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A single amino acid in coat protein of Pepper mild mottle virus determines its subcellular localization and the chlorosis symptom on leaves of pepper

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ABSTRACT

Pepper mild mottle virus (PMMoV) causes serious economic losses in pepper production in China. In a survey for viral diseases on pepper, two PMMoV isolates (named PMMoV-ZJ1 and PMMoV-ZJ2) were identified with different symptoms in Zhejiang province. Sequence alignment analysis suggested there were only four amino acid differences between the isolates: Val262Gly, Ile629Met and Ala1164Thr in the replicase, and Asp20Asn in the coat protein. Infectious cDNA clones of both isolates were constructed and shown to cause distinctive symptoms. Chlorosis symptoms appeared only on PMMoV-ZJ2-infected plants and the Asp20Asn substitution in the CP was shown to be responsible. Confocal assays revealed that the subcellular localization pattern of the two CPs was different, CP^{20Asp} was mainly located at the cell periphery, whereas most CP^{20Asn} located in the chloroplast. Thus, a single amino acid in the CP determined the chlorosis symptom, accompanied by an altered subcellular localization.

Pepper mild mottle virus (PMMoV) is a damaging pathogen of various peppers (*Capsicum* spp.) worldwide, causing significant economic losses [1–4]. In China, PMMoV was first discovered in 1994 and has since been reported in Beijing, Ningxia, Hebei, Liaoning and many other cities or provinces [4–6]. The virus belongs to the genus *Tobamovirus* (family *Virgaviridae*), a group of viruses with single-stranded, positive-sense RNA genomes encapsidated in rigid rod-shaped particles by a single type of coat protein [7]. The PMMoV genome has 6356–6357 nucleotides with a 5′-cap and a 3′-tRNA-like structure, and encodes four viral proteins [8, 9], including 126 kDa and read-through 183 kDa RNA-dependent RNA polymerase (RdRp) proteins that are required for genome replication and which are translated from the genomic RNA [10, 11]. The 126 kDa protein possesses methyltransferase and helicase activities and functions as a suppressor of RNA silencing [12, 13]. The other two viral proteins, a 30 kDa cell-to-cell movement protein and a 17 kDa coat protein (CP) are translated from 3′ co-terminal subgenomic RNAs [9, 10]. PMMoV is transmitted through seeds and soil and it can also be spread by sap transmission during cultural practices such as pruning and harvesting [14, 15]. Because of its wide distribution and remarkable stability in water, PMMoV has been used as a potential viral indicator for human fecal pollution in aquatic environments and water treatment systems [16, 17].

Peppers are one of the most important vegetable crops globally and about one third of the total production is in China. In a survey for virus diseases on pepper, forty samples with leaf crinkle and/or chlorosis were collected from pepper fields in Zhejiang province, China. Infection of PMMoV in these samples was detected by RT-PCR using primers (P1/P2) specific for the PMMoV CP gene. Total RNAs were

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Abbreviations: CP, coat protein; PC, plastocyanin; PMMoV, Pepper mild mottle virus; RdRp, RNA-dependent RNA polymerase; UGPase, UDP-glucose pyrophosphorylase.

The GenBank accession numbers: MN616926 (PMMoV-ZJ1), MN616927 (PMMoV-ZJ2).

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One supplementary table and two supplementary figures are available with the online version of ths article.

then extracted with TRIzol (Invitrogen, Carlsbad, USA), and first-strand cDNA was synthesized with primer M_4-T using ReverTra Ace (Toyobo, Osaka, Japan) and then used as template for PCR using KOD Fx Neo (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Specific DNA fragments were amplified from 22 of 40 samples and the presence of PMMoV infection was further confirmed by sequencing the PCR products.

Among the 22 infected samples, there were two obviously different types of leaf symptom. Some leaves had chlorosis while others showed only mild or no chlorosis. The full-length sequences of PMMoV isolates from a single example of each symptom type were determined. The majority of the genome was amplified using primers (P3-P6) designed from the published sequence of a PMMoV isolate (GenBank: NC_003630) (Table S1). The sequences of the 5′ and 3′ ends of genomic RNA were then determined by 5′ and 3′ rapid amplification of cDNA ends (RACE), using a SUPERSWITCH RACE cDNA Synthesis Kit (Sonice Biotechnology, Hangzhou, China) according to the manufacturer's instructions. In consequence, we obtained two complete sequences of PMMoV, named PMMoV-ZJ1 (GenBank: MN616926) from a sample without leaf chlorosis and PMMoV-ZJ2 (GenBank: MN616927) from a sample with chlorosis. The sequences were both 6356 nucleotides long and had the typical genome organization of the genus *Tobamovirus* (Fig. 1a). The 5′- and 3′- nontranslated regions (NTRs) consisted of 69 and 198 nucleotides, respectively. There were only four nucleotides that were different between the two sequences, and these were at positions 854, 2256, 3559 and 5742. These nucleotides were respectively U, A, G and G in PMMoV-ZJ1 and G, G, U, and U in PMMoV-ZJ2. Nucleotides 854, 2256 and 3559 are in the open reading frame of the replicase, while nucleotide 5742 is in the CP ORF. Each nucleotide difference caused a substitution of the encoded amino acid. In PMMoV-ZJ1, Val, Ile and Ala occur at amino acid positions 262, 629 and 1164 in the replicase, and Asp at aa position 20 in the CP while for PMMoV-ZJ2 these were respectively Gly, Met, Thr and Asn (Fig. 1a).

Next, to determine whether the sequence differences caused the different symptoms, we constructed infectious cDNA clones of the two isolates, using the method previously described [18]. For construction, two overlapping segments covering the entire genome of PMMoV were first amplified from the cDNA of both isolates using primer pairs P7/P8 and P9/P10 (Table S1). The pCB-301-CH plasmid used as the backbone was digested with *Stu* I and *Sma* I [19]. Then, the digested vector and the two fragments of PMMoV were reconstructed with the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China) according to the manufacturer's protocol. In this way, the full-length PMMoV cDNA was inserted between an upstream 35S promoter and a downstream hepatitis delta virus ribozyme and 35S terminator in the binary vector pCB-301-CH [20]. The constructs were named pCB-PMMoV-ZJ1 and pCB-PMMoV-ZJ2. To confirm the infectivity of the two clones, *Agrobacterium tumefaciens* strain EHA105 containing pCB-PMMoV-ZJ1 or pCB-PMMoV-ZJ2 was infiltrated into leaves of *N. benthamiana*. Leaf crinkle symptoms appeared on the upper leaves of *N. benthamiana* nine days after inoculation with either clone (Fig. S1, available in the online version of this article). On the plants infected with PMMoV-ZJ2, but not on those infected with PMMoV-ZJ1, there was also chlorosis on the infected leaves (Fig. S1). In addition, seedlings of pepper (*Capsicum annuum* L. No. 1 Zhejiao) were also inoculated with the two clones. Because agro-infiltration does not provide efficient infection of pepper [21], sap of *N. benthamiana* leaves infected with PMMoV-ZJ1/2 were inoculated onto leaves of pepper seedlings at the two true leaf stage. Very mild symptoms were observed on upper, uninoculated leaves of pepper infected with PMMoV-ZJ1 at three weeks past inoculation (wpi) and particularly at six wpi, and there was more obvious chlorosis on leaves infected with PMMoV-ZJ2 (Fig. 1b and S2). Western blotting using PMMoV CP antibody showed that both isolates had successfully infected, and that the accumulation of CP in ZJ1-infected pepper was greater than that in ZJ2 (Fig. 1c). The expression levels of the PMMoV RdRp and CP genes were then analysed by RT-qPCR (using primer pairs P19/P20 and P21/P22) and with *C. annuum* beta tubulin gene as an internal control (primer pair P23/P24). The results showed that the expression levels of both RdRp and CP in ZJ2-infected pepper were about 65% of those in ZJ1-infected plants (Fig. 1d).

The results above indicate that the sequence differences between the isolates may cause the difference in symptoms. We therefore produced mutated clones of PMMoV-ZJ1 individually replacing the nucleotides at each of the variable sites: PMMoV-ZJ1-1 (T854G), PMMoV-ZJ1-2 (A2256G), PMMoV-ZJ1-3 (G3559A), and PMMoV-ZJ1-4 (G5742A) (Fig. 1a). Primers used for construction are listed in Table S1. Clones were then inoculated onto *N. benthamiana* and pepper plants as before. Plants infected with PMMoV-ZJ1-1, PMMoV-ZJ1-2 or PMMoV-ZJ1-3 had mild symptoms on systemically-infected leaves of either pepper or *N. benthamiana* similar to PMMoV-ZJ1 but plants infected with PMMoV-ZJ1-4 had chlorosis on their upper leaves, similar to that caused by PMMoV-ZJ2 (Fig. 1b, S1 and S2). PMMoV CP and RNAs accumulated less in ZJ1-4-infected pepper than in ZJ1-infected plants (Fig. 1c, d). The results therefore demonstrate that a single amino acid mutation at residue 20 in the CP was responsible for the induction of chlorosis caused by PMMoV-ZJ2 infection.

The chlorosis phenotype in plants infected with viruses is often associated with the disruption of chloroplast ultrastructure [22, 23]. Indeed, chloroplast clustering and distorted grana were observed in pepper leaves infected with PMMoV-ZJ1, as compared with the MOCK plant. Overall there were fewer chloroplasts in leaves infected with ZJ1-4 or ZJ2 than in those infected with ZJ1 (Fig. 2). This suggested that PMMoV infection can damage the chloroplast ultrastructure, and that CP^{20Asn} caused more serious damage to the chloroplasts.

To further examine the association of CP^{20Asn} with chloroplasts, we investigated the subcellular localization of CP^{20Asp} and CP^{20Asn} using laser-scanning confocal microscopy. Constructs

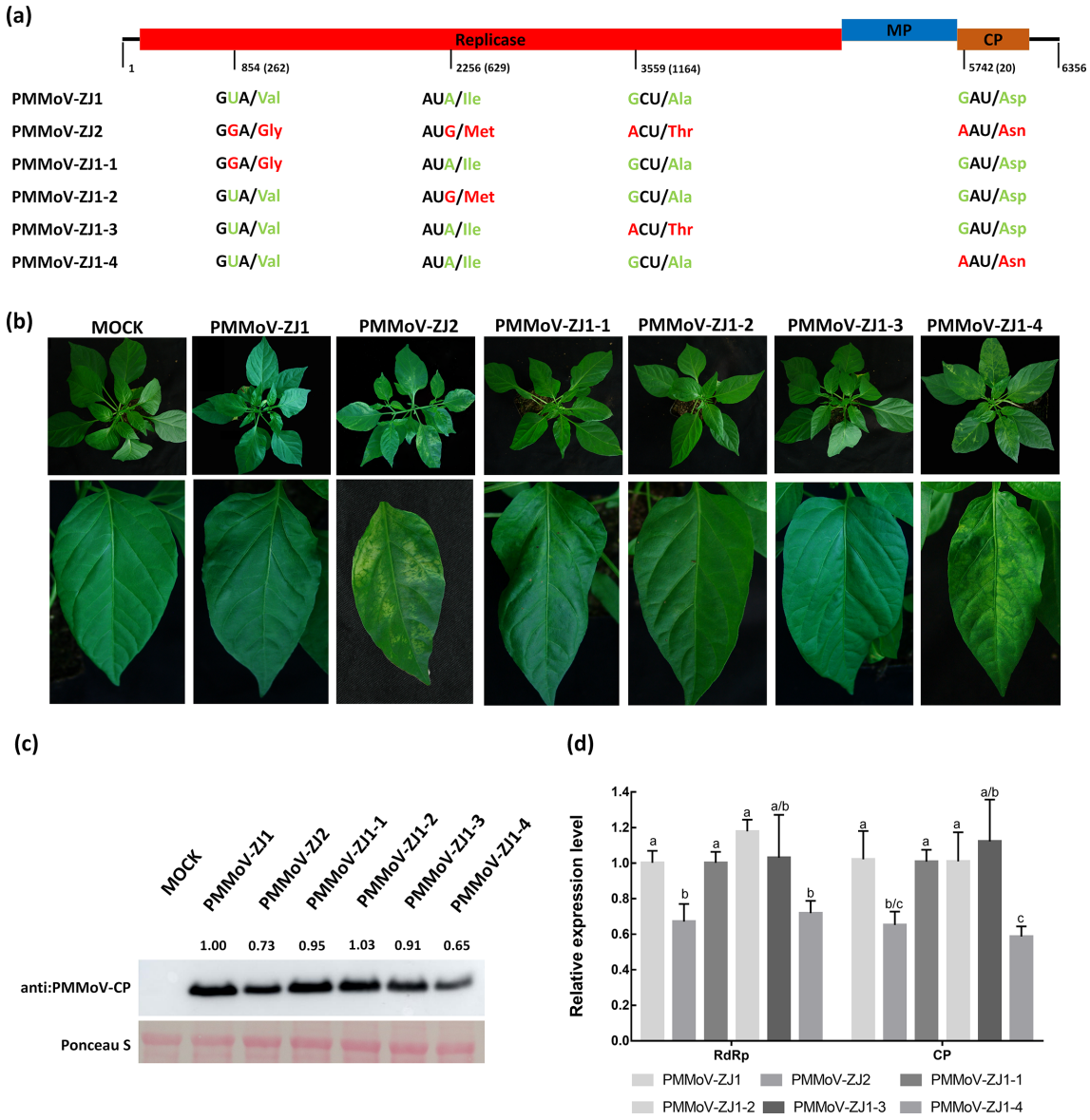

Fig. 1. Symptoms in *Capsicum annuum L.* inoculated with PMMoV-ZJ1, PMMoV-ZJ2 and its chimeric mutant viruses. (a) Schematic representation of the PMMoV genome and the four substitutions between ZJ1 and ZJ2. Red letters are nucleotides or amino acids in the ZJ2 isolate, while green letters are those in ZJ1. Bold numbers indicate the position of nucleotide, and numbers in brackets indicate the position of amino acid. (b) Symptoms induced in pepper by PMMoV isolates ZJ1, ZJ2 and its chimeric mutant at 3 wpi. (c) Accumulation of PMMoV CP in peppers inoculated with PMMoV-ZJ1, PMMoV-ZJ2 and its PMMoV mutants at 3 wpi were detected by Westen blot. The relative intensity of the blot signal quantified by ImageJ is shown above the lanes. (d) The accumulation of the PMMoV RdRp and CP gene in peppers inoculated with PMMoV-ZJ1, PMMoV-ZJ2 and its PMMoV mutants was analysed by RT-qPCR at 3 wpi. *Capsicum annuum* beta tubulin gene was used as an internal control. Bars are means±SD from three biological replicates. Different letters on histograms indicate significant differences (P<0.05).

expressing GFP-fused CP^{20Asp} or CP^{20Asn} were prepared using the pCV-GFP-N1 binary vector described before and infiltrated into leaves with *Agrobacterium tumefaciens* strain EHA105. At 3 days post infiltration (dpi) of 3-week-old *N. benthamiana* plants, green fluorescence was visible at the cell periphery, and was partially distributed around the chloroplast in cells expressing GFP-fused CP^{20Asp}. In cells expressing GFP-fused CP^{20Asn}, there was much stronger fluorescence on the chloroplasts (Fig. 3a). In order to further confirm the localization of the two CPs, protoplasts were prepared from *N. benthamiana* leaves agro-infiltrated with 35S:CP^{20Asp}:GFP or 35S:CP^{20Asn}:GFP for confocal observation. In the CP^{20Asp}:GFP treatment, fluorescence occurred at the cell periphery and was seen surrounding chloroplasts but in the CP^{20Asn}:GFP treatment, the GFP fluorescence overlapped the autofluorescence of the chloroplast (Fig. 3b). The accumulation

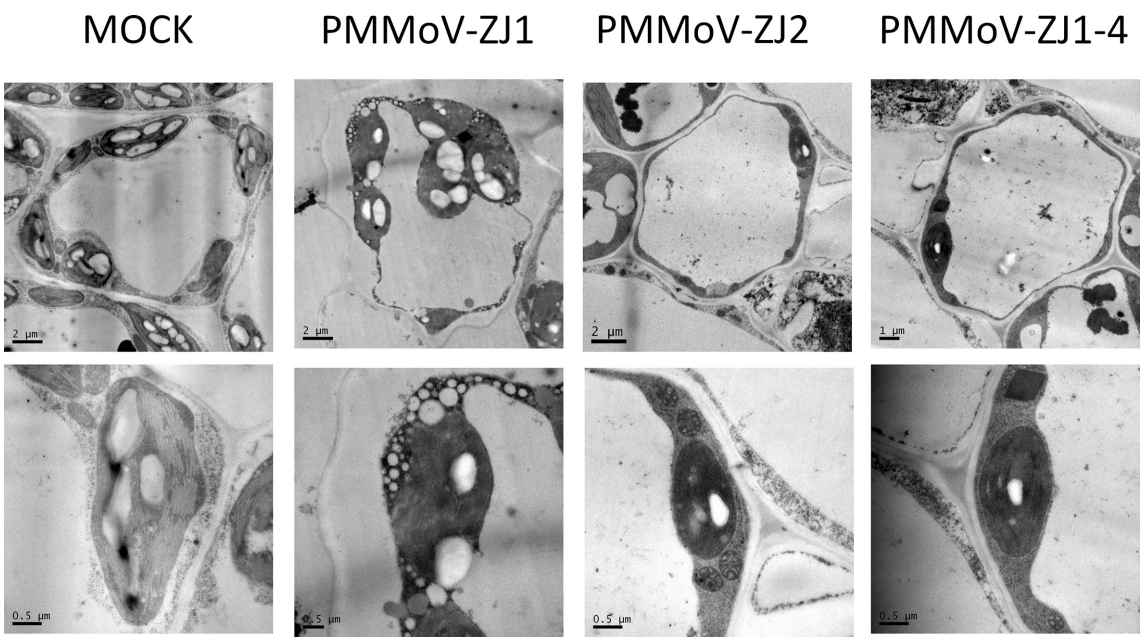

Fig. 2. The ultrastructure of chloroplasts from *Capsicum annuum L.* inoculated with PMMoV-ZJ1, PMMoV-ZJ2 or PMMoV-ZJ1-4. Leaf samples were prepared for transmission electron microscopy from healthy and PMMoV-infected pepper (3 wpi). Leaf sections (1 mm×3 mm) were fixed in 2.5% glutaraldehyde by infiltration under vacuum, rinsed with 0.1M phosphate-buffered saline (PBS) and fixed in 1% osmium tetroxide for 2 h. They were washed with 0.1M PBS 5–8 times and dehydrated through an ethanol series, and embedded in Quetol 812 resin at 70 °C for 48 h. Thin sections (70–100 nm) were cut and stained. The samples were observed using a transmission electron microscope (Hitachi H-7650) at 80 kV.

of CP in the total protein and chloroplast protein fractions of *N. benthamiana* leaves infected with PMMoV-ZJ1, ZJ2 or ZJ1-4 were also analysed by Western blot. The chloroplast protein extraction was based on the previously-published method [24]. UDP-glucose pyrophosphorylase (UGPase) and plastocyanin (PC) were used as cytoplasmic and chloroplast markers, respectively. No UGPase bands were detected in the chloroplast protein fraction, suggesting that it did not contain cytoplasmic components. CP^{20Asp} of PMMoV-ZJ1 accumulated at a higher level than CP^{20Asn} of PMMoV-ZJ2 and PMMoV-ZJ1-4 in total protein extracts. However, in the chloroplast protein fraction, CP^{20Asp} accumulation was less than that of CP^{20Asn} (Fig. 3c). These results indicate that the single amino acid at position 20 in the PMMoV CP determined its location and that the chloroplast localization of CP^{20Asn} may be the reason that it caused more serious damage to the chloroplast and induced the chlorosis symptom.

Many reports have shown that viral CP is related to the development of chlorosis or symptom severity during infection by tobamoviruses. Thus, the chlorotic symptom is induced by nucleotide deletions in the CP gene of TMV (U1 strain) [25], while in the CP of tomato mosaic virus L11Y three amino acid changes (E33S, A86T and E97K) determined chlorosis [26]. Additionally, a M17T substitution in the N-terminal domain of the CP was responsible for a difference in symptom severity induced by youcai mosaic virus [27]. We here found that the single amino acid at position 20 in the PMMoV CP determined the viral chlorosis symptom (Fig. 1). Previous reports have shown that some point mutations in the PMMoV replicase can affect symptoms and the invasive viability of the virus by altering its silencing suppressor function or its localization pattern [2, 28]. The three amino acid positions in the replicase reported here did not obviously affect viral infection, suggesting that the substitutions do not influence the function of the replicase (Fig. 1). It has been reported that the nucleotide substitution rate (subs/site/year) in the replicase is 2.6×10^{-4}, which is higher than the value of 1.8×10^{-4} for the CP [29]. The relatively conserved CP may reflect its important role in the PMMoV infection process.

The chloroplast carries out photosynthesis and it is the crucial site for the synthesis of major phytohormones and the production of reactive oxygen species. Insufficient energy supply and large amounts of ROS may result from chloroplast degradation, and this would be harmful to viral replication [30, 31]. In addition, the chloroplast can serve as a potential replication compartment and protect the dsRNA intermediate [30]. Here, PMMoV-ZJ1-4 accumulated less than PMMoV-ZJ1 in infected pepper plants (Fig. 1c, d) but data are needed on the precise nature of the association between PMMoV-ZJ1-4 and chloroplasts.

Subcellular localization of CP^{20Asp} was similar to that reported for the CP of TMV and PMMoV isolate S-47, at the cell periphery [28, 32]. In addition, CP^{20Asp} can also be observed around the chloroplast. The subcellular localization pattern of CP^{20Asn} was clearly different: the GFP fluorescence of CP^{20Asn}

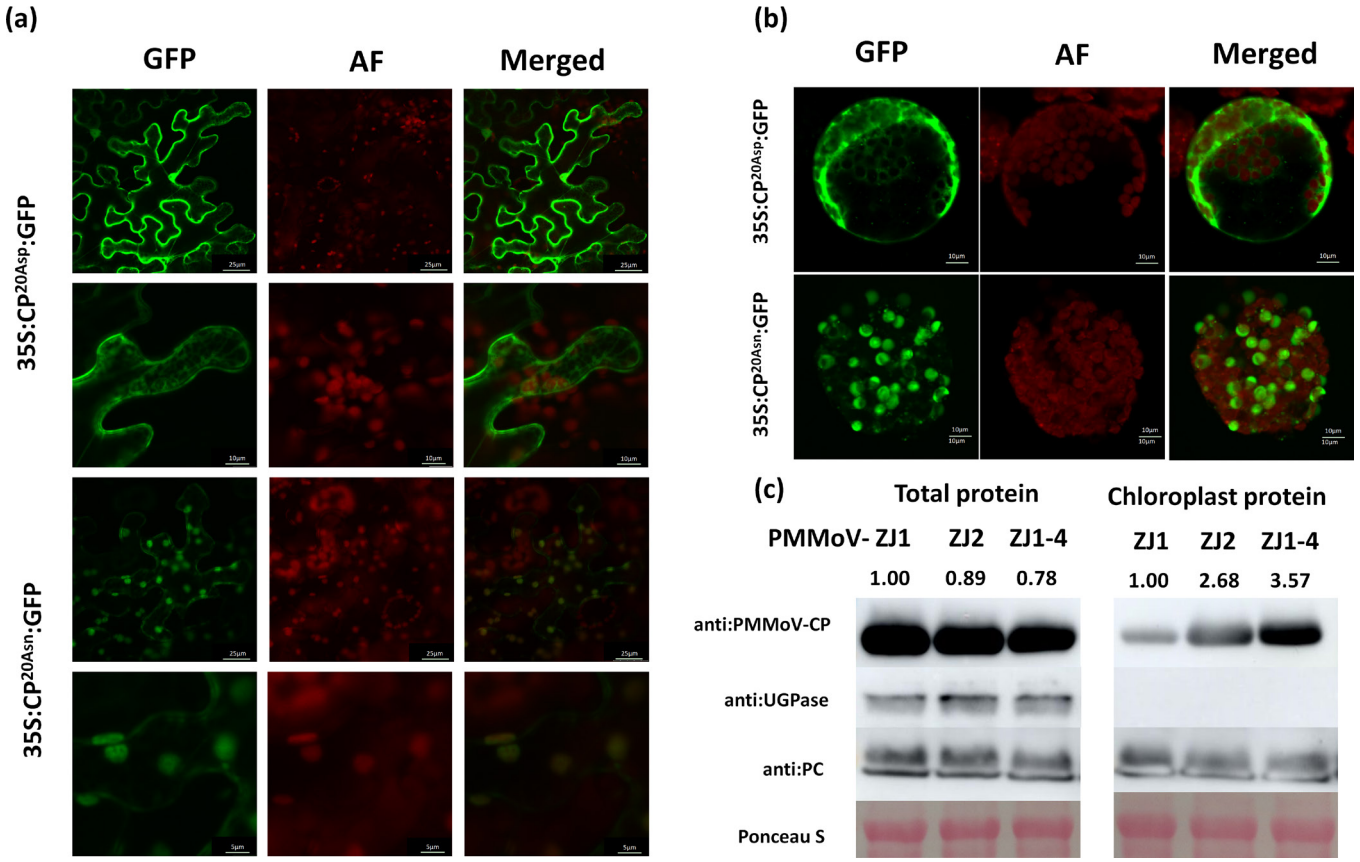

Fig. 3. Patterns of accumulation of CP^{20Asp}:GFP and CP^{20Asn}:GFP in *N. benthamiana*. (a) Subcellular localization of CP^{20Asp}:GFP and CP^{20Asn}:GFP in transiently transformed epidermal cells of *N. benthamiana* leaves. (b) Subcellular localization of CP^{20Asp}:GFP and CP^{20Asn}:GFP in protoplasts. Protoplasts were prepared from *N. benthamiana* leaves agro-infiltrated with 35S:CP^{20Asp}:GFP or 35S:CP^{20Asn}:GFP at 60 hpi. AF, chloroplast autofluorescence. (c) Western blot analysis of PMMoV CP in PMMoV-infected *N. benthamiana* chloroplasts. Total protein and chloroplast protein were extracted from PMMoV-ZJ1 or PMMoV-ZJ-4 infected *N. benthamiana* leaves at 8 dpi. All samples were separated on 12% SDS-PAGE gels at 90V for about 2 h, then transferred onto nitrocellulose membrane. RuBisCo large subunit stained by Ponceau S was used as the loading control. The relative intensity of the blot signal quantified by ImageJ is shown above the lanes. UGPase, UDP-glucose pyrophosphorylase (cytoplasm marker); PC, plastocyanin (chloroplast marker).

was detected predominantly in the chloroplast and there was less at the cell periphery (Fig. 3a, b). Western blot of total and chloroplast proteins in PMMoV-infected *N. benthamiana* confirmed that CP^{20Asn} accumulated much more in the chloroplasts (Fig. 3c), and this may be the cause of the chloroplast abnormalities. Plant virus infection induces mosaic or chlorosis symptoms by targeting the chloroplasts in geminiviruses and cucumber mosaic virus (CMV) [33, 34]. Coat protein gene sequences of two CMV strains reveal a single amino acid change correlating with chlorosis induction [35]. Furthmore, the CMV CP-Fd I (a chloroplastic factor) interaction is the primary determinant for the induction of chlorosis [36]. It is so far unknown how the PMMoV CP^{20Asn} moves to the chloroplast but it is possible that host interacting factors are also involved.

In our survey for viral diseases of pepper, two PMMoV isolates causing different symptoms (ZJ1 and ZJ2) were found in Zhejiang province. Compared with PMMoV-ZJ1, PMMoV-ZJ2 caused chlorosis on leaves of pepper but with less virus accumulation. The emergence of PMMoV-ZJ2 may be the result of co-evolution between PMMoV and its host plant. On the one hand, PMMoV-ZJ2 is a strain with enhanced pathogenicity and more severe symptoms, but on the plant side there has arisen an unclear but effective defense mechanism that limits virus titers. It will be worthwhile to monitor the epidemic character of these two isolates and further changes that may happen in the field.

In conclusion, we found that a single amino acid in CP of PMMoV determined its subcellular localization and the chlorosis symptom on plants. Meanwhile, more efforts are needed to illuminate the relationship between the subcellular localization and the chlorosis symptom.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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