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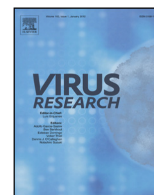

Development of apple latent spherical virus-based vaccines against three tospoviruses

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ABSTRACT

Apple latent spherical virus (ALSV) is characterized by its relatively broad host range, latency in most host plants, and ability to induce gene silencing in host plants. Herein, we focus on the above characteristic of ALSV and describe our development of ALSV vector vaccines against three tospoviruses, namely, *Impatiens necrotic spot virus* (INSV), *Iris yellow spot virus* (IYSV), and *Tomato spotted wilt virus* (TSWV). DNA fragments of 201 nt of three tospovirus S-RNAs (silencing suppressor (NS_S) and nucleocapsid protein (N) coding regions for each tospovirus) were inserted into an ALSV-RNA2 vector to obtain six types of ALSV vector vaccines. *Nicotiana benthamiana* plants at the five-leaf stage were inoculated with each ALSV vector vaccine and challenged with the corresponding tospovirus species. Tospovirus-induced symptoms and tospovirus replication after challenge were significantly suppressed in plants preinoculated with all ALSV vector vaccines having the N region fragment, indicating that strong resistance was acquired after infection with ALSV vector vaccines. On the other hand, cross protection was not significant in plants preinoculated with ALSV vectors having the NSs region fragment. Similarly, inoculation with an ALSV-RNA1 vector having the N region fragment in the 3′-noncoding region, but not the NSs region fragment, induced cross protection, indicating that cross protection is via RNA silencing, not via the function of the protein derived from the N region fragment. Our approach, wherein ALSV vectors and selected target inserts are used, enables rapid establishment of ALSV vector vaccines against many pathogenic RNA viruses with known sequences.

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1. Introduction

Tospoviruses (genus *Tospovirus*, family Bunyaviridae) are RNA viruses with a spherical structure (80–120 nm in diameter) protected by a lipid membrane envelope of host origin (Tsompana and Moyer, 2008). The tospovirus genome consists of three single-stranded RNA segments––L, M, and S––according to size (large, medium, and small, respectively). Each segment codes for different protein: L-RNA for RNA-dependent RNA polymerase (RdRp) involved in virus replication; M-RNA for the viral movement protein (NS_M) and glycoproteins (G_N/G_C); S-RNA for the silencing suppressor (NS_S) and the nucleocapsid protein (N) (Plyusnin et al., 2012). Tospoviruses are among the most distributed plant viruses worldwide, and infected plants show characteristic symptoms such as necrotic ring spots and necrotic spots on fruits and leaves accompanied by shoot apex necrosis (Pappu et al., 2009). Tospovirus infection has a serious impact on the agricultural and horticultural industries in Japan (Kuwabara et al., 2010). Vegetables, flowers, and ornamental plants are most vulnerable, and further damage as a consequence of the expanding distribution of thrips (class Insecta, order Thysanoptera) as vectors is of serious concern. Preventive measures against tospovirus epidemics currently rely on stringent control of infected materials, such as prevention and extermination of thrips and transport prohibition of infected plants (Pappu et al., 2009; Tsompana and Moyer, 2008).

In Japan, cross protection has been studied extensively as a key component of preventive measures against plant viral diseases (Ichiki et al., 2005; Kosaka and Fukunishi, 1997; Kosaka et al., 2006; Wang et al., 2006). Cross protection is the phenomenon whereby the activity of a primary virus, when introduced into the host plant via inoculation, prevents the replication of a subsequent challenge virus. This means that inoculation of plants with an attenuated virus can prevent infection and replication of the corresponding virulent virus through this phenomenon. The first attenuated virus registered as a pesticide in Japan was CUBIO ZY-02 (attenuated *Zucchini yellow mosaic virus* (ZYMV)), which played an important role

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in reducing a ZYMV-caused annual loss of ≥5000 million yen in cucumber crops in Japan (Kosaka et al., 2009). Attenuated *Cucumber mosaic virus* (CMV), which harbors satellite RNA, is also used to protect tomato plants from CMV-caused diseases (Sayama et al., 1993). However, development of an attenuated virus is not always straightforward, and identifying effective isolates that cause cross protection is time consuming and labor intensive. 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.

Two cross protection mechanisms have been suggested: a protein-mediated mechanism involving the coat protein (CP) of the primary virus, and an RNA-mediated mechanism involving RNA silencing induced by the primary virus (Beachy, 1999; Gal-On and Shiboleth, 2006; Lin et al., 2007). RNA silencing is a biological process in which RNA molecules inhibit gene expression in a sequence-dependent manner (Hannon, 2002). This process is conserved in all eukaryotes. In RNA-virus-infected plants, viral dsRNA, produced by RdRp, is processed into small interfering RNA (siRNA, 21–24 nt) by a Dicer-like protein and then incorporated into an RNA-induced silencing complex (RISC). The RISC complex cleaves the target virus RNA bearing the sequence complementary to the incorporated siRNA, thereby suppressing virus infection (Voinnet, 2005). Furthermore, the silencing signal spreads to neighboring cells, which leads to a systemic silencing effect (Mlotshwa et al., 2002; Voinnet and Baulcombe, 1997). Thus, RNA silencing plays a crucial role in defense against viral infection in plants (Baulcombe, 2004; Vance and Vaucheret, 2001; Waterhouse et al., 2001). Unlike animals, plants do not have an immune system. However, this RNA-silencing-based defense mechanism against virus infection has some resemblance to the immune mechanism in animals: dsRNA, siRNA, and the RISC complex in the plant defense system can be compared with antigen, antibody, and killer cells in the animal immune system (Lecellier and Voinnet, 2004; Plasterk, 2002; Voinnet, 2001).

Apple latent spherical virus (ALSV) has spherical virus particles (25 nm in diameter) containing two ssRNA species (RNA1 and RNA2) and three capsid proteins (Vp25, Vp20, and Vp24) (Li et al., 2000). The virus was first isolated from apple plants, but has a wide experimental host range. ALSV causes latent infection in most host plants, including *Arabidopsis thaliana*, a widely used model in plant science, and plants belonging to the genus *Nicotiana* and families Solanaceae (i.e., potato and eggplant), Cucurbitaceae, and Leguminosae, as well as fruit trees of the Rosaceae (Igarashi et al., 2009). In addition, ALSV distribute evenly in the whole plant, thereby inducing systemic virus-induced gene silencing (VIGS), and are thus useful vehicles for introducing VIGS in many plant species used in gene function analysis (Igarashi et al., 2009; Sasaki et al., 2011; Yaegashi et al., 2007; Yamagishi and Yoshikawa, 2009).

On the basis of the above characteristics of ALSV, namely, a broad host range, high latency, and its ability to induce systemic gene silencing, we thought that ALSV vectors would serve as powerful tools in disease prevention in plants. More precisely, we predicted that vaccines against virulent viruses could be prepared by constructing ALSV vectors carrying a genomic fragment of a virulent virus (hereafter referred to as ALSV vector vaccines). Provided that this strategy is effective in exerting protective effects in plants, many vaccines against various viruses can be prepared in a short period, simply by replacing one DNA insert with a different insert derived from a different target RNA virus. In this study, we describe the construction of ALSV vector vaccines against three tospoviruses, *Impatiens necrotic spot virus* (INSV), *Iris Yellow spot virus* (IYSV) and *Tomato spotted wilt virus* (TSWV), and the successful induction of strong cross protection by ALSV vector vaccines upon challenge with virulent viruses.

2. Materials and methods

2.1. ALSV vectors

pEALSR1 (RNA1-based vector) and pEALSR2mL5mR5 (RNA2-based vector) (Fig. 1), previously developed in our laboratory (Li et al., 2004), and pEALSR13′MCS (Fig. 1), which was prepared by inserting a multiple cloning site into the region outside the RNA1 ORF, were used in this study.

2.2. Tospoviruses

An INSV isolate from *Eustoma* in Shiga Prefecture, an IYSV isolate, and TSWV isolate TS-1 were kindly provided by Dr. Shin-ichi Fuji (Faculty of Bioresource Sciences, Akita Prefectural University), Dr. Tomohide Natsuaki (Faculty of Agriculture, Utsunomiya University), and Dr. Yoshitaka Kosaka (Biotechnology Research Department, Kyoto Prefectural Agriculture Forestry and Fisheries Technology Center), respectively.

2.3. Plants

ALSV vector vaccines were propagated in *Chenopodium quinoa* (Li et al., 2000). ALSV vector vaccine-induced cross protection against three tospovirus species was examined in *Nicotiana benthamiana*. Cross protection induced by ALSV vector vaccines against TSWV was also tested in *Eustoma grandiflorum.*

2.4. Construction of ALSV vector vaccines

A 201-nt DNA fragment of the NSs coding region in S-RNA (+ sequence, hereafter referred to as the NSs insert) and a 201-nt DNA fragment of the N coding region in S-RNA (complementary sequence, hereafter referred to as the N insert) were synthesized for each tospovirus (Table 1). These DNA fragments were ligated into pEALSR2mL5mR5 via the *Xho*I and *Bam*HI sites (Fig. 1). A solution (1 μg/μl) of pEALSR2mL5mR5 carrying either the N insert or the NSs insert was mixed with an equal quantity of a pEALSR1 solution (1 μg/μl), and the 3rd, 4th, and 5th true leaves of *C. quinoa* at the six-leaf stage were inoculated with 8 μl of this mixture using the carborundum method (Li et al., 2004). Upper leaves with chlorosis symptoms were collected as the sources of ALSV vaccine vectors. An ALSV vaccine vector carrying the NSs insert derived from INSV and another carrying the N insert derived from INSV were denoted INSV:NSs and INSV:N, respectively. Similarly, vectors carrying the NSs or N insert derived from IYSV were denoted IYSV:NSs or IYSV:N, respectively, and those carrying the NSs or N insert derived from TSWV were denoted TS:NSs and TS:N, respectively.

INSV inserts were also inserted into pEALSR13′MCS. Briefly, DNA fragments including the 201-nt NSs insert or a 201-nt N insert were amplified using the following primer pairs designed according to the S-RNA genomic sequence (accession number AB109100): IN:NSs-SalI (+) primer (TACATGTCGAC$_{1091}$ATTGTCCATTCACTTCTTGA$_{1110}$) and IN:NSs-MluI (−) primer (TACATACGCGT$_{1272}$CGGAAGACTTTTCAAGGTTT$_{1291}$) for the NSs insert; IN:N-SalI (+) primer (TACATGTCGAC$_{2341}$TTCACTGACTTCTTTACCAA$_{2322}$) and IN:N-MluI (−) primer (TACATACGCGT$_{2160}$ATCAAGTCTTCGGAAAGTCA$_{2141}$) for the N insert. The underlined sequences in the primers indicate the corresponding restriction enzyme sites (*Sal*I or *Mlu*I), and the numbers shown in the primer sequences indicate the nucleotide position of the genomic sequence. PCR products were treated with *Sal*I and *Mlu*I (both from TaKaRa, Ohtsu, Japan), and ligated into *Sal*I/*Mlu*I-digested pEALSR13′MCS. The resulting pEALSR13′MCS vector carrying the NSs insert (R1-IN:NSs) and that carrying the N insert (R1-IN:N) were mixed with pEALSR2mL5mR5 for inoculation of *C. quinoa* plants.

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