the early growth stage of an apple seedling in which total RNAs were inoculated to cotyledons by biolistic inoculation. A, The apple seedling (17dpi) which was inoculated to total RNAs from infected tissues to the cotyledons in the stage of Figure 1A. B, Detection of ALSV-RNA from 1st and 2nd true leaves of apple seedling. H, healthy leaf sample.

Our results suggest that the cotyledons of germinated seeds were very sensitive for virus inoculation because biolistic inoculations to the true leaves of apple seedlings resulted in a low infection rate (Table 1).

Tab. 1 Infection efficiency of viruses to apple seedlings by three inoculation methods

Inoculation Methods	Virus	Inocula	Inoculation sites	No. of infected / Inoculated plants
Rubbing	ALSV	Purified virus	Cotyledons	0/17 (0%)
Stem slashing	ALSV	Purified virus	Stem	0/39 (0%)
Biolistic inoculation	ALSV	Total RNAs	True leaves	2/48 (4.2%)
	ALSV	Total RNAs	Cotyledons*	36/38 (95%)
	ALSV	Virus RNAs	Cotyledons*	39/41 (95%)
	ACLSV	Total RNAs	Cotyledons*	6/7 (86%)

*Cotyledons of germinated seeds

In conclusion, biolistic inoculation to germinated apple seeds is an efficient inoculation method of apple viruses. The method achieves high and reproducible infection efficiency and could be applied to other virus -fruit tree combinations.

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