***Helitrons*: Their Impact on Maize Genome Evolution and Diversity**

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Abstract. Gene movement by the newly-described *Helitron* family of transposable elements apparently has significantly impacted the evolution of this genome and has contributed to the lack of intra-specific gene collinearity between different maize inbred lines. The abundance of these elements and the extent of diversity among them remain largely undetermined. Several hypotheses have been proposed to explain their transposition and mechanism by which these elements prolifically capture and mobilize gene sequences, but each lacks supporting experimental evidence. A more complete understanding of this process requires molecular and genetic evidence of *Helitron* activity in modern maize genome. Establishment of *in vivo* and *in vitro* systems to assay transposition activity would also aid our understanding of this new class of transposable elements.

1 Introduction and Overview of *Helitrons*

Helitron refers to a recently-described class of transposable elements that has a number of important and unique features. *Helitrons* appear to underlie much of the +/- polymorphisms or “intraspecific violation of genetic colinearity” recently observed in maize genome sequencing projects. This chapter reviews the short documented history of *Helitrons* and highlights many of the unresolved questions concerning this intriguing class of elements

Helitrons were first hypothesized from computer analysis of the fully sequenced genomes of *C. elegans*, rice and Arabidopsis (Kapitnov and Jurka 2001). These investigators proposed that *Helitrons* comprise a repetitive class of DNA representing ~2% of the total genome. An autonomous *Helitron* was predicted by computer simulation of the consensus sequence of non-autonomous elements. The autonomous element was proposed to contain coding information for a HEL protein composed of a rolling-circle replication initiator and DNA helicase domains needed for transposition. It also contains a replication protein A (RPA)-like protein with single-stranded DNA-binding activity. These proteins are associated with bacterial

transposons which transpose via rolling circle (RC) replication (Khan 2000; Tava-koli, Comanducci, Dodd, Lett, Albiger, Bennet 2000).

Helitrons are novel because they bear unique characteristics heretofore not shared by known class 1 (transposition through DNA) and class 2 (transposition through RNA) eukaryotic transposable elements. Despite their abundance, *Helitrons* have remained elusive because of the small size of invariant terminal sequences and the polymorphic nature of internal sequences. The latter characteristic is uniquely attributable to the presence of different gene sequences apparently captured by these elements.

Helitrons lack terminal repeats and do not duplicate host sequence upon insertion. The 5' and 3' terminal ends of *Helitrons* characterized by Kapitonov and Jurka, (2001) contain sequences TC and CTRR, respectively. They consistently insert between the bases of dinucleotide AT and contain a non-conserved short palindrome sequence located 15-20 nucleotides upstream of their 3' end capable of forming a hairpin structure (Kapitonov and Jurka 2001).

Because an autonomous *Helitron* was not found from computer searches of the *C. elegans*, rice and Arabidopsis genomes, there exists the possibility that *Helitrons* are no longer active in these organisms and the isolated *Helitron* sequences are simply evolutionary remnants of once-active elements. That *Helitrons* are active in modern genomes came from three recent reports of mutants caused by *Helitron* insertions. Two maize mutants, *sh2-7527* and *bal-ref* are caused by insertion of non-autonomous *Helitrons* (Lal, Giroux, Brendel, Vallejos, Hannah 2003; Gupta, Gallavotti, Stryker, Schmidt, Lal 2005). The mutants likely were caused by relatively recent *Helitron* insertions since (1) the insertion mutants do not contain any other alteration inactivating the gene (a signature of the evolutionary decay of functional genes) and (2) loss of *Sh2* or *Bal* leads to germination and seed production deficiencies, respectively. Natural selection pressure would significantly limit propagation of these mutants in the absence of human selection. These studies then provided the first evidence that the present-day maize genome harbors an active *Helitron*. Furthermore, the reports of nearly identical *Helitrons* inserted in different regions of the maize genome indicate that these elements were active and have moved very recently and may still be active (Lai, Li, Messing, Dooner 2005; Gupta et al. 2005). A 2005 plant mutant was recently reported in morning glory, *Ipomoea tricolor*. Isolated in the 1940's, the white flower mutant termed pearly-s-mutant was recently shown to be caused by a *Helitron* insertion into the dihydroflavonol 4-reductase B gene involved in anthocyanin biosynthesis (Choi, Hoshino, Park, Park, Iada 2007). Intriguingly and unlike maize mutants, this *Helitron* contains non-functional remnants of sequences encoding helicase and nuclease/ligase.

Helitrons have now been found via sequence analysis in many organisms (reviewed in Kapitonov and Jurka 2007). These include other plants, invertebrates, fungi and fish (Choi et al. 2007; Kapitonov and Jurka 2001; Lal et al. 2003; Poulter, Goodwin, Butler 2003). Massive abundance of *Helitrons* has been recently reported in a mammalian genome, the brown bat *Myotis lucifugus*, where they constitute ~3% of the genome (Pritham and Feschotte 2007).

2 Mass Movement of *Helitron* Mediated Gene Sequences Bestow Intra-specific Violation of Gene Colinearity in Maize.

Compared to *Helitrons* in other organisms, maize *Helitrons* appear unique in their ability to incorporate portions of host gene sequences into the transposable element (reviewed in Kapitinov and Jurka 2007). In doing so *Helitrons* contribute to the lack of gene colinearity between different maize inbred lines: The maize genome exhibits tremendous diversity which has played a vital role in its domestication. Plant breeders have successfully exploited this diversity for crop improvement. Intriguingly, data emerging from analysis of multiple regions of the genome indicate that significant differences in gene composition and colinearity are widely prevalent among different maize inbred lines. (Fu and Dooner 2002; Song and Messing 2003; Brunner, Pea, Rafalski 2005). This so-called, “intraspecific violation of gene colinearity” or “+/- polymorphism” has been shown to be primarily due to the presence/absence of gene piece rich fragments between different inbred lines. The lack of gene colinearity between maize lines and the possible contribution of *Helitrons* to this variation were first reported by Lai et al., 2005 who sequenced the *bronze* (*bz*) region of maize inbreds McC and B73 and discovered the presence of two gene piece-bearing *Helitrons* in McC and their absence in B73. This accounted for the haplotype variability at the *bz* locus between these two inbred lines. In a parallel study, the extent of lack of gene colinearity between inbred lines B73 and Mo17 was investigated by using oligonucleotide probes (Morgante, Brunner, Pea, Fengler, Zuccolo, Rafalski 2005). Here, 20% of the detected 20,656 genes were found in only one of the inbred lines. These differences were primarily caused by segments of gene piece-rich insertions rather than deletions among the two lines. Detailed sequence analysis of nine insertions indicated that eight were caused by *Helitrons*. If this limited data set has any bearing at the genomic level, one would assume that *Helitrons* may have captured and mobilized thousands of gene pieces across the maize genome. The complete sequencing of maize inbred B73 genome will not provide the complete picture of the extent and diversity of gene piece movement by *Helitrons*, since *Helitron* identification and the demarcation of their precise boundaries often entails “+/- polymorphism” studies between different inbred lines. Nevertheless, precise identification and annotation of *Helitrons* is important because the majority of the elements identified thus far represents non-autonomous *Helitrons* bearing segments of multiple genes. This, no doubt, will complicate the proper annotation of wild type genes in the sequenced maize genome.

3 Isolated Maize *Helitrons* Represent a Sub-class of *Helitrons*.

Twenty two maize *Helitrons* displaying +/- polymorphisms have been reported to date (Table 1). Fourteen were found initially through characterization of +/- DNA polymorphisms between maize lines while eight were discovered through sequence similarity to the original 14 elements. Subsequent analysis showed that all eight

HELITRON NAMES		3' TERMINUS	FIRST REFERENCE
Hel1	<i>sh2-7527*</i>	CTCCGTAGC-AACGCACGGACATTCAC-----CTAG	Lal et al., 2003
	<i>ba1-Ref*</i>	TTCCGTAGC-AACGCACGGGATATAC-----CTAG	Gupta et al., 2005
	<i>Rp1B73**</i>	CCCCGTTCG-AACGCACGGGCACTCAG-----CTAG	Gupta et al., 2005
	<i>ZeinBSS53**</i>	-TTTCGTGGCTAACGCACGGGCACATAC-----CTAG	Gupta et al., 2005
	<i>Hel1-19**</i>	TCCCGTGGC-AACGCACGGGCACGAAC-----CTAG	Lal et al., unpublished
	<i>HelA-1*</i>	TCCCGTGGC-AACGCACGGGCACCTCAC-----CTAG	Lai et al., 2005
	<i>HelA-2**</i>	TTTCGTGGC-AACGCACGGGCACCTCAC-----CTAG	Lai et al., 2005
	<i>GHIJLM9002*</i>	AACCGTAGC-AACGCACGGGCAITCAA-----CTAG	Morgante et al., 2005
	<i>NOPQ9002*</i>	CCCCGTTCG-AACGCACGGGCACCTCAC-----CTAG	Morgante et al., 2005
	<i>NOPQ73_14578**</i>	CCCCGTTCG-AACGCACGGGCACCTCAC-----CTAG	Brunner et al., 2005
	<i>NOPQM17_14594**</i>	CCCCGTTCG-AACGCACGGGCACCTCAC-----CTAG	Brunner et al., 2005
	<i>NOPQ73_9002**</i>	CCCCGTTCG-AACGCACGGGCACCTCAC-----CTAG	Brunner et al., 2005
	<i>Mo17NOPQ_14577**</i>	CCCCGTTCG-AACGCACGGGCACCTCAC-----CTAG	Brunner et al., 2005
	<i>RST9002*</i>	TCCCGTGGC-AACGCACGGGCACCTCAC-----CTAG	Morgante et al., 2005
	<i>U9002*</i>	TTACGTAGCGAA-GCACGGGCACATAC-----CTAG	Morgante et al., 2005
	<i>HI9002*</i>	TCCCGTTCG-AACGCACGGGCACCTCAC-----CTAG	Morgante et al., 2005
	<i>Hel-BSS53-zICI*</i>	TTCCGTGGC-ATTCGCACGGGCACATAC-----CTAG	Xu and Messing, 2006
	<i>Hel1-4*</i>	TTCCGTGGC-ATTCGCACGGGCACCTAA-----CTAG	Wang and Dooner, 2006
	<i>Hel1-5*</i>	AGTCGTGGC-AACGCACGGGCACCTGA-----CTAG	Wang and Dooner, 2006
Hel2	<i>TUVW9002*</i>	-----AGGGCCCGATAGGGCCCTTTCTATTCTAG	Brunner et al., 2005
	<i>HelB*</i>	-----GGGGCCCGATAGGGCCCTT-CATGTTCTAG	Lai et al., 2005
	<i>Hel1-6*</i>	----AGGGGGCAGGAAGTGG-CCCT-CTGATCTAG	Wang and Dooner, 2006

Table 1. Classification of maize *Helitrons* based on the conservation of 3' terminal sequence
*discovered initially by +/- polymorphism

**discovered by similarity of either terminal or internal sequence to other *Helitrons*

“derived” elements are associated with +/- polymorphism at their allelic sites or in paralogous genes (references in Table 1). The characterized *Helitrons* clearly define two closely related families as judged by the similarity of their 3' terminal sequence (Table 1). And, placement of gaps in the two families of 3' sequences allows for the detection of relatedness between the two families.

The major sub-family consists of 19 members while the minor group is represented by three *Helitrons*. As noted above, sequence similarity can be found in the 3' termini and, importantly, relatedness of these maize *Helitrons* can be seen by the presence of conserved sequences in a 14 bp motif in the 5' termini (data not shown).

While termini of maize *Helitrons* are strikingly similar, internal sequences exhibit little sequence similarity. Like other transposable elements, the conserved terminal ends of these non-autonomous *Helitrons* suggest that their movement may be mediated by the transposition protein provided in trans by a master or autonomous member of the family. Searches for candidate autonomous *Helitrons* have identified several instances in which sequences bearing similarity to DNA helicases are localized in close proximity to Replication protein A, a characteristic of the proposed autonomous *Helitron* (Gupta et al. 2005; Morgante et al. 2005). However, the presence of premature in-frame stop-codons or large insertions shows that the isolated sequences represent inactive elements. In addition, these putative, mutationally-silenced autonomous elements do not exhibit sequence similarity to the 3' conserved terminal ends of isolated maize *Helitrons*. This suggests that these inactive remnants of proposed autonomous *Helitrons* may not be closely related to the *Helitrons* in Table 1. The completion of the B73 genome sequence will determine if this inbred

harbors a functional autonomous *Helitron*. However, a functionally autonomous *Helitron* may not be necessary for *Helitron* transposition if the helicase and replicase activity are provided by host genes. To date there is no experimental evidence that links the coding sequences of the proposed autonomous *Helitrons* to transposition activity. In fact there is no direct experimental evidence that helicase and replicase activities are even needed for transposition.

The complete divergence in internal sequence of the characterized maize non-autonomous *Helitrons* makes it difficult to search for other members in the genome. The highly repetitive nature of genomic DNA hybridizing to *Helitron* termini (Lal et al. 2003) suggests that there are hundreds to thousands of other family members. Accordingly, the terminal ends of the *Helitrons* have been used to effectively search for other family members in the maize genome database (Gupta et al. 2005). The scheme used to discover *Helitrons* is outlined in Fig. 1.

The initial search entails detection in a single clone of sequences highly similar to the *Helitron* 5' and 3' termini. The intervening sequence then is viewed as a candidate *Helitron*. Intervening sequences are then perused for the presence of gene piece-rich sequences since characterized non-autonomous maize *Helitrons* are rich in gene piece sequences. However, maize *Helitrons* that do not ferry gene sequences have recently been reported (Wang and Dooner 2006) so the presence of gene sequences within a *Helitron* is not invariant. Definitive validation that a sequence fulfilling the criteria outlined above is, in fact, a *Helitron* requires demonstration of +/- polymorphism. One approach, accounting for the isolation of the majority of *Helitrons*, involves parallel sequencing and comparison of homologous regions in different inbreds. As an alternative, more cost effective strategy, Gupta et al., (2005) searched maize GSS, EST and HTGS clones for sequences aligning with sequences flanking the putative *Helitron*. Primers were then designed to scan different inbred lines for "empty" sites. This approach has identified 336 putative *Helitrons* in B73. Of these, 22 exhibit +/- polymorphisms just within the B73 inbred (Lal, unpublished). Since B73 is an inbred, the +/- polymorphism here likely reflects comparison of paralogous loci.

4 The Mechanism by Which *Helitrons* Capture and Mobilize Gene Sequences Remains Unknown

While the occurrence of host gene fragments embedded within transposable elements has been reported in the past it was generally regarded as a rare incidence. However, a recent report of mass movement of gene sequences by a Mutator like family of transposable elements in rice termed MULEs indicates that this process is far more prevalent than initially anticipated (Jiang, Bao, Zhang, Eddy, Wessler 2005). This may play a major role in genome evolution. The MULE family of elements has captured and mobilized more than 1000 gene fragments in the rice genome (Jiang et al., 2005). However, the mechanism by which MULE and *Helitron* super families of transposable elements acquire gene fragments may differ. For example, 20% of the gene fragments captured by MULEs bear no apparent wild-type counterpart in the rice genome but display strong similarity to intact genes from other plants. This suggests that the host gene was destroyed during the acquisition process. In contrast, Morgante et al (2005) demonstrate that the wild type progenitor of four different gene fragments captured by a *Helitron* exist in the maize genome. These investiga-

Fig. 1. Strategy for Discovering Maize *Helitrons*: The upper left and right panels display the structure of autonomous and non-autonomous maize *Helitrons*. An autonomous *Helitron* is predicted by computational analysis of related repeat sequences in the genomes of Arabidopsis, rice and *Caenorhabditis elegans* (Kapitnov and Jurka, 2001). The coding regions of DNA helicase (HEL) and single stranded DNA binding protein (REP) of an autonomous *Helitron* are marked. The exons captured by the non-autonomous *Helitron* are displayed by colored blocks. The terminal ends of the *Helitron* are displayed by pattern filled boxes and the loop near the 3' terminus represents the palindrome sequence. The A and T nucleotide immediately flanking the insertion site of the *Helitron* are indicated.

tors speculate that *Helitron* transposition involves replication and strand replacement that preserves the integrity of the donor host sequence. On the other hand, the mechanism of gene capture may not differ for the two types of elements. Given that only one of the two alleles of a gene participates in the events of gene capture, alteration in one allele (and not in the other) creates a heterozygous condition. Given heterozygosity and assuming no selection, it would simply be random chance whether the mutant, non-functional allele or the wildtype allele becomes fixed in the population following gene capture.

The similarity of *Helitron* transposition proteins, helicase and nuclease/ligase, to host genes led Kapitnov and Jurka (2001) to postulate that these genes also were recruited by the *Helitron* from the host genome. Further understanding of this process will require understanding of the mechanism by which these elements capture and mobilize host gene sequences.

While *Helitron* gene piece capture has been reported in several organisms, *Helitrons* of maize are clearly the most prolific at incorporating gene pieces. Several hypotheses have been forward to explain the mechanism of gene piece capture by

Helitrons (reviewed in Kapitinov and Jurka 2007). For example, it was proposed that transposition is initiated at the 5' end of the element and the inefficient recognition of the 3' palindrome sequence as a termination signal by the transposition machinery results in the capture of 3' flanking host sequences (Feschotte and Wessler 2001). The strong conservation of the 3' end and not the 5' end of a family of non-autonomous *Helitrons* led Morgante et al. (2005) to propose that transposition is initiated at the 3' end of the *Helitrons* and the capture of 5' host flanking sequence occurs during transposition via an unknown mechanism. Kapitinov and Jurka (2007) have proposed that gene piece incorporation occurs through double stranded breaks within the transposing *Helitron* and the random incorporation of "filler" DNA coming from the host genome.

4.1 *Helitrons* and Bacterial Integrins Share a Number of Common Features

A number of parallels between *Helitrons* and bacterial integrins suggests that integrins may be the bacterial counterpart of *Helitrons* (Fig. 2). Like *Helitrons*, integrins are mobile, lack terminal repeats and do not make host duplications upon insertion. Like *Helitrons*, integrins capture genes (Hall and Callis 1995). Integrins have the capability to acquire gene sequences from mobile elements called gene cassettes via site specific recombination involving circular intermediates. Analogous to *Helitrons*, bacterial integrins consist of conserved terminal sequences encompassing a variable region primarily consisting of captured gene cassettes. The captured gene cassettes, like gene pieces in *Helitrons* are oriented in the same direction. This allows them to be transcribed by the same promoter located in the 5' conserved sequence. Also like *Helitrons*, different gene cassettes captured by integrins are often co-transcribed as a single unit. In integrins, the site specific recombination during gene capture is mediated by an integrase gene located in the conserved 5' terminus. Whether *Helitrons* capture gene sequences using mechanisms similar to integrins needs further investigation; however sequences bearing similarity to integrase have not been reported to date in maize *Helitrons* (Gupta et al. 2005).

4.2 Potential Role of *Helitrons* in Maize and Gene Evolution and their Impact on Host Gene Expression

Integron capture of genes for antibiotic resistance in bacteria has been noted in a number of cases. Expression of the captured gene then provides an obvious advantage to bacteria exposed to the toxin. In contrast, no clear cut example of *Helitron* gene capture conditioning advantages to the *Helitron* or to the host organism is available. In fact, all characterized *Helitron*-captured maize genes are truncated and likely non-functional (Lal et al. 2003; Gupta et al. 2005, Lai et al. 2005; Morgante et al. 2005; Brunner et al. 2005). *Helitron* capture of an almost intact copy of the maize gene encoding cytidine deaminase was recently reported (Xu and Messing, 2006). The captured gene lacks its promoter, exon 1 and a major portion of intron 1. While the truncated open reading frame and the introns have been conserved, the lack of detectable transcript of this gene suggests that the gene may not be active. While no *a priori* reason exists for the absence of intact gene capture in maize, their nonexistence in extant databases is curious. Intact gene capture is possible, at least in

Fig. 2. General structural similarity between Class 1 integrons and gene carrying non-autonomous maize *Helitrons*: The upper and lower panels display the basic structure of a Class 1 integron and a maize non-autonomous *Helitron* respectively. The letter P marks the position of the promoter. The 5' and 3' conserved terminal sequences (CS) are indicated. The integrase (*int1*) and sulfonamide resistance (*sul1*) are also indicated in the integron structure. The color coded arrows display the coding sequences and direction of the captured genes.

Aspergillus nidulans (Cultrone, Dominguez, Drevet, Scazzocchio, Fernandez-Martin 2007).

The expression of captured genes by *Helitrons* can potentially have significant impact on host genome expression. For example, the *wp* mutation in soybean is caused by an insertion of *Tgm-Express1* transposon in the flavanone-3- hydroxylase gene. Similar to *Helitrons*, *Tgm-Express1* contains fragments of different genes which are transcribed giving rise to multiple, alternatively-spliced, chimeric transcripts. Intriguingly, the *wp* mutant also displays a significant increase in both seed weight and protein content. Since the genes pieces captured by *Tgm-Express1* represent enzymes involved in diverse metabolic pathways, it was postulated that their transcript may somehow modulate the expression of their wild type genes causing pleiotropic effect of the *wp* mutant (Zabala and Vodkin, 2005 Zabala and Vodkin 2007).

A potentially significant role for *Helitrons* is the synthesis of new genes. The gene pieces captured by *Helitrons* are often transcribed into eclectic transcripts conjoining coding regions of several different genes. These chimeric transcripts are sometimes alternatively spliced, generating several different transcripts via differential utilization of splice sites during pre-mRNA processing (Lal et al. 2003; Morgante et al. 2005; Brunner et al. 2005). For example, the *Helitron* insertion in maize mutant, *sh2-7527* generates several, alternatively-spliced isoforms, at least two of which are caused by alternative retention of a wild type *Sh2* intron (Lal et al. 2003). Since

mutations acting from a distance are known to influence splice site recognition, it is possible that change in the context of captured gene fragments induces alternatively splicing events (Lal et al., 1999).

Promoters located inside or outside *Helitrons* can initiate transcription. Expression of the *sh2-7527* gene uses the *Sh2* promoter to produce a chimeric transcript containing sequences of several genes. In contrast, transcription initiation of captured genes within a *Helitron* has been suggested (Morgante et al., 2005; Brunner et al., 2005). Similarly, transcription of a maize cytochrome P450 monooxygenase captured by a *Helitron* seems to be initiated inside the element (Lal and Hannah, unpublished results). In this regard, *Helitrons* parallel pack-MULEs since transcription initiation within many pack-MULEs has been documented (Jiang et al., 2004). The possibility that genes captured by *Helitrons* may lead to evolution new genes by promoter shuffling was recently reported of xanthine α -ketoglutarate-dependent dioxygenase (*xanA*) gene captured by *Helitron-N1_AN* in *Aspergillus nidulans* (Cultrone et al., 2007). Apparently, the 5' region of this gene including its tightly regulated promoter is composed of a *Helitron*. It is proposed that the 3' portion of a *Helitron* inserted upstream to the ancestral was deleted and the rolling circle replication initiating from the intact 5' end of the *Helitron* provided read-through, which terminated in the coding sequence of the downstream *xanA* gene.

4.3 Future Prospects and Overview

The newly discovered *Helitrons* constitute a vastly unexplored family of novel transposable elements. Observations to date suggest that maize contains a particular subfamily of *Helitrons*. In contrast to a “generic” *Helitron*, the maize members bear much sequence similarity in termini. This feature will greatly aid in the quick identification of *Helitrons* in large-scale sequencing projects. Maize *Helitrons* also appear to be unique in their ability to prolifically capture gene pieces.

Existing data point to two fundamentally different ways that *Helitrons* could significantly alter the maize genome and gene expression. First, the ability to capture genes provides a simple mechanism to alter gene dosage. The presence of a gene in one inbred and its absence at the homologous site in another inbred provides an uncomplicated explanation for heterosis. So far however, no case of complete gene capture has been documented in maize so the gene dosage/heterosis model awaits validation. The availability of complete maize genome sequence in the near future will provide a valuable resource to investigate whether *Helitrons* mediate the mobilization of active genes, and if they do, whether it has occurred to an extent that might impact heterosis.

Second, the ability to place segments of different genes into a common transcribed region provides the opportunity to create new genes and functions. While this aspect of *Helitron* function awaits validation, it does suggest that “hybrid” transcripts will be found in large EST collections. Studies are in progress.

Despite strong evidence that these elements have moved recently, there is no direct genetic proof of their activity in the present day maize genome. Analysis of *Helitron* movement in the two maize mutants caused by *Helitron* insertion may provide clues concerning mechanisms of transposition. Also, a more complete understanding of the process by which two short conserved terminal ends of maize non-autonomous elements mediate movement and integration of large and diverse seg-

ments of DNA may aid in designing novel strategies for transformation and transgenic expression for crop improvement.

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