Transient expression of the coat protein of *Apple chlorotic leaf spot virus* inhibits the viral RNA accumulation in *Nicotiana occidentalis*

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Abstract

The coat protein of *Apple chlorotic leaf spot virus* (ACLSV-CP) plays a crucial role in infectivity and efficient viral RNA accumulation in host cells (J. en. Virol, 88, 2007). In this study, the effect of ACLSV-CP on viral RNA accumulation in *Nicotiana occidentalis* was investigated. The CP, CPm40 (an amino acid (aa) substitution of Ala to Ser at aa position 40), CPm75 (a substitution of Phe to Tyr at aa position 75), and CPm40m75 (two aa substitutions at positions 40 and 75) of ACLSV (P205) were transiently expressed in *N. occidentalis* leaves by agroinfiltration. Immunoblot analysis showed that CP and CPm40m75 accumulated in infiltrated tissues, in contrast to CPm40 and CPm75 which were not detected, suggesting that the stable accumulation of CP is important for effective viral RNA accumulation. However, co-agroinfiltration of an infectious ACLSV cDNA clone (pBICLSF) or pBICLSF-based CP mutants (pBICLCPm40, pBICLCPm75, and pBICLCPm40m75) with a vector expressing CP (pBE2113-CP) showed no viral genomic RNA accumulations were found in any leaves infiltrated with these constructs. The inhibition of ACLSV-RNA accumulation was found only in leaves co-expressed with CP protein, but not with a frame-shift mutant of CP, a movement protein (P50), and a frame-shift mutant of P50.

Keyword: *Apple chlorotic leaf spot virus*, coat protein, protein stability, coat protein mediated resistance (CP-MR), agroinfiltration

Introduction

Apple chlorotic leaf spot virus (ACLSV) is distributed world-wide and is known to infect *Rosaceae* fruit tree species, including apple, peach, pear, plum, cherry, and apricot (Lister, 1970; Martelli et al., 1994; Yoshikawa, 2001). ACLSV is one of the causative agents of apple top-working disease in Japan (Yanase, 1973). The virus is classified into the *Trichovirus* genus, *Flexiviridae* family, and has filamentous particles approximately 600–700 nm in length, which contain a single-stranded, plus-sense RNA and multiple copies of a single coat protein of 21 KDa (CP) (Yoshikawa & Takahashi, 1988). Complete nucleotide sequences have been reported for isolates P863 and PBM1 from plum, the BAL1 from cherry, and P205, A4, B6, and MO5 from apple (German et al., 1990 & 1997; Sato *et al*., 1993; Yaegashi et al., 2007b). The genome of an apple isolate of ACLSV (P-205) consists of 7552 nucleotide (nt) and encodes three genes, including a 216 KDa replication-associated protein (Rep), a 50 KDa movement protein (P50), and a CP (Sato et al., 1993).

Based on the phylogenic analysis of CP amino acid sequences from ACLSV isolates from apple trees in Japan, we showed that ACLSV isolates are separated into two major clusters in which five amino acids at positions 40, 59, 75, 130, and 184 were highly conserved into Ala^{40}-Val^{59}-Phe^{75}-Ser^{130}-Met^{184} or Ser^{40}-Leu^{59}-Tyr^{75}-Thr^{130}-Leu^{184} within each cluster. Furthermore, we showed that the combinations of two amino acids at the positions 40 and 75 are crucial for effective virus replication in host plant cells, suggesting that ACLSV-CP plays important roles for effective viral replication, in addition to virion assembly (Yaegashi et al., 2007b).

In this study, we examined the stability of mutant CP with an amino acid substitution (CPm40; Ala to Ser at position 40, CPm75; Phe to Tyr at position 75; which is fatal to viral infectivity and replication) by agroinfiltration. The results showed the marked reduction of protein stability of CPm40 and CPm75, indicating that the stable accumulation of CP is important for effective viral RNA accumulation. On the other hand, we also showed that transient expression of ACLSV-CP inhibits viral RNA accumulation in cells. Our data showed that there are two conflicting roles of CP related to the ACLSV replication cycle.

Materials and methods

Plasmid: The binary plasmid, pBE2113-P35T (empty vector), pBE2113-CP (an expression vector for wild-type CP), pBE2113-FSCP (an expression vector for a frame-shift mutant of CP mRNA), pBE2113-P50 (an expression vector for P50), and pBE2113-FSP50 (an expression vector for a frame-shift mutant of P50 mRNA) were described previously (Yaegashi et al., 2007a; Yoshikawa et al., 2000). To construct binary vectors for expression of CPm40 (an amino acid (aa) substitution of Ala to Ser at aa position 40), CPm75 (a substitution of Phe to Tyr at aa position 75), and CPm40m75 (two aa substitutions at positions 40 and 75), each cDNA were amplified from pCPm40, pCPm75, or pCPm40m75 (Yaegashi et al., 2007b) by polymerase chain reaction (PCR) with KOD plus (TOYOBO) and two primers; ACCPBam (+)(5'-CGCGGATCCATGGCGGCGGCAGTGCTGAAC-3', *Bam*HI site is underlined) and ACCP(-) (5'-ACTAAACGCAAAGATCAG-3'). These PCR products were double-digested with *Bam*HI and *Sac*I, and replaced with a corresponding region of pBE2113-CP. The resulting plasmids were denoted as pBE2113-CPm40, pBE2113-CPm75, or pBE2113-CPm40m75, respectively. The cDNA clones of ACLSV-P205 (pBICLSF) and its mutants (pBICL⊿Rep, pBICLCPstop, pBICLCPm40, pBICLCPm75, pBICLCPm40m75) were described previously (Yaegashi et al., 2007b). All constructs were introduced into *Agrobacterium tumefaciens* strain C58C1 by a freeze-thaw method.

Agroinfiltration: The agroinfiltration method was described previously (Yaegashi et al., 2007a & b). The 5th and 6th leaves of the 7-8 leaf stage *Nicotiana occidentalis* plants were used for agroinfiltration. The agrobacteria suspensions were prepared at OD_{600}=2.0. When two agrobacteria containing two different constructs were co-infiltrated, equal volumes of each suspension were mixed prior to infiltration. The infiltrated plants were kept at 25 ℃.

Immunoblot analysis: Total protein samples from *N. occidentalis* leaves were electrophoresed in a 12.5% polyacrylamide-SDS gel and transferred electrophoretically to a PVDF membrane (Millipore). The membrane was incubated with an antiserum against ACLSV particles, followed by an anti-rabbit IgG (H&L)-alkaline phosphatase linked antibody (Cell Signaling) and immersed in development solution containing a Fast Red TR salt (Sigma) and a naphthol AS-MX phosphate (Sigma).

Northern blot analysis: For Northern blot analysis, denatured RNAs were separated on the 1% agarose gel containing 6 % formaldehyde and transferred to Hybond N+ membrane (GE healthcare). After UV-crosslinking, the membrane was hybridized with a digoxigenin (DIG) labeled RNA probe complementary to plus-strand RNA containing the CP coding region (nt positions 6888 to 7552). The hybridized membrane was immunodetected with an anti-DIG Fab fragment coupled to alkaline phosphatase (Roche), and visualized with a chemiluminescent substrate, CDP-star (GE Healthcare) on X-ray films.

Results and discussion

Stable accumulation of CP may be important for viral RNA accumulation: We already showed that an amino acid substitution of Ala to Ser at aa position 40 or Phe to Tyr at aa position 75 is fatal to viral infectivity and effective RNA replication (Yaegashi et al., 2007b). To analyze whether these amino acid substitutions have an effect on the stability of CP mutants, CP, CPm40, CPm75, and CPm40m75 (two aa substitutions at positions 40 and 75) were transiently expressed in *N. occidentalis* leaves by agroinfiltration with agrobacteria carrying pBE2113-CP, pBE2113-CPm40, pBE2113-CPm75, and pBE2113-CPm40m75, respectively (Fig. 1A).

To analyze the accumulation level of CP and CP-mRNA, infiltrated leaves were collected at 24, 48, or 72 days post infiltration (dpif). Immunoblot analysis showed that both CP and CPm40m75 accumulated in infiltrated tissues at 24, 48, or 72 dpif, in contrast to proteins of CPm40 and CPm75 which were not detected (Fig. 1B, top panel). On the other hand, northern blot analysis showed that there was no obvious difference in the accumulation levels of CP-mRNA among CP, CPm40, CPm75, and CPm40m75 (Fig. 1B, bottom panel).

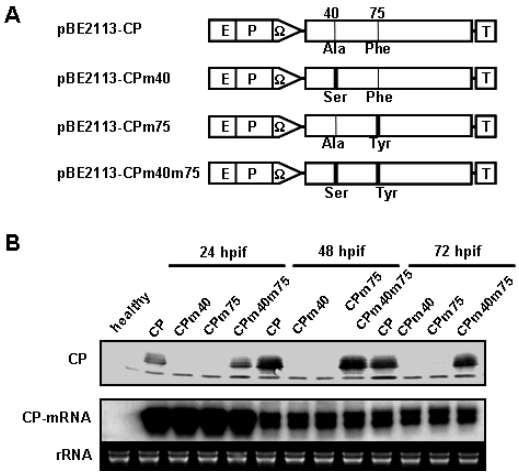

Fig. 1 Transient expression of mutant CPs in *Nicotiana occidentalis* by agroinfiltration. (A) Schematic representation of Ti plasmid constructs expressing CP and mutant CPs (CPm40, CPm75, CPm40m75). The pentagon labeled E, P, and Ω represents the transcriptional enhancer, 35S promoter of *Cauliflower mosaic virus* (CaMV), and the translational enhancer of the *Tobacco mosaic virus* 5'untranslated region, respectively. The box labeled T represents the nopaline synthase terminator. Bold lines in the CP boxes indicate the position of amino acid substitutions from Ala to Ser at 40 or Phe to Tyr at 75. (B) Immunoblot analysis of mutant CPs (Top panel) and northern blot analysis of CP-mRNA (bottom panel) in *N. occidentalis* leaves infiltrated with agrobacteria carrying a vector shown in (A) at 24, 48, 72 hours post infiltration (hpif). Ethidium bromide stainings of rRNA are shown as a loading control for northern blot analysis.

These results indicated that an amino acid substitution at aa position 40 or at aa position 75 reduces CP stability in *N. occidentalis*, and that the stable accumulation of CP is important for effective viral RNA replication and accumulation. It is not clear why CPm40 and CPm75 could not accumulate in *N. occidentalis* cells. The chemical structures of Ala and Phe are similar to those of Ser and Tyr, respectively, although the latter have no hydroxyl group in a side chain. Possibly, both CPm40 and CPm70 may not be able to form a highly stable structure in plant cells.

Transient expression of CP inhibits viral RNA accumulation: The results described above suggested that the reduction of viral replication of pBICLCPm40 and pBICLCPm75 in host cells may be due to instability of mutant CPs. Thus, we speculated that the stable accumulation of CP may enable pBICLCPm40 and pBICLCPm75 to replicate effectively in host cells. To test this hypothesis, agrobacteria containing a plasmid vector expressing CP (pBE2113-CP) and each pBICLSF based mutant shown in Fig. 2A were co-infiltrated into leaves of *N. occidentalis*. Northern blot analysis showed that viral genomic RNA did not accumulate in leaves infiltrated with a mixture of agrobacteria carrying pBE2113-CP and pBICL⁄rep, pBICLCPstop, pBICLCPm40, or PBICLCPm75 at 4 dpif (Fig. 2B). Similar results were obtained from northern blot analysis of viral genomic RNA in leaves co-infiltrated with a mixture of agrobacteria carrying pBE2113-P35T or pBE2113-P50 and each of the pBICLSF-based mutants (data not shown). These results indicated that the transient expression of CP could not complement the replication of pBICLCPm40 and pBICLCPm75. Intriguingly, when the leaves were co-infiltrated with a mixture of agrobacteria carrying pBE2113-CP and pBICLSF or pBICLCPm40m75, the accumulation levels of viral genomic RNA from pBICLSF and pBICLCPm40m75 were lower than that in leaves co-infiltrated with a mixture of agrobacteria carrying pBE2113-P35T and pBICLSF (Fig. 2B). The result supports a previous report showing that CP-expressing transgenic *N. occidentalis* was resistant to ACLSV (Yoshikawa et al., 2000). In the present study, we used an agroinfiltration method for viral inoculation which allowed us to analyze the RNA accumulation level in one cell (Voinnet et al., 2000; Yaegashi et al., 2007b). Therefore, we think the inhibitory effects of CP on ACLSV infection might be accounted for by the reduction of viral RNA accumulation in a cell.

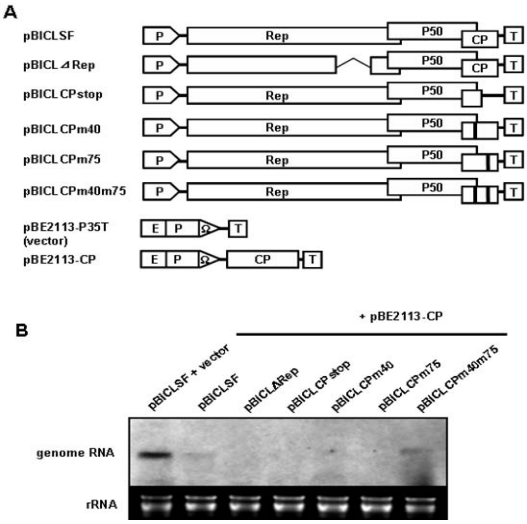

Fig. 2 The effect of transient expression of CP on accumulation of viral RNA from pBICLSF-based CP mutants. (A) Schematic representation of pBICLSF-based mutants and transient expression vector used for co-agroinfiltration. Bold lines in the CP box of pBICLCPm40, pBICLCPm75, and pBICLCPm40m75 indicate the amino acid substitutions at positions 40 (Ala to Ser) and 75 (Phe to Tyr). The representations of the pentagon labeled E, P, and Ω and the box labeled T are shown in the legend of Fig. 1. Rep, 216 KDa replication associated protein; P50, 50 KDa movement protein; CP, coat protein. (B) Northern blot analysis of viral genomic RNA extracted from leaves infiltrated with a mixture of agrobacteria carrying pBICLSF and pBE2113-P35T (vector), or pBICLSF-based mutants and pBE2113-CP at 4 days post infiltration. Ethidium bromide staining of rRNA is shown as a loading control.

Inhibition of viral genomic RNA accumulation by CP expression: To test whether the inhibitory effect of transient expression of CP on viral genomic RNA accumulation is mediated by a protein or mRNA, pBICLSF and each vector expressing CP, a frame-shift mutant of CP (FSCP), P50, or a frame-shift mutant of P50 (FSP50) shown in Fig. 3A was co-infiltrated into leaves of *N. occidentalis*.

Infiltrated leaves were collected at 4 dpif and an accumulation of viral genomic RNA was analyzed by northern blot analysis. The result showed that the accumulation level of viral genomic RNA in leaves co-infiltrated with pBICLSF and CP was lower than that in leaves co-infiltrated with pBICLSF and FSCP, P50, or FSP50 (Fig. 3B), indicating that the suppression of RNA accumulation is mediated by expression of CP, not by mRNA. It has been reported that transgenic plants expressing viral CP confer resistance to a homologous virus, referred to as CP-mediated resistance (CP-MR; Beachy, 1999). Several mechanisms of CP-MR are proposed, i.e., expression of CP interferes with disassembly of the challenge virus because transgenic plants expressing CP of *Tobacco mosaic virus* (TMV) are resistant to TMV particle inoculation, but not to TMV RNA inoculation (Register & Beachy, 1988; Bendahmane & Beachy, 1999). In the case of *Potato virus X* (PVX), PVX CP interacts with the origin of assembly and restricts replication, or it interferes with translation of the replicase because transgenic plants expressing CP are resistant to inoculation with both PVX particles and PVX RNA (Hemenway et al. 1998; Spillane et al. 1997).

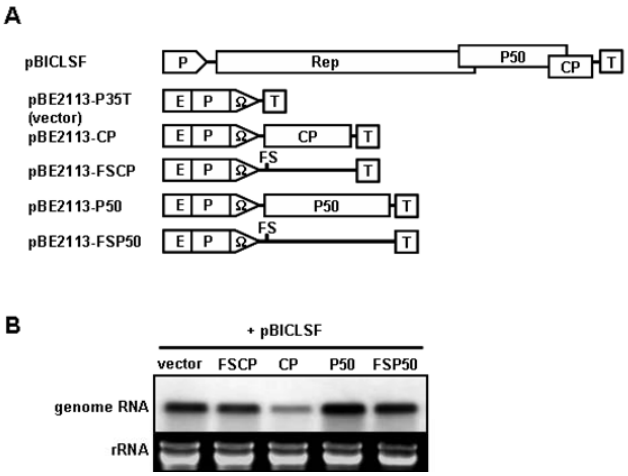

Fig. 3 Suppression of viral RNA accumulation by transient expression of CP. (A) Schematic representation of pBICLSF and transient expression vector used for co-agroinfiltration. The representation of the pentagon labeled E, P, and Ω and the box labeled T are shown in the legend of Fig. 1. (B) Northern blot analysis of viral genomic RNA extracted from leaves infiltrated with a mixture of agrobacteria carrying pBICLSF and each transient expression vector shown in (A) at 4 days post infiltration. Ethidium bromide staining of rRNA is shown as a loading control.

In this study, we show two conflicting sets of data; (1) stable accumulation of CP is important for effective viral genomic RNA accumulation, and (2) transient expression of CP inhibits viral genomic RNA accumulation. We think that ACLSV replication may be regulated by the level of CP accumulation and/or the timing of CP expression. Viral CP is known to have many functions including viral replication, symptom modulation, cell-to-cell movement, systemic spread, and suppression of RNA silencing, in addition to virion formation (Callaway *et al*., 2001; Thomas *et al*., 2003; Lu *et al*., 2004). Understanding the function(s) of ACLSV-CP in viral replication will elucidate the actual mechanism of positive and negative effects of CP on viral genomic RNA accumulation.

Acknowledgements

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