Heterologous encapsidation of recombinant pea early browning virus

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The coat protein gene of pea early browning virus (PEBV) was replaced with that of another tobavirus, tobacco rattle virus (TRV strain PPK20). The recombinant virus multiplied efficiently in the systemic host Nicotiana benthamiana and, on the local lesion host Phaseolus vulgaris, produced symptoms typical of PEBV rather than TRV showing that viral coat protein is not a determinant for lesion morphology. Both viral RNAs were encapsidated by TRV coat protein although the shorter particles (encapsidated RNA-2) did not form a discrete population. Evidence is presented to suggest involvement of nucleotide sequences upstream of the coat protein gene in virus particle assembly.

Introduction

The tobravirus group of plant viruses comprises three distinct members, pea early browning virus (PEBV), tobacco rattle virus (TRV) and pepper ringspot virus (PRV) (Harrison & Robinson, 1986). These viruses have a bipartite, positive-sense, ssRNA genome, the larger RNA (RNA1) encoding the replicase and cell-to-cell spread proteins and the smaller RNA (RNA2) encoding the virus coat protein usually together with one or more other proteins of unknown function (Bergh et al., 1985; Cornelissen et al., 1986; Angenent et al., 1986; Hamilton et al., 1987; MacFarlane et al., 1989; Goulden et al., 1990). Tobraviruses exhibit considerable variation in the composition of their coat proteins; in consequence, particularly for TRV, a large number of serologically different virus isolates have been identified (Harrison & Woods, 1966; Robinson & Harrison, 1985).

Previous studies have shown that under greenhouse conditions pseudorecombinant tobraviruses can be generated having RNA1 from one virus isolate and RNA2 from a different isolate of the same virus. Viable pseudorecombinants cannot be formed having genome parts from different viruses, for example RNA1 from TRV and RNA2 from PEBV. Indeed, the delineation of the three tobraviruses is based partly on the incompatibility of their genome components (Lister, 1968; Frost et al., 1967). However, more recently it has become apparent that at least two of the tobraviruses can undergo recombination in planta. A number of TRV isolates have been described in which part of the RNA2 has been replaced by sequences from PEBV (Robinson et al., 1987). These isolates induce disease symptoms typical of TRV and can form pseudorecombinants only with other TRV isolates. However, these anomalous isolates are serologically identical to PEBV. The mechanism for the generation of such recombinant viruses is not known although a copy-choice model has been proposed in which a replicase molecule bound to a partially extended genome RNA from one virus switches to a second viral RNA template during minus-strand synthesis (Angenent et al., 1986). In contrast to the situation with TRV, recombinant isolates of PEBV carrying part of TRV RNA2 have not been described.

It might be expected that the direction of RNA exchange between two coinfecting viruses would be dependent on the relative population levels of each virus, a ratio which would alter according to the host plant in which the viruses were present. The absence of recombinant PEBV isolates might, therefore, be the result of horticultural practices where there is a predominantly one-way transport of PEBV-infected material into areas containing mainly TRV-infected plants. Alternatively, perhaps PEBV replicase is less prone to template switching than is TRV replicase, or more prosaically the PEBV coat protein may not encapsidate TRV RNA thus limiting the spread of such recombinants in the field. To investigate this phenomenon in more detail we have used recombinant DNA techniques to replace the coat protein gene of the SP5 isolate of British PEBV with that of the PRN-serotypic PPK20 isolate of TRV (Ploeg et al., 1992). Here we describe the infectious nature and symptomatology of this engineered recombinant virus.
Methods

Clone construction. The construction of pCaN1 and pCaN2, full-length infectious cDNA clones of PEBV, has been described (MacFarlane et al., 1992). A Clal site was introduced into pCaN2 at position 488 [23 nucleotides (nt) upstream of the coat gene protein] using a synthetic oligonucleotide (5' AACTCGGTATGCCGATACGTAAA). A BamHI site was introduced at position 1167 (28 nt downstream of the coat protein gene) using a second oligonucleotide (5' TACATT GGATCCGTTAGGGAG). Mutagenesis was performed using the method of Kunkel (1985) and correctly modified clones were checked by restriction enzyme mapping and DNA sequencing. The clone carrying both newly introduced restriction sites was designated the coat protein gene clone pCaN2CP9.16. Clone pCaN2CP9 was created by replacing the 945 bp HindIII-NheI fragment containing the 5' non-coding region and 5' 460 bp of the coat gene protein from pCaN2 with the same fragment from pCaN9.16. This clone carries the Clal site introduced upstream of the coat protein gene but lacks the BamHI site introduced into pCaN2CP9.16 downstream of the coat protein gene.

The synthesis, cloning and mutagenesis of cDNA corresponding to the coat gene of TRV strain PPK20 will be described elsewhere (A. Mathis, A. T. Ploeg, D. J. F. Brown & J. F. Bol, unpublished). Clone pK42.15 contains the TRV coat gene protein flanked 20 nt upstream by a Clal site. The complete TRV coat protein gene can be excised from this clone as a Clal-BglII fragment. pCaN2CP9.16 was digested with Clal and BamHI and purified away from the PEBV coat protein gene by electrophoresis from an agarose gel. The TRV PPK20 coat protein gene was isolated in a similar manner and ligated into pCaN2CP9.16 to create the hybrid PEBV/TRV clone pCaN2CPK20. The PEBV coat protein is 212 amino acids in length with a calculated Mr of 23244. TRV strain PPK20 coat protein is 204 amino acids in length with a calculated Mr of 22330. Computer analysis shows that these two proteins share 38% amino acid sequence identity (A. Mathis, A. T. Ploeg, D. J. F. Brown & J. F. Bol, unpublished).

Inoculation of plants. Plasmid clones pCaN2, pCaN2CP9, pCaN2CP9.16 and pCaN2CPK20 were purified by centrifugation through caesium chloride-ethidium bromide gradients, precipitated with ethanol and resuspended in water. Two lg of each clone was combined with 2 lg of pCaN1 and sterile distilled water in a total volume of 10 ml and gently rubbed onto a single Nicotiana benthamiana leaf which had been dusted with carborundum. In addition, plants were inoculated manually with TRV strain PPK20 (provided by D. J. F. Brown, Scottish Crop Research Institute) or the SP5 isolate of British PEBV. The plants were examined at 7 days post-inoculation (p.i.) for symptoms of virus infection.

Local lesion assay. Systemically infected leaves of N. benthamiana plants inoculated either with the infectious cDNA clones, TRV or PEBV were homogenized in 5 ml/g 30 mm-sodium phosphate buffer pH 7. The homogenate was then inoculated onto French bean (Phaseolus vulgaris L. var. The Prince) and Chenopodium amaranticolor. Local lesions were examined at 4 days p.i.

Northern blotting. RNA was extracted from virus-infected plants using the method of Verwoerd et al. (1989) and analysed by Northern blotting as described previously (MacFarlane et al., 1992). PEBV-specific probes were derived from clones pT7FL3 and pT72 which, respectively, carry the full-length 7.0 and 3.4 kb cDNAs of RNAs 1 and 2 (MacFarlane et al., 1991). TRV-specific probes were derived from clone pK42.15 which carries a 1.5 kb cDNA fragment comprising the virus coat gene protein and downstream sequences (A. Mathis, unpublished).

Western blotting. Samples of virus-infected leaves were frozen in liquid nitrogen, ground to a powder and extracted with PBS. A sample of the soluble protein fraction was separated by electrophoresis in a 15% polyacrylamide gel (Laemmli, 1970), transferred to nitrocellulose and probed with either a 100-fold dilution of a polyclonal rabbit antiserum raised against the SP5 isolate of British PEBV or a 1000-fold dilution of a polyclonal rabbit antiserum raised against TRV strain PRN (provided by D. J. Robinson, Scottish Crop Research Institute).

Immunosorbent electron microscopy (ISEM). This was carried out as described by Roberts et al. (1984). The lengths of trapped particles stained with 1% uranyl acetate were measured from electron micrographs using a Summagraphics digitizer linked to a Tektronix 4051 microprocessor.

Stability assay of recombinant virus. N. benthamiana plants were inoculated as described above with pCaN1 plus pCaN2, pCaN1 plus pCaN2CP9.16, or pCaN1 plus pCaN2CPK20. The plants were harvested at 9 days p.i., cut up with scissors and divided into two batches. One batch was homogenized by grinding in a mortar together with 5 ml/g sodium phosphate buffer pH 7. The second batch of leaf material was extracted using a modification of the method of Jupin et al. (1990). The leaf tissue was homogenized in a mortar together with 5 ml/g TM buffer (100 mm-Tris-HCl, 10 mm-MgCl2, pH 7.5). Cell debris was removed by low-speed centrifugation and aliquots of both supernatants were inoculated onto C. amaranticolor plants. The remainder of the supernatant extracted in TM buffer was incubated at 37 °C for 30 min and then aliquots were inoculated onto C. amaranticolor. The stability of unencapsidated RNA to these treatments was tested by mixing purified TRV PPK20 RNA with both the phosphate-buffered and TM-buffered homogenates derived from non-inoculated N. benthamiana plants. RNA was added to a final concentration of 50 ng/ml and 1 ug RNA was inoculated onto each C. amaranticolor plant. Plants were examined for the production of necrotic lesions at 5 days p.i. As an additional test of particle stability, virus was purified from N. benthamiana plants which had been inoculated with a homogenate of plants previously infected with pCaN1 and pCaN2CPK20 DNAs. Virus particles were purified by precipitation with polyethylene glycol followed by repeated cycles of low-speed and high-speed centrifugation as described by MacFarlane et al., (1989). The virus preparation was resuspended in 30 mm-sodium phosphate pH 7.0 and stored at 4 °C for 10 months. Subsequently the virus preparation was inoculated onto N. benthamiana, RNA was isolated from the plants at 6 days p.i. and analysed by Northern blotting.

Results

Biological properties of the PEBV recombinant

All the viruses tested in this work produced similar small chlorotic lesions on C. amaranticolor. TRV strain PPK20 produced the most severe symptoms with the inoculated leaf often being killed by 4 days p.i. TRV PPK20 produced pinpoint necrotic lesions on P. vulgaris (Fig. 1 a) whereas PEBV strain SP5 (Fig. 1 b), cloned PEBV (pCaN1/pCaN2), the intermediate cloned PEBV (pCaN1/pCaN2CP9.16) and the cloned PEBV/TRV hybrid (pCaN1/pCaN2CPK20) (Fig. 1 c) all produced large (3-4 mm diameter) necrotic lesions. PEBV SP5 produced symptoms of extensive leaf curling and stunting in the systemic host N. benthamiana whereas TRV PPK20 produced only limited leaf curling together with some vein necrosis of the systemically infected leaves. The intermediate cloned PEBV (pCaN1/
Recombinant PEBV: heterologous encapsidation

Fig. 1. Local lesions in P. vulgaris var. The Prince inoculated with TRV strain PPK20 (a), PEBV strain SP5 (b) and pCaN1/pCaN2CPK20 (c).

Fig. 2. Northern blot of RNA from individual N. benthamiana plants. Lanes M, mock-inoculated; lanes 1, inoculated with pCaN1/pCaN2; lanes 2, pCaN1/pCaN2CP9.16; lanes 3, pCaN1/pCaN2CPK20. The blot was probed with cDNA to PEBV RNAs 1 and 2 (a) or to TRV PPK20 RNA2 (b). The positions of RNAs 1 and 2 are marked by arrowheads.

pCaN2CP9.16) and the cloned hybrid (pCaN1/pCaN2CPK20) produced symptoms in N. benthamiana similar to those caused by PEBV SP5.

Analysis of viral RNAs and virus serology

Northern blot analysis showed that plants inoculated with each cloned virus contained both RNA1 and RNA2 in systemically infected leaves. pCaN1/pCaN2CPK20-inoculated plants contained amounts of viral RNAs similar to pCaN1/pCaN2CP9.16- and CaN1/pCaN2-inoculated plants (Fig. 2a). In Western blots both pCaN1/pCaN2- and pCaN1/pCaN2CP9.16-inoculated plants contained virus coat protein which reacted with
PEBV-specific antiserum but not with TRV-specific antiserum. Conversely, pCaN1/pCaN2CPK20-inoculated plants reacted strongly with TRV-specific antiserum whereas pCaN1/pCaN2- and pCaN1/pCaN2CP9.16-inoculated plants did not (Fig. 3). Some cross-reactivity occurred with the TRV PRN antiserum recognizing the PEBV coat protein. The TRV PRN antiserum is known to be less specific than the PEBV antiserum and has been shown to trap PEBV particles in ISEM tests (I. Roberts, unpublished). It was also apparent that during SDS-PAGE the TRV PPK20 coat protein migrated more slowly than that of PEBV SP5 whereas the predicted $M_r$ of the PPK20 protein is slightly smaller than that of PEBV.

**Calculation of virus particle length**

Virus particles were identified in plants inoculated with each of the different viruses. Lengths were calculated by comparison with micrographs of catalase-coated grids. Data are presented graphically (Fig. 4) and mean particle lengths for each virus or cDNA clone combination are

Fig. 4. Frequency length distributions of virus particles in *N. benthamiana* plants inoculated with PEBV strain SP5 (a), pCaN1/pCaN2 (b), TRV strain PPK20 (c), pCaN1/pCaN2CP9.16 (d), pCaN1/pCaN2CPK20 (e) or pCaN1/pCaN2CP9 (f). The x-axis denotes particle length in nm. Each bar spans 4 nm. The y-axis denotes the number of particles observed within each size class.
Table 1. Mean particle lengths of natural and cloned virus isolates

<table>
<thead>
<tr>
<th>Virus isolation</th>
<th>Mean length (nm)</th>
<th>s.d.</th>
<th>No. observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEBV SP5</td>
<td>L: 212.5</td>
<td>6.16</td>
<td>85</td>
</tr>
<tr>
<td>PEBV SP5</td>
<td>S: 99.9</td>
<td>6.18</td>
<td>118</td>
</tr>
<tr>
<td>TRV PPK20</td>
<td>L: 189.0</td>
<td>4.65</td>
<td>117</td>
</tr>
<tr>
<td>TRV PPK20</td>
<td>S: 104.0</td>
<td>5.53</td>
<td>183</td>
</tr>
<tr>
<td>pCaN1/pCaN2</td>
<td>L: 213.6</td>
<td>8.0</td>
<td>173</td>
</tr>
<tr>
<td>pCaN1/pCaN2</td>
<td>S: 101.4</td>
<td>7.21</td>
<td>119</td>
</tr>
<tr>
<td>pCaN1/pCaN2CP9,16</td>
<td>L: 214.2</td>
<td>7.58</td>
<td>180</td>
</tr>
<tr>
<td>pCaN1/pCaN2CP9,16</td>
<td>S: ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pCaN1/pCaN2CPK20</td>
<td>L: 200.3</td>
<td>6.15</td>
<td>139</td>
</tr>
<tr>
<td>pCaN1/pCaN2CPK20</td>
<td>S: ND</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pCaN1/pCaN2CP9</td>
<td>L: 215.0</td>
<td>7.11</td>
<td>94</td>
</tr>
<tr>
<td>pCaN1/pCaN2CP9</td>
<td>S: ND</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* ND, Not determined.

The mean lengths of PEBV L particles (encapsidated RNA1) and S particles (encapsidated RNA2) were previously determined to be about 215 nm and 105 nm respectively (Harrison, 1973). These values are in close agreement with data derived for L particles of the PEBV SP5, pCaN1/pCaN2, pCaN1/pCaN2CP9 and pCaN1/pCaN2CP9.16 viruses and for the S particles of the PEBV SP5 and pCaN1/pCaN2 viruses examined in this work. Similarly, the published mean lengths of TRV L and S particles are 185 to 196 nm and 50 to 115 nm, respectively (Robinson & Harrison, 1989). The wide range ascribed to the S particle mean length reflects the great variation in the size of RNA2 from different isolates of TRV. The measurements obtained in this work for TRV strain PPK20 are in good agreement with the published figures. The mean particle length of L particles in plants inoculated with pCaN1/pCaN2CPK20 was 200.3 nm which is shorter than that obtained for PEBV strain SP5 and larger than that obtained for TRV strain PPK20. Plants inoculated with pCaN1/pCaN2CP9, pCaN1/pCaN2CP9.16 or pCaN1/pCaN2CPK20 did not contain S particles with a tightly clustered mean length but rather had S particles covering a wide size range. The stability of this distribution was assessed by analysing the particle length profile of pCaN1/pCaN2CPK20 after a second passage in N. benthamiana. In this experiment the wide variability in the length of S particles was retained (data not shown). Attempts were made to construct clones having only the novel ClaI site upstream of the coat protein gene or the BamHI site downstream of the coat protein gene. We were able to make a clone carrying only the 5' ClaI site (pCaN2CP9) which did, nevertheless, retain the variable S particle length profile (Fig. 4f). Also the distribution of L and S particles differed according to the source of the inoculum. Plants inoculated with homo-

Table 2. Stability of homologous and recombinant virus

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Phosphate buffer, t = 0 min</th>
<th>TM buffer, t = 30 min</th>
</tr>
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<tbody>
<tr>
<td>Water</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Water + PPK20 RNA</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pCaN1 + pCaN2</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pCaN1 + pCaN2CP9.16</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pCaN1 + pCaN2CPK20</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

* (-) No lesions; (+) 50 to 100 lesions; (++) 200 to 500 lesions. Numbers are lesions present on half-leaves of two C. amaranticolor plants. Inoculation procedure is described in the text.

Stability of recombinant PEBV

Northern blot analysis clearly showed the accumulation of full-length RNA1 and RNA2 molecules in pCaN1/pCaN2CPK20-inoculated plants. Experiments were then devised to show that the viral RNAs were encapsidated in the heterologous coat protein. Plants inoculated with the cloned DNAs were homogenized in either of two buffers and the extracts were inoculated onto other plants. Homogenization in phosphate buffer is used routinely prior to mechanical transmission of PEBV. The second buffer (TM) has been shown to promote rapid degradation of unencapsidated viral RNA but not to affect encapsidated RNA (Jupin et al., 1990). The results of this experiment are shown in Table 2. One µg of unencapsidated TRV PPK20 RNA produced between 50 and 100 lesions on C. amaranticolor when initially mixed with phosphate or TM buffer but was completely inactivated after 30 min incubation in TM buffer. However, homogenates of plants inoculated with pCaN1/pCaN2, pCaN1/pCaN2CP9.16 or pCaN1/pCaN2CPK20 retained their infectivity after prolonged incubation in TM buffer. In a further experiment virions were purified from plants inoculated with a pCaN1/pCaN2CPK20-infected homogenate. The purified virions retained their infectivity when stored for 10 months at 4 °C and plants inoculated with the purified virus preparation contained normal amounts of both viral RNAs (data not shown). These results show that, although there is an apparent lack of discretely sized S particles in pCaN1/pCaN2CPK20-inoculated plants, sufficient numbers of stably encapsidated, full-length S particles are present to cause a productive infection.
Discussion

The results presented in this paper demonstrate that a recombinant of PEBV carrying the coat protein gene of TRV is able to replicate and spread systemically in N. benthamiana. The PEBV recombinant produced large necrotic lesions when inoculated onto P. vulgaris which resembled those induced by both PEBV SP5 and the PEBV cDNA clones pCaN1/pCaN2. The two most studied natural recombinants of TRV are I6, which was originally isolated from soil obtained from Italy and is serologically related to British PEBV, and N5, which was obtained from narcissus in Scotland and is serologically related to Dutch PEBV (van Hoof et al., 1966; Harrison et al., 1983). These naturally occurring recombinant isolates of TRV behave similarly, producing pinpoint lesions typical of TRV on P. vulgaris suggesting that the symptoms are determined by RNA1 (Robinson et al., 1987).

Initially it appeared that the PEBV recombinant constructed in this work might not be stably encapsidated. However, passaging and virus purification experiments show that both RNA1 and RNA2 are stably incorporated into L and S particles. An initial analysis of particle length distribution also showed the absence of discrete S particles in plants inoculated with clones pCaN1/pCaNCP9.16. This clone has ClaI and BamHI restriction sites inserted upstream and downstream, respectively, of the PEBV coat protein gene and was used as an intermediate in the construction of the recombinant PEBV/TRV RNA2 clone. It is possible that one or both of these introduced sequences is located within the origin-of-assembly sequence (OAS) of RNA2 and in some way reduces the efficiency of encapsidation of the RNA. The precise position of the OAS has not been determined for any tobravirus. For PEBV RNA2, subgenomic (sg) RNAs for the coat protein and the downstream 29K protein have been found in virus-infected plants (MacFarlane et al., 1991; Johnsen et al., 1991). The coat protein sgRNA is encapsidated; however the situation is not known for the 29K protein sgRNA, making it impossible to assign a likely position for the RNA2 OAS. This work has shown that PEBV RNA1 can be encapsidated efficiently by TRV coat protein, suggesting that the OAS of both viruses are likely to share a common secondary structure and perhaps also a similar primary sequence.

Examination of the nucleotide sequence downstream of the coat protein gene of PEBV SP5 and TRV PPK20 in the region of the introduced BamHI site does not reveal any significant homology. However, the ClaI site introduced upstream of the coat protein gene interrupts a sequence of about 40 nt which is highly conserved among all tobaviral RNA2 sequences published to date (Fig. 5) [sequence data: PEBV SP5 (Goulden et al., 1990); TRV PPK20 (A. Mathis, A. T. Ploeg, D.J.F. Brown & J. F. Bol, unpublished); PRV TCM (Angenent et al., 1986); TRV PSG (Cornelissen et al., 1986); PRV CAM (Bergh et al., 1985)]. This conserved region is about 50 to 65 nt downstream of the initiation site of the coat protein sgRNA and may not, therefore, function solely as part of the subgenomic promoter. A clone (pCaN2CP9) carrying only the ClaI site mutation upstream of the coat protein gene retained the diffuse S particle length profile, lending support to our suggestion that this sequence might be (part of) the RNA2 OAS.

This work also shows that whereas the mean lengths of L particles present in plants infected with PEBV SP5 or TRV PPK20 were in close agreement with published measurements, L particles from plants inoculated with clones pCaN1/pCaN2CPK20 had an intermediate length. The finding that particles of PEBV RNA1 encapsidated in vivo in the TRV coat protein are shorter than the same RNA encapsidated in PEBV coat protein raises questions about the extent to which PEBV and TRV share the same particle structure. Goulden et al. (1992) have proposed a model for the structure of tobravirus particles based on a comparison of amino acid sequences of a number of tobravirus coat proteins with that of tobacco mosaic virus, for which a very detailed structural map is already available. The exact nucleotide length of TRV strain PPK20 RNA2 is not known, thus making it impossible to calculate an expected particle length for PEBV RNA1 encapsidated in TRV PPK20 coat protein. However, Goulden et al. (1992) have shown that the amino acid residues which are likely to interact with viral RNA are conserved between the tobravirus coat proteins suggesting that the number of ribonucleotides binding to each coat protein subunit will be the same among all the tobraviruses. The reduction in particle length of heterologously encapsidated PEBV RNA1 might show a smaller helical pitch in the TRV particle compared to that of PEBV, perhaps reflecting the smaller size of the TRV coat protein.

Fig. 5. Alignment of nucleotide sequences upstream of tobaviral coat protein genes. Virus strains are indicated to the left of each sequence. Hyphens indicate gaps inserted to maximize homologies. Asterisks mark nucleotide differences between adjacent sequences. The location at which the ClaI site was inserted into pCaN2CP9.16 is indicated by underlining. The coat protein gene initiation codon is double underlined. References for the sequences are given in the text.
molecule or a decrease of the azimuthal tilt of the TRV coat protein subunits relative to the long axis of the virus particle. This suggests that the protein–protein interactions in the particles of the two viruses are subtly different and might necessitate the construction of slightly different structural models for the different tobaviruses.

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References


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