Silencing of active transposable elements in plants

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In plant genomes the vast majority of transposable elements (TEs) are found in a transcriptionally silenced state that is epigenetically propagated from generation to generation. Although the mechanism of this maintenance of silencing has been well studied, it is now clear that the pathways responsible maintaining TEs in a silenced state differ from the pathways responsible for initially targeting the TE for silencing. Recently, attention in this field has focused on investigating the molecular mechanisms that initiate and establish TE silencing. Here we review the current models of how TEs are triggered for silencing, the data supporting each model, and the key future questions in this fast moving field.

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Introduction

Plant genomes are littered with TEs and their derived fragments, ranging from 15% TE in the 135 MB *Arabidopsis thaliana* genome to 85% of the TE-expanded 2500 MB *Zea mays* genome. Although abundant, the vast majority of these TEs are not actively producing mRNAs or transposing, either due to their short fragmented nature or their transcriptionally-repressive silenced state. This highly efficient silencing of particularly the full-length autonomous TEs functions to neutralize the inherent mutagenic potential of TE activity produced from transposition events.

Although a great deal is known about how TE silencing is both maintained and reinforced (see [1–3] for more detail), very little is understood about how TEs arrived to this heterochromatic state in the first place. The exact duplicate nature of TE sequences demonstrates that TEs have invaded plant genomes and had *boom* periods of

activity before the eventual *bust* of transcriptional silencing (for a recent example, see [4]). This *Current Opinion* focuses on recent research aimed at determining how an active TE is initially triggered for silencing, and how silencing marks are established and reinforced, resulting in the end-point state of maintenance of heterochromatic silencing.

Initiation of silencing

Two routes of initiation of silencing can occur, defined by whether or not existing small interfering RNAs (siRNAs) produced from previously silenced TE copies in the genome show sequence homology to the active copy.

Homology-dependent initiation of silencing

Plant genomes harbor tens of thousands of silenced TEs. These silenced TEs can be used to target active homologous TEs for RNA-directed DNA methylation (RdDM) and epigenetic silencing (see [5] for a review of RdDM). The well-studied RdDM pathway in plants can be split into upstream and downstream acting phases based on two RNA Polymerase II (Pol II) derivatives that target silenced TEs for non-protein coding transcription, called Pol IV and Pol V. In the upstream phase, Pol IV is recruited to previously silenced TEs through the transcriptionally repressive histone post-translational modification of histone H3 lysine 9 methylation (H3K9me) [6]. The TE transcript product of Pol IV is converted into double-stranded RNA by the protein RNA dependent RNA Polymerase 2 (RDR2) and subsequently cleaved into specifically sized 24-nucleotide (nt) siRNAs by Dicer-like 3 (DCL3) (Figure 1A). In all plants investigated, 24-nt siRNAs produced from silenced TEs are highly abundant [7–9]. In the downstream phase of the RdDM cycle, the 24-nt siRNAs are loaded into ARGONAUTE 4 (AGO4) and AGO6 and direct these proteins to nascent transcripts still associated with their chromatin template produced by Pol V. Pol V transcripts serve as a RNA scaffold on the chromatin that allows for siRNA-based silencing information to direct chromatin modification. Interaction between AGO4 or AGO6 and the Pol V transcript results in recruitment of a chromatin-modifying protein complex that directs H3K9me and DNA methylation of the TE locus (Figure 1A) (see [10] for a review).

One function of these omnipresent 24-nt siRNAs is to act as a *homology sensor*, useful for identifying TEs with similar sequences to ensure that matching active TEs become silenced (Figure 1B). Multiple examples of the ability of the 24-nt siRNAs to silence active homologous TEs exist. For example in *Arabidopsis*, when TEs are activated or an *Arabidopsis* TE is transgenically placed

Figure 1

Pol IV RNA-directed DNA methylation and homology-dependent silencing. **(A)** The Pol II derivatives Pol IV and Pol V function in a RNA-directed DNA Methylation (RdDM) pathway to reinforce TE methylation through the activity of 24-nt siRNAs. In the upstream phase of this pathway Pol IV, RDR2 and DCL3 function to produce the 24-nt siRNAs from TEs associated with H3K9me. In the downstream phase of this pathway the interaction between a Pol V scaffolding transcript and a 24-nt siRNA-loaded AGO4 or AGO6 protein results in DNA methylation and H3K9me of the target TE. **(B)** Previously silenced and degenerated 'cryptic' TEs are abundant in plant genomes and are concentrated at the pericentromere. Pol IV transcribes these heterochromatic TEs, and the resulting non-coding RNAs are cleaved into 24-nt siRNAs by the activity of RDR2 and DCL3. The 24-nt siRNAs can function as a *homology sensor* to identify other TEs with similar sequences and initiate or reinforce their silencing by targeting H3K9me and DNA methylation. DNA methylation is shown as red/CG, blue/CHG and green/CHH sticks, where H = A,T or C.

back into the TE-silenced genome, the Pol IV/RDR2/DCL3 (Pol IV-RdDM) components are necessary for the efficient re-silencing of these TEs [11,12,13^{••}]. Likewise, in maize silencing of the *Mutator* TE family occurred when one TE copy underwent a rearrangement and created a long hairpin RNA producing abundant 24 to 25-nt siRNAs that resulted in the *trans*-silencing of active *Mutator* TEs at

different loci [14]. Therefore, 24-nt Pol IV siRNAs produced from previously silenced or defective TE copies can act to *trans*-silence active copies (Figure 1B), and the silenced TEs within a plant genome provide the plant with an innate immunity against TE activity. An analogy has been made of the plant genome converting active TEs into cryptic (silenced and degenerated) or 'zombie' TEs,

keeping them present in the genome for the use of silencing any active TEs of similar sequence [15]. Major questions in the Pol IV-RdDM pathway remain, such as how much homology is required between a 24-nt siRNA and its target transcript, and how Pol V is recruited to active TEs?

Homology-independent initiation of silencing

Recent research has shown a large number of TE horizontal transfers [16,17], which provide the TE the opportunity to colonize and proliferate in a new genome by circumventing the homology-based silencing pathway. New TE insertions may be silenced upon integration, as double-strand breaks are known to signal the recruitment of a small RNA-based silencing mechanism [18], however this has not been demonstrated to regulate TE activity. The common consensus is that when the homology-based silencing fails (due to lack of TE homology or failure of the Pol IV-RdDM pathway), before the TE is transcriptionally silenced, silencing must begin at the post-transcriptional level. Thus, TE mRNA degradation into 21 to 22-nt siRNAs via the plant's endogenous RNAi mechanism (which in Arabidopsis utilizes RDR6, AGO1, DCL2 and DCL4) [19,20] is the first trigger to eventual non-homology based TE silencing (Figure 2). In addition, RNAi represents a back-up system to post-transcriptionally silence TE activity when transcriptional silencing is

lost, either during high stress, in Arabidopsis mutants, or developmental time points with TE transcriptional activity (see Box 1) [21–23]. The RNAi mechanism represents a self-reinforcing amplification loop that generates highly abundant 21 to 22-nt siRNAs from TE mRNAs (Figure 2). The products of RNAi, which are generated by RDR6, are secondary siRNAs able to work in *trans* and target other TE mRNAs with similar sequences, taking advantage of the repetitive nature of TEs to silence entire active TE families at once rather than individual elements. The double-stranded RNA products of RDR6 are cleaved by DCL4 and DCL2, which produce 21-nt and 22-nt siRNAs respectively [24,25]. The balance of 21 to 22-nt siRNAs is critical, as cleavage of a mRNA by a 22-nt siRNA is a known trigger for additional RDR6 activity and production of more secondary siRNAs [26,27]. The activity of DCL4 and production of 21-nt siRNAs, is an important decelerator to the system, preventing secondary siRNA production from spreading to similar gene sequences and degrading all mRNAs [28–30]. However, even without 22-nt siRNAs, targeting by multiple 21-nt siRNAs has been shown in some cases to result in RDR6 activity and secondary siRNA production [31].

One major question is how RNAi is triggered to degrade TE mRNAs and not genic mRNAs. In non-plant species,

Figure 2

Multiple mechanisms trigger post-transcriptional silencing of TEs. TE mRNAs may be processed by a number of non-mutually exclusive pathways that lead to reduced translation. TE RNAi is initiated by microRNAs and potentially other mechanisms that result in the cleavage of TE mRNAs. Some cleaved TE mRNAs are then copied into double-stranded RNA by RDR6. DCL2 and DCL4 function to degrade TE double-stranded RNA and produce the abundant TE secondary siRNAs that direct additional rounds of RNAi, creating a loop of TE transcription and degradation.

inefficient RNA splicing and incorrect termination trigger a transcript to be degraded by RNAi [32–35], and these may contribute to TE RNAi in plants (Figure 2). A major breakthrough in understanding the triggering of TE RNAi in plants came with the identification of TE mRNAs as microRNA targets. MicroRNAs are individual small RNAs specifically processed from stem-loop mRNA precursors that function to cleave and translationally repress complementary (usually genic) mRNA transcripts [36]. In a recent publication, known and new microRNAs were demonstrated to target the first cleavage event of some TE mRNAs (Figure 2) [37^{••}]. Although microRNAs are typically 21-nt, some microRNAs are 22-nt and trigger RDR6 production of double-stranded RNA from the targeted TE mRNA, initiating the cycle of RNAi (Figure 2). Although a conceptual breakthrough for identifying how TE mRNAs are selected for RNAi, how much this mechanism contributes to triggering RNAi compared to other potential mechanisms outlined in Figure 2 remains to be determined.

If a microRNA, secondary siRNA or other small RNA is required to initiate RNAi on a TE mRNA, this begs the question of where this complementary small RNA comes from in a case when a new TE enters a genome via horizontal transfer or transgenesis? One idea is that a TE mRNA can be triggered for RNAi without the requirement of an existing matching small RNA. A second idea that has gained popularity in non-plant organisms is that there are low level ‘primal’ or ‘scan’ small RNAs constantly produced that survey the transcriptome, randomly sampling mRNAs for cleavage and silencing. If a TE produces a mRNA substrate that is identified through targeting by a scanning small RNA, TE siRNAs can be generated and selectively enriched by RDRs (or the ping-pong pathway for animal piRNAs) to generate the high level of repeat siRNAs required for TE silencing. In fission yeast AGO1 and in *C. elegans* 21U-RNAs perform cellular mRNA sampling and amplification of secondary siRNAs only occurs when antisense or foreign RNA substrate is available [38–40]. The cryptic microRNAs identified by [37^{••}] that target TEs may represent this transcriptome surveillance mechanism in plants. Alternatively, a small amount of TE transcription may be allowed (even in TE-silenced cells), and due to the nested configuration of TEs these overlapping sense/antisense transcripts may be cleaved to produce a low level of small RNAs. These could then scan the transcriptome for sense TE mRNAs, initiating RNAi and post-transcriptional silencing of TE mRNA transcripts. Future experiments are required to determine if or how transcriptome scanning for TE mRNAs is accomplished in plants.

Establishment of TE chromatin modification

For transcriptionally active TEs that are subject to RNAi, the mechanism of how this post-transcriptional level silencing transitions to chromatin modification and the

start of transcriptional silencing has been the focus of several recent publications. One model for this mechanism suggests that the first chromatin modification takes place only after a threshold copy number of elements is reached and enough mRNA is produced to overwhelm the DCL2 and DCL4 RNAi proteins, thus resorting to the DCL3 cleavage into 24-nt siRNAs (Figure 3) [41^{••}]. These 24-nt siRNAs then direct RdDM similar to their function when generated by Pol IV/RDR2. Therefore, this could represent a mechanism by which the plant could ‘measure’ the amount of aberrant transcript, choosing to switch to transcriptional silencing if the TE produces too much transcript and overwhelms RNAi. Although this mechanism is attractive, it does not account for loci that are highly expressed and targeted by RNAi that do not become methylated, nor regions of the genome that display transcript RNAi and RdDM without the presence of DCL3-dependent 24-nt siRNAs (such as gene-regulating *trans*-acting siRNA (tasiRNA) loci) [42].

A second mechanism has been recently reported whereby transcriptionally active TEs targeted by RNAi, and tasiRNA loci, are directly targeted for a form of RdDM that is not dependent on DCL3 or 24-nt siRNAs. In this mechanism, the 21 to 22-nt siRNAs that are directly produced from the RNAi of Pol II transcripts (by DCL2 and DCL4) can participate in a Pol II expression-dependent form of RdDM (Figure 3) [13^{••},42]. Expression-dependent RdDM functions in both TE and virus establishment of silencing [13^{••},43]. In the case of TE silencing, mutants were used that are defective in the maintenance of TE silencing, thus stalling and extending this establishment phase of silencing with the purpose of studying this phase of silencing with increased resolution. This work found that the 21 to 22-nt siRNAs are directly incorporated into the AGO6 protein, and similar to its role in Pol IV-RdDM (driven by 24-nt siRNAs), these 21 to 22-nt siRNAs direct AGO6 to chromatin targets using the Pol V scaffolding transcripts (Figure 3) [44[•]]. This pathway was named RDR6-RdDM because of its dependence on the 21 to 22-nt siRNAs produced by RDR6, distinguishing it from the 24-nt driven Pol IV-RdDM [13^{••}]. Why some 21 to 22-nt siRNAs are incorporated and direct AGO6 and how Pol V is recruited to active TEs (providing specificity for TE targets of this pathway) are both currently unknown. In addition, this AGO6 mechanism may only represent one submechanism of the emerging field of (Pol II) expression-dependent DNA methylation [45]. For example, a protein called NERD was identified that participates in a hybrid pathway that utilizes both RNAi (RDR1, RDR6, DCL2, DCL4) and Pol IV-RdDM (Pol IV and DCL3) components to silence some noncoding RNA, newly arisen pseudogenes and a few TEs [46]. Although different specific pathways have been identified, the similarities between these non-mutually exclusive mechanisms are critically important and constitute a major breakthrough

Figure 3

Establishment of RNA-directed DNA methylation. Once RNAi has been triggered, two models exist for how DNA methylation is established. These models differ in their dependence on DCL3 and which sized siRNA (21 to 22-nt vs 24-nt) targets the Pol V scaffolding transcript in the nucleus, establishing RNA-directed DNA methylation. PTGS = post-transcriptional gene silencing.

and advancement in this field: They define alternative RdDM mechanisms that are not solely dependent on Pol IV or RDR2, thus demonstrating that the establishment of TE chromatin marks can occur independently of the Pol IV-RdDM mechanism that is responsible for homology sensing and reinforcement of TE silencing.

Reinforcement of silencing marks

TE heterochromatic marks can be positively reinforced through the activity of the Pol IV-RdDM pathway and 24-nt siRNAs (Figure 1) (see [5] for a recent review). Both Pol IV and Pol V are recruited to regions of the genome with previously established H3K9me or DNA methylation [6,47], and therefore this mechanism acts in two important ways. First, for regions that are associated with heterochromatic marks but not fully silenced, this mechanism acts as a self-reinforcing amplification loop to strengthen DNA and histone methylation patterns and keep TEs in a perpetually silenced state. Second, Pol IV-RdDM can spread the silencing marks to adjacent regions [48]. In Arabidopsis, which lacks the transcription-independent spreading of H3K9me by Heterochromatin Protein 1 [49,50], the Pol IV-RdDM pathway is probably the major mechanism responsible for the spread of heterochromatin. Both the reinforcement and spreading characteristics of Pol IV-RdDM are particularly important for the transcriptional silencing of small TEs and TEs near genes. In the case of small TEs, there may not be enough nucleosome binding to maintain or 'hold' this TE region in a silenced state over

time without the requirement for RdDM reinforcement. For TEs near genes, Pol IV-RdDM is required to define TE chromatin edges and sharp boundaries between heterochromatic TEs and euchromatic genes [51,52]. The Pol IV-RdDM pathway is therefore required to maintain transcriptional repression of a subset of small and euchromatic TEs in the Arabidopsis and maize genomes [53–55].

Maintenance of TE silencing

Similar to animals, plant TE DNA methylation and H3K9me (established by the RdDM pathways described above) can be copied and epigenetically transmitted from cell to cell across mitotic divisions without the requirement to be reset by additional RdDM. This epigenetic information is carried within the chromatin during cell division and can be propagated without the requirements of small RNAs. The protein MET1 acts to replicate patterns of symmetrical CG DNA methylation during cell division, while the proteins CMT2 and CMT3 act to maintain the methylation of CHH and CHG sites (where H = A, T or C) guided by the H3K9me mark (Figure 4) [56,57]. These proteins thus ensure that each cell has the same DNA methylation as the progenitor cell. Some methylation, particularly in the CG context, can be efficiently copied at TEs to very high (>95%) levels [58]. Many TEs in both the maize and Arabidopsis genomes are maintained in a transcriptionally silenced state only by the propagation of CG methylation and H3K9me, and do not require RdDM to reinforce this silencing [44,58,59,60], although they

Figure 4

Maintenance of TE epigenetic silencing. TE DNA methylation in the CG sequence context (red) is propagated via the DNMT1 homolog MET1. TE CHG (blue) and CHH methylation (green) are maintained by CMT3 and CMT2, which are recruited to previously silenced TEs through their interaction with the heterochromatic mark H3K9me. In plants, TE DNA methylation is propagated from cell-to-cell, as well as from one generation to the next, resulting in *trans*-generational epigenetic inheritance of TE silencing. H = A, T or C.

will often produce Pol IV-dependent 24-nt siRNAs (see *Homology-Dependent Initiation of Silencing* section). In addition, a recent paper has identified a subset of TEs that may represent the evolutionarily transition from requiring reinforcement by Pol IV-RdDM to only requiring maintenance silencing. The authors termed these TEs 'double-locked' for their requirement of both Pol IV-RdDM and histone modification [61].

In plants, the propagation of chromatin information is not only passed across mitotic divisions, but is also maintained from one generation to another [62,63]. Although the *trans*-generational epigenetic silencing of TEs in plants is well established, in animals this topic remains controversial due to debate on the level of completeness of erasure of chromatin marks during animal reproduction and embryogenesis (reviewed in [64]). The lack of erasure of chromatin marks during development in plants means that the progress made towards silencing a TE where one generation left off can be picked-up and added to by the next generation (if the silencing occurred in the in the correct cell types, see Box 1). Thus, many of the RdDM mechanisms described above are not apparent on a generation-to-generation basis when TEs are maintained in a silenced state, but are only apparent when TEs reactivate or are transferred into a new genome. Once deeply silenced, TE sequences will begin to accumulate mutations due to the lack of selective pressure for their activity, and the TE will degrade into a cryptic or unrecognizable sequence.

Remaining active despite multiple mechanisms of repression

In wild-type cells with functional homology-dependent and -independent silencing mechanisms, occasionally

some TE copies are able to remain active. For example, the ONSEN retrotransposon acquired a heat-responsive regulatory element that leads to TE activation upon heat stress despite functional silencing mechanisms [12,23]. Another example is the Arabidopsis *Evade* TE, which was discovered because once activated it escapes corrective reestablishment of silencing and transposes into genes, even though there are closely related silenced family members present in the genome [76]. The ability of *Evade* to avoid silencing is probably due to the lack of TE siRNAs that it or its closely related family members produce until transpositions result in a higher copy number. Additionally the encapsulation of its mRNA into *gag* protein particles sequesters it from 21 to 22-nt siRNAs [41]. In other cases, the lack of silencing is puzzling because silenced members of the same TE family within the same genome are producing abundant Pol IV-dependent 24-nt siRNAs that should *trans*-silence the active copy. For example, in maize individual *Mutator* family TE copies are able to remain active despite abundant 24-nt siRNAs generated from silenced and fragmented homologous copies [77]. The activity of these single elements could potentially result in a period of high activity for the TE family. These booms in amplification occasionally (on evolutionary timescales) occur, leaving their relics and signatures of recent activity in plant genomes [78]. Therefore, a major question in the field is how can individual elements remain active in the same genome with other family members that are silenced?

Multiple mechanisms of TE evasion of silencing have been proposed, such as by the TE encoding a suppressor of gene silencing or by the TE becoming an adaptive benefit to the regulation of a critical gene [79–81].

Box 1 Role of Development in TE Silencing

Although this *Current Opinion* article focuses on the mechanisms and pathways responsible for TE silencing, all of these pathways are not functional in all tissues or developmental time points. Plant development exerts a spatial and temporal level of regulation on TEs. For example, the RDR6-RdDM pathway outlined in Figure 3 is dependent on the function of AGO6, which is only expressed in meristematic tissues [44], while reinforcement by Pol IV-RdDM and maintenance of DNA methylation occur more broadly across different organ and tissue types. Data has demonstrated that the shoot apical meristem and floral meristem, which produce the germ cells, are critical for the proper silencing of TEs [65], thus ensuring that the initiation or establishment of TE silencing accomplished in one generation is epigenetically passed to the next generation.

In addition to the developmental-specific timing of the silencing of TEs, there are particular points in normal plant development that undergo developmental relaxation of TE silencing (DRTS) [66]. In the wild-type pollen grain, the pollen vegetative nucleus undergoes a loss of heterochromatin and global reactivation of TE expression, followed by transposition [67,68]. In the pollen vegetative nucleus, TEs undergo the CHH hypermethylation associated with transcriptional activity and expression-dependent RdDM [69]. In addition, the analogous central cell in the female gametophyte shows signatures of chromatin decondensation [70], and after fertilization the endosperm displays DRTS [71,72]. Both the pollen vegetative nucleus and endosperm are terminally differentiated tissues, and it has been proposed that the DRTS events play a role in providing information content about TEs (small RNAs or other) to the germinal sperm cells or embryo [68,71]. It has been suggested that the DRTS events are preparing the critical germ cells for a confrontation with potentially active TEs upon cross fertilization, which will then be consolidated into epigenetically silenced heterochromatin [73], similar to the TE silencing/hybrid dysgenesis mechanism found in *Drosophila* [74]. Similarly, a DRTS event in maize leaves at the time of transition from juvenile to adult vegetative growth has been suggested to provide TE information back to the meristem to efficiently silence TEs where it matters most: first, for the production of future haploid gametophytes with only one copy of each gene and second, for the next generation [75]. However, in all of these cases there is no clear understanding if the DRTS events result in a lasting or heritable reinforcement or triggering of TE silencing. Critical questions to define the functional role of DRTS events (if any) remain unanswered.

Although examples of many of these mechanisms exist [82,83], the mechanism that has gained the most attention is TE integration into an active gene, thus hiding and/or preventing the TE from silencing. In the case of the *Mutator* TEs mentioned above, the position of the element and proximity to a gene is critical for evasion of silencing [84]. In *Arabidopsis* a systematic cataloging of active *Mutator* TEs identified insertion sites in genic 3' untranslated regions as safe havens to avoid silencing [85]. The authors proposed that the TE copies were insulated from silencing due to the selective pressure to maintain the gene in an active chromatin configuration. Because different TE families have different insertion site preferences, the ability to circumvent TE silencing may be higher for some DNA TEs (such as *Mutator*) that target genic regions for insertion, and lower for LTR retrotransposons that typically target heterochromatin.

A major question that remains is whether a given TE copy inserted near a gene can avoid silencing altogether, or whether silencing is targeted but then reversed? For example, the TE may be completely resistant to de novo chromatin modification, potentially due to the downstream Pol V chromatin-modifying complex not being targeted to this genic region. This could account for the production of Pol IV-dependent 24-nt siRNAs by the silenced TE family members, but their lack of activity on the genic TE insertion. Alternatively, Pol IV-RdDM may target a genic TE copy for silencing, but subsequently this silencing is over-written by DNA and histone demethylases to maintain a euchromatic environment around the gene. In *Arabidopsis*, the histone demethylase IBM1 and DNA glycosylases (which remove methylated DNA) ROS1, DME, DML2 and DML3 are required to keep genic sequences near TE insertion sites in the proper euchromatic environment [86–88]. It will be critical to understand if and how TE copies can avoid silencing, or what regulates the demethylation machinery to overwrite TE silencing, as the answer to this question will provide insight on how to manipulate TE, transgene and endogenous gene expression.

Conclusion

Three years ago the known mechanisms responsible for regulating TEs were confined to Pol IV-RdDM and the maintenance of DNA methylation. More recently, multiple independent groups have identified diverse mechanisms that the plant uses to repress transcriptionally active TEs, adding a new level of detail and function to post-transcriptional silencing pathways. Although examples of multiple molecular mechanisms responsible for silencing TEs are described here, how these individual mechanisms are ordered and link together is currently unknown. Models have been generated that attempt to assemble these pathways in a linear fashion [5,43,89], but currently these pathways exist as individual modules that may fit together in a number of different arrangements. Future research is required to assemble the progression of silencing of an active TE.

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