A single silent substitution in the genome of *Apple stem grooving virus* causes symptom attenuation

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Among randomly mutagenized clones derived from an infectious cDNA copy of genomic RNA of *Apple stem grooving virus* (ASGV), we previously identified a clone, pRM21, whose *in vitro* transcript (ASGV-RM21) does not induce any symptoms characteristic of the original (wild-type) cDNA clone (ASGV-wt) in several host plants. Interestingly, ASGV-RM21 has only a single, translationally silent nucleotide substitution, U to C, at nucleotide 4646 of the viral genome within open reading frame (ORF) 1. Here, we characterize and verify this unprecedented silent-mutation-induced attenuation of symptoms in infected plants. Northern and Western blot analyses showed that less ASGV-RM21 accumulates in host plants than ASGV-wt. In addition, two more silent substitutions, U to A and U to G, constructed by site-directed mutagenesis at the same nucleotide (4646), also induced attenuated symptoms. This is the first report that a single silent substitution attenuates virus-infection symptoms and implicates a novel determinant of disease symptom severity.

*Apple stem grooving virus* (ASGV) is the type species of the genus *Capillovirus*, a group of plant viruses with a monopartite genome consisting of a 6·5 kb, single-stranded, plus-sense RNA that is 5'-capped and 3'-polyadenylated. ASGV RNA contains two overlapping open reading frames: ORF1 (6·3 kb) and ORF2 (1·0 kb), which encode proteins of molecular mass 241 and 36 kDa, respectively (Yoshikawa et al., 1992; Ohira et al., 1995) (Fig. 1). The larger ORF1 encodes an apparently chimeric polyprotein containing at least two conserved regions: the replicase (Rep) region contains several domains (methyltransferase, papain-like protease, NTP-binding helicase and RNA-dependent RNA polymerase domains) characteristic of viral Rep, and the coat protein (CP) region is located at the C-terminal end and contains motifs typical of CP (Yoshikawa et al., 1992; Ohira et al., 1994, 1995). ORF2 encodes a protein with conserved motifs for both movement proteins (MP) and viral proteases (Yoshikawa et al., 1992; Ohira et al., 1995). The expression strategies of these ORFs are not yet well understood. By using an antiserum against purified ASGV particles, the expected 241 kDa polyprotein for ORF1 was not detected in a plant infected with ASGV; only a small protein thought to be the CP was detected (Yoshikawa & Takahashi, 1992). These results suggest that CP is expressed from subgenomic RNA rather than polyprotein processing of the 241 kDa protein. The mechanism of expression of ORF1 thus remains to be elucidated. Construction of a full-length cDNA clone, pITCL, of ASGV lily strain (formerly *Citrus tatter leaf virus* lily strain: Nishio et al., 1989; Yoshikawa et al., 1993; Ohira et al., 1995) that produces infectious viral RNA transcripts (ASGV-wt) *in vitro* has been described (Ohira et al., 1995), allowing mutagenesis of the ASGV genome.

We randomly mutagenized pITCL using a DNA repair-deficient mutator *Escherichia coli* strain (Greener et al.,...
An in vitro transcript (ASGV-RM21) of one of the resultant clones, pRM21, was found to induce almost no symptoms in inoculated plants (Chenopodium quinoa) and unexpectedly had only one silent nucleotide substitution within ORF1 (Lu et al., 2001). The silent mutation was U to C (T to C for cDNA) at nucleotide 4646 of the ASGV genome, which is located downstream from the RNA-dependent RNA polymerase domain of the replicase region within ORF1 (Fig. 1). Here, we extensively analyse this clone, pRM21, and several site-directed mutagenized clones.

Infecctivity assays of each ASGV cDNA clone were performed as follows. RNA transcribed in vitro was immediately used to inoculate C. quinoa seedlings at the five- to ten-leaf stage after adjusting the concentration of the transcribed RNA to 1 μg μl⁻¹, as described by Ohira et al. (1995). Plants were tested for virus infection by RT-PCR with an AMV RNA PCR kit (Takara) using total RNA extracted by Isogen (Nippon Gene) and ASGV-specific primers. The amplified cDNA of infecting viruses was detected by agarose gel electrophoresis and sequenced with a DNA sequencer following the manufacturer’s protocol (Applied Biosystems). RNA transcribed in vitro from pITCL (ASGV-wt) induced symptoms characteristic of ASGV. These consist of severe chlorotic and necrotic local lesions on inoculated C. quinoa leaves, seen about 1 week post-inoculation (p.i.) (Fig. 2a) and chlorotic mottling and malformation on upper leaves (Fig. 2b). In contrast, ASGV-RM21, which has a silent nucleotide substitution within ORF1, usually induced no symptoms (data not shown) or occasionally very mild symptoms, with only a few, small chlorotic spots on inoculated leaves (Fig. 2c) and no symptoms on upper leaves (Fig. 2d). Dwarfing was observed in the plants infected with ASGV-wt, but no dwarfing occurred in those infected with ASGV-RM21 (Fig. 2e). RT-PCR analysis confirmed the presence of ASGV-RM21 progeny in the asymptomatic upper leaves (data not shown). The attenuated symptoms caused by ASGV-RM21 were observed also in additionally tested plants including Nicotiana glutinosa and Vigna sesquipedalis cv. Kurodane-Sanjaku. In N. glutinosa plants, ASGV-wt induced mosaic, chlorotic spots or faint ring spots with etched surface on the systemically infected leaves (Fig. 2f), while ASGV-RM21 infected asymptotically (Fig. 2g). In V. sesquipedalis, ASGV-wt produced necrotic spots on the upper leaves (Fig. 2h), but ASGV-RM21 showed no infectivity (Fig. 2i). The stability of the ASGV-RM21 mutation was tested after four serial passages in C. quinoa plants using crude leaf extracts as an inoculum. Inoculated plants invariably produced attenuated symptoms identical to those induced by the original ASGV-RM21 (data not shown). The complete nucleotide sequences of the progeny viruses were determined using 26 internal primers. No nucleotide alteration during these passages was detected, indicating that the single nucleotide mutation of ASGV-RM21 is stable. In order to confirm that the single U to C substitution at nucleotide 4646 is responsible for the attenuated symptoms with ASGV-RM21, the C at nucleotide 4646 of pRM21 was mutagenized back to the wild-type T nucleotide using a QuikChange site-directed mutagenesis kit (Stratagene). As expected, transcripts of the resultant construct produced identical symptoms to ASGV-wt (data not shown). Similarly, when the T at nucleotide 4646 of pITCL was mutagenized into C, transcripts of the resultant construct produced no symptoms, as in the case of pRM21 (data not shown).

To find whether the attenuated symptoms with ASGV-RM21 were accompanied by an altered amount of virus, the accumulation of viral CP and RNA was analysed by Western and Northern blot analyses, respectively, in plants inoculated with ASGV-wt or -RM21. Fresh plant tissue (0-5 g) of either inoculated or upper leaves was collected from each plant, and the amounts of ASGV CP were compared by SDS-polyacrylamide gel electrophoresis and Western blot analysis (Sambrook & Russell, 2001a). The blotted CP was detected using anti-ASGV antibody and an ECF Western Blotting Reagent Pack (rabbit) (Amersham). In both inoculated and upper leaves, less of the viral CP expressed by ASGV-RM21 progeny accumulated as compared with ASGV-wt progeny (Fig. 3a). Northern blot analysis was performed by fractionating total RNA extracted from infected plants, as described by Verwoerd et al. (1989), in 1 % agarose/formaldehyde gels (Sambrook & Russell, 2001b). Blotted viral RNA was detected with an alkaline phosphatase-labelled DNA probe (cDNA of ORF2 region) prepared by PCR and with a chemiluminescent ECL substrate following the manufacturer’s protocol (Amersham) and analysed by LAS 1000 (Fujiﬁlm) and Image Gauge software version 3.45 (Fujiﬁlm). ASGV-RM21 progenies produced less 6·5 kb genomic RNA than ASGV-wt progenies (Fig. 3b). In addition to the 6·5 kb genomic RNA, 2·0 and 1·0 kb bands were also detected. Using probes that covered the entire ORF2 or only the 5’ half of ORF2, we concluded that the 2·0 and 1·0 kb bands were subgenomic RNA for ORF2 and CP, respectively (data not shown). This finding is in agreement with Magome et al. (1997), who reported 2·0 and 1·0 kbp dsRNA species in ASGV-infected plants.

To determine the effect of the nucleotide species at nucleotide 4646 in the ASGV genome, the T at nucleotide 4646 of the original pITCL was mutagenized into two other possible nucleotides, A and G, and the pathogenicity of the resultant constructs (pT4646A and pT4646G, respectively) was analysed. The transcripts from pT4646A (ASGV-U4646A) induced almost no symptoms, as observed for ASGV-RM21 (Fig. 2j and 2k). The transcripts from pT4646G (ASGV-U4646G) induced clearly attenuated symptoms in infected C. quinoa plants (Fig. 2l and 2m). Northern (Fig. 3c) and Western (data not shown) blot analyses indicated that both of these mutants accumulated less in host plants than ASGV-wt. These results indicate that any change of nucleotide 4646 U in the ASGV genome results in reduced virus accumulation and attenuated pathogenic phenotypes, although such changes are translated silently in the ORF1 polypeptide.
The relationship between viral genetic information and the symptoms induced by the virus in host plants has long attracted much attention. Reduced virus replication or reduced cell-to-cell or long-distance movement, or combinations of these factors, may cause attenuated symptoms. Amino acid alterations in viral replicase causing attenuated

**Fig. 2.** Comparison of the pathology of the original ASGV-wt (a, b, f and h) and mutants, ASGV-RM21 (c, d, g and i) in *Chenopodium quinoa* (a, b, c and d), *Nicotiana glutinosa* (f and g; transmitted light image of the reverse side) and *Vigna sesquipedalis* (h and i) at 14 days p.i. (e) Growth of *C. quinoa* plants (21 days p.i.) inoculated with water (mock) (left), ASGV-wt (right) or -RM21 (middle). (j)–(m) Symptoms of the other mutants, ASGV-U4646A (j; inoculated-, and k; upper-leaf), or ASGV-U4646G (l; inoculated-, and m; upper-leaf) on *C. quinoa*.
symptoms have been well documented (Nishiguchi et al., 1985; Holt et al., 1990; Lewandowski & Dawson, 1993; Hagiwara et al., 2002). In addition, it has been reported that alteration of the amino acids in CP or MP modifies symptom development (Shintaku & Palukaitis, 1992; Banerjee et al., 1995; Rao & Grantham, 1995; Fujita et al., 1996; Moreno et al., 1997; Andersen & Johansen, 1998; Szilassy et al., 1999; Takeshita et al., 2001; de Assis Filho et al., 2002). It has also been implied that symptom development is a complex process involving interactions between viral genes or their products and host plant factors. The viral RNA 3'- and 5'-noncoding sequences can also affect the expression of disease symptoms (Rodríguez-Cerezo et al., 1991; Zhang et al., 1994; van der Vossen et al., 1996). In this study, RM21 and the other two substitution mutations at nucleotide 4646 of the ASGV genome, which are responsible for the attenuated pathogenic phenotype, are translationally silent. To our knowledge, this is the first example of silent mutations in the coding region of a plant viral sequence affecting viral symptoms.

As for the mechanism of the reduced accumulation of the viruses with a mutation in nucleotide 4646, one can argue that the nucleotide is located in the promoter region of the subgenomic RNA for MP, since the mutation is located upstream from the coding region (ORF2) of the putative MP. Although the promoter for MP subgenomic RNA has not yet identified, a homologous sequence, 5’-GCTNGAN(2)TTNGAGAN(2)TTNGGN(2)AN(14)AATG-3’, is located upstream from both the MP and CP genes, and might be part of the subgenomic promoter of both genes (Magome et al., 1997). Nucleotide 4646 is 104 and 141 bases upstream from the homologous sequence and the initiation codon of MP, respectively. If the MP subgenomic promoter is somehow affected by the mutation, then the transcription of MP subgenomic RNA should be affected. Therefore, we quantified the amount of genomic, MP subgenomic, and CP subgenomic RNA species using the data shown in Fig. 3(b) (RNA signal intensity in inoculated leaves). The relative abundance (genomic RNA = 100) of genomic RNA:MP subgenomic RNA:CP subgenomic RNA was 100:12:18 for ASGV-wt and 100:13:20 for ASGV-RM21, meaning that the relative abundance of MP subgenomic RNA compared to that of genomic RNA is not specifically reduced in ASGV-RM21, although the absolute abundance of all three RNA species is reduced equally. This suggests that the mutation at nucleotide 4646 does not affect MP subgenomic RNA transcription.

Another possible explanation of the reduced accumulation of RM21 is that nucleotide 4646 might be involved in the expression or activity of viral replicase, thereby affecting virus replication. A stable stem–loop structure was predicted near nucleotide 4646 of the ASGV-wt genomic RNA using the program MFOLD (Zuker & Jacobson, 1998), and altered stem–loop structures were predicted for genomes of ASGV mutants having any of three substitutions at nucleotide 4646 (data not shown). The mechanism of symptom attenuation caused by a single nucleotide substitution which is translationally silent might be related to the alteration of the secondary structure of the genome.
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