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# Transfection of heteroduplexes containing uracil · guanine or thymine · guanine mispairs into plant cells

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## Abstract

We have compared the fate of U · G mispairs or analogous T · G mispairs in DNA heteroduplexes transfected into tobacco protoplasts. The heteroduplex DNA consisted of tomato golden mosaic virus DNA sequences in the *Escherichia coli* vectors pUC118 or pUC119. After transfection, the mismatched U residues were lost with an efficiency of greater than 95%, probably as a result of the uracil-DNA glycosylase pathway for excision of U residues in any sequence context. In contrast to the preferential removal of the mispaired U residues, biased removal of T residues from analogous heteroduplexes was not seen in the transfected plant cells. Also, we investigated the effect of extensively methylating one strand of the heteroduplex DNA used for transfection. Surprisingly, such methylation resulted in highly biased loss of the mismatched base from the 5-methylcytosine-rich strand of T · G-containing heteroduplexes.

## Introduction

Mismatched U residues from U · G pairs are removed from DNA heteroduplexes transfected into bacterial or mammalian cells with an efficiency of greater than 95% [8, 14, 34]. This is probably the result of the uracil-DNA glycosylase pathway for excision of U residues in any sequence context, whether properly paired with A residues or mispaired [24, 34]. T residues analogously mispaired with G residues in SV40 DNA heteroduplexes are corrected to C.G pairs in transfected mammalian cells with an efficiency of more than 92% [7]. A thymine-DNA glycosylase and possibly another DNA-binding protein specific for T · G mispairs has been implicated in this process [36, 42]. Other types of DNA mismatch repair systems not specifically directed to excision of mispaired T residues have been demonstrated in bacteria, yeast, fruit flies, toads, and mammals *in vivo* or *in vitro* [5, 9, 17, 19, 20, 38]. In addition, there is a specialized DNA repair pathway in

† Deceased. We dedicate this paper to the memory of this young scientist.

*E. coli* that is highly specific for removal of the mispaired T rather than the mispaired G of T · G mismatches but only at certain sequences, most notably, 5′-CC(A/T)GG-3′ sites, which are the targets, in *E. coli* K strains, for methylation of the second C residue [18, 23, 43]. In contrast, mammalian cells perform T-directed repair of T · G mismatches within a variety of sequence contexts [7].

The directed excision of U residues from U · G mispairs and T from T · G mispairs is presumed to function as a means of correcting spontaneous deamination at C and 5-methylcytosine ($m^5C$) residues in DNA [11, 12, 24, 41]. In this study, we tested whether U residues in U · G mispairs are selectively lost from heteroduplex DNA transfected into plant cells as they are when introduced into bacterial or mammalian cells. Also, we predicted that because vascular plants have 4–7 times higher levels of genomic $m^5C$ than do mammals [13, 40], they might remove T residues from T · G mismatches with ≥90% efficiency as mammalian cells do. We tested these hypotheses by transfecting tobacco protoplasts with heteroduplexes containing U · G or T · G mispairs in DNA sequences from a geminivirus, tomato golden mosaic virus (TGMV [4, 22]), inserted into a bacterial plasmid. Lastly, because adenine methylation in only one strand of a DNA heteroduplex introduced into *E. coli* can direct mismatch repair to the unmethylated strand [21, 26], we also used heteroduplexes methylated in C residues of one strand or in neither strand for transfection of the tobacco protoplasts.

## Materials and methods

### *Synthesis of heteroduplex DNA*

Single-stranded (ss) phage DNA was obtained from TGMV/pUC118 or pUC119 phagemid recombinants pTGA26 and pTGA41 [6, 39]. Four pmol of this template was annealed with 80 pmol of purified, 5′-phosphorylated synthetic oligonucleotides [44] for synthesis of heteroduplexes *in vitro* in a reaction catalyzed by the Klenow fragment of *E. coli* DNA polymerase I and T4 DNA ligase and then covalently closed circular (CCC) DNA molecules were purified as previously described [6, 34]. Generally, 1–4 μg of DNA was recovered and this DNA consisted, almost exclusively, of CCC molecules, most of which were relaxed circles as determined by gel electrophoresis in the presence of ethidium bromide [32]. To show that more than 90% of this DNA consisted of molecules that had incorporated the mutant oligonucleotide primer, we demonstrated, by nitrocellulose filtration after alkaline treatment and neutralization [32], that the formation of CCC molecules was reduced more than 90% if the primers were used in their unphosphorylated (5′-OH) form. Also, when we used a primer that was the same as primer C (Table 1) except that it had additional arbitrary bases at the 5′ end (5′-CCCG-3′), giving a single-stranded 5′ tail upon annealing to the TGMV insert, the yield of CCC DNA decreased by 95%.

### *Transfection and restriction analysis*

Protoplasts of *Nicotiana tabacum* were used for transfection with the DNA heteroduplexes [6, 28, 29]. DNA isolated from the cells 8 days after transfection [25] was incubated with restriction enzymes (10 units of enzyme per μg of DNA), electrophoresed, and subjected to Southern blot analysis with a $^{32}$P-labeled riboprobe specific for TGMV A DNA [6]. Blots were autoradiographed and examined by microdensitometry. Complete digestion was verified with λ DNA as an internal control as previously described [6].

## Results

Tobacco protoplasts were transfected with recombinant TGMV/pUC118 or pUC119 DNA (pTGA26 and pTGA41, respectively, Fig. 1) containing mismatches in TGMV sequences. These recombinant DNAs have approximately one-and-a-half full-length copies of the duplex form of the TGMV A genome [3] and differ in their orienta-

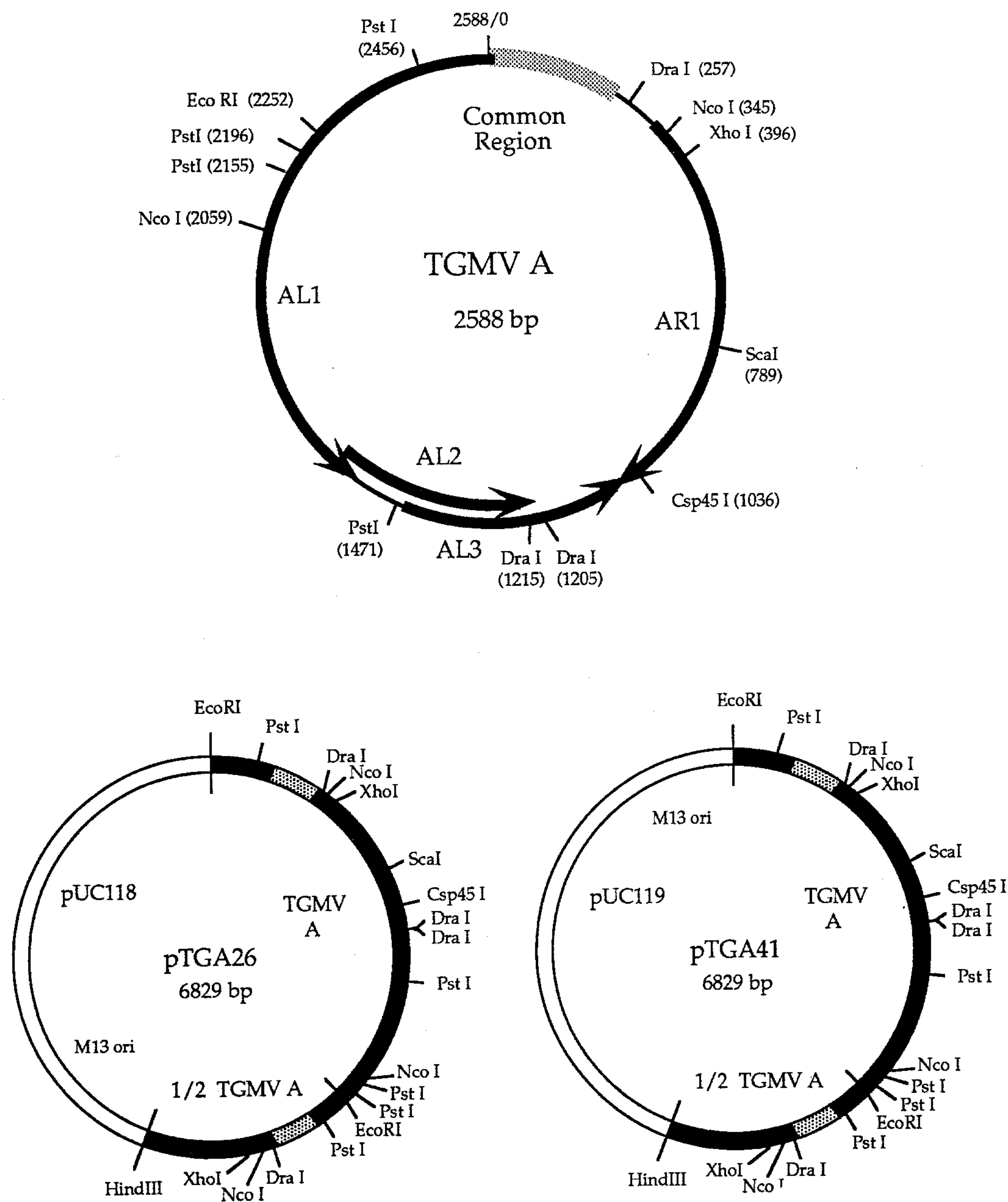


*Fig. 1.* Diagrams of TGMV DNA A and phagemids pTGA26 and pTGA41. The ds replicative form of TGMV DNA A is shown. The arrows indicate the positions of open reading frames and the hatched box represents the common region (CR), a sequence present in both TGMV DNA A and DNA B. Open reading frame AR1 encodes the viral coat protein. Phagemids pTGA26 and pTGA41 [37] contain one-and-a-half copies of TGMV DNA A (shaded) including two copies of its CR (hatched boxes) in pUC118 or pUC119 phagemid vectors (unshaded). The M13 origin of replication in the pUC plasmids is indicated. In pTGA26, the viral strand of TGMV DNA A is contained within the viral strand of the phagemid, whereas in pTGA41, the complementary strand of TGMV DNA A is within the viral strand of the phagemid.

tion of the viral strand of TGMV A DNA (the one normally packaged in virions in plant cells [15]) with respect to the viral strand of the recombinant (the one packaged in the M13 virions upon cotransfection with M13 helper phage [39]). TGMV DNA A encodes all the viral proteins

required for viral DNA replication [31]. It also contains the so-called common region (CR), in which *cis*-acting elements required for DNA replication are located [30]. When the inoculum DNA contained a duplication of the CR, a much higher yield of TGMV A DNA monomers in tobacco protoplast transfectants was obtained than when only one copy of the CR was present. In protoplasts transfected with pTGA26 or pTGA41, ca. 2.5 kb double-stranded (ds) circular TGMV A DNA monomers containing only one copy of the TGMV A genome and no vector sequences are released and replicated [35]. Also, the ss DNA form of the TGMV A monomer usually accumulates in these transfectants [37].

The pTGA26 or pTGA41 DNA used for transfection had mutations (C→U or C→T) that were introduced into one strand of the TGMV coat protein gene's open reading frame (AR1) via primer oligonucleotides used for synthesis of the heteroduplex CCC DNA from the ss phagemid template DNA (Table 1, Fig. 1). Deletion of AR1 does not interfere with accumulation of viral ds DNA in transfected protoplasts [37]. Protoplasts were propagated for 8 days after treatment with the heteroduplexes. Total DNA was then extracted and purified, digested with restriction endonucleases diagnostic for the sequence that contained the mispair, and subjected to blot hybridization with an *in vitro* synthesized RNA probe specific for TGMV A DNA.

Three batches of heteroduplexes, each with a U · G mispair at a different position, were used for transfection. For the heteroduplex made with primer N (Table 1), a C→U mutation was introduced into one strand at the *Sca* I site. The wild-type (WT) TGMV insert released from pTGA26 gives 921 and 682 bp fragments upon hydrolysis with *Sca* I and *Pst* I. If the progeny DNA molecules inherit the mutation, they will be resistant to digestion by *Sca* I (*Sca* $I^R$) and so the above double digestion would give a 1603 bp fragment instead of the 921 and 682 bp *Sca* I-sensitive (*Sca* $I^S$) fragments. As seen in lane 3 of Fig. 2, TGMV DNA sequences from cells transfected with this U · G mismatch-containing heteroduplex

*Table 1.* Heteroduplex regions of transfecting DNAs[1].

| Type of mispair | Type of recombinant DNA | Name of primer | Sequence of primer/template region | Wild-type | Enzyme site |
|---|---|---|---|---|---|
| U·G | pTGA26 | N | 5′-CCGTTGCAGTAUTTGGCTCA-3′<br>3′-GGCAACGTCATGAACCGAGT-5′ | *Sca* I | AGTACT |
| U·G | pTGA26 | H | 5′-TAGATTUGAATTTTCAACGT-3′<br>3′-ATCTAAGCTTAAAAGTTGCA-5′ | *Csp* I | TTCGAA |
| T·G | pTGA26 | G | 5′-TAGATTTGAATTTTCAACGT-3′<br>3′-ATCTAAGCTTAAAAGTTGCA-5′ | *Csp* I | TTCGAA |
| U·G | pTGA41 | J | 5′-TTCTCCTUGAGGAAGTTTGCC-3′<br>3′-AAGAGGAGCTCCTTCAAACGG-5′ | *Xho* I | CTCGAG |
| T·G | pTGA41 | I | 5′-TTCTCCTTGAGGAAGTTTGCC-3′<br>3′-AAGAGGAGCTCCTTCAAACGG-5′ | *Xho* I | CTCGAG |
| G·T | pTGA41 | K | 5′-TTCTCCTCGGGGAAGTTTGCC-3′<br>3′-AAGAGGAGCTCCTTCAAACGG-5′ | *Xho* I | CTCGAG |
| T·G | pTGA26 | M | 5′-CCGTTGCAGTATTTGGCTCA-3′<br>3′-GGCAACGTCATGAACCGAGT-5′ | *Sca* I | AGTACT |
| G·T | pTGA26 | C | 5′-AAACTTCCTCGGGGAGAATA-3′<br>3′-TTTGAAGGAGCTCCTCTTAT-5′ | *Xho* I | CTCGAG |

[1] The sequence of the heteroduplex in the region of the primers used for their *in vitro* DNA synthesis is shown. The primer is the top strand of the depicted duplex region and its mutant base at the indicated restriction site is underlined.

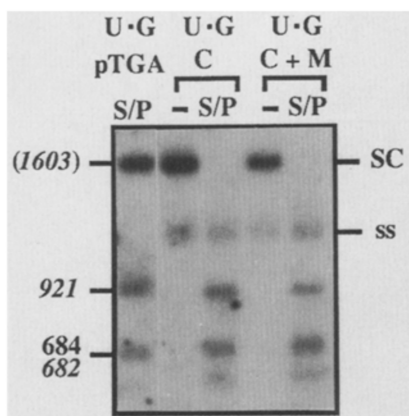


*Fig. 2.* Restriction analysis after transfection with pTGA26 DNA containing a U · G mispair at a *Sca* I site. Tobacco protoplasts were transfected with pTGA26 DNA synthesized *in vitro* and containing a mispair (U · G) at the *Sca* I site (Table 1). DNA isolated from transfected protoplasts was electrophoresed without digestion (-) or following digestion with *Sca* I and *Pst* I (S/P) and subjected to Southern blot analysis using mixed TGMV DNA A riboprobes corresponding to both the viral and complementary strands. The diagnostic fragments for restriction resistance (1603 bp) and restriction sensitivity (921 and 682 bp) at the originally mispaired site are indicated by italics. The positions of supercoiled (SC) and single-stranded (ss) TGMV DNA are shown at the sides of the panel. The lane marked pTGA contained nonmethylated U · G-containing pTGA26 DNA (pure inoculum) used for transfection (10 ng) and its *Sca* $I^R$ 1603 bp band represents the partial restriction resistance of the mismatched (U · G) restriction site in the inoculum DNA [33]. Lanes marked C contained 1 $\mu$g and those marked C + M contained 2 $\mu$g of DNA protoplasts transfected with pTGA26 DNA that was nonmethylated or partially hemimethylated (one fourth of C residues in one strand replaced with $m^5C$ residues), respectively. A 684 bp *Pst* I fragment coelectrophoreses with the 682 bp *Sca* I/*Pst* I fragment but the important position to note is that of the 1603 bp fragment which is seen only in the inoculum heteroduplex DNA as denoted by the parentheses.

gave no detectable 1603 bp fragment. Microdensitometric analysis of the relative signal intensities in the 1603 bp *Sca* $I^R$ band region and the 921 bp *Sca* $I^S$ band indicated that less than 5% as much signal was in the former region as in the latter.

It was possible that extensive substitution of C residues with $m^5C$ residues might influence the loss of bases from the U-containing strand upon transfection (see below). Therefore, by incorporation of a 1:3 mixture of $m^5$dCTP and dCTP during the synthesis of CCC DNA used for transfection, we prepared heteroduplex DNA in which the same strand contained $m^5C$ residues and the mismatched U at the *Sca* I site. This had no effect on the *Sca* I sensitivity of the TGMV sequences in the transfectants (Fig. 2).

To test whether the specific elimination of the U residue from U · G mispairs occurs with a similar high efficiency in other sequence contexts, protoplasts were transfected with a U · G-containing heteroduplex having the mutant U residue at a *Csp*45I site (Table I, primer H). Again, little or no DNA was detectable at the position of the band (1714 bp) representing the mutant genotype (resistance to *Csp*45I) associated with the U-containing strand in the inoculum DNA (Fig. 3A, lane 4). Similar results were obtained with a third type of heteroduplex that contained a mispaired U residue, but this time, in the complementary strand of TGMV A DNA rather than in the viral strand and at the *Xho* I site (Table I, primer J and Fig. 1; data not shown).

For comparison to the U · G heteroduplexes, T · G-containing pTGA26 or pTGA41 DNA was used in parallel transfections. With a T · G mispair in exactly the same position as the above-described U · G mispair at the *Csp*45I site, we obtained quite different results (Table 1; Fig. 3A, lane 6). In this case, similar amounts of the diagnostic fragment for restriction resistance (1714 bp *Nco* I digestion fragment) and those for restriction sensitivity (1023 bp and 691 bp *Nco* I/*Csp* 45I double-digestion fragments) were seen. Such partial restriction resistance (1603 as well as 1075 bp fragments) was also obtained after transfection with another heteroduplex containing a T residue mispaired with a G residue but at the *Xho* I site (Fig. 3B, lane 2). With a number of T · G heteroduplexes (Table 1), whether the T residue was in the viral or complementary strand of the TGMV or pTGA sequences and whatever the sequence adjacent to the mispair, similar results were obtained and the ratio of signal in a diagnostic restriction-resistant band to that in a restriction-sensitive band varied from 0.9 to 1.3 (Fig. 2 and 3; and data not shown).

Because adenine hemimethylation at the *E. coli* replication fork directs bacterial DNA mismatch

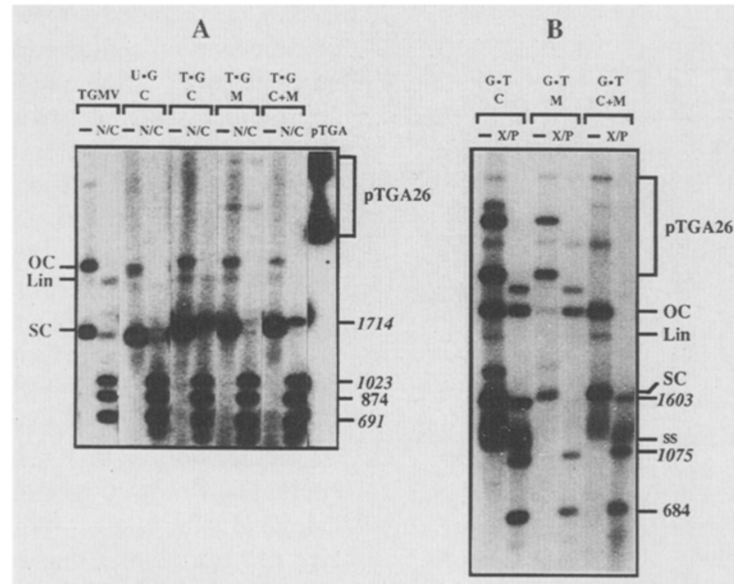


*Fig. 3.* Restriction analysis after transfection with pTGA26 heteroduplexes containing a U · G or T · G mispair at a *Csp*45I or *Xho* I site. Transfection analysis was as in Fig. 2 except for the different heteroduplexes (Table 1) and restriction endonucleases (*Nco* I and *Csp*45I, N/C; *Xho* I and *Pst* I X/P) used. Lanes marked C, M, and C + M contained 1, 5, and 1.5 (A) or 2, 4, and 4 (B) $\mu$g of DNA from protoplasts transfected with plasmid that was unmethylatated, hemimethylated (complete C replacement with $m^5C$ in one strand) or partially hemimethylated (one fourth of C residues replaced in one strand), respectively. The lane marked TGMV contained DNA (60 ng) from a TGMV-infected plant. The italicized numbers indicate the size in bp of bands diagnostic for restriction resistance (1714 or 1603 bp) or sensitivity (1023 and 691, or 1075 bp) at the original site of the mismatch. In this figure, the low-mobility region of the gel is also shown. The low-mobility bands in this region (labeled pTGA26) seen in the undigested samples probably consist mostly of high-molecular-weight inoculum DNA corresponding to supercoiled and open circular forms of pTGA26. Apparently multimeric forms of TGMV A DNA are also present. The low-mobility bands in undigested DNA samples varied in relative amounts from one transfection to another. The position of bands electrophoresing as linear (Lin) or open circular (OC) TGMV ds DNA are indicated. Lanes 3 and 4 in panel A come from a different gel than the rest of the lanes; no 1714 bp band is visible in lane 4 above the midpoint of the SC band in lane 3 as in lanes 6 and 8. The minor doublet bands in lane 4 ($\leq 10\%$ of the intensity of the 1023 bp band) at the midpoint of the SC band are of unknown origin and were also seen in DNA from cells transfected with analogous U · G heteroduplexes methylated in the U-containing strand (data not shown). The light band seen in panel B in the X/P lanes below the 684 bp band is the 528 bp *Xho* I/*Pst* I fragment.

repair to excise the mismatched residue from the unmethylated strand [1, 26], we tested the effect of methylating C residues in only one strand of TGMV DNA on repair of its T · G mismatches. When cytosine-hemimethylated pTGA26 or pTGA41 T · G containing heteroduplexes (obtained by replacing dCTP in the *in vitro* primer extension reaction with $m^5$dCTP) were used to transfect tobacco protoplasts, little or no signal was detected in the region where the restriction-resistant fragment (derived from the methylated strand) would electrophorese, whereas such a band was prominent in parallel transfections with the analogous nonmethylated heteroduplex (Fig. 3A, lane 8; Fig. 3B, lane 4; and data not shown). We also prepared these heteroduplexes with replacement of only 25% of the C residues in the *in vitro* synthesized strand by using a mixture of $m^5$dCTP and dCTP (1:3) in the primer extension reaction. All of these partially hemimethylated heteroduplexes gave a higher percentage of restriction resistance at the test site than did the analogous heteroduplexes completely substituted with $m^5C$ in the mutant strand (Fig. 3A, lane 10; Fig. 3B, lane 6; and data not shown). However, the partially hemimethylated heteroduplexes gave a lower percentage of restriction-resistant ds TGMV DNA than did nonmethylated heteroduplexes.

## Discussion

In bacterial cells, uracil-directed repair at U · G mismatches is extremely efficient due to the uracil-DNA glycosylase pathway for base excision [34]. Uracil-DNA glycosylase is found in plants [16] as well as in diverse other organisms [24, 27] and is likely to be responsible for the highly (more than 95%) efficient loss of uracil residues from U · G mispairs seen in these plant transfections (Figs. 2 and 3) and in analogous mammalian cell transfections [8, 14]. Such uracil excision repair had to occur before the first round of replication of the TGMV DNA sequences to explain the lack of detectable TGMV A progeny molecules with the genotype of the strand containing the U residue from the U · G mismatch.

Uracil excision repair systems are thought to play their most important role in counteracting spontaneous deamination of C residues in DNA [24]. The base-specific removal of T residues rather than G residues from T · G mispairs in all tested DNA sequences introduced into mammalian cells [42] and from only certain 5 bp sequences in *E. coli* cells [2] is thought to reflect a similar need to compensate for the even greater propensity of $m^5C$ residues in DNA to undergo spontaneous deamination [10–12, 24]. Like vertebrates, all vascular plants methylate an appreciable fraction of their C residues and they typically methylate much more of this base (up to 33% of their C residues) than do mammalian cells [13, 40]. It is, therefore, surprising that we found in tobacco cells no significant bias toward loss of T residues mismatched with G residues from a variety of transfected T · G-containing heteroduplexes. A caveat in these experiments is that we do not know whether there was no mismatch repair or mismatch repair with no base preference. However, it is clear that, in these experiments, no repair akin to the 92% efficient T-specific excision at T · G mismatches described for viral DNA transfecting mammalian cells could be seen [7]. Therefore, it is unclear how plant cells might counteract spontaneous deamination at their abundant genomic $m^5C$ residues.

The other unexpected result in this study was the finding that hemimethylation of the transfecting heteroduplexes directed the loss of T · G mismatches from the methylated strand rather than from the unmethylated strand as is found in *E. coli* cells [21, 26]. This preferential loss of the mispaired base in the methylated strand upon transfection of tobacco protoplasts occurred in a number of sequence contexts when either the viral or complementary strand of TGMV DNA A was methylated. The loss of the mismatched base from the methylated strand, such that its associated phenotype was not seen in progeny TGMV A DNA monomers, occurred despite the fact that cytosine methylation in these progeny molecules is undetectable [6]. The very small amounts of adenine methylation and cytosine methylation [1, 2] introduced by the *E. coli* cells into pTGA26 and pTGA41 phagemid strands used for *in vitro* synthesis of the transfecting ds DNA gave no detectable bias for the genotype of either strand when synthesis of the CCC DNA utilized dCTP instead of $m^5$dCTP. In contrast, the *in vitro* synthesized DNA strands with 25–100% of their C residues replaced by $m^5C$ were counterselected in the transfected tobacco protoplasts. It is unlikely that this result was due to an extensive DNA repair mechanism directed against $m^5C$ residues. With approximately one third of the cytosine residues in the DNA of the tobacco cells being naturally methylated [40], such a repair mechanism, if it existed, would be likely to kill these host cells. Rather, we propose that the strand that is extensively methylated at C residues is selectively lost during the stage when the TGMV DNA insert is released from a transfecting pTGA26 or pTGA41 DNA molecule [35] or immediately thereafter. That methylation may be interfering with release of the TGMV insert from pTGA26 and pTGA41 DNAs and directing the choice of a single template strand for synthesis of ds TGMV A molecules [35] to the nonmethylated strand is consistent with the decrease in the yield of progeny TGMV A DNA molecules observed when the transfecting DNA is hemimethylated [6].

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