

Analysis of the Spatial Distribution of Identical and Two Distinct Virus Populations Differently Labeled with Cyan and Yellow Fluorescent Proteins in Coinfected Plants

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ABSTRACT

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Apple latent spherical virus (ALSV) expressing yellow and cyan fluorescent proteins (ALSV-YFP and ALSV-CFP) was used to investigate the distribution of identical virus populations in coinfecting plants. In *Chenopodium quinoa* plants inoculated with a mixture of ALSV-YFP and ALSV-CFP, fluorescence from YFP and CFP was always distributed separately in both inoculated and upper uninoculated leaves. Inoculation of each ALSV-YFP and ALSV-CFP to different leaves of a *C. quinoa*

plant resulted in the separate distribution of each virus population among different upper leaves. When *C. quinoa* leaves were first inoculated with ALSV-CFP and then ALSV-YFP was reinoculated into the same leaves at various times after the first inoculation, ALSV-YFP infected only tissues where ALSV-CFP infection had not been established. The spatial separation was also found in *Nicotiana benthamiana* leaves coinoculated with *Bean yellow mosaic virus* (BYMV)-YFP and BYMV-CFP. In contrast, both YFP and CFP fluorescence signals were observed in the same tissues of *N. benthamiana* leaves mixed infected with ALSV-YFP and BYMV-CFP. YFP fluorescence from ALSV-YFP in mixed-infected leaves was brighter and longer than in leaves infected with ALSV-YFP singly.

In nature, mixed infection of different plant viruses is a common phenomenon in plants, especially in perennial plants like fruit trees. When two or more different viruses have infected the same plants, the viruses may replicate independently, or interact with each other either antagonistically or synergistically, depending on their combinations (6). In addition to mixed infections of different viruses, the virus genome that exists in a single-virus-infected plant is known to be heterogeneous, i.e., a mixture of sequence variants. For example, it is reported that all isolates of *Apple stem grooving virus* from several fruit trees contain at least two to four sequence variants that differ considerably from each other in nucleotide sequence (12,13,28). Similar genome heterogeneity of an infecting virus in a single plant was reported in *Apple chlorotic leaf spot virus* and *Apple stem pitting virus*, and it may be a universal phenomenon in fruit tree viruses (3,26). At present, there is no information on the distribution of sequence variants in infected leaves, e.g., on whether each sequence variant could replicate in the same cells and tissues or could be distributed separately in the different cells and tissues. The molecular marker of green fluorescent protein (GFP) from jellyfish, *Aequorea victoria*, a 27-kDa monomer protein (17), has been widely used for monitoring gene expression and protein localization because it is nontoxic to plants, brightly fluorescent, very stable in cells, and nondestructive (15). GFP has been used to investigate the localization of plant viruses in infected plants because the cell-to-cell and long-distance movement of viruses

can be observed directly in living cells and tissues by using GFP-tagged plant viruses (2,4,14,15,19,25). Furthermore, two different fluorescent proteins can be used for double-tagging techniques to analyze the interaction between two virus strains or two different viruses coinfecting the same plants. It has been recently reported that populations of identical, but differently labeled potyviruses, exhibited spatial separation in mixed-infected plants (5).

Apple latent spherical virus (ALSV) has an isometric virus particle of 25 nm in diameter and is classified into a new genus *Cheravirus* (9). ALSV has been isolated from apple trees in Japan without causing any obvious disease (11). The virus contains two single-stranded RNA species (RNA1 and RNA2) and three capsid proteins (Vp25, Vp20, and Vp24) (9,11). Recently, infectious cDNA clones of ALSV-RNAs were constructed and modified into viral vectors for expressing foreign genes in plants (10). The ALSV vector that expresses GFP has been used for demonstrating that a movement protein and the three capsid proteins are all indispensable for the cell-to-cell movement of the virus (27).

In this study, ALSV vectors expressing cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) were constructed and used to investigate the distribution of identical virus populations in coinfecting plants. We also used *Bean yellow mosaic virus* (BYMV) expressing CFP or YFP to investigate the distribution of different virus populations in mixed-infected plants. The results indicated that identical but differently labeled virus populations (ALSV-YFP and ALSV-CFP or BYMV-YFP and BYMV-CFP) were always distributed separately in coinfecting leaves. On the other hand, infection by different virus combinations (ALSV-YFP and BYMV-CFP) showed that both viruses were distributed in the same tissues of infected leaves. Furthermore, coinfection of ALSV and BYMV resulted in the synergistic effects consisting of severe symptoms and high ALSV accumulation.

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MATERIALS AND METHODS

Construction of the ALSV-RNA2 vectors expressing YFP and CFP. ALSV expressing YFP (ALSV-YFP) or CFP (ALSV-CFP) was constructed from ALSV vectors (pEALSR1 and pEALSR2L5R5) (10). cDNA fragments containing YFP and CFP genes were amplified from pEYFP-1 and pECFP-1 (CLONTECH, Japan), respectively, by polymerase chain reaction (PCR) using EX Taq DNA polymerase (TAKARA, Japan) and primer pairs XhoGFP (5'-CCCTCGAGATGGTGAGCAAGGGCGAGGA-3', containing a *Xho*I site [underlined]) and BamGFP (5'-CGGGATCCCTTGTACAGCTCGTCCA-3', containing a *Bam*HI site [underlined]), respectively (10). The amplified cDNA fragments of YFP and CFP genes were double-digested with *Xho*I and *Bam*HI, and ligated to pEALSR2L5R5 restricted with the same enzymes. The resulting pEALSR2-based vectors containing YFP or CFP genes were designated pEALSR2L5R5YFP or pEALSR2L5R5CFP, respectively (Fig. 1A).

Construction of the BYMV-based vectors expressing YFP and CFP. CFP and YFP gene fragments were produced by PCR using two primers, Xba-EGFP (5'-CTAGTCTAGAAATGGTGA-GCAAGGGCGAGGAGCTG-3', containing a *Xba*I site [underlined]) and EGFP-Nhe (5'-CTAGCTAGCCTTGTACAGCTCGT-CCATGCCGAGAG-3', containing a *Nhe*I site [underlined]) as described before. Each of the amplified products was digested with *Xba*I and *Nhe*I sites of pIbG:Vec (23). The resulting pIbG:Vec containing CFP or YFP genes was designated pIbG:CFP and pIbG:YFP, respectively (Fig. 1B).

Viral inoculation. Both pEALSR2L5R5YFP and pEALSR2L5R5CFP were purified from large-scale cultures of *Escherichia coli* DH5a using a Qiagen plasmid mini kit (Qiagen, Japan). Each plasmid was mixed with purified pEALSR1 at a concentration of 1 µg/µl each, and mechanically inoculated to leaves of *Chenopodium quinoa* (6 true leaf stage). After a week, upper leaves (0.1 g) with symptoms were homogenized in 0.4 ml of inoculation buffer (0.1 M Tris-HCl [pH 7.8], 0.1 M NaCl, and 5 mM MgCl₂·6H₂O) and then mechanically inoculated to host plant leaves.

In the case of BYMV-based vectors, pIbG:FL, pIbG:YFP, and pIbG:CFP were inoculated to *Nicotiana benthamiana* by a PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA) as described previously (21,22). After a week, upper leaves (0.1 g)

with mild mosaic symptoms were homogenized in 0.4 ml of 0.1 M phosphate buffer (pH 7.0) and then mechanically inoculated to *N. benthamiana* leaves.

Fluorescence microscopy. Infected leaves of *C. quinoa* and *N. benthamiana* plants were observed under a fluorescent stereoscopic microscope (KEYENCE VB-G25, Japan) with the following filter sets: CFP-B filter sets (excitation filter 436/20 nm, and barrier filter 480/30 nm), and YFP-B filter sets (excitation filter 490/20 nm, and barrier filter 540/40 nm). Digital micrographs were taken with a digital camera (Olympus DP70, Olympus, Japan). The images acquired with CFP and YFP filters were superimposed using Photoshop (Adobe).

ELISA. Virus accumulations in inoculated leaves were assessed by an enzyme-linked immunosorbent assay (ELISA). Infected samples were homogenized with carbonate-bicarbonate buffer, pH 9.6, and trapped for 1 h at 37°C in ELISA plates. After incubation, the antibodies against ALSV or BYMV coat proteins diluted to 1/2,000 in Tris-buffered saline containing Tween 20 (TTBS) were added and incubated overnight at 4°C. The goat anti-rabbit immunoglobulin G, diluted to 1/2,000 in TTBS, was added and incubated for 1 h at 37°C. Finally, *p*-nitrophenylphosphate (1 mg/ml) in 10% diethanolamine, pH 9.6, was added, and the absorbance was measured with a microplate reader model 550 (Bio-Rad).

RESULTS

Distribution of identical but differently labeled virus populations in coinoculated plants. The mixtures of ALSV-CFP and ALSV-YFP were mechanically inoculated to leaves of *C. quinoa* plants, and the inoculated leaves were observed under a fluorescent microscope with the filters for CFP or YFP. Both CFP and YFP fluorescent spots first appeared on inoculated leaves at 3 days postinoculation (dpi) (Fig. 2A-(i)). Fluorescence spots were then enlarged as ring-shaped ones after 5 dpi (Fig. 2A-(ii) and (iii)) consistent with those of ALSV-GFP infection (27). When CFP images were superimposed on YFP images, fluorescence spots of CFP and YFP were always separated and never overlapped each other in coinoculated leaves, even if fluorescence spread over the whole area of leaves (Fig. 2A-(iii)). In upper uninoculated leaves, CFP and YFP fluorescence was initially found on veins and then spread over the whole area of leaf tissues.

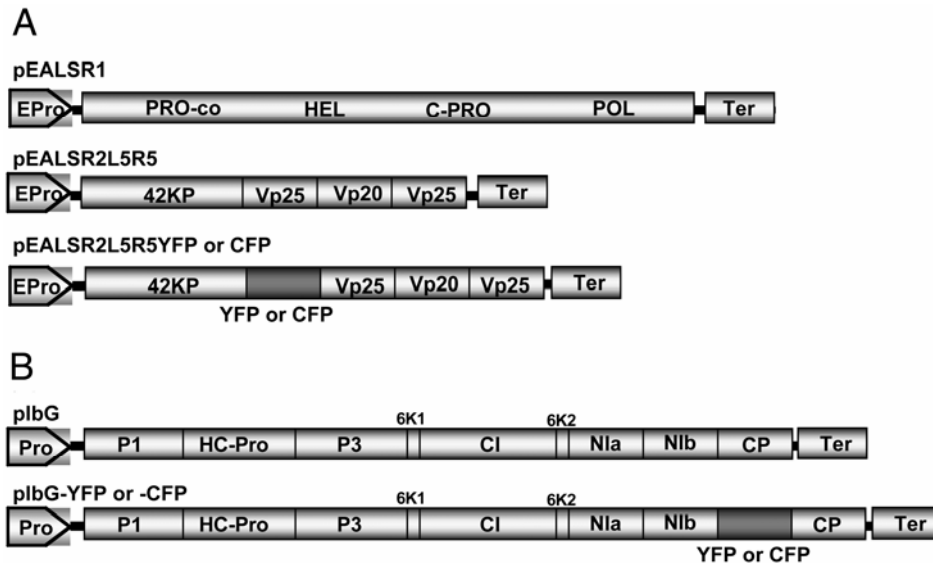


Fig. 1. Schematic representation of full-length infectious cDNA clones of the **A**, *Apple latent spherical virus* (ALSV) and **B**, *Bean yellow mosaic virus* (BYMV)-based vectors. Cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) genes were inserted between 42KP and Vp25 genes of the ALSV vector, and between nuclear inclusion b (Nib) and coat protein (CP) genes of BYMV vector. EPro, enhanced CaMV 35S promoter; Pro, CaMV 35S promoter; Ter, nopaline synthase terminator.

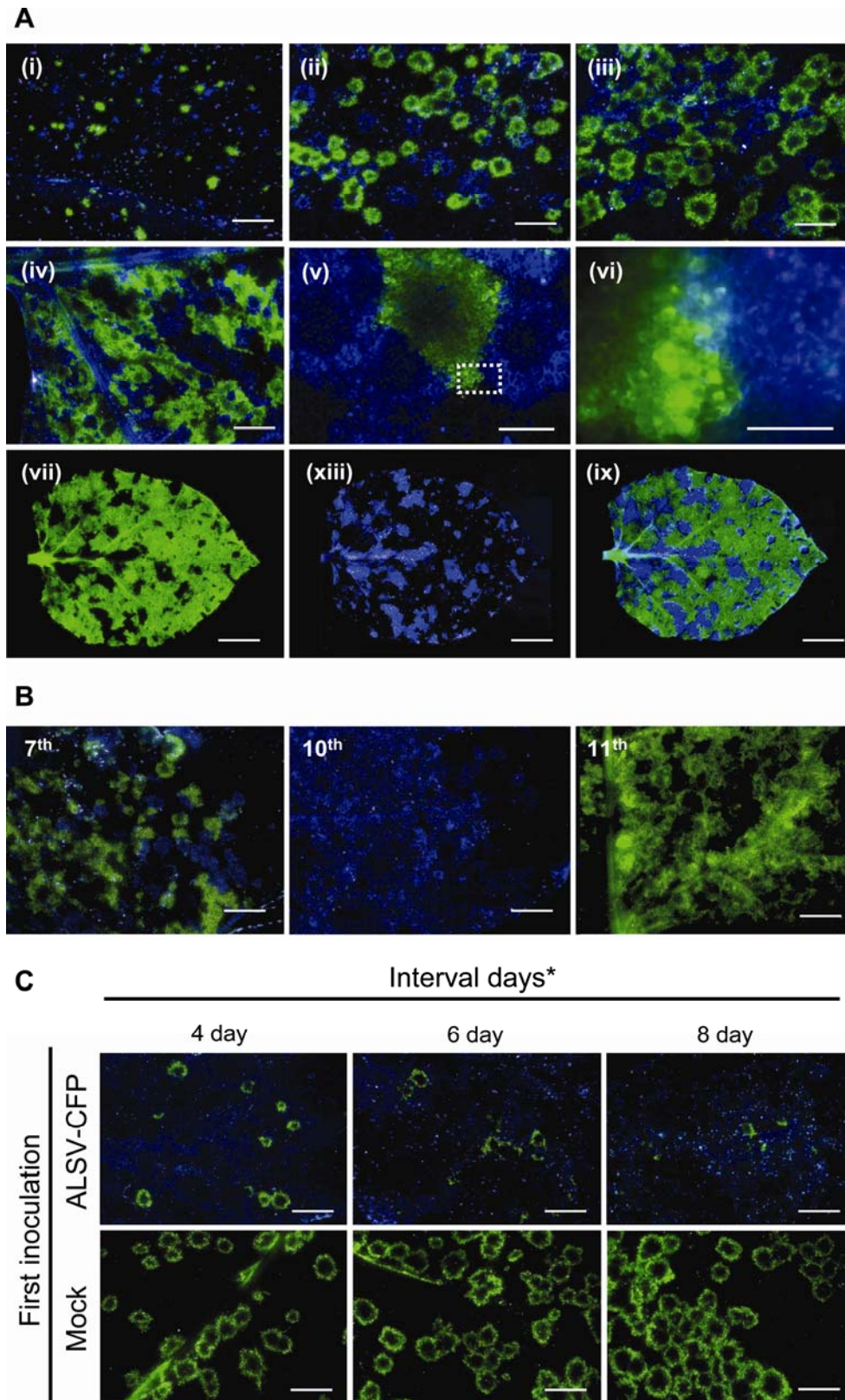


Fig. 2. Analysis of the distribution of identical, but differently labeled virus populations in coinoculated plants. **A**, The fluorescence of cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) on inoculated leaves at 3 days postinoculation (dpi) (i), 5 dpi (ii), 6 dpi (iii), and upper uninoculated leaves at 11 dpi (iv, v, and vi) of *Chenopodium quinoa* plants infected with *Apple latent spherical virus* expressing CFP (ALSV-CFP) and YFP (ALSV-YFP). A border area between populations of ALSV-YFP and ALSV-CFP in (v) is shown in (vi). The fluorescence of YFP (vii) and CFP (viii) on an upper leaf of *Nicotiana benthamiana* mixed-infected with *Bean yellow mosaic virus* expressing CFP (BYMV-CFP) and BYMV-YFP. The picture (ix) is a merged image of (vii) and (viii). **B**, Distributions of ALSV-CFP and ALSV-YFP among different upper leaves of a *C. quinoa* plant at 10 dpi. A *C. quinoa* plant was inoculated with ALSV-CFP to the 3rd and 5th true leaves and ALSV-YFP to the 4th and 6th leaves. Both YFP and CFP fluorescence signals were detected in a 7th leaf (left panel). CFP and YFP were separately distributed on the 10th (center panel) and on the 11th leaves (right panel), respectively. **C**, YFP spots at 7 days after ALSV-YFP inoculation on *C. quinoa* leaves that were first inoculated with ALSV-CFP or buffer only (Mock), and then secondly inoculated with ALSV-YFP at 4, 6, or 8 days after first inoculation. Bars: **A**, 2 mm in (i) to (iv), 500 μ m in (v), 200 μ m in (vi), and 5 mm in (vii) to (ix); **B and C**, 2 mm.

Fluorescence signals of CFP and YFP in upper leaves were also distributed separately and never overlapped each other (Fig. 2A-(iv)). Observation of the adjacent regions between CFP and YFP fluorescence showed that only one or two cell layers of the border might have contained both CFP and YFP fluorescence (Fig. 2A-(v) and (vi)). When a mixture of BYMV-CFP and BYMV-YFP

was inoculated to *N. benthamiana* plant, spatial separation of virus populations was also found in upper leaves (Fig. 2A-(vii), (viii), and (ix)). These results indicated that populations of identical, but differently labeled viruses (ALSV-CFP and ALSV-YFP or BYMV-CFP and BYMV-YFP), replicated predominantly in discrete areas and remained distributed separately. Our data

TABLE 1. Interference between *Apple latent spherical virus* expressing cyan fluorescent proteins (ALSV-CFP) and ALSV expressing yellow fluorescent proteins (YFP) on *Chenopodium quinoa* leaves which were first inoculated with ALSV-CFP and then with ALSV-YFP at different intervals

Interval days between first and second inoculation	Source of first inoculation	Numbers of YFP spots ^a			
		Leaf positions			
		4th	5th	6th	7th
4	Mock	135	193	1,002	511
	ALSV-CFP	40	58	89	112
6	Mock	445	496	545	1,202
	ALSV-CFP	130	169	166	137
8	Mock	262	366	750	1,447
	ALSV-CFP	41	41	12	0

^a The sum of YFP spots per two leaves counted at 7 days after ALSV-YFP inoculation.

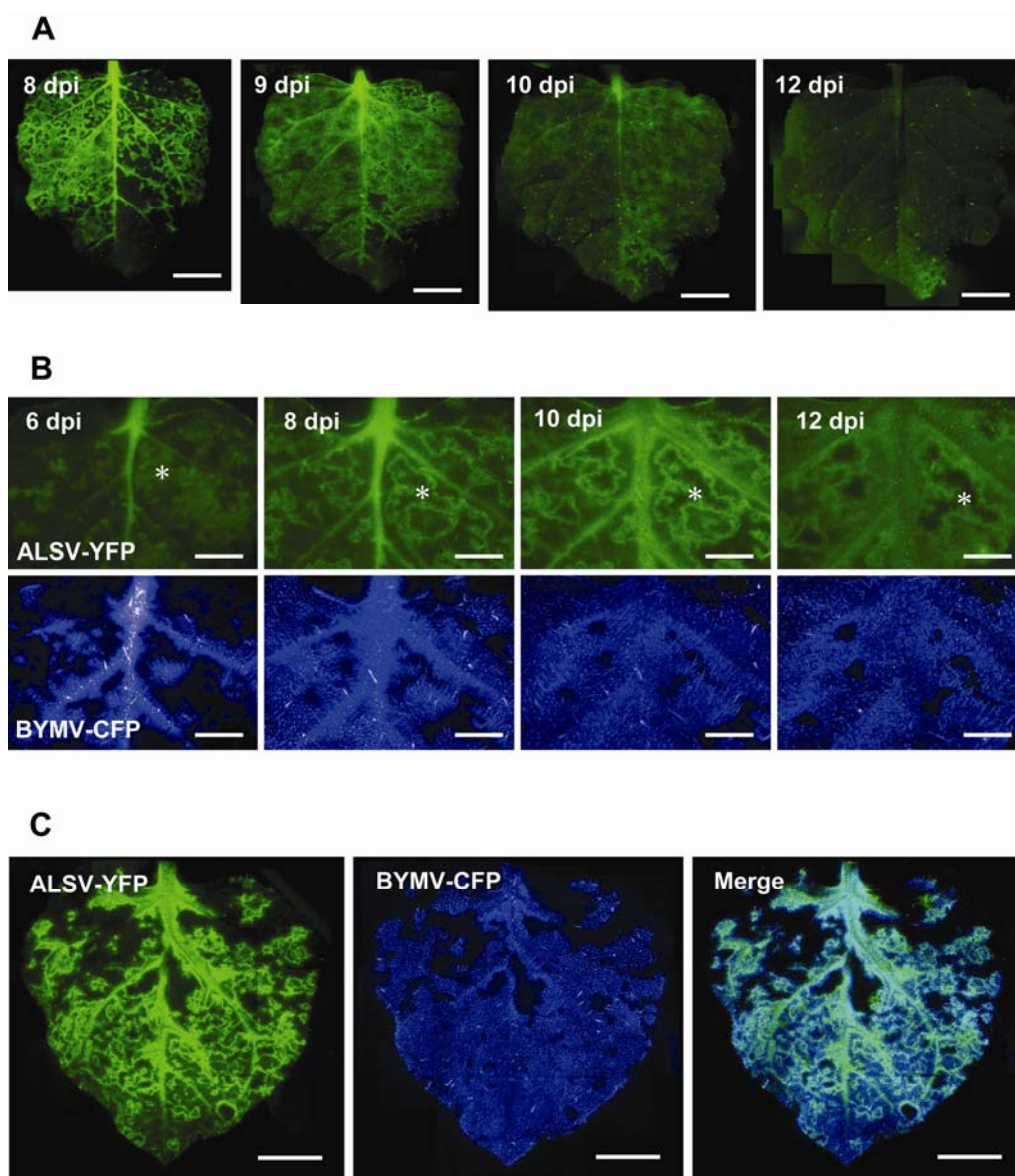


Fig. 3. Distribution of *Apple latent spherical virus* expressing yellow fluorescent protein (ALSV-YFP) and *Bean yellow mosaic virus* expressing cyan fluorescent protein (BYMV-CFP) in coinfecting plants. **A**, YFP fluorescence on the same inoculated leaf of an *Nicotiana benthamiana* plant infected with ALSV-YFP at 8, 9, 10, and 12 days postinoculation (dpi). **B**, Distribution of YFP and CFP of an upper leaf of an *N. benthamiana* plant coinfecting with ALSV-YFP and BYMV-CFP. The same uninoculated upper leaf was photographed at 6, 8, 10, and 12 dpi. Asterisks indicate the same position. **C**, Distribution of YFP and CFP in an upper leaf of *N. benthamiana* coinfecting with ALSV-YFP and BYMV-CFP at 12 dpi. Bars: **A and C**, 5 mm, and **B**, 2 mm.

were consistent with the spatial separation of three different potyviruses and a *Potato virus X* (PVX) population in mixed-infected *N. benthamiana* plants (5).

Separate distribution of ALSV-CFP and ALSV-YFP among different upper leaves. In the next experiments, ALSV-CFP and ALSV-YFP were separately inoculated to the 3rd and 5th true leaves and to the 4th and 6th true leaves of *C. quinoa* plants (7 true leaf stage), respectively. Observation by fluorescent microscope showed that both CFP and YFP fluorescence signals were found in a separated area in a 7th true leaf at 10 dpi (Fig. 2B). However, in the 8th to 15th true leaves after 10 dpi, each CFP and YFP fluorescence was observed in different leaves, i.e., ALSV-CFP was distributed in the 8th, 10th, 12th, and 14th leaves, and ALSV-YFP was detected in the 9th, 11th, 13th, and 15th leaves (Fig. 2B). The experiment was repeated two times and the results were essentially identical. These results clearly indicated that the spatial separation of ALSV-CFP and ALSV-YFP occurred among different leaves of infected *C. quinoa* plants.

Interference between ALSV-CFP and ALSV-YFP. To investigate the interference between ALSV-CFP and ALSV-YFP in infected leaves, ALSV-CFP was first inoculated to the 4th to 7th true leaves of *C. quinoa* plants (8 true leaf stage), and secondly ALSV-YFP was inoculated to the same leaves at 4, 6, and 8 days after first inoculation. Other sets of *C. quinoa* plants were inoculated with buffer only (mock) followed by a second inoculation with ALSV-YFP. The development and distribution of each virus population in inoculated leaves were observed at 7 days after a second inoculation by fluorescent microscope. The YFP fluorescence with roughly circular dimensions (YFP spots) was observed in all cases of mock-inoculated leaves (Fig. 2C; Table 1). The sizes of YFP spots in mock-inoculated leaves were almost identical irrespective of the interval days between first and second inoculation (Fig. 2C). In contrast, YFP spots on leaves preinoculated with ALSV-CFP were always fewer in number and smaller in size than those on mock-inoculated leaves, and these YFP spots were only found in tissue areas where CFP fluorescence was not observed (Fig. 2C). When the interval between the first and second inoculations was 8 dpi, ALSV-YFP infection was extremely reduced in the 6th and 7th leaves compared with those in mock-inoculated leaves (Table 1). The results indicated that pre-infection of ALSV-CFP interferes with effective infection of ALSV-YFP. In other words, ALSV-YFP could not infect the tissue area where ALSV-CFP infection had already been established.

Distribution of different virus populations in coinfecting plants. When *N. benthamiana* plants were inoculated with ALSV-YFP, the YFP fluorescence was observed initially on veins in upper leaves at 5 dpi and then found on the whole tissues of upper leaves (Fig. 3A). However, the intensity of YFP fluorescence was

so weak compared with that in infected *C. quinoa* plants, and the fluorescence rapidly disappeared after 10 dpi (Fig. 3A). When ALSV-YFP and BYMV-CFP were mechanically inoculated to each half leaf of *N. benthamiana*, only a few YFP and CFP spots were found in inoculated leaves at 3 dpi, and the intensity of fluorescence was too weak to analyze their distribution in inoculated leaves. In upper leaves, CFP and YFP fluorescence signals were detected initially on veins at 5 dpi (data not shown) and then spread to other area of leaves at 6 to 12 dpi (Fig. 3B). The YFP fluorescence in mixed-infected leaves was always brighter and maintained longer than in leaves infected with ALSV-YFP singly. In Figure 3, the exposure time for YFP fluorescence in a leaf infected with ALSV-YFP singly (Fig. 3A) was four times longer than those in a leaf mixed infected with ALSV-YFP and BYMV-CFP (Fig. 3B). The distribution pattern of CFP fluorescence almost coincided with that of YFP fluorescence, strongly suggesting that ALSV-YFP and BYMV-CFP could exist in the same tissues in upper leaves of *N. benthamiana* plants (Fig. 3C).

Synergistic interaction between ALSV and BYMV. The accumulation levels of ALSV and BYMV in the inoculated and uninoculated upper leaves of *N. benthamiana* plants infected with a single virus or two viruses were examined by ELISA at 14 dpi. ALSV accumulation levels in leaves doubly infected with ALSV and BYMV were two to threefold higher than those in leaves infected with ALSV singly (Fig. 4). On the other hand, there were no differences in BYMV accumulation levels between BYMV-infected leaves and the two virus-infected (ALSV and BYMV) leaves (Fig. 4).

To investigate why ALSV accumulation levels were higher in mixed-infected plants than those in single-infected plants, the helper component proteinase (HC-Pro) sequence of BYMV was ligated to the region between the 42K and Vp25-encoding sequences of the ALSV-RNA2 vector genome (Fig. 1A), and the resulting ALSV-BYHCPro was inoculated to *C. quinoa* plants (8 true leaf stage) by mechanical inoculation. Infected *C. quinoa* plants showed more severe mosaic symptoms in the upper leaves at 12 to 14 dpi (data not shown) compared with those in plants infected with ALSV. ELISA of inoculated leaves at 14 dpi indicated that the accumulation level of ALSV-BYHCPro was two to three times higher than that of ALSV (data not shown), strongly suggesting that the HC-Pro of BYMV might be a factor for enhancement of ALSV accumulation levels in mixed-infected plants.

DISCUSSION

The results presented in this paper showed that populations of identical, but differently labeled viruses (ALSV-CFP and ALSV-YFP or BYMV-CFP and BYMV-YFP), replicated predominantly

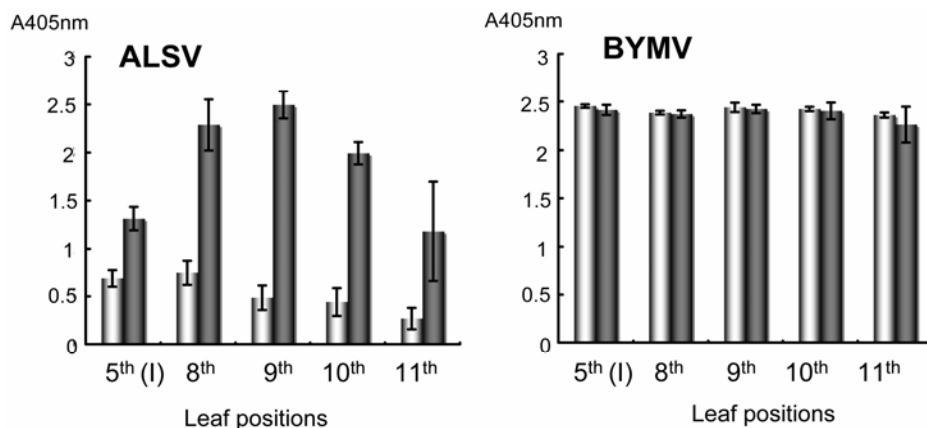


Fig. 4. Enzyme-linked immunosorbent assay (ELISA) of the accumulation of *Apple latent spherical virus* (ALSV) and *Bean yellow mosaic virus* (BYMV) in *Nicotiana benthamiana* leaves infected with a single virus (light gray columns) or two viruses (dark gray columns). Inoculated leaves (5th true leaves) and upper uninoculated leaves (8th to 11th true leaves) were assayed at 14 days postinoculation.

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LITERATURE CITED

in discrete areas and remained separately distributed in both inoculated and upper uninoculated leaves in coinoculated plants. This was consistent with the spatial separation of three different potyviruses and PVX using viruses differently labeled with GFP and DsRed (5). The separated distributions of identical viruses in coinfecting leaves were also analyzed by electron microscopy for *Alfalfa mosaic virus* and in situ hybridization for *Cucumber mosaic virus* (7,24). Thus, the spatial separation between populations of identical viruses and strains in infected plants might be a common phenomenon, though the mechanism(s) of the spatial separation is still unknown.

In plants infected with ALSV-YFP and ALSV-CFP, both YFP and CFP fluorescence signals were observed in a few border cells between two different fluorescent cell clusters (Fig. 2A-(v) and (vi)). A similar observation was reported in infected tissues with populations of identical but different labeled potyviruses at border areas of two neighboring, different colored cell clusters (5). The border cells containing both YFP and CFP fluorescence signals might have been the cells in which ALSV-YFP and ALSV-CFP initiated infection to simultaneously, because a secondary virus could not infect the cells where a primary virus infection has already been established as discussed below.

In our studies, the spatial separation of ALSV-CFP and ALSV-YFP also occurred among leaves of infected *C. quinoa* plants when ALSV-CFP and ALSV-YFP were separately inoculated to different leaves of plants (Fig. 2B). In other words, the spatial separation occurs not only at the cellular level in nonvascular tissues but also within the vascular tissue in infected *C. quinoa* plants. It is thought that ALSV-CFP replicated in the 3rd and 5th true leaves might have moved to upper leaves through phloem bundles differently from those utilized by ALSV-YFP in the 4th and 6th true leaves. When ALSV-CFP moved to the upper leaves faster than ALSV-YFP and established the infection in vascular tissues, the slower migrating virus (ALSV-YFP) might not have been able to infect the upper leaves.

When leaves of *C. quinoa* plants were first inoculated with ALSV-CFP, and then ALSV-YFP was secondly inoculated to the same leaves, ALSV-YFP infection was strictly restricted to the tissues where ALSV-CFP infection had not been established (Fig. 2C). The results indicated that a secondary virus (ALSV-YFP) could not infect the tissues where a primary virus (ALSV-CFP) infection had been established. The spatial separation and interference between ALSV-CFP and ALSV-YFP in this paper is thought to indicate visually the phenomenon of cross-protection (16).

In contrast, both YFP and CFP fluorescence signals were observed in the same area of *N. benthamiana* leaves mixed infected with ALSV-YFP and BYMV-CFP (Fig. 3B and C), strongly suggesting that both viruses were replicated in the same tissues of infected leaves. In addition, YFP fluorescence in leaves mixed infected with ALSV-YFP and BYMV-CFP was brighter than in leaves infected with ALSV-YFP singly, and it also remained longer than that in single infection. The enhancement of YFP fluorescence is thought to be due to the increase of ALSV-YFP accumulation in mixed-infected leaves, because the accumulation level of ALSV in leaves infected with ALSV and BYMV was two- to threefold higher than in leaves infected with ALSV singly (Fig 4). These results indicated that the synergistic interaction between ALSV and BYMV occurred in mixed-infected *N. benthamiana* plants as reported for other members of the genus *Potyvirus* (18,20). The synergistic interaction between ALSV and BYMV reported here is thought to be caused by the suppression of posttranscriptional gene silencing with the HC-Pro encoded by the BYMV genome (1,8). This was clearly demonstrated by the inoculation of *C. quinoa* plants with ALSV-BYHCPro containing a BYMV HC-Pro gene in which the accumulation level of ALSV-BYHCPro was two- to threefold higher than that in leaves infected with ALSV (data not shown).

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