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Preventive and curative effects of *Apple latent spherical virus* vectors harboring part of the target virus genome against potyvirus and cucumovirus infections

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ABSTRACT

Apple latent spherical virus (ALSV)-based vectors experimentally infect a broad range of plant species without causing symptoms and can effectively induce stable virus-induced gene silencing in plants. Here, we show that pre-infection of ALSV vectors harboring part of a target viral genome (we called ALSV vector vaccines here) inhibits the multiplication and spread of the corresponding challenge viruses [*Bean yellow mosaic virus*, *Zucchini yellow mosaic virus* (ZYMV), and *Cucumber mosaic virus* (CMV)] by a homology-dependent resistance. Further, the plants pre-infected with an ALSV vector having genome sequences of both ZYMV and CMV were protected against double inoculation of ZYMV and CMV. More interestingly, a curative effect of an ALSV vector vaccine could also be expected in ZYMV-infected cucumber plants, because the symptoms subsided on subsequent inoculation with an ALSV vector vaccine. This may be due to the invasion of ALSV, but not ZYMV, in the shoot apical meristem of cucumber.

Introduction

Apple latent spherical virus (ALSV), originally isolated from an apple tree in Japan and classified in the genus *Cheravirus*, is a small spherical virus 25 nm in diameter, which is composed of a two-segmented single-stranded RNA genome (RNA1 and RNA2) and three coat proteins (Vp25, Vp20, and Vp24) (Li et al., 2000). Although apple is the only known host of ALSV in nature, it has a relatively wide range of experimental hosts including *Arabidopsis thaliana*, solanaceous plants (tobacco, tomato, potato, etc.), cucurbits (cucumber, melon, squash, luffa, etc.), legumes (soybean, azuki bean, pea, etc.), and fruit trees of the Rosaceae family (apple, pear, peach, etc.) (Igarashi et al., 2009; Sasaki et al., 2011). ALSV latently infects most of these host plants without causing any obvious symptoms.

We previously reported that ALSV-based vectors effectively induced stable virus-induced gene silencing (VIGS) of endogenous genes for long periods in plants (Igarashi et al., 2009; Sasaki et al., 2011; Yaegashi et al., 2007; Yamagishi and Yoshikawa, 2009). RNA silencing, a sequence-specific gene inactivation mechanism first reported in plants in 1990 (Napoli et al., 1990), is induced by double-stranded RNA (dsRNA) and is universally conserved in eukaryotes (Hannon, 2002). Infection of plants by an RNA virus leads to synthesis of replicative dsRNA by an RNA-dependent RNA polymerase. This dsRNA is degraded into 21–25 nucleotide (nt) small interfering RNA (siRNA) by Dicer (in plants, by Dicer-like). The siRNA is incorporated into an RNA-induced silencing complex, resulting in degradation of RNA with sequence complementary to the siRNA (Voinnet, 2005). In addition, siRNA moves into adjacent cells, eventually leading to its systemic spread (Mlotshwa et al., 2002; Voinnet and Baulcombe, 1997). RNA silencing in plants can be compared to the immune system in animals (Lecellier and Voinnnet, 2004; Plasterk, 2002; Voinnet 2001), and RNA silencing plays an important role as a defense mechanism against viral infection in plants possessing no immune mechanism (Baulcombe, 2004; Vance and Vaucheret, 2001; Waterhouse et al., 2001).

Zucchini yellow mosaic virus (ZYMV), belonging to the genus *Potyvirus*, causes serious damage in cucurbit crops worldwide (Lecoq and Desbiez, 2008). The virus is transmitted by aphids in a non-persistent manner and causes symptoms including severe mosaic, malformation of leaves and fruits, and withering of plant tissue,

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particularly in cucurbits (Lecoq and Desbiez, 2008). Though cucumber mosaic disease is caused by a single infection by ZYMV, the disease becomes severe on superinfection by two or three viruses including ZYMV, *Cucumber mosaic virus* (CMV), and *Watermelon mosaic virus* (WMV). To alleviate damage to crops by ZYMV, cross protection using attenuated ZYMV is employed for control of the viral disease (Kosaka and Fukunishi, 1997; Kosaka et al., 2006; Lecoq and Desbiez, 2008; Wang et al., 2006). Recently, an attenuated strain of ZYMV (ZYMV 2002), formulated as a biotic pesticide (CUBIO ZY-02), came on the market and has been used in cucumber plants in Japan (Kosaka et al., 2009). Development of attenuated plant pathogenic viruses, however, is not always straightforward, and the production and screening of such viruses require much time and labor, and may be difficult, depending on the target viral species. For that reason, the practical use of attenuated viruses for control of viral diseases is very limited. Furthermore, cross protection is often not sufficient when the challenge virus is phylogenetically distant from the primary virus, and thus, each attenuated virus has to be established individually for each virulent virus.

Ratcliff et al. (1999) reported RNA mediated cross-protection between viruses, in which primary inoculation of *Tobacco rattle virus* (TRV) vector with green fluorescence protein (GFP) exhibited cross protection against challenge inoculation of *Potato virus X* (PVX) vector carrying GFP sequence, and also primary inoculation of PVX vector with GFP induced cross protection against challenge of *Tobacco mosaic virus* (TMV) with GFP. As described above, ALSV is not only a virus with very weak pathogenicity that latently infects most host plant species, but it also efficiently induces systemic VIGS in infected plants. Here, we report the production of recombinant ALSV, in which part of a pathogenic virus (BYMV, ZYMV or CMV) gene was introduced into an ALSV vector (we called an ALSV vector vaccine for the sake of convenience in this paper), and that plants preinoculated with this ALSV vector vaccine showed strong resistance against challenge inoculation with a pathogenic virus as found between TRV-GFP and PVX-GFP, and PVX-GFP and TMV-GFP (Ratcliff et al., 1999). Furthermore, a curative effect can also be expected in virus-infected plants because the disease symptoms subside on subsequent inoculation with an ALSV vaccine.

Results

Preparation of ALSV vector vaccines harboring a genome fragment of BYMV, ZYMV, or CMV

Two ALSV vectors (pEALSR1 and pEALSR2mL5mR5) developed earlier in our laboratory (Li et al., 2004) and pEALSR2mL5mR5-3′MN, which was prepared by inserting a multiple cloning site into the region outside the RNA2 ORF, were used in this study (Fig. 1).

We first constructed two types of ALSV vector vaccines harboring a 240-nt DNA fragment of the P3 region in BYMV-RNA (Table 1). For one, a DNA fragment was inserted between the *Xho* I and *Bam* HI sites, and for the other, the same DNA fragment was inserted in the 3′-noncoding region of ALSV-RNA2 (Fig. 1). These ALSV-RNA2 vectors with pEALSR1 were used to inoculate *Chenopodium quinoa* to produce ALSV vector vaccines [ALSV-BY:P3 and ALSV-BY:P3(−)] (Table 1). These ALSV vector vaccines infected *N. benthamiana* systemically without causing the appearance of any symptoms.

With the aim of producing an ALSV vector vaccine harboring a ZYMV genome fragment, we used pEALSR2mL5mR5 because this vector has been found to induce effective VIGS of several endogenous genes in herbaceous and fruit tree plants (Igarashi et al., 2009; Sasaki et al. 2011). Each region of the ZYMV genome was amplified by PCR (Table 1) and ligated into the *Xho* I and *Bam* HI sites in pEALSR2mL5mR5, as shown in Fig. 1. The resulting ALSV-RNA2 vectors were used to produce 10 ALSV vector vaccine candidates against ZYMV (ALSV-Z:P1, ALSV-Z:Hc-Pro, ALSV-Z:P3, ALSV-Z:Cl, ALSV-Z:NIa, ALSV-Z:Nib, ALSV-Z:CP, ALSV-Z:CP117, ALSV-Z:CP66, and ALSV-Z:CP33) (Table 1). Similarly, three ALSV vector vaccines provided with parts (207–240 nt) of the CMV genome (corresponding to RNA1, 2, and 3; namely ALSV-C:1a, ALSV-C:2a, and ALSV-C:CP) and an ALSV vector vaccine with a ligated chimeric sequence of 150 nt each of the ZYMV and CMV genome fragments (ALSV-ZC300) were produced (Table 1). All these ALSV vector vaccines caused systemic infection of cucumber plants without symptoms (Table 1).

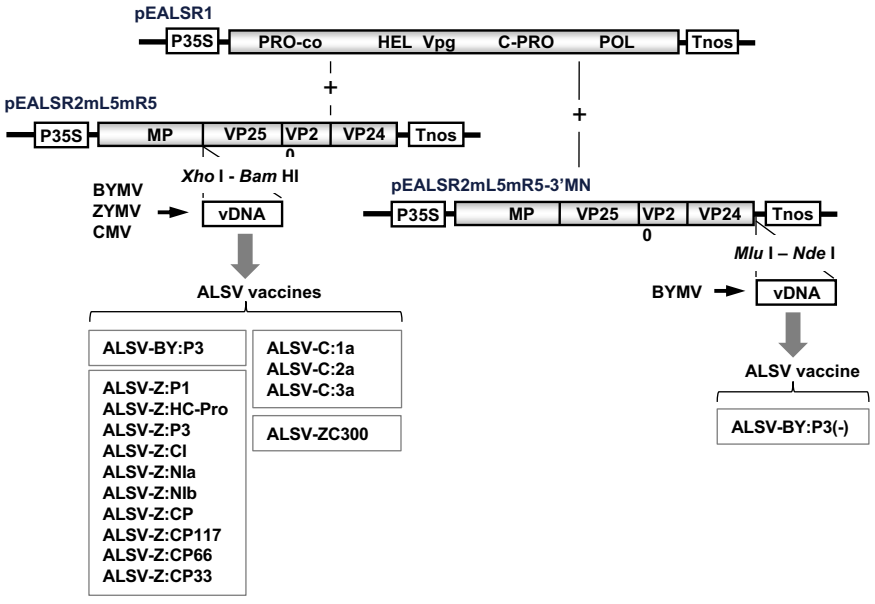

Fig. 1. Construction scheme for ALSV vector vaccines against BYMV, ZYMV, and CMV. DNA fragments amplified from each viral genome were ligated into a cloning site of ALSV-RNA2 vector (pEALSR2mL5mR5 or pEALSR2mL5mR5-3′MN) and used to inoculate *C. quinoa* to produce ALSV vaccines.