

Transposable elements in *Escherichia coli* antimicrobial resistance

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ABSTRACT

Transposable elements are capable of switching their positions on the genome thereby causing gene arrangements and contributing to genome evolution. The aim of this review is to specifically discuss the role of transposable elements in transferring antimicrobial resistance genes in *E. coli*, thus contributing to increase in virulence and conferring the possibility of multidrug resistance. Different types of transposable elements such as transposons and integrons and their profound influence on *E. coli* antimicrobial resistance are the focus of this review.

Keywords: Transposable Elements; *E. coli*; Antimicrobial Resistance; Integrons

1. INTRODUCTION

In 1983 Barbara McClintock earned her Nobel prize after discovering jumping genes or transposons in maize. At the time of the discovery of transposons, there was much speculation about their credibility because they conferred elasticity to the genome, much contradictory to the genetic stability proven by Gregor Mendel [1]. But now, almost seven decades later, owing to the astonishing advances in molecular techniques, the structure and function of the transposons and their role in genomic evolution have been proven beyond any doubt [2].

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2. TRANSPOSABLE ELEMENT(S)

{(TE(s)) are DNA sequences that are capable of changing their positions within the genome, creating new mutations and may change the cell's genome size and often result in the duplication of the individual TE [2]. In general, they are considered as junk DNA and they make up a large proportion of the C-value of the eukaryotic cell and are useful in research to modify the DNA inside the living organisms [3]. TEs possess two main characteristics that differentiate them from other genetic elements. As they are mobile, they are capable of changing their genetic environment and in doing so they alter the genetic environment of the locus into which they insert [4]. Since they have the intrinsic ability to multiply during the transposition process, they are automatically amplified, with a large copy number, restricted only by the carrying capacity of their environment (*i.e.* the genome) [1]. Transposons often persist in genomes over millions of years. This requires an exquisite balance between replication and suppression of their activity. In fact, transposons often have specific targeting mechanisms that exploit "safe havens" in the genome, such as noncoding or transcriptionally repressed regions, that help them survive for longer periods [5].

Several types of transposable elements are present. One of those is mobile genetic elements which includes two classes based on their mechanism of transposition, and can best be described as either "copy and paste" (for Class I TEs) or "cut and paste" (for Class II TEs) [6].

2.1. Class I (Retro-Transposons)

They function by copying themselves from DNA to RNA by transcription and *vice versa* by reverse transcription. The resulting DNA copy will subsequently be inserted into the genome at a different position. The reverse transcriptase enzyme will initiate the reverse transcription, which is often coded for by the TE itself. Retro-transposons behave very similarly to retroviruses, such as HIV [6].

2.2. Class II (DNA Transposons)

In this case, an RNA intermediate is not needed for the cut-and-paste transposition mechanism of Class II TEs which are catalyzed by various types of transposase enzymes [4]. Some of those transposases do not need any specific target site to bind. In contrast, others can only bind to specific sequence targets. The transposase produce a staggered cut at the target site, forming sticky ends. The transposase will then remove the DNA transposon and ligates it into the target site [6]. The DNA polymerase fills in the resulting gaps from the sticky ends and DNA ligase closes the sugar-phosphate backbone. The final outcome is target site duplication and the production of short direct repeats at the insertion sites of DNA transposons. As a consequence, gene duplications can result and is an important evolutionary process [7].

The cut and paste mechanism is not always needed for all DNA transposons. In certain instances, replicative transposition is noted upon the replication of the transposon to a new target site. There is always the possibility for TEs to lose their ability to synthesize either reverse transcriptase or transposase through mutation without losing their ability to jump through the genome [8]. In bacteria, transposons have an additional function often to confer antibiotic resistance through the presence of an additional gene on a plasmid. There is always the possibility for transposons to jump from chromosomal DNA to plasmid DNA and *vice versa*, producing antibiotic or in many instances multi drug resistance genes belonging to the Tn family in bacteria [9]. In case the transposable elements do not have the additional genes, they are known as insertion sequences. Some of the common insertion sequence elements in *E. coli* are listed in **Table 1**.

Transposable elements are one of the major forces that drive prokaryote genome evolution. Analyses of TE evolutionary dynamics revealed extensive variability in TE density between prokaryote genomes, even closely related [10]. To explain this variability, a model of recurrent invasion/proliferation/extinction cycles has been proposed by Cerveau *et al.* [10], wherein they examined different parameters that influence these cycles in two of the simplest TE classes: insertion sequences and group II introns. In particular, they have focused on TE trans-

Table 1. *E. coli* insertion sequences.

Element	# in <i>E. coli</i>	# in F factor
IS1	8	-
IS2	5	1
IS3	1	2
IS4	1	-

position efficiency (mechanisms and regulation), ability to transfer horizontally (through plasmids and phages), and impact on genome evolution (gene activation/inactivation and structural variation).

Currently, there is an intense interest in studying the evolution of TEs and their effects on the bacterial genome. There may be a universal common ancestor for all TEs or may have evolved in an independent manner from one another and spread to other kingdoms through horizontal gene transfer [11]. Some of them may benefit the host while others are considered like viruses as selfish DNA parasites. As a matter of fact, some TEs share similarities in their genome to viruses, indicating possible common ancestor as viruses. Bacteria have developed a specific mechanism of gene deletion to prevent excessive TE activity, which may lead to the destruction of its chromosome. TEs may have the ability to transpose to conjugative plasmids and some have integrons which are genetic elements able to catch and express genes from other sources. Integrons have the integrase enzyme that can integrate gene cassettes. Around 40 antibiotic resistance genes have already been identified on cassettes, and hence have virulence genes. The objective of this review is to delineate the involvement of transposable elements in *E. coli* antimicrobial resistance.

3. ANTIMICROBIAL RESISTANCE IN *E. COLI*

Antimicrobial drugs have played an important role in alleviating illness and death associated with infectious diseases in animals and humans. However, selective pressure exerted by antimicrobial drug use and also irrational use of antibiotics also have been the major factors behind the emergence and spread of drug-resistance traits among pathogenic and commensal bacteria [12]. In addition, resistance has developed after invention of every major class of antimicrobial drugs, varying in time from as short as 1 year (penicillin) to >10 years (vancomycin) [13].

The development of multi-drug resistance in *E. coli* is of major concern worldwide. Although prevalence rates for resistant *E. coli* strains are significantly distinct for various populations and environments and the impact of resistance to antimicrobial drugs is ubiquitous [14]. Erb *et al.*, [15] reviewed the emergence trends in *E. coli* an-

timicrobial resistance from 1970 to 2006 and observed that the prevalence of resistance to specific antibiotics was highly variable in different populations and in different countries, ranging from 0% to 100%. The prevalence of resistance to a wide range of antibiotics reported in studies from Middle and South America, Spain, and Turkey was higher than that reported in the USA and Central Europe. Moreover, a tendency towards higher prevalence rates of resistance in recent years was observed.

Widespread antibiotic resistance has been recognized in *E. coli* isolates from human, animal and environmental sources [16]. A very recent study by Adachi *et al.*, [17] showed the occurrence of fluoroquinolones and fluoroquinolone-resistance genes in *E. coli* in the aquatic environment in Japan. This is the first report of plasmid-mediated quinolone resistance determinant *qnrS1* in the aquatic environment. Another recent study by Ryu *et al.*, [18] on antimicrobial resistance and resistance genes in *E. coli* strains isolated from commercial fish and seafood in Korea showed that Class 1 and Class 2 integrons were associated with conferring resistance of these strains to a wide range of antibiotics. Another bothersome area is the infectious *E. coli* of animal origin that are multi drug resistant. Because of the intensive use of antimicrobial agents in food animal production, meat is frequently contaminated with antimicrobial-resistant *E. coli*. Humans can be colonized with *E. coli* of animal origin and because of resistance to commonly used antimicrobial agents, these bacteria may cause infections for which limited therapeutic options are available. This may lead to treatment failure and can have serious consequences for the patient. Furthermore, *E. coli* of animal origin may act as a donor of antimicrobial resistance genes for other pathogenic *E. coli* [16]. There are numerous reports that address most recent findings and indicate the severity and magnitude of antimicrobial resistance in *E. coli* from a wide spectrum of environments and enumerating them here would neither be possible nor pertinent. The aim of this review is to focus on the role of transposable elements and the underlying molecular mechanisms that aid in development of versatility in *E. coli* in acquiring and disseminating antimicrobial resistance.

4. THE ROLE OF MOBILE GENETIC ELEMENTS IN *E. COLI* ANTIMICROBIAL RESISTANCE

Bacterial evolution has been largely influenced by the high plasticity of bacterial genomes, thus leading to their adaptation to most ecosystems. This ability to exchange and rearrange genomic sequences to gain new traits has been extensively documented with the development of resistance to antibiotics [19]. Today, the increasing rate of antibiotic resistant bacteria is a major public health

issue [20]. During the last decade, several studies have highlighted the environmental resistance as a source of resistance genes of clinical interest [21-24].

A variety of genetic elements are strongly associated with the exchange and transfer of antimicrobial resistance genes among gram-negative bacteria. Transposons can transfer resistance genes intracellularly. On the other hand, integrons are genetic elements that can acquire several resistance genes in the form of gene cassettes. These genetic elements sometimes co-exist and work cooperatively. For example, integrons, which are not mobile by themselves, can acquire an ability to transfer when they are incorporated into transposons [25]. In this section, we are reviewing the individual contribution of integrons and transposons in acquiring antimicrobial resistance and genomic evolution of *E. coli*.

5. TRANSPOSONS IN *E. COLI*

E. coli is known to harbour a wide range of transposons such as Tn3, Tn5, Tn7, Tn9 and Tn10, each conferring resistance to antibiotics, namely, Tn3 to ampicillin, Tn5 to kanamycin, Tn7 to trimethoprim, streptothricin, spectinomycin, streptomycin, Tn9 to chloramphenicol and Tn10 to tetracycline respectively [26]. Some of the common *E. coli* transposons and their antibiotic resistance are listed in **Table 2**. The Tn5 transposon is known to harbour a gene cassette of three genes conferring resistance to three antibiotics: the *ble* gene for bleomycin resistance, the *neo* gene for neomycin and the *str* gene for streptomycin resistance [27].

Among the numerous and important classes of transposons are the conjugative transposons. As their name implies, conjugative transposons are mobile elements that possess the genetic machinery to facilitate their own transfer between bacterial cells. Referring back at some pioneering work in this field about two decades ago, several prototypes of conjugative transposons have been described [35]. An earlier misconception was that these transposons were largely found only on Gram-positive bacteria but later on it was observed that the transposon groups Tn916 and Tn1545 transfer readily to a wide

Table 2. Common *E. coli* transposons.

Transposable Element	Antibiotic Resistance	References
Tn3	Ampicillin	[28]
Tn5	Kanamycin	[29]
Tn7	Trimethoprim, Streptothricin, Spectinomycin, Streptomycin	[30]
Tn9	Chloramphenicol	[31]
Tn10	Tetracycline	[32]
Tn903	Kanamycin	[33]
Tn1681	Heat Stable (ST) Toxin	[34]

variety of gram-positive and gram-negative bacteria in the laboratory. In one report, *Enterococcus faecalis* readily transferred plasmid-borne Tn916 to *Alcaligenes eutrophus*, *Citrobacter freundii*, and *E. coli*, conferring selectable levels of tetracycline resistance [1]. It has been assumed that acquiring antimicrobial resistance mediated by transposons bestows upon the bacteria a fitness impact enabling it to survive better under selection pressure. *E. coli* is known to have acquired resistance to chloramphenicol, tetracycline and kanamycin from genes carried on transposons [36]. Therefore, as is evidenced by the above reports, starting from earlier antibiotics such as tetracycline, kanamycin and chloramphenicol to the new generation trimethoprim and sulfonamides, a wide array of transposons are involved in imparting resistance [37]. The broad-spectrum antibacterial trimethoprim is heavily used worldwide, most commonly in combination with sulphonamides. Trimethoprim inhibits the enzyme dihydrofolate reductase (Dfr). *E. coli* most commonly becomes resistance acquiring one of over 30 known *dfr* genes encoding resistant variants, most of which belong to the *dfrA* category [38]. Recently, Labar *et al.*, [39] found the regional dissemination of a trimethoprim-resistance gene cassette via a successful transposable element. They observed a 50% increase in the prevalence of trimethoprim resistance among fecal *E. coli* from healthy Nigerian students between 1998 and 2005, a trend to increase that continued in 2009. In this study 16.3% isolates carried transposons conferring trimethoprim resistance, indicating transposition as a principal mechanism for disseminating *dfrA7* among *E. coli* from Nigeria and Ghana.

With the recent innovations in technology for analyzing the molecular genetics of bacterial pathogens, understanding of the role of transposons in the dissemination of antimicrobial resistance has expanded significantly. Although a lot of attention has been focused on transposon mediated transfer of antibiotic resistance, the wide spread involvement of integrons in conferring multi drug resistance is being strongly asserted in recent studies. Integrons are genetic elements that can collect several resistance genes in the form of gene cassettes. These genetic elements sometimes co-exist and work cooperatively. For example, integrons, which are not mobile by themselves, can obtain an ability to transfer when they are incorporated into transposons. It thus becomes very important to focus on this class of transposable elements, which seem to have relied initially on transposons to mobilise them, but have rapidly evolved in their ability to rapidly disseminate wider range of resistance to various antimicrobials in *E. coli*. Hence the next section is aimed at documenting some of the recent evidences for integron mediated antimicrobial resistance in *E. coli* from different environments.

6. ROLE OF INTEGRONS IN ANTIMICROBIAL RESISTANCE

Integrons contain an *intI* gene encoding a site-specific recombinase belonging to the integrase family and a recombination site *attI*. A gene cassette includes an open reading frame and, at the 3'-end, a recombination site *attC*. Integration or excision of cassettes occurs by a site-specific recombination mechanism catalyzed by the integrase [40]. At least six classes of integrons have been determined according to their *intI* gene. Classes 1, 2 and 3 are the most studied and are largely associated with the dissemination of antibiotic resistance. Most gene cassettes encode antibiotic resistant determinants [41]. Integrons seem to play a major role in the spread of multidrug resistance in Gram-negative bacteria but recently they have been described in Gram-positive bacteria [20]. Moreover, the finding of super-integrons with gene cassettes coding for other determinants (biochemical functions, virulence factors) in different Gram negative bacteria suggests that integrons are probably involved in bacterial genome evolution [40]. More than 100 gene cassettes may be present, in contrast to resistance integrons where less than ten cassettes are present. Many species harbour super-integrons, which are species-specific, whereas particular resistance integrons can be found in a variety of species. In the last few years, a variety of new gene cassettes have been described. Many of these encode resistance against newer generation antibiotics such as cephalosporins and carbapenems [39].

7. TYPES OF INTEGRONS

With relation to acquisition of antimicrobial resistance, two major groups of integrons have been described: "chromosomal integrons" (CIs), and "mobile integrons" (MIs). They are located on the chromosome of hundreds of bacterial species; in fact, *in silico* analysis showed that 17% of sequenced bacterial genomes exhibit such genetic arrangements [38]. MIs are not self-transposable elements but are found on mobile genetic elements such as plasmids and transposons, which aid in their dissemination among bacteria. MIs contain a limited number of GCs (less than 10 GCs) [42]. The GCs described to date in these MIs usually encode antibiotic-resistance determinants. MIs are therefore sometimes also called "resistant integrons" (RIs) or "multidrug resistance integrons" (MRIs).

7.1. Integrons and Antibiotic Resistance in *E. coli* from Various Environments

Antibiotic pressure has probably played a key role in the MIs selection and dissemination in bacteria. More than 130 GCs conferring resistance to antibiotics and more than 60 GCs of unknown functions have been described

in MIs [43]. Genes involved in resistance to almost all antibiotic families are embedded in GCs, including beta-lactams, aminoglycosides, trimethoprim, macrolides, chloramphenicol, fosfomycin, lincosamides, rifampicin and quinolones. Studies have suggested that MIs were more prevalent in bacterial communities subjected to direct or indirect antibiotic pressure in clinical, agricultural, and environmental settings [44-48]. Recent *in vitro* studies have shown that antibiotics are able to induce integrase transcription, both in CIs and MIs, *via* the SOS response. The SOS response is a global regulatory network controlled by the transcriptional repressor LexA and induced by stress leading to direct or indirect DNA damage, such as damage resulting from exposure to some widely used antibiotics such as fluoroquinolones, beta-lactams, trimethoprim, aminoglycosides [49-51]. The activation of the SOS response in bacteria results in integrase over expression, which leads to the raise of GCs recombination events.

Clinical, veterinary and environmental surveys have shown that bacteria harboring MIs are often associated with multidrug-resistant (MDR) phenotypes [52]. Recently, many studies have been conducted and have documented the involvement of integrons in acquisition of antibiotic resistance in *E. coli*, out of which some of the most recent findings are being discussed in this section. Integrons have been implicated in aiding antibiotic resistance in *E. coli* in a wide range of environments such as polluted water, poultry, food, hospitals, etc.

7.2. Integrons in *E. coli* from Polluted Waters

Chen *et al.*, [53] carried out a study on Class I integrons in antibiotic resistance in *E. coli* strains isolated from the Minjiang River drainage basin in Fujian Province, which is one of China's most intensive livestock and poultry production areas. According to this study, a total of 666 (24%) *E. coli* isolates carried at least one of the virulence genes *elt*, *fedA*, *astA*, *fasA*, *estA*, *stx(2e)*, *paa*, and *sepA*. Forty-one percent of the isolates harbored class 1 integrons, and these isolates had a significantly higher probability of resistance to tobramycin, cefoperazone, cefazolin, ciprofloxacin, norfloxacin, azitromycin, and rifampin than isolates with no Class 1 integron detected. Frequencies of resistance to selected antibiotics were as high as or higher than those in fecal, wastewater, and clinical isolates in published surveys undertaken in China, North America, and Europe. Another recent study [54] conducted on *E. coli* isolates from the Dongjiang River in South China showed that 87.5% of the isolates were resistant to at least three antibiotics. The presence of integrons (Classes 1 and 2) was frequently observed (82.3%) while no class 3 integron was found. Twenty-one resistance genes of 14 gene cassette arrays and 10

different families of resistance genes were identified in these integrons. The above two recent studies have clearly documented the role of integrons in acquiring antimicrobial resistance.

Koczura *et al.*, [55] studied the antimicrobial resistance of integron-harboring *E. coli* isolates from clinical samples, wastewater treatment plant and river water. They used multiplex PCR assay to determine the frequency of integron presence in *E. coli* isolates cultured from wastewater treatment plant (WWTP) (integrons were detected in 11% of *E. coli* isolates), river water upstream (6%) and downstream (14%) the discharge of WWTP, and clinical specimens (56%). Antimicrobial resistance of the integron-positive isolates, determined by disk diffusion method, varied between *E. coli* of different origin. Isolates from the downstream river, compared to those cultured from upstream river, were more frequently resistant to kanamycin, cephalotin, cotrimoxazole, trimethoprim, and fluoroquinolones. Moreover, they displayed broader resistance ranges, expressed as the number of classes of antimicrobials to which they were resistant.

7.3. Integrons in *E. coli* from Animal Sources and Poultry

Soufi *et al.*, [56] studied the prevalence and diversity of integrons and associated resistance genes in *E. coli* isolates from poultry meat in Tunisia and observed a high rate of resistance to a wide range of antibiotics and were found to harbour various integrons. Sixty percent of isolates harbored integrons (Class 1, 30 isolates; Class 2, 5 isolates). Class 2 integrons contained in all cases the *dfrA1-sat1-aadA1-orfX* gene cassette arrangement. Nine gene cassette arrangements have been detected among Class 1 integrons, containing different alleles of *dfrA* (five alleles) and *aadA* (2 alleles) genes, which encode trimethoprim and streptomycin resistance, respectively. An uncommon gene cassette array (*sat-psp-aadA2-cmlA1-aadA1-qacH-IS440-sul3*) was identified in three class 1 integron-positive isolates. The study concluded that *E. coli* isolates of poultry origin could be a reservoir of antimicrobial-resistance genes and of integrons. Kadlec and Schwarz [57] conducted a study in a veterinary farm in Germany on identification of integron-associated resistance genes among *E. coli* isolates from horses, pigs and dogs. They reported the involvement of both Classes I and 2 integrons associated with resistance to a wide range of antibiotics. Another study [58] reported the association of tetracycline resistance with Class I integrons in *E. coli* isolates from beef cattle. All these findings show us the alarming incidence of multiresistant, integron harbouring *E. coli* in the veterinary environment.

7.4. Integrons in *E. coli* from Human Sources

Recently, Najibi *et al.*, [59] studied the distribution of Class 1 integrons among enteropathogenic *E. coli*. They investigated the incidence of and resistance gene content of Class 1 integrons among enteropathogenic *E. coli* (EPEC) and non-EPEC and to investigate intraspecies genetic diversity of EPEC strains isolated from children with diarrhea in Iran. They reported that 82% EPEC isolates and 11 68.7% non-EPEC isolates harbored the *int1* gene specific to the conserved integrase region of Class 1 integrons. A recent study [60], on EPEC in children with diarrhea in Iran, showed the presence of 61% of atypical *E. coli* isolates resistant to six different antibiotics, all harbouring Class 2 integrons. Highly resistant *E. coli* strains are not only found in humans with disease but have, also, been reported in the faeces of healthy humans. When Vinue *et al.*, [61] studied the prevalence and diversity of integrons and associated resistance genes in faecal *E. coli* isolates of healthy humans in Spain, they found integrases associated with Class 1 and/or Class 2 integrons belonging to seven different gene cassettes, conferring resistance to a wide spectrum of antibiotics in 29% of the *E. coli* isolates. This leads to a worrisome speculation that individuals in the community could be a reservoir of integron-containing *E. coli* isolates and improper sanitation could easily cause the rapid dissemination of these resistant strains.

All the above mentioned reports draw our attention not only to the presence of antibiotic resistance conferring integrons in *E. coli* from a wide range of environments, but also highlight the vast molecular diversity of the integron gene cassettes present in these strains. The molecular diversity is a key factor that gives these strains a selective advantage for rapid dissemination in the environment.

8. INSERTION SEQUENCE COMMON REGION ELEMENT (ISCR)

Having discussed in detail the role of transposable elements in antimicrobial resistance, one is bound to highlight the recent concept of insertion sequence common region element (ISCR) and their role as a gene capture system in organisms. ISCR elements differ from the insertion sequences by lacking terminal inverted repeats (IRs), do not generate directly repeated sequence on insertion and are thought to be transposed by a mechanism defined as rolling-circle (RC) transposition [62]. ISCR elements are closely related to an unusual family of insertion sequences called the IS91 family. ISCR elements, as a novel gene-capturing system, are capable of mobilizing any piece of adjacent DNA sequences [63]. This powerful gene mobilization mechanism serves as a highly mobile vector or milieu in transferring antibiotic resistance genes between different species of bacteria

[64]. Nineteen members of the ISCR family have been discovered until now in many Gram-negative pathogens. The majority of these elements are found to be closely associated with antimicrobial resistance genes that are not necessary components of the host genome, suggesting that ISCR elements may be responsible for the rapid transmission of bacterial multi-drug resistance in the environment [65]. ISCR elements are now recognized as powerful antibiotic resistance gene capture and movement systems that also possess the ability to construct extended clusters of antibiotic resistance genes on plasmids as well as on chromosomes [66]. As a result, there is a lot of focus on these elements as major contributors to bacterial antimicrobial resistance and research in this area in the future would definitely provide deeper insights into this versatile phenomenon of genome evolution.

9. CONCLUDING REMARKS

E. coli, usually a commensal bacterium of humans and animals is a highly versatile and ubiquitous bacterium. Pathogenic variants cause a wide range of infections such as intestinal and extraintestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis and septicemia [67]. Surveillance data show that resistance in *E. coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine [68]. The past two decades have witnessed major increases in emergence and spread of multidrug-resistant bacteria and increasing resistance to newer compounds such as fluoroquinolones and certain cephalosporins [14]. Intensive molecular analyses have shown multiple mechanisms in which *E. coli* attain the antimicrobial resistance, the chief contributions to this genomic fitness being horizontal gene transfer in terms of conjugative plasmids, transducing phages and transposable elements carrying resistance genes. Of the various contributing genetic elements, this review aims at assessing the recent information available on the role of transposable elements in dissemination of multidrug resistance traits to *E. coli*. The transposons and integrons, owing to their genomic plasticity have contributed a great deal to the fitness quotient and robustness of *E. coli* to survive in varying environments. The information presented here is an indication of the magnitude of genomic variation that has resulted due to the transposable elements and also fortifies the role of these elements in genomic evolution, thoroughly justifying the predictions of Barbara McClintok that transposons will play a major role in genomic diversity and evolution.

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