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Yuji Kishima · Shiko Yamashita · Tetsuo Mikami

Immobilized copies with a nearly intact structure of the transposon Tam3 in *Antirrhinum majus*: implications for the *cis*-element related to the transposition

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Abstract In this study we have focused on two copies of the transposon Tam3 isolated from an *Antirrhinum majus* plant which has flower variegation due to the excision of Tam3 from the *nivea* locus. These two copies possess a high homology, over 95%, to an active Tam3 element found in the *nivea*$^{\text{recurrence:Tam3}}$ allele. Although somatic excision of the Tam3 copy from the *nivea* locus can be detected at 15°C by Southern blotting, neither of the two copies showed any sign of the excision. Both of the immobilized copies were also found in five varieties from different *A. majus* sources, all of which contain common fragments. The results suggest that the two copies have been fixed in the genomes of many *A. majus* varieties. Structural differences between these immobilized copies and the known active copy were mainly observed in the subterminal regions, including the terminal inverted repeats. The immobility of the two Tam3 copies might be due to mutations within the end regions of essential *cis*-elements in Tam3 transposition, as reported for Ac and En/Spm.

Key words *Antirrhinum majus* · Subterminal region · Tam3 · Terminal inverted repeats · The immobilized copy · Transposon

Introduction

Transposons are peculiar DNA segments that have a talent to move on chromosomes (McClintock 1947, 1948). Since plant mutations induced by transposons are useful for gene isolation, the exploration of gene function and the production of various alleles (Gierl and Saedler 1992), the elements showing an ability to transpose have been thoroughly studied. Analyses have been made with respect to transposases and other open reading frames encoded in the autonomous elements, as well as to *cis*-acting sequences, and DNA modifications regulating the transposition of the elements (for a review see Saedler and Gierl 1996). These studies, therefore, have been focused on active transposable elements linked with unstable phenotypes, while many other copies homologous to these active elements have been neglected.

Tam3 in *Antirrhinum majus* is one of the well known cut-and-paste-type transposons (Sommer et al. 1985; Coen et al. 1989). Almost half of the genes isolated by transposon tagging in *A. majus* have been obtained by using Tam3, indicating that Tam3 is highly active. Martin et al. (1989) estimated that about 20–30 copies of Tam3 are present in the *A. majus* haploid genome. They also reported that six out of eight Tam3 copies had restriction maps different from the map for the known active one, and more particularly changed within 400 bp from each end. In addition, they suggested that alterations of the end regions affect the frequency of transposition.

To explore the *cis*-elements required for transposition, transgenic plants with truncated transposable elements have been employed so far (Coupland et al. 1988, 1989). As Martin et al. (1989) suggested, however, if endogenous Tam3 copies possess varying transposition activities, it is possible to find *cis*-elements for transposition through a comparative analysis of those copies. In the present paper, two stable Tam3 copies which possess a nearly intact structure were isolated from a plant with Tam3 activity in *A. majus*. By analyzing these two copies we attempted to identify structural alterations related to the failure of transposition.

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Y. Kishima (✉) · S. Yamashita · T. Mikami
Laboratory of Genetic Engineering, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan
Fax: + 81-11-716-0879

Materials and methods

Plant materials and DNA isolation

We used *A. majus* plant from HAM5 (our designated name) as the line containing active Tam3 to give a variegated flower phenotype.

This line was derived from the *nivea*$^{\text{recurrence:Tam3}}$/stabilizer^{-} line supplied by Dr. Cathie Martin (John Innes Center, Norwich). DNA was prepared from the above plants grown at 25°C for the first 3 months and from the same plants grown for 5 months after transfer to 15°C. Growth at 15°C increases the excision frequency for Tam3. The other lines which we used were: HAM3 (*nivea*$^{\text{recurrence:Tam3}}$/stabilizer^{+}, supplied by Dr. Cathie Martin), HAM7 (Magic Carpet Kongou; Takii seed company), HAM8 (F_1 Butterfly Blonde; Takii seed company), HAM11 (Princess Purple Eye; Takii seed company), and HAM12 (Kousei Yellow; Takii seed company). These genetic backgrounds are different from HAM5, except in the case of HAM3 which is isogenic with HAM5. Plants were grown continuously at 25°C. The DNA was isolated from young organs containing leaves and inflorescence meristems according to the method of Martin et al. (1985).

Construction of the genomic library and screening of the Tam3 clones

DNA, extracted from a single plant of the HAM5 line grown at 25°C, was partially digested with DNaseI and fractions of the size 6–10 kb were collected as described by Sambrook et al. (1989). To make blunt ends of DNA, the fractions were treated with T4 DNA polymerase, and then the *Eco*RI linker was added to the ends. Excess *Eco*RI linker was removed by a Sepharose CL-4B gel (Pharmacia). The fractions with an *Eco*RI linker were ligated into the *Eco*RI site of λ ZAP II vector (Stratagene). Packaging of the ligates was performed by a λ in vitro packaging module (Amersham). The resultant library containing approximately 1 000 000 clones was amplified using the plate lysate method (Sambrook et al. 1989). A 3788-bp *Kpn*I fragment including a full-length Tam3 (3630 bp) was used as a probe. The probe was labeled with [^{32}P]-CTP by the random priming method (Amersham). Selected positive plaques were converted into pBluescript phagemid according to the manufacturer's instruction (Strategene).

Excision assay by Southern blotting

The *Eco*RI-*Bgl*I fragment adjacent to the Tam3 sequence in each clone was prepared as a probe for the excision assay. The flanking fragments were purified by squeezing the liquid from frozen agarose. To identify the somatic excision of Tam3 at *nivea*$^{\text{recurrence:Tam3}}$, a cloned CHS gene provided by Dr. Cathie Martin (John Innes Center) was used. DNA prepared from HAM5 plants grown at 25°C and 15°C, and from the other lines, was digested with *Hin*dIII or *Eco*RI, and transferred to BIODINE B (Pall) after 0.8% agarose-gel electrophoresis. Southern hybridizations were carried out using the ECL gene-detection system (Amersham).

Sequencing of the Tam3 copies

We analyzed the structure of three Tam3 copies; the two copies from the clones isolated in this study and one from the *nivea*$^{\text{recurrence:Tam3}}$ clone provided by Dr. Cathie Martin (John Innes Center, Norwich). Sequencing was performed by an auto-sequencing system (Li-Cor) with a BcaBest auto-sequencing kit (Takara). The resultant data have been deposited in the DDBJ database (S-17: accession no. AB005454; S-40: accession no. AB005455).

Results

Isolation of immobilized Tam3 copies

Initially, we isolated 50 independent Tam3 clones. To select full-length Tam3 copies among them, we looked for clones that generate a 3.2-kb Tam3 fragment after *Bgl*I digestion, because *Bgl*I cleavage sites are present near both ends of an autonomous Tam3 copy (Hehl et al. 1991). Subsequent to the selection, 18 clones which were expected to have full-length Tam3 sequences were obtained.

We examined the full-length copies in terms of excision activity. At both the *pallida* and the *nivea* locus it has been reported that the frequency of somatic excision of Tam3 at 15°C was 1000-times higher than that at 25°C (Carpenter et al. 1987). This was also observed in our DNA sample prepared from the plant carrying the *nivea*$^{\text{recurrence:Tam3}}$ allele. When probing with *nivea* (chalcone synthase gene), a 9.2-kb *Eco*RI fragment containing a Tam3 sequence (3.6 kb) was hydridized in the sample grown at 25°C, while in the sample at 15°C a 5.6-kb fragment without Tam3 appeared in addition to the 9.2-kb fragment, indicating that Tam3 had been somatically excised (Fig. 1 A). Among the selected Tam3 clones, such low-temperature-dependent excision was commonly detected in a number of the clones when Tam3-flanking sequences in these clones were used as probes (data not shown). However, the probes from two clones, S-17 and S-40, failed to detect the band shift in the 15°C sample (Fig. 1 B, C). Both

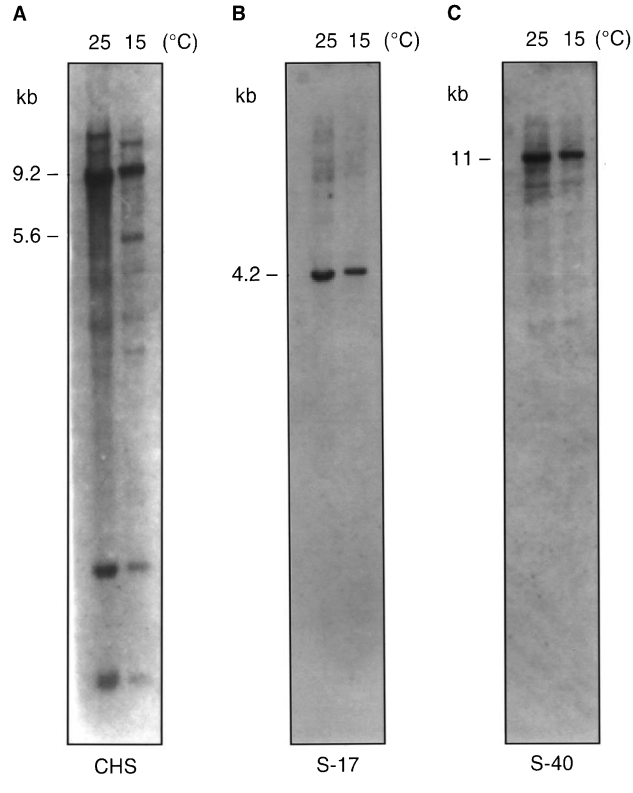

Fig. 1A–C Southern blot of the genomic DNAs from HAM5 plants grown at 25°C and 15°C. **A** An *Eco*RI digest was probed with the *CHS* gene. **B** A *Hin*dIII digest was probed with a Tam3-flanking sequence from the S-17 clone. **C** An *Eco*RI digest was probed with a Tam3 flanking sequence from the S-40 clone

a 4.2-kb *Hin*dIII fragment in S-17 and an 11-kb *Eco*RI fragment in S-40 should contain stable Tam3 sequences, because *Hin*dIII does not cleave the insert in S-17, and *Eco*RI does not cleave the insert in S-40. Such a state of Tam3 was thought to be due to either temperature insensitivity or to a loss of excision activity in the Tam3 copy. To test these two possibilities, we carried out another hybridization experiment. Figure 2 illustrates that the HAM3, 5, 7, 8, 11 and 12 lines harbor fragments identical to those found in the S-17 and S-40 clones from HAM5. As these lines have different genetic backgrounds, it could be judged that the Tam3 copies in these fragments are no longer active or else have remarkably reduced excision frequencies. The two Tam3 copies must be stable in many varieties of *A. majus*. Interestingly, HAM11 shows a heterozygous form of the segment corresponding to the probe from S-40, giving a 7.4-kb fragment without Tam3 in addition to an 11-kb fragment (Fig. 2A). This was not due to somatic excision, since the intensities of the two bands are equal and the plant was grown at 25°C. Thus, the blotting pattern from HAM11 suggests that the Tam3 copy in the S-40 clone was active in the past.

Structures of the immobilized copies

In both the S-17 and the S-40 clones, the Tam3 region was subjected to sequencing. In spite of the inability to transpose, the two overall sequences (S-17: 3577 bp; S-40: 3607 bp) revealed over 95% homology (total mismatching nucleotide number; S-17: 145, S-40: 154) to the autonomous Tam3 sequence reported by Hehl et al. (1991). We failed to find conspicuous alterations, such as deletions, insertions or rearrangements, from these sequences. Structural differences between the two immobile Tam3 copies and the active copy were due to small-scale alterations of less than 30 bp, including base changes, small deletions, and insertions. Most of these changes are thought to be the result of mutation events that originated in the authentic Tam3 elements.

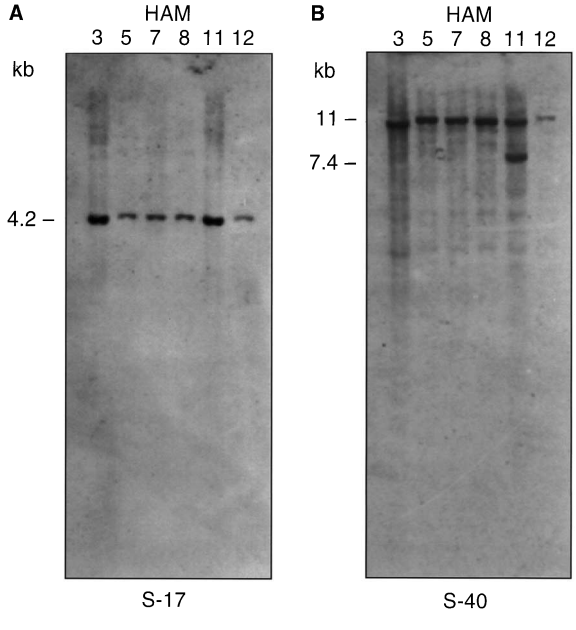

Fig. 2A, B Southern blot of the genomic DNAs from six different varieties of *A. majus* grown at 25°C. **A** A *Hin*dIII digest was probed with a Tam3 flanking sequence from the S-17 clone. **B** An *Eco*RI digest was probed with a Tam3 flanking sequence from the S-40 clone

Figure 3 shows a comparison of the sequences from each end of the three Tam3 copies. About half the mutations which occurred in the immobilized copies mapped within 300 bp from the 5′ end and 600 bp from the 3′ end. Differences found in the terminal inverted repeats (TIRs) seem to be critical, because TIRs are regarded as an influential factor for the mobility of maize transposons (Schiefelbein et al. 1988; Hehl and Baker 1989; Healy et al. 1993). According to the reported Tam3 sequence, the TIRs are composed of TAAAGATGTGAA (Fig. 3A), while the sequences of the 3′ end in both immobilized copies include different residues (Fig. 3 B). The 9th and 10th nucleotides from the 3′ end in the S-17 copy are replaced by C and G, and the 9th nucleotide in the S-40 copy is C instead of A, resulting in TTGCCATCTTTA (S-7) and TTCCCATCTTTA (S-40). Moreover, in both copies, the residue next to the 5′ TIR was converted from T to C. The other pronounced alterations were found in the end regions of the S-17 copy, where three deletions over 10 bp long are present (Fig. 3 A, B). Two of them are located within 100 bp from the ends. On the other hand, the end regions in the S-40 copy are considerably conserved, except for a 25-bp deletion which occurred between 3122 and 3147 bp from the 5′ end in the reference Tam3 (Fig. 3 B). The direct duplications adjoining both ends of each copy are completely preserved as GTTCAAAC in S-17 and AATCCAAG in S-40 (Fig. 3 A, B).

The rest of the region is relatively conserved among the three copies. Structural alterations in the sequence from 300 bp to 3100 bp were scored at 61 and 82 sites in S-17 and S-40, respectively (data not shown). All mutations resulted from base substitutions, small deletions, and insertions of less than 10 bp. A putative transposase gene for Tam3 lies in a 2.5-kb region in the middle of the element. The sequences in S-17 and S-40 corresponding to the transposase gene show strong resemblances to the reference Tam3, with a frequency of 98.5 and 97.7%, respectively. However, their coding regions definitely lose the ability to produce transposase protein due to frame-shift mutations. The loss of functional transposase genes in S-17 and S-40 probably does not account for their immobility, since active, non-autonomous elements which have lost transposase genes generally can transpose via the transposase from autonomous elements (Döring and Starlinger 1984).

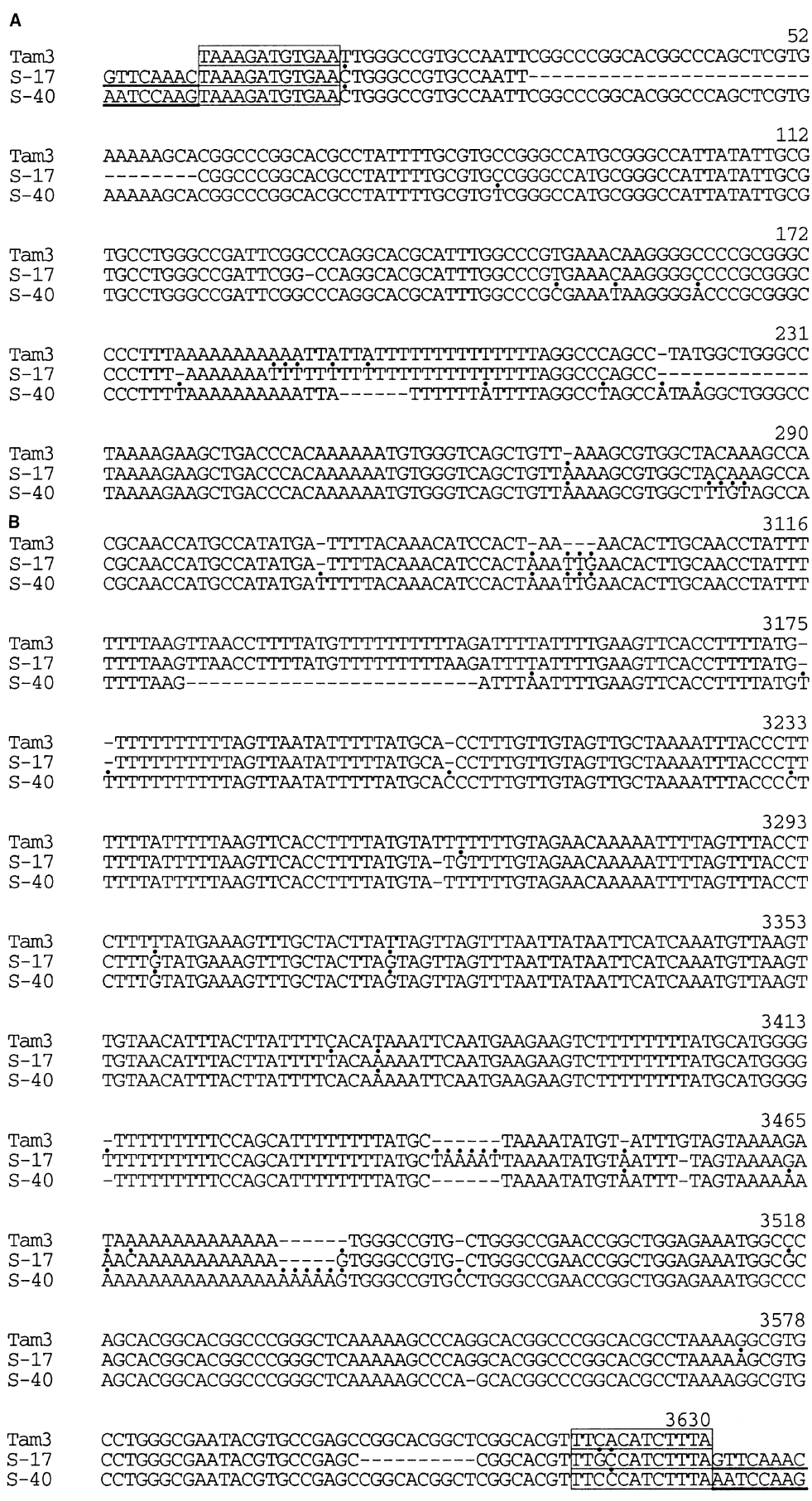

Fig. 3A, B Sequence comparison of both end-regions from the three Tam3 copies. **A** An extent of 300 bp from the 5′ end of the element, including direct duplications. **B** An extent of 600 bp from the 3′ end of the element, including direct duplications. *Nucleotide below dot* shows mismatching with a reference sequence of Tam3 (Hehl et al. 1991). *Broken lines* indicate deletions. TIRs are *boxed* and the target duplications are *underlined*. *Figure* at right side in each row shows the nucleotide number from the 5′ end of the reference Tam3. According to our determination of the Tam3 sequence from *nivea*$^{\text{recurrence:Tam3}}$, an adenine should be added between 685 bp and 686 bp from the 5′ end in the sequence according to Hehl et al. (1991). Consequently, the length of the autonomous Tam3 from *nivea*$^{\text{recurrence:Tam3}}$ is estimated as 3630 bp

Discussion

Despite the presence of active elements, the two Tam3 copies from the S-17 and the S-40 clones are incapable of transposition, and are stable in the genomes of *A. majus* varieties. Surprisingly, however, the structures of the two immobilized copies resembled the active Tam3. Compared with the other transposons reported so far, it appears to be a unique phenomenon that the immobilized copies preserve their intactness. Usually, non-autonomous elements have altered structures in the internal region, but can transpose non-autonomously (Döring and Starlinger 1986). In the present study, the structural differences detected between active and immobilized Tam3 copies allow us to assume the presence of *cis*-acting determinants for transposition.

The alteration most likely related to the immobility is the mismatched sequences in the TIRs. Internal base changes in the TIR were found at the 9th and 10th residues from the 3′ end. Replacements of the TIRs in Ac resulted in weak or no excision in transgenic plants (Hehl and Baker 1989; Healy et al. 1993). Deletion of the TIRs in En/Spm can also reduce the excision frequency of the element (Schiefelbein et al. 1988). Thus, the correct sequence of both terminal repeats is thought to be essential for transposition.

The other possible cause of the immobility appears to be deletions in the subterminal regions found in both clones. The stable mutations at the *a-m2* and the *a2-m1* alleles in maize were caused by defective En/Spm elements that do not excise even in the presence of the autonomous elements (Schwarz-Sommer et al. 1985; Masson et al. 1987; Menssen et al. 1990; Thatiparthi et al. 1995). The defective element in this allele lacks the 5′ subterminal region containing the *tnpA*-binding motifs. In heterologous plants, Ac elements with modified subterminal regions showed immobilization and a reduced frequency of excision (Coupland et al. 1988, 1989). These results provided evidence that the subterminal regions are intrinsic *cis*-elements for transposition.

In the two Tam3 copies reported in this study, the aberrations in the TIRs and/or their adjacent sequences are likely to be involved in their immobilization. If so, these regions may include binding motifs for the transposase and/or host factor(s), as found in Ac/Ds and En/Spm (Kunze and Starlinger 1989; Feldmar and Kunze 1991; Trentmann et al. 1993; Becker and Kunze 1996, 1997; Levy et al. 1996; Gierl et al. 1988).

It is noteworthy that all the five *A. majus* varieties which we examined possess the two immobilized Tam3 copies. The two copies, therefore, must have lost the ability to excise before these strains were bred. This fact leads us to suggest that temporary epigenetic modification, such as methylation, is not concerned with these immobilizing events, but certain mutations are. A considerable number of Tam3 copies might have lost the ability to excise during differentiation of the *Antirrhinum* species. Such fossilized transposons can provide alternative genetic markers for phylogeny and linkage studies, and can also serve as tools for understanding the mechanisms of transposition.

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