The polymerase in its labyrinth:

mechanisms and implications of RNA recombination

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The wide variety of RNA viruses, and the diseases associated with them, may result in part from the capacity of RNA genomes to evolve through genetic recombination. Here we address the mechanism of RNA recombination, and ask questions about its prevalence and purpose in nature.

A growing body of evidence suggests that genetic recombination occurs in many RNA viruses. We limit the discussion here to RNA viruses that use RNA as their sole genetic material; therefore, these recombination events must happen at the RNA level, without utilizing a DNA intermediate. The following questions arise. Which RNA viruses undergo recombination and at what frequency? Is the ability to recombine a universal property of RNA viruses? What is the mechanism of RNA recombination, and how does it differ from the known mechanisms of DNA recombination? Is the biological purpose of RNA recombination, if there is one, discernible?

The incidence of homologous recombination among RNA viruses

The term 'homologous recombination' is often used to refer to intratypic recombination, in which the parental genomes are identical with the exception of the marker loci. Intertypic recombination, between viruses of different serotypes that typically differ in sequence by 10–15%, is sometimes referred to in the literature as 'nonhomologous recombination'. For our purposes, however, we shall categorize both intratypic and intertypic recombination events as essentially 'homologous'. We shall reserve the term 'nonhomologous' for those recombination events in which little or no homology between parental molecules is evident at or near the crossover site.

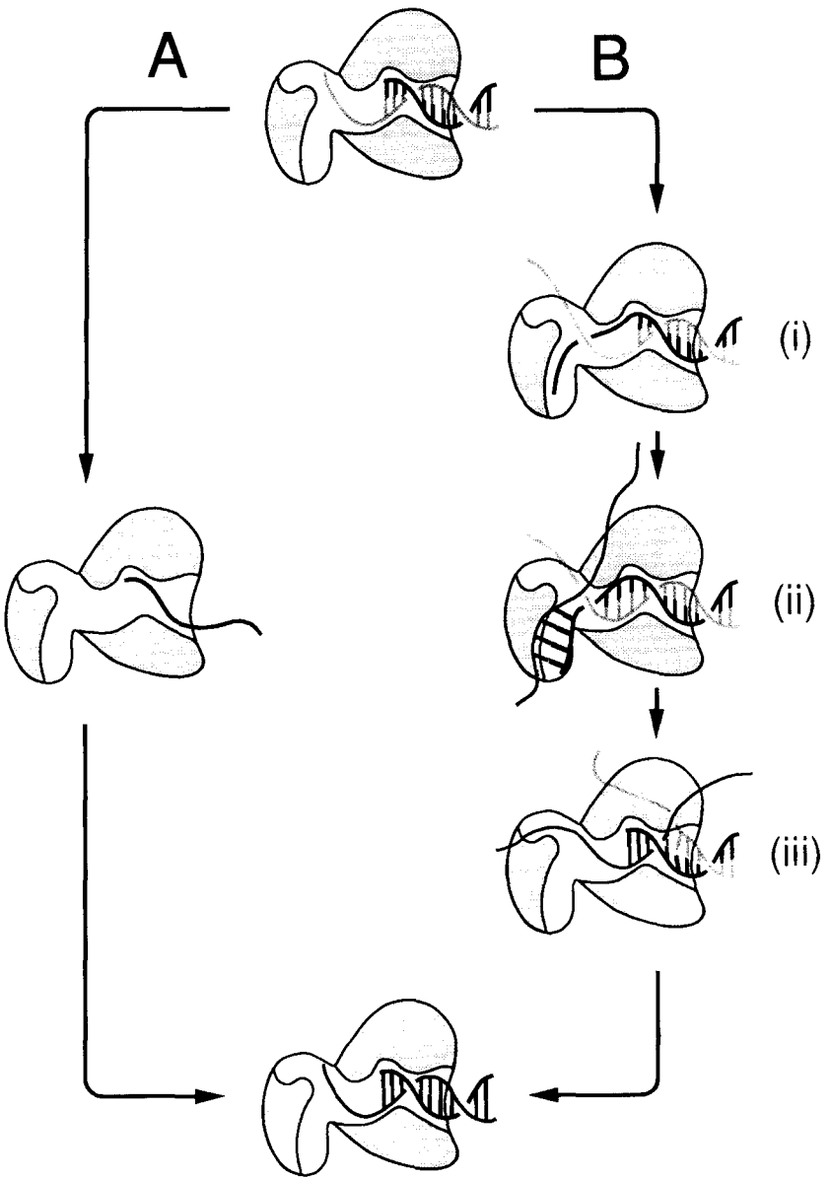
The first evidence of recombination in RNA viruses was found in poliovirus[1] and foot-and-mouth disease virus[2]. Initially it appeared that homologous recombination might be limited to the picornaviruses, because several studies failed to find evidence for recombination in other viruses, including Sindbis virus, vesicular stomatitis virus, and RNA phage[3]. More recent studies, however, have firmly established a high frequency of recombination in mouse hepatitis virus, a coronavirus, both in tissue culture and in the animal host[4]. The occurrence of RNA recombination has also been established in two positive-strand plant viruses, brome mosaic virus and cowpea chlorotic mottle virus[5,6].

Several methods have been used to measure homologous RNA recombination; each of these methods has limitations, and all rely on the production of viable recombinant viruses. Recombination frequency is typically measured by a virus yield test. Cells are co-infected under permissive conditions with two different parental viruses bearing genetic markers such as temperature sensitivity or drug resistance. The progeny viruses are then assayed under restrictive conditions to detect recombinants, and the recombination frequency is determined by dividing the yield of recombinants by the sum of the yields of the parental viruses. The sensitivity of this assay is limited by the typically high reversion rate of RNA viruses[3]; the recombination rate must be higher than the reversion rate to be detectable. The 'infectious center' test detects cells harboring recombinant molecules by plating cells from a mixed infection on confluent cell monolayers, followed by growth at the restrictive temperature. This assay is qualitative in the sense that it does not measure the total number of recombinant viruses, but sensitive in that it detects any cell containing a recombinant virus. Biochemical analysis can be used to confirm the presence of recombinant RNA from plaque isolates obtained from mixed infections; methods employed have included analysis of RNase T_1 digestion patterns and RNase protection. These biochemical methods can reveal multiple crossovers within a single genome, but have the limitation that relatively few recombinant molecules can be screened at one time, and frequencies of recombination cannot be readily determined.

It remains unclear whether the capacity to recombine is a universal property of all RNA viruses. There are no laboratory examples of homologous recombination among alphaviruses, negative-strand viruses or RNA phage, and yet each of these has been demonstrated to produce nonhomologous recombinants, as will be discussed below. Furthermore, the naturally isolated western equine encephalitis virus has recently been shown to be a recombinant alphavirus[7]. The apparent lack of homologous recombinants in some viruses could be explained in several ways. First, it is conceivable that some RNA viruses simply do not undergo recombination at any significant frequency. It is also possible, however, that the prevalence of RNA recombination has been underestimated because complementation of parental viruses and the high reversion frequency (10^{-3} to 10^{-4}) of RNA viruses mask low-frequency recombination events. Finally, even though recombinant molecules may be generated during replication they may not be viable, and therefore not detected by any of the laboratory methods discussed above.

Work is in progress in this laboratory and others to develop sensitive methods for the *in vitro* detection of recombinant RNA molecules in cytoplasmic RNA derived from mixed infections by use of the polymerase chain reaction (PCR) following reverse transcription. Direct detection will allow one to distinguish between the recombination event itself and the subsequent selection for viability, thereby shedding light on the underlying molecular requirements for recombination. Furthermore, it may allow one to detect recombination between RNA viruses previously reported not to undergo genetic exchange.

FIG 1



Two alternative models for copy-choice recombination are depicted (structures adapted from Ref. 18). Both models begin with normal synthesis, with the nascent (black) strand positioned in the polymerization site and fully base paired with the donor (pink) template strand. Template switching could then occur via either (A) a nonprocessive (dispersive) mechanism involving dissociation of one or both RNA strands from the replication complex, or (B) a processive mechanism in which the replication complex remains continuously associated with the RNA, ultimately resulting in synthesis on the acceptor template (red). Although the processive model may initially appear complex, it is kinetically more efficient than the nonprocessive model, and also invokes a lower-energy recombinational intermediate. Thus it is worth considering whether or not the dynamics of the polymerization process are likely to accommodate such a mechanism. From a mechanistic standpoint, *E. coli* DNA polymerase I Klenow fragment is arguably the best-understood polymerase and therefore provides a conceptual basis for understanding the capabilities common to all polymerases. The crystallographic studies of this DNA-dependent DNA polymerase as well as extensive kinetic studies[18,39] have shown that in order for the 3'–5' exonuclease activity to excise a misincorporated base, the DNA must slide approximately eight nucleotides relative to the protein, and three or four nucleotides at the 3'-end of the nascent strand must unpair and be positioned in a groove of the exonuclease domain. This suggests, surprisingly, that a degree of transient unpairing of the nascent 3'-end is a relatively frequent event during the polymerization process. This may have relevance to the propensity of certain viral polymerases to switch templates during synthesis. The processive model shows transient sliding and unpairing of the 3'-end of the nascent strand (i) permitting pairing with an alternative (red) template molecule (ii). The recombinational intermediate is then shown sliding back to the polymerization site with concomitant template strand exchange (iii). Pairing of the nascent strand with the acceptor template strand behind the replication complex (not shown) before the strand-switching event would greatly increase the local concentration of acceptor template and thereby facilitate the homologous recombination process. None of the known RNA-dependent RNA polymerases, DNA-dependent RNA polymerases, or reverse transcriptases has 3'–5' exonuclease activity. It is these proteins that seem to show the highest propensity for template switching (see text). Thus we speculate that the absence of the exonuclease domain (with its single-stranded nucleic acid-binding groove) could in fact promote efficient processive template switching. Note that the paths of the template strands as shown would technically bisect the protein, but are depicted thus for simplicity; the reader can imagine alternative pathways for the template RNA strands that would avoid this conceptual difficulty.

The mechanism of homologous RNA recombination

Is recombination site specific? No evidence for site specificity was found among 15 intertypic recombinant poliovirus isolates[8]. Statistical analysis of these and 25 other intertypic recombinant poliovirus isolates again found no particular consensus sequence surrounding the crossover sites[9]. Although early studies of recombination in coronaviruses suggested a bias toward recombination sites near the 5' end of the genome, subsequent studies have uncovered recombinants with crossover sites randomly distributed throughout the genome[10]. This provides further support for the idea that homologous RNA recombination is not a site-specific event, and any site bias observed

in a particular study is more likely to be a result of selective pressure rather than an indication of inherent site selectivity in the recombination event.

Recombination could conceivably occur either post-replicatively, by a pathway involving strand scission and ligation or by a copy-choice mechanism during viral replication. *Trans*-splicing, yielding recombinant RNA molecules and mediated by a self-splicing yeast group II intron, has been observed *in vitro*[11]. However, this reaction is highly sequence specific and thus is unlikely to provide a universal mechanism of RNA recombination. A protein-mediated strand scission and ligation mechanism similar to that found in DNA recombination systems would not need to be sequence specific. However, no such multienzyme pathway has yet been demonstrated to operate on RNA viruses. Strong genetic evidence has shown that recombination in poliovirus occurs via a copy-choice mechanism in which the viral RNA polymerase switches templates during negative strand synthesis[8]. Intertypic poliovirus recombinants show significantly elevated homology between the parental RNAs at the 3' side (of the positive sense strand) of the crossover sites, consistent with copy-choice recombination during negative strand synthesis, mediated by homology between the 3' end of the nascent negative strand and the plus strand template of the other parent RNA[9]. In the absence of any evidence to the contrary, the copy-choice mechanism is widely regarded as being operative in many if not all examples of RNA recombination, both homologous and nonhomologous[7,12–14].

Several models have been proposed to address the question of how parental RNA template molecules of the same polarity might be aligned through base pairing to potentiate template switching during poliovirus RNA replication[15,16]. Template switching may occur by a nonprocessive mechanism such as that illustrated in Fig. 1A, via dissociation of the polymerase from the template but not from the nascent RNA strand, as suggested by Cascone *et al.*[14] for recombination between satellite RNAs of turnip crinkle virus. Another nonprocessive mechanism would involve complete dissociation of the polymerase from the RNA, as proposed by Makino *et al.*[17] for coronavirus recombination (not shown). Alternatively, one can envisage a processive mechanism in which the viral polymerase switches templates without dissociation (Fig. 1B). We have based our ideas for this processive model on *E. coli* DNA polymerase I Klenow fragment, the only polymerase for which a three-dimensional structure is known[18]. No direct evidence has yet been obtained to support or distinguish these models. In any case, more detailed knowledge of the *in vivo* structure of the replicative intermediate in each of these viruses, as well as the intrinsic properties of the viral replication apparatus, will be required to understand fully the intricacies of the copy-choice recombination strategy.

Nonhomologous RNA recombination

The list of examples of nonhomologous RNA recombination continues to grow. It is clear that nonhomologous recombination, though infrequent, does occur and occasionally results in competitive progeny in suitable evolutionary niches. Nonhomologous recombination sometimes even results in the transduction of cellular sequences. Monroe and Schlesinger[19] found two independent examples of Sindbis virus 'defective interfering' (DI) particles containing cellular $tRNA^{Asp}$ sequences at their 5'-ends. Munishkin *et al.*[20] found a similar linkage between sequences from Qβ phage and $tRNA^{Asp}$.

The most commonly observed incidence of nonhomologous recombination is the production of DI particles during high multiplicity viral passages. These deleted genomes probably arise by a template-switching mechanism during replication. Sequence analysis of the crossover sites for DI genomes rules out a simple model in which deletions arise by synthesizing RNA across the base of a hairpin structure in the RNA template. This does not exclude the involvement of fortuitous base pairing in the formation of more complicated recombinational intermediates[15].

Although we have treated the discussion of the incidence and mechanism of homologous and nonhomologous recombination as separate issues, we envisage a general recombination pathway that could account for all types of RNA recombination, based on a simple correlation between the stability of base pairing in the recombinational intermediate and the frequency of recombination. In poliovirus the frequency of homologous intertypic recombination in which the parents differ in sequence by 10% is 100-fold lower than that for completely homologous intratypic recombinants[8]. The frequency of nonhomologous recombination has never been quantified, but is probably quite low. Nonhomologous recombination could be promoted by fortuitous RNA structures, supported by viral or cellular sequences, but these regions of structure would be relatively short. Thus we can think of intratypic, intertypic and nonhomologous recombination as representing a continuum of decreasing base pairing probability and correspondingly decreasing probability of successful recombination.

Purpose and consequences of RNA recombination

Ideas about the origin and purpose of RNA recombination range from its being an unavoidable side reaction of RNA-dependent RNA polymerization to its providing an indispensable source of viral genetic diversity[21]. If we assume that there is an evolutionary advantage to genetic recombination, the dynamics of RNA virus populations in different environments should affect the relative importance of recombination in the generation of diversity, as shown in Fig. 2. RNA viruses are well known for their extremely high mutation rates[3], so it can be argued that new mutations should provide ample genetic diversity to feed the fires of evolution in a population of RNA viruses. On the other hand, in small founding populations subjected to large selective pressures, recombination should display an increased advantage. Finally, the more loci that are involved in the generation of a specific trait, the more crucial recombination might be in the creation of a particular genotype conferring that trait[22,23].

Recombination may have other consequences in addition to generating diversity. In the absence of recombination, the potential exists for deleterious mutations to accumulate, leading to decreased fitness of the population. This phenomenon is known as Muller's

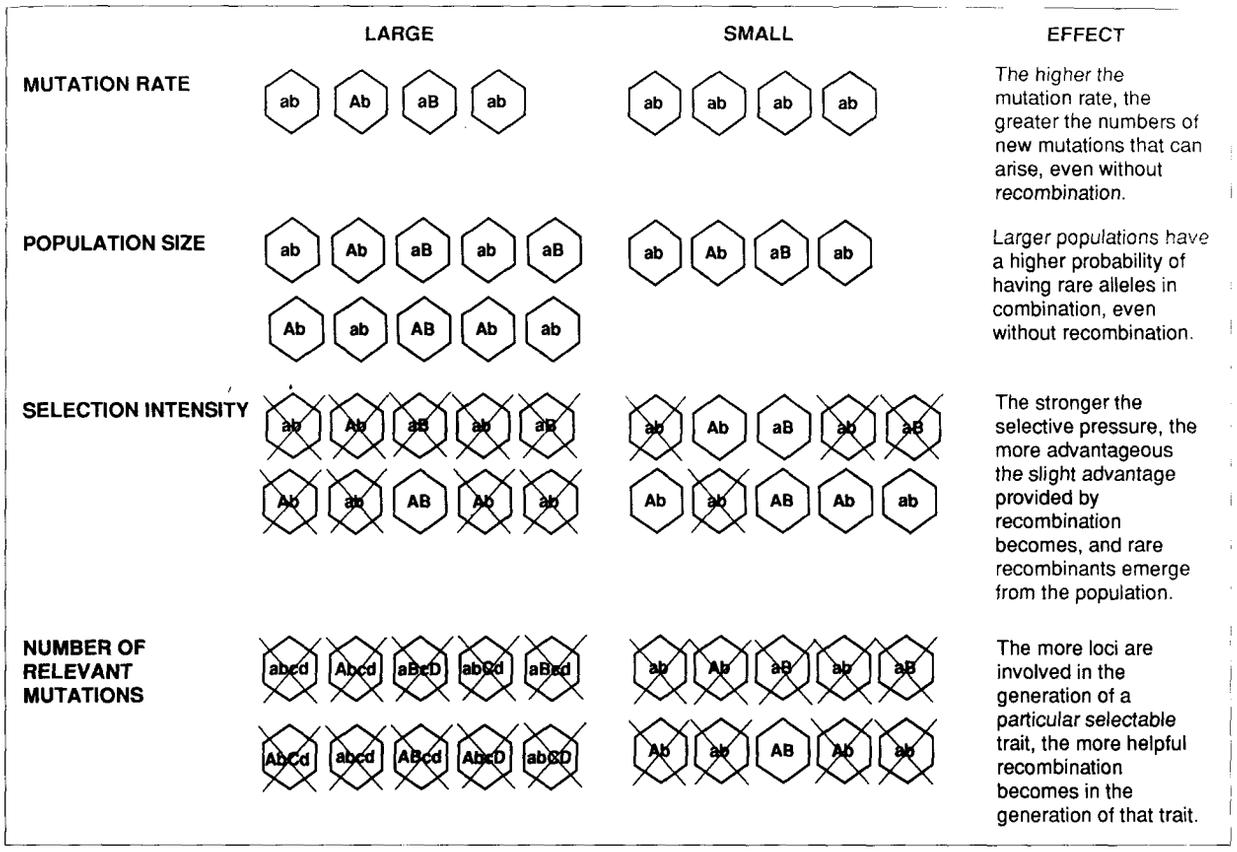


FIG 2

Factors affecting the relative importance of recombination in the creation of new genotypes. Low mutation rates, small populations, high selection intensities, and large numbers of relevant mutations are all factors that can increase any importance of recombination in generating diversity.

ratchet[24,25]. The ability to recombine may thus be advantageous in repairing lesions created by mutation. In particular, however, the recombination frequency must be higher than the rate of reversion in order to provide a significant repair function. Although the frequency of mutation (and therefore reversion) is quite high for RNA viruses, it is conceivable that at least some RNA viruses recombine at a sufficiently high frequency to make repair an important function of recombination.

RNA viruses that are clearly the result of RNA recombination events have been observed outside the laboratory environment. For example, approximately 1 in 10^7 humans, after vaccination with the oral poliovirus vaccine, contract paralytic poliomyelitis[26]. Although many of the members of this small unfortunate group were immunocompromised before they received the vaccine, the evolution and outgrowth of neurovirulent polioviruses in all of these individuals is surely an example of a small founding viral population under strong negative selection. Of the viruses that have been characterized from these individuals, many have proven to be recombinant polioviruses[27]. As mentioned previously, western equine encephalitis virus is an example of a successful recombinant RNA for which a large number of mutations may be involved in fitness. Sequence analysis of western equine encephalitis virus revealed it to be a 'homologous' recombinant between Sindbis virus and a fairly distant relative, eastern equine encephalitis virus[7]. Thus, it is clear that RNA recombination can play a role in the evolution of RNA viruses.

Sequence comparisons of other RNA viruses also strongly suggest that recombination events in the past between distantly related viruses have contributed to the evolution of RNA viruses. In each case the argument for a recombinational origin rests on the idea that convergent evolution to such a high degree of homology would have been highly unlikely. Examples include mouse hepatitis virus, which contains sequences from influenza C[28], certain plant viruses and alphaviruses[29], and viroids[13].

Thus, whether or not RNA recombination has been selected over evolutionary time, the ability of RNA viruses to recombine has affected their evolution. To determine whether the ability to recombine is a selectable trait, it will be necessary to know whether it is even possible for RNA-dependent RNA polymerization to occur without template switching. This question can be addressed in two ways: by knowing whether there is natural variation among the RNA viruses for the ability to recombine, and by understanding the mechanism of template switching during RNA polymerization. Both lines of investigation are being actively pursued in this laboratory and others.

Template switching by other nucleic acid polymerases

Does template switching occur during the action of polymerases besides RNA-dependent RNA polymerases?

If so, does it have any biological importance, or is it simply a side reaction of polymerization?

Copy-choice recombination between DNA genomes has been invoked in only a few instances in recent years. In *Haemophilus influenzae*, a high percentage of transfected novobiocin-resistant plasmids rapidly acquire sequences conferring novobiocin sensitivity from the chromosomal DNA. The information transfer is dependent on recombination genes, and since there is no concomitant transfer of radioactivity from the prelabeled chromosomal DNA, the authors consider that both plasmid and chromosomal sequences are copied during plasmid replication[30]. Another case in which template switching by a DNA-dependent DNA polymerase has been suggested is in the DNA recombination event leading to heavy chain switching in immunoglobulin genes[31]. These authors observed that this recombination was extremely error-prone, sometimes leading to two distinct sequence populations resulting from a single recombination event in a clonal population. They infer that recombination occurred by a copy-choice mechanism on the assumption that this would be more error-prone than conventional homologous recombination. However, these results could also be reconciled with a breaking–rejoining model in which the recombination intermediate was resolved in an error-prone manner. Another report of copy-choice DNA recombination in the generation of deletions between transposable elements has been recently retracted[32]. However, *in vitro* reactions with bacteriophage T4 DNA polymerase that can be reconciled with template switching have been reported[33].

In the case of DNA-dependent RNA synthesis, the *in vivo* switching of template strands by *E. coli* RNA polymerase was invoked by Birge and Low in 1974 to explain a genetic phenomenon they termed 'conjuduction'[34]. The observation was that active β-galactosidase could be synthesized in merodiploids bearing two different noncomplementing *lacZ* alleles in a *recBC*⁻ background, but only when a functional *recA* allele was present. The *recA*-dependent event, however, did not result in the formation of stable *lacZ*⁺ colonies. Birge and Low postulated the *recA*-dependent formation of a 'transcribable intermediate', on which *E. coli* RNA polymerase could switch template strands at the Holliday junction, giving rise to a recombinant, functional mRNA[34]. The absence of genetic selections for most strand-switching events during DNA-dependent RNA synthesis would surely have limited the cases in which these events were detected.

The occurrence of homologous and nonhomologous recombination in retroviruses is well documented. Reverse transcriptases are DNA- or RNA-dependent DNA polymerases, and template-switching events have been invoked to explain three different aspects of retroviral replication and recombination. First, during every infectious cycle, the synthesis of both positive and negative strands of the DNA provirus requires a transfer of the replication complex from one RNA template to another. This template switch is undoubtedly facilitated by the degradation of the first RNA template by RNase H as the proviral DNA is synthesized, promoting base pairing with a second template[35,36]. Second, Hu and Temin[37] found that the generation of recombinant retroviral RNAs in a single replicative cycle was dependent on the presence of heterozygotic virions; a simple mixed infection of two parental viruses was not sufficient to generate recombinants. Thus, a virion core-associated event, probably reverse transcription, is responsible for generating recombinant retroviruses[37]. A third example of possible template-switching events is the transduction of cellular sequences by retroviruses. Although the mechanism of these nonhomologous recombination events remains the subject of some controversy, convincing evidence has been provided that at least some of these events occur by recombination, presumably replicative, between viral and cellular RNAs[38] (also discussed by H. Temin in March *TIG*).

Thus, increasing amounts of data and speculation from other fields suggest that both RNA- and DNA-dependent polymerases can switch either RNA or DNA templates at some frequency. Whether these copy-choice events are mediated by dispersive events or by processive mechanisms (Fig. 1), it is clear that such recombination events could have profound effects on the inheritance and the expression of the genomes involved.

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Spatial regulation of photosynthetic development in C4 plants

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Leaf development in C4 plants requires the morphological and functional differentiation of two photosynthetic cell types (bundle sheath and mesophyll). Photosynthetic reactions are split between bundle sheath and mesophyll cells, with each cell type accumulating a specific complement of photosynthetic enzymes. Current evidence suggests that in order to activate this cell-specific expression of photosynthetic genes, bundle sheath and mesophyll cells must interpret positional information distributed locally around each vein.

Leaf development in all vascular plants is initiated within the shoot apical meristem. Clonal analysis has shown that in most plants, subsequent patterns of cell division are often highly variable, indicating that cells remain pluripotent until late in development[1]. Despite this developmental plasticity, the final arrangement of cell types, particularly in ordered structures such as monocot leaves, is always the same. This review summarizes our current understanding of how photosynthetic differentiation is regulated during leaf development in C4 plants.

C4 carbon fixation

Although most plants that photosynthesize use the C3 photosynthetic pathway, the C4 system of carbon fixation has evolved a number of times and is represented in both monocot and dicot species[2]. Whereas in C3 plants only one photosynthetic cell type differentiates, photosynthetic reactions in C4 plants are split between two morphologically distinct cell types. In C3 plants, CO_2 is fixed into C3 acids through the ribulose bisphosphate carboxylase (RuBPCase)-mediated carboxylation of RuBP in the Calvin cycle. This carboxylation reaction is inhibited by O_2, which competes with CO_2 at the active site of RuBPCase, in the energy-wasting process of photorespiration. C4 plants minimize photorespiration by concentrating levels of CO_2 at the active site of RuBPCase. This is achieved through the metabolic co-operation of neighbouring bundle sheath and mesophyll cells. CO_2 is first fixed into C4 acids by an O_2-insensitive carboxylase (phosphoenolpyruvate carboxylase, PEPCase) in the mesophyll cells and then transferred to the bundle sheath. Subsequent decarboxylation in the bundle sheath releases CO_2 to be fixed in the Calvin cycle, as in C3 plants (for reviews on C4 physiology see Refs 3–5).

C4 function depends upon the differentiation of Kranz anatomy, in which photosynthetic bundle sheath and mesophyll cells form successive layers around the veins[6]. At maturity, bundle sheath and mesophyll cells exhibit dimorphic chloroplasts[7]. Mesophyll cell chloroplasts resemble those seen in C3 plants, that is, their photosynthetic vesicles are stacked into structures known as grana, whereas bundle sheath chloroplasts are agranal, and have reduced photosystem II activity. Three variations of the C4 scheme exist, which differ in the C4 compound shuttled between mesophyll and bundle sheath cells (malate or aspartate) and in the decarboxylating enzyme used in bundle sheath cells

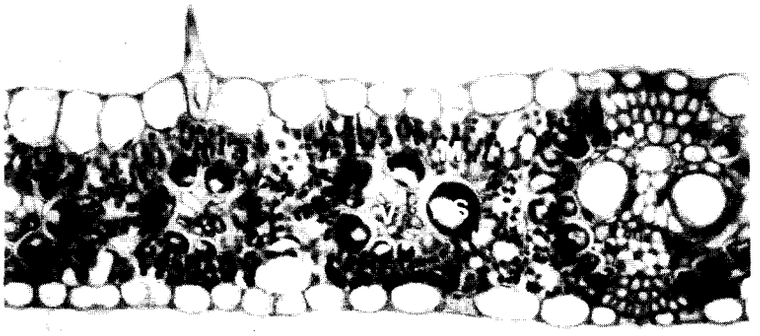

FIG 1
Transverse section of a maize leaf showing the arrangement of photosynthetic cell types. Leaf tissue was embedded in paraffin and stained with Fast Green M, mesophyll cell; BS, bundle sheath cell; V, vascular tissue; E, epidermal cell.