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Abstract

Cauliflower mosaic virus (CaMV) is transmitted by aphids using the non-circulative transmission mode: when the insects feed on infected leaves, virus particles from infected cells attach rapidly to their stylets and are transmitted to a new host when the aphids change plants. Mandatory for CaMV transmission, the viral helper protein P2 mediates as a molecular linker binding of the virus particles to the aphid stylets. P2 is available in infected plant cells in a viral inclusion that is specialized for transmission and named the transmission body (TB). When puncturing an infected leaf cell, the aphid triggers an ultra-rapid viral response, necessary for virus acquisition and called transmission activation: The TB disrupts and P2 is redistributed onto cortical microtubules, together with virus particles that are simultaneously set free from virus factories and join P2 on the microtubules to form the so-called mixed networks (MNs). The MNs are the predominant structure from which CaMV is acquired by aphids. However, the P2 domains involved in microtubule interaction are not known. To identify P2 regions involved in its functions, we generated a set of P2 mutants by alanine scanning and analyzed them in the viral context for their capacity to form a TB, to interact with microtubules and to transmit CaMV. Our results show that contrary to the previously characterized P2-P2 and P2-virion binding sites in its C-terminus, the microtubule binding site is contained in the N-terminal half of P2. Further, this region is important for TB formation since some P2 mutant proteins did not accumulate in TBs but were retained in the viral factories where P2 is translated. Taken together, the N-terminus of P2 is not only involved in vector interaction as previously reported, but also in interaction with microtubules and in formation of TBs.

Keywords

Plant virus, aphid vector, transmission, cell biology, microtubules, cauliflower mosaic virus.

Introduction

Cauliflower mosaic virus (CaMV) is like hundreds of other plant viruses transmitted by aphids (reviewed in [1,2]). The aphid vectors acquire CaMV particles (virions) when feeding on infected plants and transport them attached to the aphid mouthparts (the stylets) to a new host plant, where the virions are inoculated during subsequent feeding activity. In the stylets, the icosahedral virions bind to a receptor belonging to the stylin proteines and localized at the stylet tips [3,4]. However, virions alone can not bind to the vector stylets. For this, they need P2, the virus-encoded transmission helper protein of CaMV. P2 complexes with virions to form a transmission unit that binds to the stylets [5,6]. CaMV acquisition from infected tissue is a complicated process because P2 is not complexed with virions in infected cells. Rather, virions localize mainly in distinct cytoplasmic virus-induced inclusions, so-called virus factories (VF), whereas P2 is found in transmission bodies (TB), another type of cytoplasmic viral inclusions, where it colocalizes with viral protein P3 [7–9]. A consequence of this set-up is that P2 and virions must form ad hoc transmission units when aphids probe infected plant cells [7]. This can happen either in the infected cell or in the aphid stylets, where it allows for sequential acquisition of CaMV: first attachment of P2 on stylet receptors, then binding of virions on stylet-associated P2 [10]. Both transmission pathways are facilitated by a viral-cellular reaction induced by the aphid feeding activity [11,12]. In their quest for nutrient-filled sap transported within the phloem vessels, aphids insert their stylets into cells located in the epidermal and mesophyll layers for a sampling of their contents. The stylet punctures wound but do not kill the visited cells and trigger instantaneous dissolution of the transmission bodies and the redistribution of P2 onto cortical microtubules as P2 networks. Simultaneously, virions are dispatched from the factories and associate with the P2 networks to form so-called mixed networks, composed of transmission units and free P2. Transmission units and free P2 are readily ingested by the aphids and attach to the stylets. Aphids may acquire more P2 and transmission units during subsequent punctures, and, after taking-off and alighting on another plant, inoculate them to start a new infection. The sequential acquisition and the vector-induced transmission mode, coined transmission activation, have important consequences on transmission biology (discussed in [13,14]. Remarkably, transmission activation can also be induced by abiotic stresses such as incubation with CO_2 or azide that cause formation of P2 networks [12].

Formation of the P2-virion transmission unit is mandatory for aphid transmission of CaMV, whereas interaction with microtubules is only required in the cellular context, because firstly, aphids transmit CaMV after feeding on purified P2-virion preparations, devoid of microtubules [15] and secondly, inhibiting formation of P2 networks in infected cells by drugs coincides with inhibition of transmission [12].

Little is known about the structure and functional domains of P2. Structure predictions and limited experimental data suggest that the 159 amino acids 18 kD P2 protein contains an N-terminal knob domain, composed of short β-sheets and α-helices (Fig. 1). The knob seems to be separated by a disordered stretch from the rod-like C-terminus, which contains two experimentally confirmed α-helices [15]. The α-helices of three P2 molecules form parallel coiled-coils and promote auto-assembly of P2 into a trimer. The trimers can assemble to higher-order structures, so-called paracrystals, indicating that there is a second, yet unknown self-interaction domain in the P2 molecule [15]. The α-helices are also involved in formation of the transmissible complex because they interact with the virion [5]. For this, the P2 C-terminal α-helices bind to the N-terminal α-helices of P3, which is besides a component of TBs also a virus-associated protein [16] forming a mesh on the capsid surface, composed of viral capsid protein P4 [17,18]. The N-terminus of P2 is required for interaction with the vector, because substitutions of amino acid 6 of P2 modify or abolish transmission but do not compromise formation of the transmissible complex [19]. P2 was reported to interact with microtubules in plants, in insect cells and *in vitro* [20,21]. The P2-microtubule interaction is necessary for formation of transmission bodies [21], and for efficient CaMV acquisition by aphids

[11,12]. However, the microtubule-binding domain of P2 is unknown. Likewise, besides the P3-interaction domain, no other P2 regions interacting with cellular or viral factors are identified.

We here set out to better characterize the functional domains of P2. We were in particular interested to identify the hitherto unknown microtubule and vector binding regions of P2. For this, P2 mutants were created by alanine-scanning and tested for their capacity to form mixed networks and to transmit the virus.

Material and methods

Aphids and plants

A non-viruliferous clonal *Myzus persicae* (Sulz) population was reared under controlled conditions (22/18 °C day/night with a photoperiod of 14/10 h day/night) on eggplant. Turnip plants (*Brassica rapa* cv. 'Just Right') were grown at 24/19 °C day/night with a photoperiod of 16/8 h day/night.

Cloning

For transient expression in plant protoplasts, the alanine substitutions Ala1 to Ala12 were inserted with the QuikChange mutagenesis kit (Agilent Technologies) into the pCK-P2 plasmid, which permits expression of wild type P2 under control of 35S promoter [11].

For baculovirus expression in *Sf9* cells, the P2 sequence with a his-tag added to the N-terminus [HP2, [15]] was PCR-amplified using pCK-P2 as matrix. The primers used were Apa-Start-his (5'-TCCCGGGCCCATGCACCATCACCATCACCATGGAGGTTCTAGCATTACGGGTCAACCGCATG-3') coding for an *Apa*I site, a his6-tag, a GGS linker and the N-terminus of P2 and P2-Sac2 (5'-CATCCGCGGTTAGCCAATAATATTCTTTAATCC-3') coding the C-terminus of P2 and a *Sac*II site. The PCR product was digested with *Apa*I and *Sac*II and inserted between the *Apa*I and *Sac*II restriction sites of the baculovirus transfer vector pOETIC_6xHis (Oxford Expression Technologies). This yielded a plasmid coding for HP2 under control of the polh promoter. The alanine substitutions were then introduced with the QuikChange mutagenesis kit.

For expression of P2-Ala in the viral context, the P2 sequence in the infectious CaMV-coding plasmid pGreen-35S-BJI [22] was replaced by an *Apa*I site. The different P2-Ala sequences were PCR-amplified from pOETIC-P2-Ala plasmids using primers with additional *Apa*I sites and inserted into pGreen-35S-BJI.

All inserts were verified by sequencing.

Baculoviruses and protein expression

Recombinant baculoviruses coding P2-Ala and P2-Ala-GFP mutants were obtained by recombination in *Sf9* cells using the flashBAC system (Oxford Expression Technologies) and pOETIC_6xHis plasmids. Baculovirus stocks were produced as indicated by the manufacturer. For protein production, *Sf9* cells cultivated at 28 °C in TNM-FH insect medium (Sigma) enriched with 5 % fetal calf serum, were infected at near confluence with the virus stocks and cultivated for another 48-72 h. Cells were then harvested by centrifugation, resuspended in SES buffer [23], ultrasonicated to extract the proteins, and stored at -20 °C or -80 °C until use.

Plant inoculation

Two weeks old turnip plants were rub-inoculated with 15-30 µg of pGreen plasmid coding for wild type CaMV or P2-Ala1-6 mutants. Plants were observed for symptoms every 2-3 days up to 50 days after inoculation (dpi). Subsequent inoculations were made by rub-inoculation with crude extracts prepared from infected plants as described [11]. The mutant viruses were thus serially passaged 2 to 6 times. The P2 sequence in infected plants was verified by PCR sequencing and plants containing viral sequences with additional mutations were not used for experiments.

Aphid transmission tests

For transmission experiments after membrane acquisition, aphids starved for 1 h were allowed to acquire CaMV for 15 min across stretched Parafilm membranes from suspensions containing purified CaMV particles [24], and extracts from P3- and P2-Ala mutant expressing *Sf9* cells in 15 % sucrose/SES buffer as described [7]. Then at least 10 aphids per plant were placed onto 20 one-week old turnip seedlings for at least 1 h inoculation period. Aphids were killed with insecticide as described [25]. After 3 weeks, the percentage of symptomatic plants was scored visually. For plant to plant transmission assays, 2-3 detached medium sized infected leaves with well-established, comparable symptoms were used. Starved aphids were placed on the leaves for 1 h acquisition time. Then at least 10 aphids were placed per plant on turnip seedlings for 1 h inoculation. Aphids were killed as described above and test plants observed for symptoms visually for up to 50 dpi. Plant leaves and *Sf9* extracts were checked for P2 content by Western blot and material devoid of P2 was excluded from analysis.

SDS-PAGE and Western blot

Sf9 extracts or total plant extracts, obtained by grinding frozen leaf discs in 50 mM HEPES pH 8.0, were mixed with 4x Laemmli buffer, heated for 5 min at 105 °C and separated by discontinuous 6/13.5 % SDS-PAGE [26]. Gels were stained with Coomassie Blue or proteins were transferred onto nitrocellulose membranes with a semidry blotting apparatus. Membranes were stained with Ponceau Red S and antigens revealed by Western blot using the NBT-BCIP color reaction for revelation as described [12].

Immunofluorescence microscopy

Protoplasts were fixed with 1 % glutaraldehyde, immobilized on polylysine-coated slides, permeabilized for 1 h with 0.1-0.3 % Triton-X100, bleached for 15 min with 2 mg/ml $NaBH_4$ and blocked in BB buffer (3 % BSA, 0.1 % Triton-X100, 0.05 % NaN_3 in PBS). Cells were then incubated with a 1:200 dilution of anti-rabbit P2, P4 or anti-rat P6, and with a 1:100 dilution of anti-mouse tubulin antibody in BB for 1 h at 37 °C or overnight at 4 °C. After rinsing with PBS, the slides were incubated with a 1:200 dilution of Alexa Fluor 488 or 594-conjugated goat anti-rabbit or goat anti-rat and a 1:75 dilution of Alexa 488 or 594-conjugated goat anti-mouse secondary antibodies (Thermo Fisher Scientific) in BB for 1 h at 37 °C or overnight at 4 °C. Slides were mounted for observation in Fluoromount medium containing DAPI.

Observations were made with a Zeiss LSM700 confocal microscope operated in sequential or single channel mode. Alexa Fluor 594 dye was excited with a 555 nm laser, and the mirror was set to record emission from 555-620 nm. Alexa Fluor 488 fluorophores were excited with a 488 nm laser, and emission was collected from 490-530 nm. For collecting fluorescence of DAPI-colored nuclei, a 405 nm laser was used for excitation and emission was captured from 405-530 nm. Images were captured at 0.29-0.34 µm intervals using a 63x oil-immersion objective. Raw images were processed using proprietary Zen (Zeiss) and open source Image J software (NIH) to adjust brightness and contrast.

Antibodies

The following primary antibodies were used: rabbit anti-P2 [27], rabbit anti-P3 [7] rabbit anti-P6 [9], rat anti-P6 (directed against a peptide and prepared by Eurogentec), monoclonal mouse anti-α-tubulin DM1A [28], and rabbit anti-P4 (Loewe). Alexa 488 and 594 (Thermo Fisher Scientific) and alkaline phosphatase (Santa Cruz) conjugates of secondary antibodies were used for immunofluorescence and Western blot analyses, respectively.

Protoplasts

Protoplasts were prepared from infected turnip leaves by enzymatic digestion as described [12]. For stress treatment, protoplasts were exposed to 0.02 % azide for 40 min, or to gaseous CO_2 for 15 min as described [12]. For transfection, protoplasts were prepared by enzymatic digestion and transfected with 30 µg plasmid using the PEG method as described [29]. Protoplasts were cultivated in Gamborg medium at 25 °C.

PCR and sequencing of CaMV and baculoviruses

For extraction of CaMV DNA, leaves were frozen and ground in 50 mM HEPES pH 8.0, centrifuged for 10 min at 14,000 g, and 10-100x diluted supernatants used for PCR with primers pGREEN-P2-F (5'-AAT-AGG-AAA-CGG-TGC-TTC-ATC-CTC-T-3') and pGREEN-P2-R (5'-TTG-TTA-CAG-GGG-CAA-TCA-TTG-ATG-A-3') flanking P2. For extraction of baculovirus DNA, 100 µl of virus stocks were lysed with 1 volume 10 mM Tris-HCl pH 7.6, 10 mM EDTA, 0.25 % SDS, extracted with 1 volume chloroform and the aqueous phase precipitated with sodium acetate and ethanol. DNA pellets was resuspended in 30 µl of water and aliquots used for PCR with primers pOET-F (5'-GGA-GAT-AAT-TAA-AAT-GAT-AAC-C-3') and pOET-R (5'-CAA-CAA-CGC-ACA-GAA-TCT-AGC-GC-3') flanking P2. PCR products were sequenced using the same primers.

Statistics

Fisher's exact test with 2 nominal variables (infection status and CaMV mutant) was used to compare transmission rates. Tests were run using the R implementation on https://biostatgv.sentiweb.fr/>?module=tests/fisher.

Results

We wanted to identify functional domains in the P2 sequence. For this, we used the alanine-scanning technique and replaced three wild-type (wt) amino acids by three alanine residues approximately every ten amino acids all along the P2 sequence (Fig. 1). As P2 mutations are often lethal in the viral context, thus precluding analysis [20,30], the alanine mutations were first introduced in pCK-P2, a plasmid for transient expression of P2. The plasmids were used to transfect protoplasts purified from turnip plants infected with CaMV B-JI ΔP2 [21]. This recombinant virus is deleted of the entire P2 open reading frame and does not express P2, but is otherwise fully infectious. CaMV B-JI ΔP2 infected protoplasts form *de novo* regular TB when transfected with pCK-P2 [11] and can therefore be used to study P2 functions. We used this system and immunofluorescence to determine whether the P2 alanine mutants could still form regular TBs and, as a functional test, associate with microtubules after CO_2 or azide stress, as does wt P2 [12]. Figure 2A,B shows that wt P2 formed one or several normal TBs in unstressed cells and decorated microtubules in stressed cells as reported [11,12]. The alanine mutants showed different P2 distributions (Fig. 2C-H) that are summarized in Table 1: P2-Ala1 to Ala6 accumulated in diffuse or dense perinuclear inclusions, and stress treatment induced association, to varying degrees, of the mutant proteins with microtubules: P2-Ala6 decorated microtubules, P2-Ala1 was found close to microtubules, Ala2 and Ala5 were found close to microtubules or decorating them, and P2-Ala3 and Ala4 did not colocalize with microtubules. Alanine substitutions in the second half of the P2 sequence (P2-Ala7 to Ala12) did not induce an unusual phenotype: all P2 mutants formed normal-looking TB and stress treatment induced relocalization of mutant P2 from inclusions to microtubule networks.

Because the P2 mutants P2-Ala1 to Ala6 showed, contrary to P2-Ala7 to Ala12, an aberrant P2 location and, except P2-Ala6, aberrant MT interaction, we examined them more closely to better define P2 regions necessary for TB formation and microtubule interaction. For this, we introduced

P2-Ala1 to Ala6 in pGreen-35S-B-JI, an infectious CaMV-encoding plasmid, and rub-inoculated turnip plants with the plasmids. Mosaic and leaf-curling symptoms, typical of CaMV infection, appeared in plants inoculated with wt and all mutant viruses except those inoculated with CaMV-P2-Ala4, which was not infectious, and was excluded from this part of the analysis (Table 2A). Infection rates using plasmids were comparable for wt CaMV and P2-Ala1 mutant virus, and lower for all other mutant viruses (Table 2A). Plants displayed symptoms 22 to 32 days after plasmid inoculation with all viruses except CaMV-P2-Ala2, where symptoms appeared about 20 days later (Table 2A). On subsequent serial passages using extracts from infected leaves as inoculum, the time span from inoculation to appearance of symptoms (latency) shortened considerably for all CaMV mutants. Latency periods as for wild type virus were observed after one serial passage for CaMV-P2-Ala1 and Ala2, whereas CaMV-P2-Ala3, Ala5 and Ala6 took more than one passage to reach latency periods comparable to wild type CaMV (Table 2B).

PCR analysis of the genome region containing P2 indicated that the P2 sequence was gradually deleted from the genome of CaMV-P2-Ala2, Ala3, and Ala6, whereas the P2 sequence of the P2-Ala1 and P2-Ala5 mutants was stably maintained (Table 2A and Fig. 3A). Partial and complete deletions were observed within the same infected plants and accumulated late in infection, starting 58 to 67 days after inoculation with plasmids, which corresponds to 12 (Ala2), 33 (Ala3), and 36 (Ala6) days after appearance of symptoms. Deletions were also observed after serial passages using leaf extracts that had been screened by PCR to contain the full length P2 sequence before being used for inoculation. In this case, deletions appeared 23 to 41 days after inoculation, i.e. 11 to 19 days after appearance of symptoms (Table 2B). Several PCR products of different sizes were often detected in one plant, suggesting that genomes with different deletions in P2 co-existed for a certain time. There was a tendency that deletions appeared faster during later passages. Figure 1S shows for CaMV-P2-Ala6 how P2 deletions appeared during infection and after mechanical serial passages. Sequencing of PCR products indicated partial and complete deletions of the P2 sequence from the genome. Partial deletions introduced often a premature stop codon as was the case for the P2-Ala2 (P2-Ala2-1) and P2-Ala3 (P2-Ala3-1 and -3) mutants shown in Fig. 3B. Interestingly, one of the two *Apa*I sites flanking P2 (used for insertion of P2 mutants into the viral genome) was often conserved (Fig. 3B). Besides deletions, also rearrangements or short insertions were observed occasionally.

We tested by Western blot accumulation of P2 and of key viral proteins, as a proxy to estimate the effect of the different P2-Ala mutations on P2 accumulation and on virus infection. For this we chose the capsid- and TB-associated protein P3, capsid protein P4 and the VF matrix protein P6. Concerning P2, only P2-Ala3 accumulated to wt P2 levels. P2 protein was not detected in plants inoculated with CaMV-P2-Ala1, P2 accumulation was very low in CaMV-P2-Ala5 and low in CaMV-P2-Ala2- and P2-Ala6-infected plants (Fig. 4). P3, P4 and P6 accumulated similarly in mutant and wt-infected plants, indicating that – once symptoms appeared – the mutant P2 proteins had no major effect on accumulation of these proteins and thus on infection. However, as indicated above, mechanically passaging the different viruses using tissue extracts resulted reproducibly in deletion of the P2-Ala2, Ala3 and Ala6 sequences from the genome, suggesting that the mutations destabilized the genome (Table 2).

Then we looked at the ability of the mutant P2 proteins to form TBs in the context of an authentic viral infection and, as a functional test, to interact with microtubules after stress treatment. For this, protoplasts were prepared from infected plants and processed either directly or after treatment with azide or CO_2 for immunofluorescence against P2, P4, P6 and α-tubulin (TUA) to evaluate P2 location with regard to VFs, virions and microtubules. Figure 5A shows that wt P2 accumulated in one or several TBs that were clearly distinct from the often perinuclear VFs. Stress treatment induced dissolution of TB and relocalization of wt P2 in the form of networks, whereas VFs remained unchanged. The wt P2 networks colocalized with microtubules (Fig. 5B). Also a portion of the virions formed, as judged by P4 labeling, networks under stress condition (Fig. 5C),

as reported [11,12]. No TBs or any other P2-containing structures were detected in cells infected with CaMV-P2-Ala1, in line with the results from Western blots, where no P2 protein was detected (Fig. 5A,B), and stress treatment did not induce P4 networks (Fig. 5C). P2-Ala2 accumulated in often perinuclear inclusions. The P2 inclusions labeled positive for P6, suggesting that P2 was at least partially retained in VFs in these cells (Fig. 5A). Occasionally, the inclusions stained also positive for TUA (Fig. 4B) as did wt TBs ([12] and data not shown), indicating that P2-Ala2 could interact with tubulin. Indeed, stress treatment induced P2-Ala2 networks on microtubules and also P4 networks (Fig. 5B,C). We observed also another phenotype in CaMV-P2-Ala2 infected cells, which appeared after several mechanical passages of the virus. In these plants, P2-Ala2 accumulated diffuse in the cytoplasm, but P2 interacted still with microtubules after stress treatment (Fig. 2S). Sequencing revealed that the P2 sequence contained an additional point mutation changing amino acid 10 from a tyrosine to a phenylalanine. P2-Ala3 accumulated in infected cells in one or a few inclusions of various size, some of which were apposed to the nucleus. P2/P6 double-labeling showed that some of the P2-Ala3 inclusions stained positive for P6, indicating that some P2-Ala3 was retained in VFs (Fig. 5A). The P2-Ala3 inclusions contained sometimes also TUA (Fig. 5B). Stress treatment did not induce formation of P2-Ala3 networks on microtubules; the P2-Ala3 inclusions seemed unchanged by the treatment (Fig. 5B), and also virions did not form networks after stress treatment (Fig. 5C). CaMV-P2-Ala5-infected cells displayed P2-Ala5 in often perinuclear inclusions that stained positive for P6 and were thus VFs (Fig. 5A). Stress treatment induced partial P2-Ala5 relocalization on microtubules (Fig. 5B), suggesting that TB formation, but not microtubule interaction was impaired by the mutation. Also a part of the virions localized on networks after stress treatment (Fig. 5C). CaMV-P2-Ala6 formed one or several P2-containing inclusions in infected plant cells. Many of them labeled also positive for P6, suggesting that most of P2-Ala6 was retained in VFs (Fig. 5A). Stress treatment induced P2-Ala6 and virion networks (Fig. 5B,C), indicative of microtubule interaction of P2-Ala6 and virions.

Next we tested aphid transmission of the mutant viruses in plant-to-plant transmission assays. Only CaMV-P2-Ala2, Ala5 and Ala6 were transmissible (Table 3). Transmission rates of the three mutants were significantly lower compared to wt CaMV (93±8 % for wt CaMV, 2±3 % for CaMV-P2-Ala2, 1±3 % for CaMV-P2-Ala5, 21±17 % for CaMV-P2-Ala6; $p<0.001$, Fisher's exact test). Transmission rates of CaMV-P2-Ala6 dropped at later stages of the infection, whereas they remained stable for wt CaMV (Fig. 6). This seemed to correlate with the appearance of deletions of partial or total P2 sequence and concomitant lower P2 protein levels in infected plants (Fig. 2S). CaMV genomes with full length P2 sequence and incomplete P2 sequences coexisted often for some time in infected plants, and transmission was only recorded when at least some full length P2 was present. Whether the same was true for CaMV-P2-Ala2 and Ala5, was difficult to determine because of the low transmission rates.To test whether non-transmissibility of P2-Ala3 was due to the context of the viral infection in the plant or whether it was an intrinsic property of the mutant protein, aphid transmission assays after membrane acquisition of recombinant P2, P3 and purified virions were performed. At the same time we tested also transmission after membrane acquisition of P2-Ala1 that did not accumulate in infected plants, and of P2-Ala4 that was not infectious in the viral context, and where in both cases plant-to-plant transmission experiments were not possible. Table 4 shows that none of the mutant P2 proteins supported virus transmission.

Discussion

In this work we started a functional analysis of the CaMV aphid transmission protein P2. This protein has many exciting features but it is still hardly characterized functionally or structurally. This is mainly due to its intrinsic properties: its insolubility precludes structural characterization by crystallization, its toxicity impedes production in all current recombinant protein expression systems except the *Sf9*/baculovirus system, and many P2 mutants are either unstable or lethal in the viral context, again hindering its characterization. To circumvent this problem, we here implemented successfully a protoplast system using cells infected with a P2 deletion mutant of

CaMV and provided P2 *in trans* by transfection with P2 expression plasmids. These cells display all features of a genuine CaMV infection but do not express P2 and do not contain TBs. TBs will only form after P2 is expressed from the plasmid. This approach avoided interference of P2 mutants with establishment of viral infection, as has been reported for other CaMV P2 mutants that are either non-infectious or revert to wild type [30,31]. Using this approach, we tested twelve P2 alanine scanning mutants distributed along the P2 sequence for their capacity to form TBs and to interact with microtubules after stress treatment. We found that P2-Ala7 to Ala12, all mutated in the second half of the protein, formed normal TBs. Stress induced P2 networks on microtubules for all these six mutants. This indicates that microtubule binding activity, required for P2 export from VFs towards newly forming TBs [21] and for relocalization of P2 from TBs to microtubules during vector acquisition [12], is not mediated by the C-terminal half of P2.

For the P2 alanine substitutions localized in the N-terminus (P2-Ala1 to Ala6), we found variable microtubule interactions. P2-Ala3 and Ala4 did not colocalize with microtubules. P2-Ala1 was detected close to but not on microtubules, Ala2 and Ala5 decorated microtubules or were detected close to microtubules after stress treatment. Finally, P2-Ala6 decorated microtubules like wt P2. These results indicate that the entire P2 N-terminus is involved in microtubule binding, with the region comprising amino acids 35 to 50 contributing most to it, since alanine substitutions in this region (P2-Ala3 and Ala4) abolished microtubule interaction completely. Interestingly, P2-Ala3 inclusions in infected plants contained some apparently soluble tubulin (Fig. 5B) as do wt TBs under certain conditions [12]. This suggests that P2 interaction with microtubules is somewhat different from that with soluble tubulin. Additional analysis is necessary to define more precisely P2's microtubule- and tubulin-binding domain(s).

Basically similar results concerning P2 distribution were obtained when the P2 alanine mutants were introduced in the viral genome. A notable difference was that P2-Ala1 protein could not be detected in infected cells. Apparently, it was unstable in the viral context, as has been reported before for other P2 mutants [9,32]. Further, the diffuse cytoplasmic distribution of P2-Ala2 and Ala3 was less often observed in infected plants than in transfected protoplasts. Rather, P2-Ala2 and Ala3 accumulated, like P2-Ala5 and Ala6, in VFs. The differences might be due to different translation sites of P2: P2 translated from the viral 35S RNA is produced, like all other viral proteins, in or close to VFs and in close contact with P6 and other viral proteins [33], whereas P2 from plasmid-derived mRNA is probably translated free in the cytoplasm. Whatever the reason for the discrepancies, the presence of the mutant P2s in VFs indicated that they were not exported correctly from VFs to emerging TBs [21]. One explanation is that missing or aberrant P2-microtubule interaction prevented P2 export and TB formation. Support for this interpretation comes from the observation that P2 was also retained in VFs after incubation of CaMV-transfected protoplasts with the microtubule depolymerizing drug oryzalin [21]. Alternatively or additionally, a putative P2-P6 interaction region for which there is genetic and biochemical evidence [34,35], might localize to the N-terminus of P2 and be perturbed in the mutants. There were also slight differences in microtubule interaction between transfected protoplasts and protoplasts derived from infected plants: P2 found close to microtubules but not decorating them (P2-Ala2 and Ala5) was less often observed in infected than in transfected cells. We assume again that the less strictly controlled expression of P2 in the transfected cells might account for the differences.

In aphid transmission assays using plants inoculated with the mutant viruses as virus source, CaMV-P2-Ala2 and Ala5 were barely and CaMV-P2-Ala6 moderately transmissible, compared to wt CaMV. The lower transmission rates might be explained partially by lower accumulation of the mutant proteins in infected tissue. However, the transmission rates correlated also nicely with microtubule interaction: P2 from barely transmissible CaMV-P2-Ala2 and Ala5 associated or decorated with microtubules weakly, whereas P2 from highly transmissible wt CaMV and moderately transmissible CaMV-P2-Ala6 decorated microtubules strongly. P2 from CaMV-P2-Ala3 did not interact at all with microtubules and was not transmissible, although it accumulated to

similar levels like wt P2. CaMV-P2-Ala1 and Ala4 could not be tested in plant-to-plant transmission because the mutant virus did not accumulate P2 (CaMV-P2-Ala1) or was not infectious (CaMV-P2-Ala4). Therefore these two P2 mutants and the plant-to-plant non transmissible P2-Ala3 were produced as recombinant proteins and tested for transmission in membrane feeding assays where aphids were allowed acquisition assay periods on suspensions containing mutant P2 and virions. Again, no transmission was observed. Because these P2 mutants had in common that they did not or only weakly interact with microtubules in transfected protoplasts, the results indicate that the microtubule interaction domain might overlap with the yet undefined stylet-binding domain, which is localized in P2's N-terminus [19].

An interesting observation was that the sequences of P2-Ala2, Ala3 and Ala6 were unstable in the genome and were partially or completely deleted from the viral genome whereas wt P2, P2-Ala1 and Ala5 sequences were stable. Partial deletion of the P2 sequence often introduced premature stop codons. Besides deletions also rearrangements were observed. Taken together, this indicates that expression of P2-Ala2, Ala3 and Ala6 was noxious to the virus. Wild type P2 is regarded as a toxic protein whose expression is tightly regulated [30,31,36]. According to this hypothesis, the alanine substitutions in P2-Ala2, Ala3 and Ala6 increased P2 toxicity and their elimination by deletion restored viral fitness. Expression of P2-Ala4 might have been too toxic to allow CaMV replication, explaining why it was not infectious. Finally, P2-Ala5 had similar properties like wt P2, and P2-Ala1 could not exhibit its possible toxicity because it did not accumulate in infected cells. Therefore, the latter two mutants were maintained in the genome.

It remains enigmatic why P2 is maintained in the CaMV genome at all because this protein has, except transmission, no other function during infection of the plant and because P2 deletion mutants are as infectious as wild type CaMV. One should expect that selection pressure eliminates P2 from the genome just as GFP and other transgenic insertions into viral genomes are often unstable [37,38]. One explanation is that the replication initiation site and the splice acceptor site in the P2 sequence boost replication and counterbalance costs of P2 expression. Since the observed partial and complete deletions removed also the genetic elements in the P2 sequence, they do not seem to be required for CaMV infection when there is no P2 gene or accumulation.

Complete deletions conserved one of the two *Apa*I sites used for insertion of P2 sequences. We propose the repetitive *Apa*I sequences were used as a scaffold to excise the P2 sequence in several steps, probably during reverse transcription or synthesis of the +strand DNA. A similar observation was made for the C4-184 mutant where the deletion site eliminating a large portion of the P2 sequence is surrounded by a stretch of highly similar sequences [39].

Taken together, we present here evidence for a modular organization of P2 that will be fascinating to explore further. Its C-terminus is dedicated to viral interactions, i.e. P2 self-interaction [15] and binding to P3 and virions [5]. Its N-terminus controls interactions with the environment, i.e. vector-binding and microtubule interaction. Interestingly, functions housed by the modules overlap; domains for P2 self-interaction and P2-P3 interaction are contained in the same region of the C-terminus, and those for vector-binding and microtubule interaction map to the same stretch in the N-terminus of P2.

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Author contributions

Christiane Then: conceptualization, validation, investigation, writing – original draft, writing – review & editing, visualization, supervision. Aurélie Bak: conceptualization, investigation. Alexandre Morisset: validation, investigation. Beatriz Dáder: investigation, validation, writing – review & editing. Marie Ducousso: validation, investigation. Jean-Luc Macia: validation, investigation. Martin Drucker: conceptualization, writing – original draft, writing – review & editing, visualization, formal analysis, supervision, project administration, funding acquisition.

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Figures

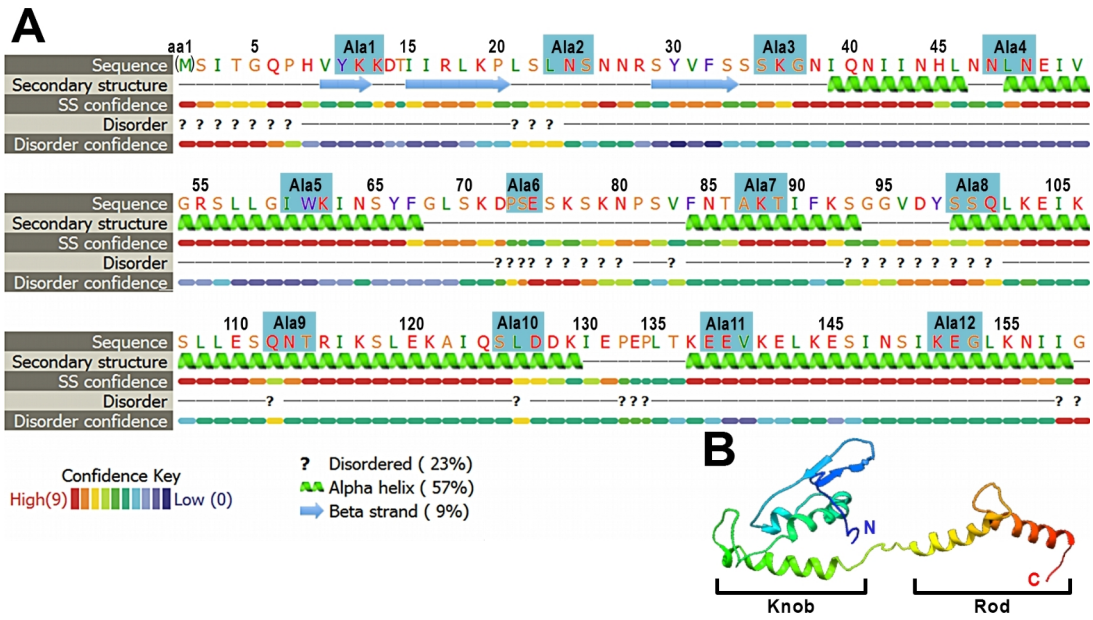

Figure 1: Structure prediction of P2. (A) Secondary structure prediction discerns between β-strands, α-helices and disordered regions. The confidence of the prediction is indicated for each amino acid. The blue boxes show the position of the alanine substitution. (B) Three-dimensional model of P2. The colors represent the order of the amino acids from the N-terminus (dark blue) to the C-terminus (red). The overall structures suggests an N-terminal knob, separated by an unstructured stretch from a rod-like C-terminus. All predictions were made with the PHYRE2 program [40].

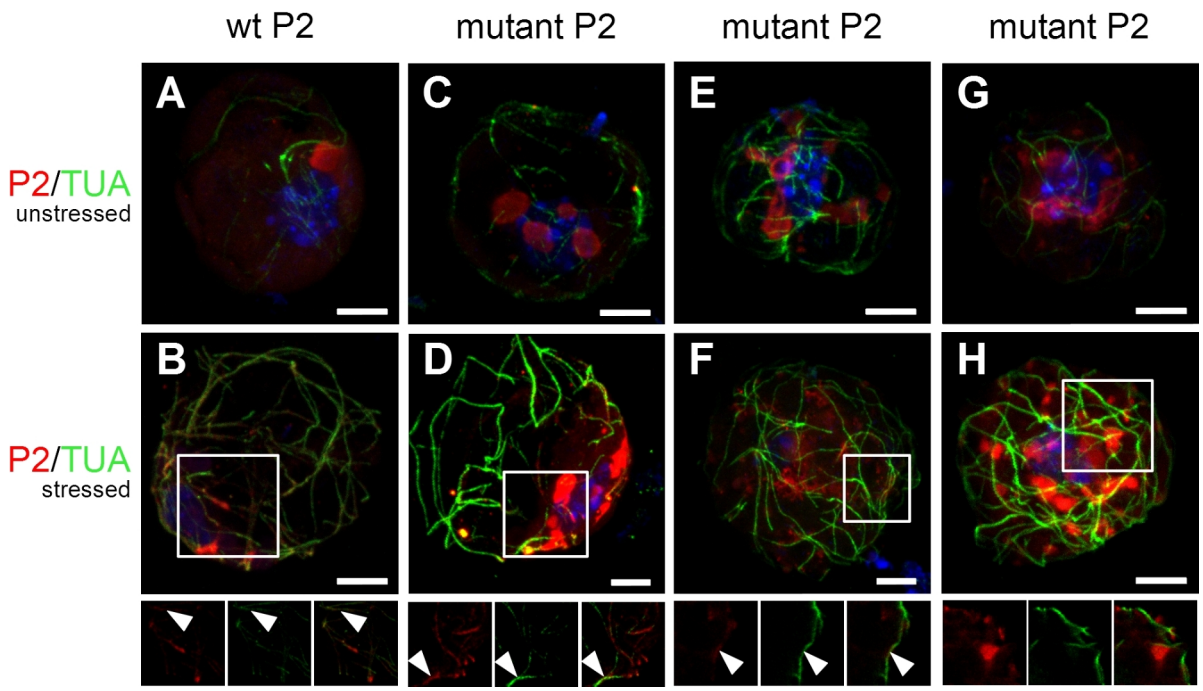

Figure 2: Ectopic expression of P2 alanine (Ala) mutants in CaMV-ΔP2-infected protoplasts. CaMV-ΔP2-infected protoplasts were transfected with plasmids coding for wt P2 or the alanine mutants P2-Ala1 to Ala12. After overnight incubation the protoplasts were processed directly (top panel) or after CO_2 stress (bottom panel) for immunofluorescence against P2 (red) and α-tubulin (TUA, green). Nuclei are counterstained with DAPI (blue). (A) Wild type P2 accumulates in a typical transmission body and associates (B) with microtubules after CO_2 treatment. (C-H) P2 alanine mutants show a wt phenotype (C), accumulate in perinuclear irregular dense (E) or less dense (G) inclusions and are, upon stress treatment still able to decorate microtubules (D,F) or are found only close to, but not on microtubules (H). Shown are confocal maximum projections (top panels) or single channels of single optical sections (small images in the bottom panel) of the regions outlined in the projections. The arrowheads point to colocalizing P2 and α-tubulin label represented in the insets in panels B, D, F and H. The following P2-Ala mutants are shown: P2-Ala8 (C,D), Ala3 (E), Ala2 (F) and Ala1 (G,H). Scale bar 5 μm.

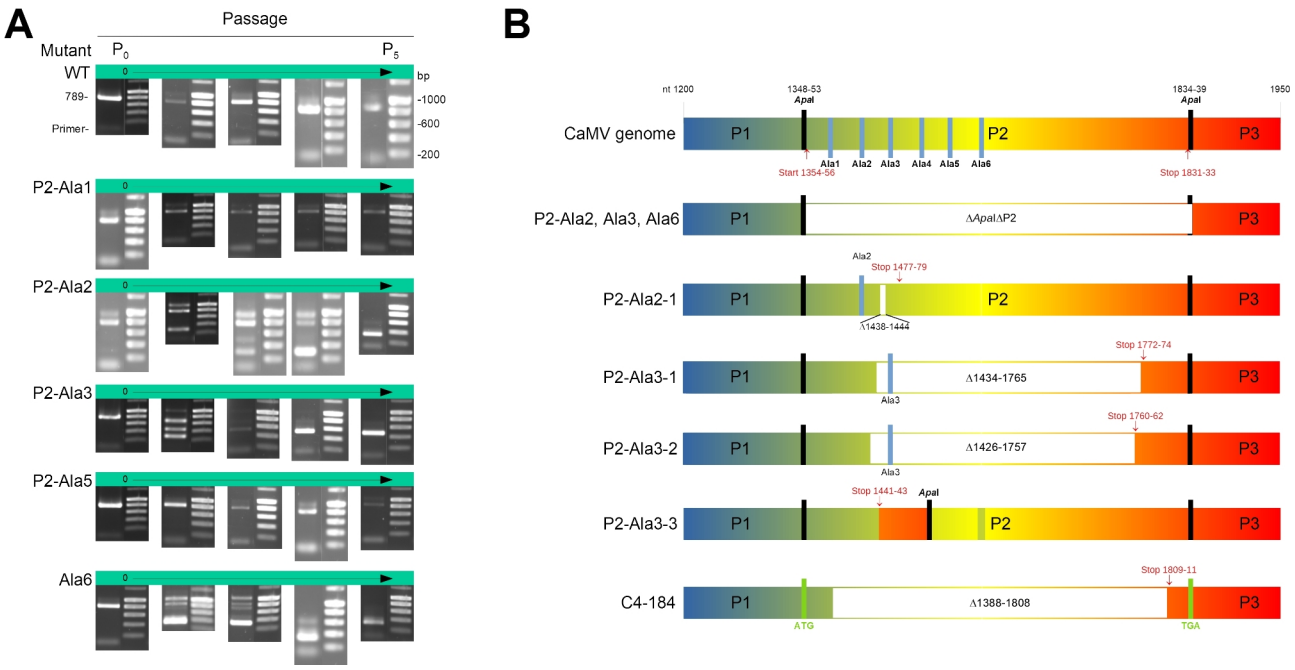

Figure 3: Deletion of the P2 sequence from the CaMV genome. (A) PCR detection of deletions. Extracts from infected plants were used in PCR with primers hybridizing at the end of P1 and the beginning of P3 of the CaMV genome. Wild type virus containing the entire P2 sequence yielded a 789 bp amplicon, and a genome deleted of the entire P2 sequence a 312 bp product. P0 corresponds to plants initially inoculated with plasmid DNA, and P5 to the last (5th) passage tested. The samples presented between P0 and P5 are from intermediate passages. Wild type P2 as well as P2-Ala1 and Ala5 were stable throughout the number of passages tested, whereas P2-Ala2, Ala3 and Ala6 sequences were deleted from the genome. Note that P2-Ala2 and Ala6 genomes contained deletions comprising almost the entire P2 sequence, whereas P2-Ala3 maintained ~150 bases of the P2 open reading frame. (B) Examples of deletions and rearrangements of the region comprising P2. PCR amplicons obtained with the same primers as in (A) were gel-purified and sequenced. The first image shows the genome region including P2, the *Apa*I restriction sites used for inserting the P2-Ala mutant sequences and the position of the alanine substitutions. Premature stop codons created by the deletions are shown in the deleted sequences. For comparison, the natural P2 deletion mutant C4-184 is shown at the bottom.

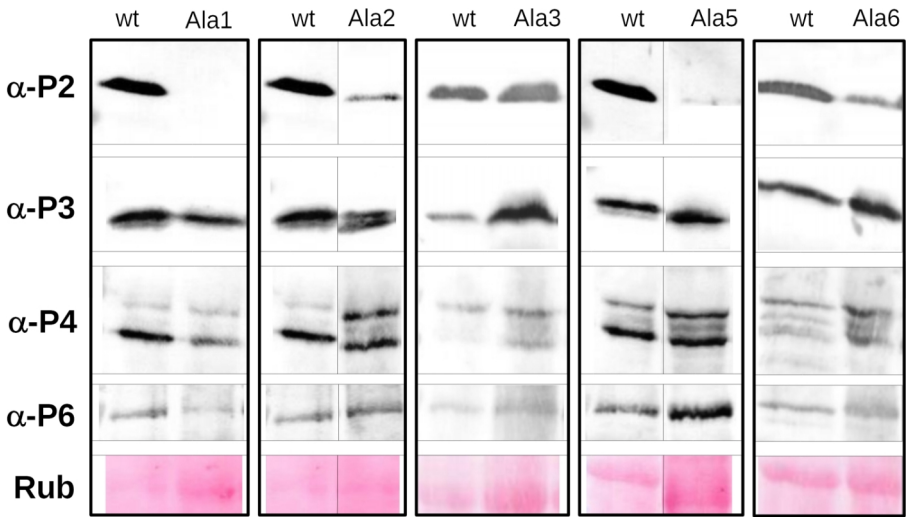

Figure 4: Expression of viral proteins. Total extracts of infected leaves prepared after at least one serial passage of the viruses were analyzed by Western blot using the indicated antisera. Wild type and mutant CaMV-infected leaves presenting similar symptoms were used for analysis. This corresponds for wild type and P2-Ala1 leaves harvested at 19 dpi, for Ala2 at 27 dpi, for Ala3 at 36 dpi, for Ala5 at 36 dpi, and for Ala6 at 40 dpi. P2-Ala1 and Ala2 extracts were analyzed on the same membrane and show therefore the same wt extract. P2-Ala3, Ala5 and Ala6 extracts were analyzed independently on separate membranes, each together with a wt extract. The black vertical lines in the P2-Ala2 and Ala5 blots indicate that wt and mutant extracts were charged on the same gel, but not on neighboring lanes. Poinceau Red stain of the Large RuBisCO subunit (Rub) served as loading control. Experiments were repeated 3 times with similar results.

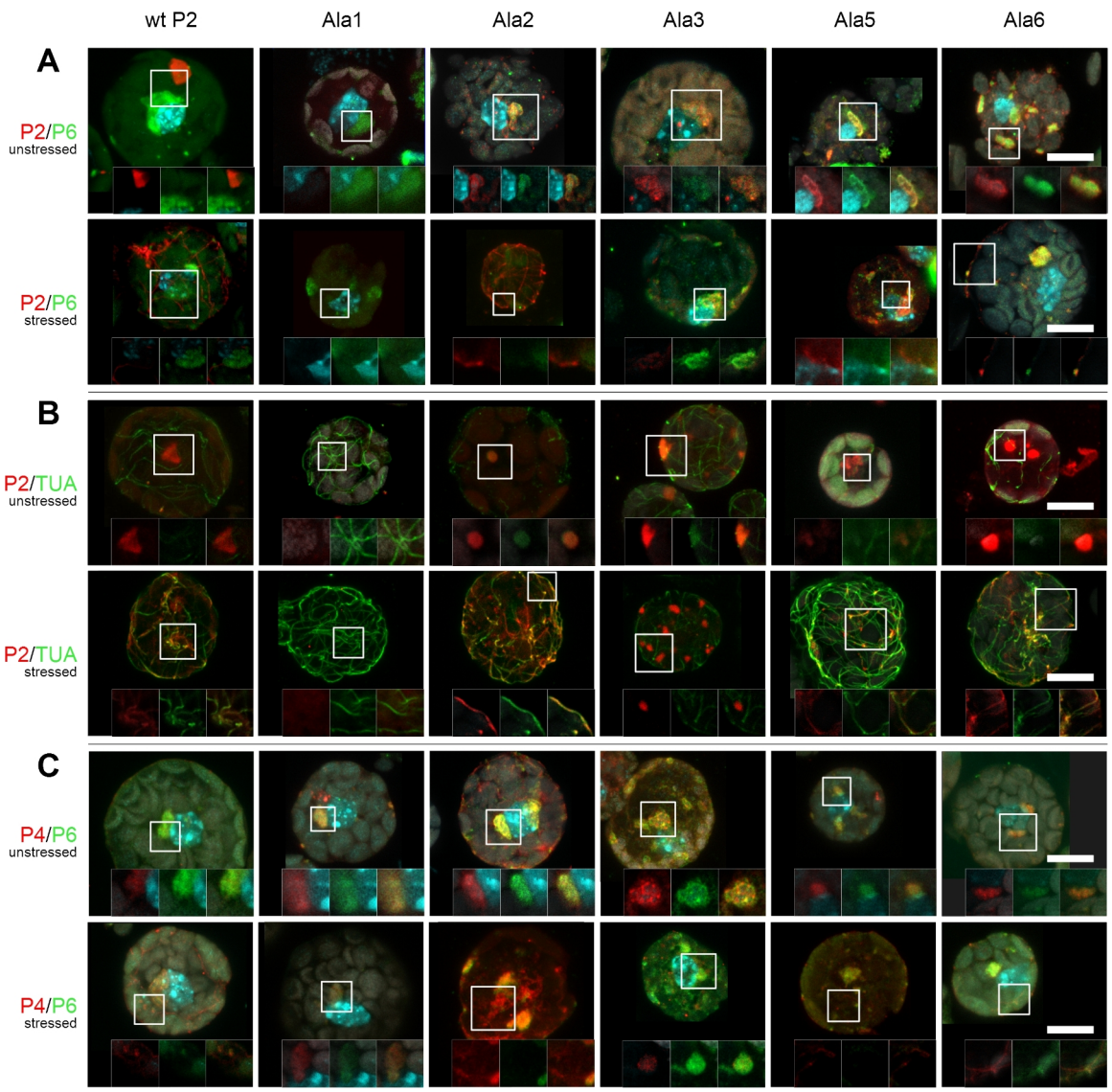

Figure 5: Phenotype of P2 alanine mutants in mutant CaMV-infected plant cells. Protoplasts purified from infected plants were processed for immunofluorescence against (A) P2 (red) and P6 (green), (B) P2 (red) and TUA (green) and (C) P4 (red) and P6 (green) either directly (unstressed) or after stress treatment. Nuclei are presented in blue (DAPI stain) in some images to show perinuclear localization of viral inclusions. Shown are confocal maximum projections and in the insets single channels of single sections of the projection. Scale bar 10 µm.

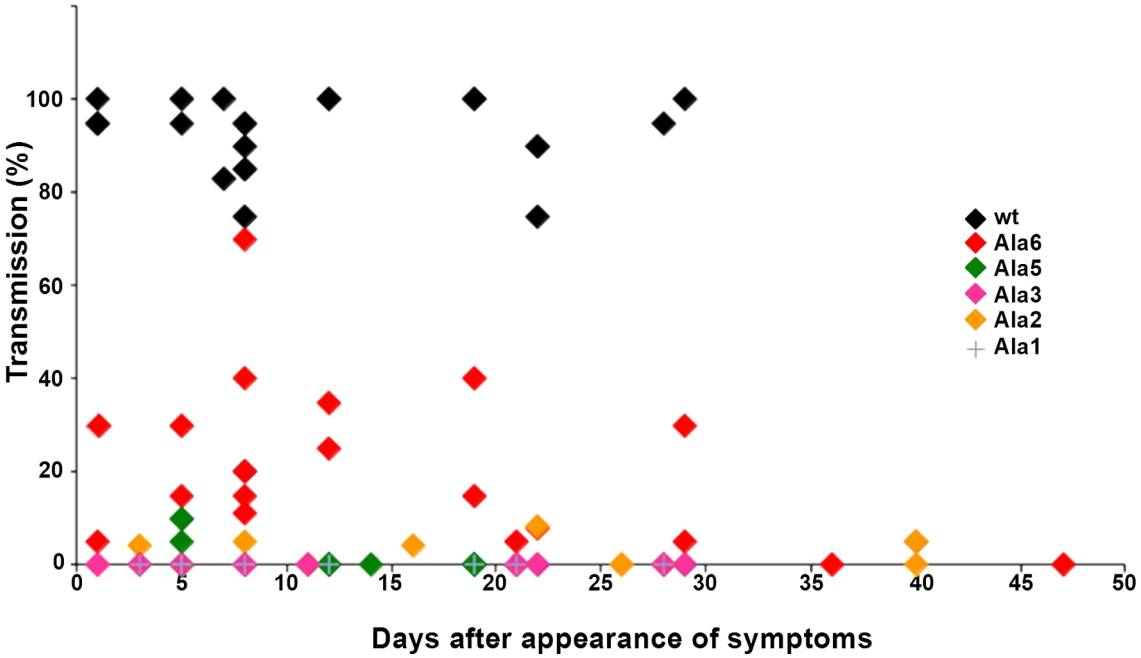

Figure 6: Transmission of P2-Ala mutants related to age of infection. Starved aphids were placed for 60 min on infected turnip leaves for virus acquisition and then transferred to healthy turnip seedlings for inoculation. Ten aphids per test plant and 20 test plants were used for each assay. Each point represents one transmission assay realized at the day after appearance of symptoms indicated in the x-axis.

Tables

Table 1: Phenotype of P2-Ala mutants. Wild type or mutant P2 were transiently expressed in protoplasts infected with CaMV B-JI ΔP2, and the distribution of P2 in untreated cells and in cells treated with CO_2 analyzed by immunofluorescence microscopy. TB, transmission body.

	Untreated cells	Cells treated with CO_2	
P2 mutant	P2 localization	P2 decorates microtubules	P2 is found close to microtubules
wild type	In normal TB	+	-
Ala1	In perinuclear aggregates	-	+
Ala2	Diffuse in cytoplasm	+	+
Ala3	Diffuse in cytoplasm	-	-
Ala4	In perinuclear aggregates	-	-
Ala5	In perinuclear aggregates	+	+
Ala6	In perinuclear aggregates	+	-
Ala7	In TB	+	-
Ala8	In TB	+	-
Ala9	In TB	+	-
Ala10	In TB	+	-
Ala11	In TB	+	-
Ala12	In TB	+	-

Table 2: Infectivity and stability of CaMV P2-Ala mutants. Turnip seedlings in the two juvenile leaf stage were for initial inoculation rub-inoculated with plasmids encoding the CaMV genome harboring wild type or mutant P2 and observed daily for symptoms. For the subsequent serial passages, extracts prepared from infected leafs were mechanically inoculated to test plants. The extracts were verified by PCR for presence of the P2 sequence before inoculation. dpi, days after inoculation, das, days after appearance of symptoms, n.a. not applicable, n.t. not tested.

A: Plasmid inoculation

CaMV mutant	Plants infected/inoculated	Infected plants (%)	Appearance of symptoms (dpi)	P2 sequence stable	Start degradation of P2 sequence	
					dpi	das
wild type	9/12	75	22-25	Yes	-	-
P2-Ala1	11/16	69	28-29	Yes	-	-
P2-Ala2	3/32	9	43-46	No	58-62	12-19
P2-Ala3	3/15	20	24-27	No	60-65	33-41
P2-Ala4	0/21	0	n.a.	n.a.	n.a.	n.a.
P2-Ala5	4/20	20	22-28	Yes	-	-
P2-Ala6	2/15	13	26-32	No	67-71	35-45

B: Inoculation with leaf extracts

CaMV mutant	Appearance of symptoms (dpi)		P2 sequence stable	Start degradation of P2 sequence (dpi)			
				1st passage		5th passage	
	1st passage	5th passage		dpi	das	dpi	das
wild type	12	n.t.	Yes	-	-	-	-
P2-Ala1	13	11	Yes	-	-	-	-
P2-Ala2	13	12	No	27	14	27	15
P2-Ala3	22	11	No	41	19	23	12
P2-Ala4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
P2-Ala5	21	11	Yes	-	-	-	-
P2-Ala6	17	12	No	34	17	23	11

Table 3: Transmission of CaMV P2-Ala mutants. Starved aphids were allowed to feed for (A) 60 min to acquire CaMV from infected turnip leaves or (B) 15 min to acquire purified CaMV particles, recombinant his-tagged P2 (HP2) and P3 by membrane feeding, and then transferred to turnip seedlings for inoculation. Ten aphids per test plant were used for plant-to-plant transmission assays and for transmission tests after membrane acquisition feeding. n.a., not applicable.

A: Plant-to-plant transmission

CaMV mutant	Infected plants/total plants	n experiments	% Transmission (min-max)
Wild type	373/403	9	93 (75-100)
P2-Ala1	0/220	6	0*
P2-Ala2	6/328	13	2 (0-8)
P2-Ala3	0/256	8	0
P2-Ala4	n.a.	n.a.	n.a.
P2-Ala5	3/496	8	1 (0-10)
P2-Ala6	83/400	10	21 (0-70)

*no P2 protein detected.

B: Transmission after membrane acquisition

P2 mutant	Infected plants/total plants	n experiments	% Transmission (min-max)
HP2	74/259	8	29 (0-95)
HP2-Ala1	0/160	5	0
HP2-Ala3	0/260	8	0
HP2-Ala4	0/200	6	0