Circle-Seq Analysis Reveals the Involvement of eccDNAs in Salt Stress Response of Bermudagrass (Cynodon dactylon)

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Abstract

Extrachromosomal circular DNAs (eccDNAs) have been identified in a wide variety of plant species and play a pivotal role in genomic plasticity, emerging as key drivers of stress adaptation. However, the functional roles of eccDNAs under environmental stress remain largely unexplored in plants. As a high-quality turfgrass, bermudagrass (*Cynodon dactylon* L.) is a pivotal species for the reclamation and improvement of saline-alkali soils. Therefore, we performed a comprehensive analysis of the eccDNAs profile in bermudagrass under salt stress. A total of 1,068 eccDNAs were identified across all chromosomes. These eccDNAs were characterized by short lengths (ranging from 100 bp to 1 kb) and low GC content. eccDNA was not entirely random but rather exhibited a certain preference in intergenic regions and coding sequences (CDS). The identification of A/T-rich motifs at the junction sites suggests that these eccDNAs likely originate from physically unstable scaffold/matrix attachment regions (S/MARs) and are generated via the microhomology-mediated end joining (MMEJ) pathway. Notably, salt stress specifically enriched eccDNAs derived from DNA transposons, including the CACTA, Harbinger, Helitron, and MITE families. Overall, our findings reveal complex extrachromosomal regulatory mechanisms in bermudagrass, offering novel insights into its genomic adaptation under environmental stress.

1 Background

Extrachromosomal circular DNA (eccDNA) is a class of nonchromosomal DNA molecules. Unlike linear chromosomal DNA, it is circular and autonomous, capable of replicating independently within cells. This autonomy endows eccDNA with unique functional capabilities, including the ability to evade certain regulatory constraints imposed by chromosomal DNA[1, 2]. The size of eccDNA varies significantly, ranging from minuscule DNA fragments of a few hundred base pairs to large circular DNA molecules spanning millions of base pairs. The formation of large eccDNA can regulate oncogene expression[3, 4], while smaller eccDNA fragments covering only gene exons can generate mature regulatory short RNAs that modulate the expression of their chromosomal counterparts[3]. eccDNA was first discovered in mammalian cells and higher plants, where it was identified as a large extrachromosomal circular molecule termed Double Minutes (DMs)[4]. Subsequent studies identified drug-resistant mouse cells harboring DMs of the *DHFR* gene, revealing the mechanism of action of eccDNA, this gene mediates cellular resistance to the drug[5]. Despite originating outside the canonical chromosomal framework, eccDNAs function as indispensable functional elements that govern gene expression programs, exacerbate genomic instability, and underpin cellular fitness and evolutionary plasticity[1]. Studies have demonstrated that eccDNAs facilitate the autonomous copy number gain of genes and distal enhancers, providing a potent mechanism for rapid gene expression rewiring and functional cellular remodeling[2, 6]. Furthermore, only a minimal fraction of eccDNAs share identical ligation sites, indicating high eccDNA heterogeneity. Given the distinct motif patterns flanking these ligation sites, their formation mechanisms likely differ[7].

Previous research on eccDNAs has predominantly focused on human biology[8], whereas explorations in plants remain relatively limited. Despite their widespread prevalence in the plant kingdom, the identification and quantification of eccDNAs pose significant technical challenges. However, with the advancement of high-throughput sequencing technology in recent years, research on eccDNA in plants has become feasible[9]. Research on eccDNAs has been documented in various plant species, including Arabidopsis (*Arabidopsis thaliana*)[10], rice (*Oryza sativa*)[11, 12], Blackgrass (*Alopecurus myosuroides*)[13] and sugarcane (*Saccharum officinarum*)[14]. In wild-type rice endo sperm, the highly active retrotransposon PopRice was identified as the origin of eccDNAs forms[15], indicating the prevalence of eccDNAs during various developmental stages of plants. Besides, eccDNAs can regulate glyphosate resistance by carrying the herbicide target enzyme gene 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)[16] in *Amaranthus palmeri*. The eccDNAs associated with tRNA abundance which controls protein synthesis under conditions of stress in Arabidopsis[10]. Additionally, in rice, eccDNAs tends to accumulate in regions associated with stress-related genes[12]. These studies highlight the significant role of eccDNAs in plants. Although numerous investigations have explored the functional roles of eccDNAs in plants, its role in plant adaptation to environmental stresses remains poorly documented, and its underlying mechanisms remain unclear.

Bermudagrass (*Cynodon dactylon* L.) is a perennial warm-season turfgrass species that serves as a key plant for the reclamation of saline-alkali soils and the acceleration of soil desalination processes[17]. While extensive research has been conducted on bermudagrass, the identification and characterization of its eccDNAs have not yet been reported. Recently, the availability of high-quality reference genomes for bermudagrass has significantly advanced our understanding of its genetic architecture[18], thereby making it feasible to identify and characterize the potential roles of eccDNAs in this species. We investigated the changes in eccDNA in bermudagrass under CK and salt stress and conducted a preliminary analysis of the mechanisms underlying eccDNA formation in bermudagrass genome. This study provides new insights into the role of eccDNA in responding to plant stress.

2 Materials and Methods

2.1 Plant materials and stress treatments

Bermudagrass cultivar '*Yangjiang*' was cultivated in the plastic pots (15 cm diameter × 20 cm height) filled with nutrient soil in the greenhouse of Yangzhou University under standard turf management conditions for about 1 month. The Hoagland solution with 200 mM NaCl were used for salt stress treatment, and Hogland solution without NaCl were regarded as CK. Each treatment consisted of three independent biological replicates. Following the respective treatments, leaf tissues were rapidly harvested, immediately frozen in liquid nitrogen, and stored at -80°C. Total genomic DNA (gDNA) was isolated using the standard CTAB method.

2.2 Linear DNA digestion and eccDNA enrichment

NEB Exonuclease V Enzyme (NEB, M0345L) was added to the genomic DNA (gDNA) to remove linear DNA. Following a continuous two-day digestion period, the remaining DNA samples were purified using VAHTS DNA Clean Beads to remove enzyme residues and degraded linear DNA fragments. The enrichment circular DNA was subsequently subjected to linear amplification via rolling circle amplification (RCA) utilizing Phi29 MAX DNA Polymerase, and the RCA products were purified using a Cycle Pure kit, resuspended in sterile nuclease-free water, and prepared for library construction and high-throughput sequencing.

2.3 Circle-Sequencing (Circle-Seq) Analysis

Circle-seq library preparation and sequencing were performed by Tubegene (China) on the Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads. Quality control of the raw data was performed using fastqc (v0.12.1). Low-quality reads were subsequently removed to obtain high-quality clean reads. High-quality clean reads were mapped to the bermudagrass reference genome using bwa (v0.7.15), and the reference genome assembly was obtained from Zhang et al[18].The identification of eccDNAs across all samples was performed using Circle-map (v1.1.4), and samtools (v0.2) software was used to get raw soft-clipped read counts of the break point. Then DESeq2 (v1.50.2) software was used to perform normalization and differentially expressed eccDNA (DE-eccDNAs) filter by *p* value and fold change. Finally, the identified eccDNAs were annotated using bedtools (v2.27.1). The MEME tool (http://meme.nbcr.net/meme/) was used to identify motifs within the 20bp flanking sequences (20 bp upstream and downstream) of the eccDNA breakpoints.

2.4 Transposition analysis

The TE annotation file was selected from all bermudagrass annotation files. All identified eccDNAs genomic coordinates were intersected with the TE annotations using the GenomicRanges and rtracklayer packages in R (v4.3.0).

2.5 Detection of eccDNAs in bermudagrass protoplasts via fluorescence in situ hybridization (FISH)

bermudagrass protoplasts were isolated by incubating fresh leaf tissues in a pretreatment buffer, followed by enzymatic digestion. Following an incubation period of 3–4 h at 28°C in the dark, the digestion was terminated by the addition of 20 mL of W5 buffer. The cell suspension was then passed through a 70-μm strainer, and the collected protoplasts were fixed for 12 h before slide preparation. Subsequently, the oligonucleotide probe was designed using Primer (v6.0) and its specificity was verified against the bermudagrass genome via Blast to ensure unique binding. Additionally, the OligoAnalyzer tool (https://www.idtdna.com/calc/analyzer) was used to evaluate and minimize potential secondary structures, such as hairpins and dimers. Finally, the slides were examined under a fluorescence microscope to capture the target signals

2.6 Statistical Analysis

All statistical computations and graphic visualizations were conducted within the R statistical environment (v4.3.0). Multiple comparisons between different conditions were performed using Duncan's multiple range test at the 0.05 significance level. Data were presented as mean ± standard deviation.

3 Result

3.1 Quantitative analysis of the number of eccDNA in bermudagrass

The Circle-seq method originally developed by Møller[19] was used to investigate the quantity of the eccDNA in bermudagrass (Supplementary Fig S1 and S2). The results showed that, in total, 827 eccDNA were identified after salt stress treatment in bermudagrass, which were significantly higher than CK of 241 (Fig. 1A and Supplementary Table S1), indicates that salt stress induces the generation of eccDNA. Although significant differences exist between CK and Salt treatments, the samples within each group also showed remarkable variation, and there was scarcely any overlap between them (Fig. 1B). This observation implies that the eccDNA exhibits a high degree of specificity, even among those from the same type of treatment.

To systematically identify the salt stress response related differentially express eccDNAs (DE-eccDNAs), a differential abundance analysis was performed between the CK and Salt groups. Based on the criteria of up-regulated ($|\log_2$ (Fold change)$| \geq 1$ and $p \leq 0.05$) and down-regulated ($|\log_2$ (Fold change)$| \leq 1$ and $p \leq 0.05$), a total of 342 up-regulated and 123 down-regulated DE-eccDNAs were identified in bermudagrass after salt stress treatment. Volcano plots reveal notable asymmetry across distinct lineages, with up-regulated eccDNAs predominating over down-regulated eccDNAs (Fig. 1C). Additionally, the heatmap displays a clear segregation between CK and Salt groups, eccDNAs that were minimally present in the CK but showed robust accumulation in Salt group (Fig. 1D). Specifically, a dramatic reprogramming of eccDNA profiles upon stress exposure, suggesting an active biogenesis of eccDNA in bermudagrass under salt stress.

3.2 Analysis of eccDNA characteristics in bermudagrass

To analyze the function of eccDNA in bermudagrass further, the eccDNAs were aligned to the tetraploid bermudagrass genome to complete annotation of the encoded genes. The annotation results showed that eccDNA were related to 221 and 776 coding genes in CK and Salt groups, respectively (Fig. 2A). Furthermore, it was found that eccDNAs can originate from a single gene or from multiple genes. Specifically, 890 eccDNAs were derived from a single gene, 56 eccDNAs were derived from two genes, 14 eccDNAs were derived from three genes, and only one eccDNA was derived from five or more genes. (Fig. 2B).

In addition, the distribution of eccDNA encompassing entire gene sequences (Full-eccDNA) was characterized across each sample (Fig. 2C and Supplementary Table S2). The results indicated that, despite their relatively low abundance within the total annotated eccDNAs, Full-eccDNAs exhibited a pronounced sensitivity to salt stress. While the count of Full-eccDNAs remained low in CK at only 4, this number increased significantly to 10 following salt treatment (Fig. 2C). Given that these Full-eccDNAs encompass complete gene sequences, they represent promising candidates for further investigation of their potential roles in the plant salt-stress response.

Besides, the results also showed that the size distribution of eccDNAs spanned a wide range, from hundreds of base pairs to megabases. However, most of the eccDNAs identified in bermudagrass were shorter, with lengths predominantly ranging from 100 bp to 1 kb (Fig. 2D). Comparative analysis revealed no significant difference in the length distribution patterns between CK and Salt. Both groups exhibited similar median lengths and distribution profiles.

3.3 Distribution of eccDNA across the chromosomal landscape in bermudagrass

To systematically evaluate the impact of salt stress on eccDNA, the distribution patterns of eccDNA across different chromosomes were investigated (Fig. 3A). The results suggested that eccDNAs are distributed both uniformly and randomly along the chromosomes, and salt stress does not alter this distribution pattern.

Further quantitative assessment of eccDNA formation frequency showed that salt stress significantly induced the accumulation of eccDNAs (Fig. 3B). Compared to CK groups, the eccDNA frequency among Salt groups exhibited a significant elevation in bermudagrass. In addition, the Salt group exhibited higher inter-sample variability, which reflected the highly heterogeneous nature of individual sample under salt stress. However, due to variations in chromosome length, a detailed analysis of the formation rates for each individual chromosome was conducted. (Fig. 3C). The findings revealed distinct differences in sensitivity to salt stress among different chromosomes. Although all chromosomes exhibited higher frequencies after salt stress compared to CK, specific chromosomes (5A1, 5A2, 7A2) demonstrated significantly higher ratio of eccDNA formation.

3.4 Mechanistic analysis of eccDNA formation in bermudagrass

To investigate the formation mechanisms and genomic origin characteristics of eccDNAs, 1,068 eccDNAs were classified into distinct genomic features, including genes (5’UTR, CDS, 3’UTR), 2 kb upstream of the genes (up2kb), 2 kb downstream of the genes (down2kb), and intergenic regions (Fig. 4A). 19.5% of eccDNAs overlapped gene regions, 10% of ID-eccDNAs mapped to up2kb regions, 8.9% to down2kb, and 61.6% to intergenic regions. A more detailed analysis of eccDNAs classified as genes showed that 69.2% overlapped to CDS region, 1.5% to 3’UTRs, and 4.3% to 5’UTRs (Fig. 4C).

Furthermore, a genomic element annotation analysis was performed on eccDNA. The results demonstrated that distal intergenic regions constitute the primary component of eccDNAs, followed by promoter regions (Fig. 4B). Notably, further categorization of promoter-derived eccDNAs revealed that the vast majority were significantly enriched within the core promoter region located within 1 kb upstream of the transcription start site (TSS) (Promoter ≤ 1 kb). This proportion was remarkable higher than those observed in distal promoter regions (Promoter 1–2 kb and 2–3 kb).

Besides, the 40-bp sequences flanking the junction breakpoints were extracted and analyzed for GC content. GC content analysis revealed that the eccDNA sequences themselves were characterized by a significantly low GC content. As illustrated in Fig. 4D, the average GC content within the eccDNA regions in both the CK and Salt groups was lower than that of the sequences located immediately upstream and downstream of the breakpoints. Moreover, motif enrichment was performed using the MEME tool to compare the start and end positions of each DNA, and the results showed that sequences of 29 bp and 21 bp were identified at the junction of the eccDNA and significant enrichment of adenine deoxynucleotides (dAMP, A) and thymine deoxynucleotides (dTMP, T) signals, but lacking motifs with high GC content (Fig. 4E). This implied that the formation of eccDNA does not occur randomly at any genomic location, but rather specifically tends to excise and circularize from regions with locally elevated AT content.

3.5 Salt stress leads to change in TE-overlapped eccDNAs in bermudagrass

TEs constitute a significant component of the genome and exhibit high activity under environmental stresses and prone to recombination. Therefore, a comprehensive analysis of all TE-overlapped eccDNAs was performed (eccTEs). The results showed that 633 eccTEs were identified across all samples (Fig. 5A), which accounting for 59.3% of the total eccDNA count (Fig. 5B). This revealed that eccTEs were dominated, and transposon regions should serve as hotspots for eccDNA formation. Interestingly, despite the high proportion of eccTEs, only 11 eccTEs were shared between CK and Salt treatments (Fig. 5C). This suggests that salt stress may specifically activate the circularization of eccTEs sequences at distinct sites.

Further analysis showed that the proportion of the eccTEs related to DNA transposons increased from 0.67% to 2.92% in bermudagrass after salt stress treatment compared to that of CK group (Fig. 5D). The genomic analysis revealed that the hAT family constituted the predominant DNA transposon family in bermudagrass, however, only the hAT family transposon related eccTEs was detected in the CK group, exhibiting a singular distribution pattern (Fig. 5E), and after salt stress treatment, beyond the eccTEs related to hAT family, the CACTA, MITE, Helitron, and Harbinger were also identified in bermudagrass (Fig. 5E). Besides, the accumulation of retrotransposons related eccTEs remained relatively stable which decreased marginally from 35.35% in CK group to 31.5% in Salt group, and the Gypsy family was the most prevalent (Fig. 5F, G). These findings indicated that salt stress could induce increase certain transposon families.

3.6 FISH detection of eccDNAs in bermudagrass

To validate the trend of salt-induced increase in eccDNAs abundance, the FISH assay was performed using bermudagrass protoplasts. *ecc690* (*Cd6A2G000690*) was selected from the identified eccDNA pool to serve as a template for probe design (Supplementary Table S3 and S4). This probe was subsequently employed to observe the spatial distribution and quantitative dynamics of the target eccDNA within the nuclei. As illustrated in Fig. 6, the spatial distribution of the specific eccDNA within the interphase nuclei of bermudagrass protoplasts was clearly visualized through the FISH assay. Under CK, only sporadic and faint fluorescence signals were observable within the cell nuclei (Fig. 6A-C). However, following salt stress, both the number and intensity of punctate foci within each nucleus increased significantly (Fig. 6D-F). These findings are highly consistent with our bioinformatic predictions.

4 Discussion

Advances in sequencing techniques have made it possible to study eccDNA in plants which had been demonstrated to play an important role in various physiological processes. As a repository of genetic diversity, it facilitates rapid responses and evolutionary adaptation in organisms when exposed to biotic or abiotic stresses[20, 21]. Currently, there are several tools that are available for identifying eccDNAs, such as Circle-Map[22], Circle_finder[23] and ECCsplorer[24]. Given that eccDNA in plants is typically short[11, 25], and circle-map has advantages to identify eccDNA from short-read data[26]. So, this software was selected in the present study. Zhuang et al[12] discovered that drought stress could induce increase of eccDNA accumulation in rice, which suggested the potential for eccDNA to serve as a practical biomarker for assessing DNA damage. In this study, a significant elevation in the number of eccDNAs in bermudagrass after salt stress treatment was observed, which indicated that environmental stress might accelerate the processes of genomic fragmentation and circularization. Different to the change of quantity, the length distribution trends of eccDNAs in Salt and CK groups were similar, and most of them were short in length (Fig. 2D). However, the eccDNAs in bermudagrass exhibited a high degree of heterogeneity, scarcely any eccDNAs were shared among different samples, even between biological replicates within the same treatment. This observation was consistent with previous study in Arabidopsis[10]. Furthermore, previous study in cancer cells have reported that the eccDNAs could encompass full-length genes, which might potentiate cellular responses to environmental stimuli[27]. Herein, 10 Full-eccDNA fragments were identified in the Salt group, a number that was significantly higher than that in the CK group (Fig. 2C), which indicated that salt stress might triggered larger-scale recombination or excision events within the bermudagrass genome, and led to the generation of more circular DNA molecules carrying intact coding frames from the genome.

4.1 Genomic origins reveal the functional preference of eccDNAs

The origins of eccDNAs were diverse and vary significantly across different species. Previous studies had demonstrated that eccDNAs are primarily distributed within introns, 5’ UTR, exon, CpG island, intergenic region, and TEs [28, 29]. A recent study on Arabidopsis revealed that eccDNAs primarily originate from centromeric and pericentromeric regions[30]. In our study, it was found that eccDNAs in bermudagrass were primarily derived from genes and intergenic regions. (Fig. 4A). Interestingly, further analysis of the eccDNAs that originated from genes revealed a significant enrichment within CDS, whereas their occurrence in 5’ UTRs and 3’ UTRs was relatively low (Fig. 4C), this was consistent with the findings in rice[12]. Therefore, these results indicated that the generation of eccDNAs was not a completely random genomic fragmentation process, but rather exhibits a distinct functional preference. Besides, previous studies have found that herbicide resistance genes in eccDNA could improve herbicide tolerance of plants via regulating the expression of specific genes[16, 31, 32]. These findings suggested that eccDNAs might function as a factor which could enable rapid amplification and expression of stress-related genes by carrying intact coding sequences in salt stress response of plant.

4.2 eccDNA formation is mediated by MMEJ and associated with S/MARs

Nowadays, studies suggested the formation of eccDNA should be a complex process with several hypothesized pathways[33, 34]. It might originate from DNA replication processes, such as the excision of chromosomal loops or mispairs of DNA fragments during replication pauses[35]. Besides, it was also been proposed that eccDNAs are formed through the ligation of double-stranded DNA fragments, a process mediated by microhomology [36, 37]. Moreover, eccDNA exhibited a double direct repeat pattern, with significant enrichment of A/T bases flanking the junction sites, suggesting that microhomologous recombination might be a potential mechanism of eccDNA formation[28, 38, 39]. In this study, motif analysis of the sequences flanking the eccDNA breakpoints revealed a significant enrichment of A/T motifs (Fig. 4E), these high frequency A/T repeats likely function as microhomology sequences, mediating the circularization of linear DNA fragments through the microhomology-mediated end joining (MMEJ) mechanism, which was consistent with previous studies. Interestingly, It was observed that the GC content of eccDNAs was significantly lower than that of the upstream and downstream flanking regions (Fig. 4d), which was inconsistent with previous findings[25]. Previous studies in human have identified scaffold/matrix attachment regions (S/MARs) as specific DNA sequences that anchor chromatin to the nuclear scaffold or matrix, these sequences are DNA fragments ranging from 300 to 3000 bp in length and were rich in A/T bases[40, 41]. Narwade et al[42] found that the majority of identified S/MARs had kinked and curved DNA signatures, the relatively weak hydrogen bonding of A-T base pairs confers greater flexibility and instability to these regions, making them prone to chain separation and consequently leading to the shedding of S/MARs to form loops. In addition, the study on Arabidopsis S/MARs revealed that these regions were predominantly located in intergenic regions[43], which is consistent with the genomic distribution of eccDNAs observed in our research (Fig. 4A). Therefore, we speculate that eccDNAs in bermudagrass may originate from S/MARs within intergenic regions and that eccDNA formation may be mediated by the MMEJ pathway.

4.3 The association between eccDNAs and transposable elements under stress

The relationship between eccDNAs and TEs has recently been studied in various organisms, such as rice[11, 15], Arabidopsis[44] and potato (*Solanum tuberosum*)[45]. The generation of eccDNAs can facilitate TE transposition and frequently originates from TE sequences, conversely, certain TEs actively participate in and enhance eccDNA formation[35]. Our study found that eccDNA was highly abundant in TE regions, particularly in long terminal repeat (LTR) regions, which was consistent with the results in spermatogenesis[46]. The hAT family was widely distributed and the most abundant class of DNA transposons in plants and animals[47, 48]. A significant enrichment of hAT family DNA transposons was observed in both the CK and Salt groups, that was consistent with previous studies. It had been reported that the insertion of a CACTA-like TE into rapeseed (*Brassica napus* L.) could function as an enhancer to upregulate the expression of genes associated with specific traits[49]. The insertion of a Harbinger-like TE upstream of *ZmCCT9* resulted in the repression of its transcription and the promotion of flowering in maize(*Zea mays* L.)[50]. In citrus (*Citrus reticulata Blanco*), the insertion of a miniature inverted-repeat transposable element (MITE) into the promoter region of *CiRWP*, a key candidate gene controlling polyembryony, had been shown to enhance its expression[51]. Similarly, a Helitron insertion in the promoter of *MYB61* had been shown to influence nitrogen use efficiency and grain yield in rice[52]. In the present study, it was found that the proportion of eccDNAs derived from DNA transposons increased significantly after salt stress treatment (Fig. 4D). Specifically, several distinct transposon families, including CACTA, Harbinger, Helitron, and MITE, were identified as major contributors (Fig. 4E). These findings suggest that salt stress-induced eccDNAs, enriched with such TE sequences, might participate in the stress response of bermudagrass, and eccDNAs might function as a functional carrier that mediated the rapid amplification of transposon-derived regulatory modules, thereby promoting the flexible response of the genome to environmental stress.

5 Conclusion

In summary, this study reveals the significance of eccDNA as a dynamic genomic response mechanism through a comprehensive analysis of bermudagrass under salt stress. Our findings indicated that salt stress significantly induced an accumulation of eccDNAs and promoted the generating of the eccDNAs that overlapped with full-length genes. The eccDNAs exhibited a distinct low GC content and an enrichment of A/T-rich motifs. Given their preferential distribution in intergenic regions, we speculate that these eccDNAs primarily originate from S/MARs and are circularized via the MMEJ mechanism. Furthermore, salt stress specifically increased the proportion of eccDNAs overlapping with DNA transposons, these eccDNAs might serve as functional regulatory factors that enhanced the expression of stress-responsive genes through the rapid amplification of *cis*-acting elements. Taken together, eccDNAs were involved in the regulation of salt stress responses in bermudagrass, and our study provided novel insights into the mechanisms of plant environmental adaptation.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Consent for publication

No application.

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Author's contribution

ZZ designed the research and wrote the manuscript. ZL and SC conducted the plant stress treatments and FISH experiments. Data visualization completed by ZZ. XL supervised the project and revised the manuscript. ZD and JF provided Fund and XY provided intellectual inputs, Supervision and Project administration. All authors have reviewed and approved the final version of the manuscript.

Availability of data and materials

The raw Circle-seq data generated in this study have been submitted to the NCBI Sequence Read Archive (SRA) database and the accession number is PRJNA1433702, and will be publicly available upon publication.

Supplementary Information

The online version contains supplementary material available at XXX.

Ethics approval and consent to participate

Not applicable.

References

1. Qi K, Liu Z, Amevor FK, Xu D, Zhu W, Li T, et al. Emerging research insights and future perspectives on the advancement of eccDNA: A comprehensive review. Biotechnol Adv. 2025;84:108693.

2. Shibata Y, Kumar P, Layer R, Willcox S, Gagan JR, Griffith JD, et al. Extrachromosomal microDNAs and chromosomal microdeletions in normal tissues. Science. 2012;336(6077):82–6.
3. Paulsen T, Shibata Y, Kumar P, Dillon L, Dutta A. Small extrachromosomal circular DNAs, microDNA, produce short regulatory RNAs that suppress gene expression independent of canonical promoters. Nucleic Acids Res. 2019;47(9):4586–96.
4. Hotta Y, Bassel A. Molecular size and circularity of DNA in cells of mammals and higher plants. Proc Natl Acad Sci U S A. 1965;53(2):356–62.
5. Alt FW, Kellems RE, Bertino JR, Schimke RT. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. Biotechnology. 1992;24:397–410.
6. Kumar P, Dillon LW, Shibata Y, Jazaeri AA, Jones DR, Dutta A. Normal and cancerous tissues release extrachromosomal circular DNA (eccDNA) into the circulation. Mol Cancer Res. 2017;15(9):1197–205.
7. Lu W, Li F, Ouyang Y, Jiang Y, Zhang W, Bai Y. A comprehensive analysis of library preparation methods shows high heterogeneity of extrachromosomal circular DNA but distinct chromosomal amount levels reflecting different cell states. Analyst. 2023;149(1):148–60.
8. Zuo S, Yi Y, Wang C, Li X, Zhou M, Peng Q, et al. Extrachromosomal circular DNA (eccDNA): From chaos to function. Front Cell Dev Biol. 2021;9:792555.
9. Yu X, Liu Z, Sun X. Single-cell and spatial multi-omics in the plant sciences: Technical advances, applications, and perspectives. Plant Commun. 2023;4(3):100508.
10. Wang K, Tian H, Wang L, Wang L, Tan Y, Zhang Z, et al. Deciphering extrachromosomal circular DNA in Arabidopsis. Comput Struct Biotechnol J. 2021;19:1176–83.
11. Ni H, Yong-Villalobos L, Gu M, López-Arredondo DL, Chen M, Geng L, et al. Adaptive dynamics of extrachromosomal circular DNA in rice under nutrient stress. Nat Commun. 2025;16(1):4150.
12. Zhuang J, Zhang Y, Zhou C, Fan D, Huang T, Feng Q, et al. Dynamics of extrachromosomal circular DNA in rice. Nat Commun. 2024;15(1):2413.
13. Fu W, MacGregor DR, Comont D, Saski CA. Sequence characterization of extra-chromosomal circular DNA content in multiple blackgrass (*alopecurus myosuroides*) Populations. Genes (Basel). 2023;14:1905.
14. Huang Y, Ding W, Zhang M, Han J, Jing Y, Yao W, et al. The formation and evolution of centromeric satellite repeats in Saccharum species. Plant J. 2021;106(3):616–29.
15. Lanciano S, Carpentier MC, Llauro C, Jobet E, Robakowska-Hyzorek D, Lasserre E, et al. Sequencing the extrachromosomal circular mobilome reveals retrotransposon activity in plants. PLoS Genet. 2017;13(2):e1006630.
16. Molin WT, Yaguchi A, Blenner M, Saski CA. The eccDNA replicon: a heritable, extranuclear vehicle that enables gene amplification and glyphosate resistance in *amaranthus palmeri*. Plant Cell. 2020;32(7):2132–40.

17. Hanna WW, Anderson WF. Development and impact of vegetative propagation in forage and turf bermudagrasses. Agron J. 2008;100(S3):S–103.
18. Zhang B, Chen S, Liu J, Yan YB, Chen J, Li D, et al. A high-quality haplotype-resolved genome of common bermudagrass (*Cynodon dactylon L.*) provides insights into polyploid genome stability and prostrate growth. Front Plant Sci. 2022;13:890980.
19. Møller HD. Circle-seq: isolation and sequencing of chromosome-derived circular DNA elements in cells. Methods Mol Biol. 2020;2119:165–81.
20. Spier Camposano H, Molin WT, Saski CA. Sequence characterization of eccDNA content in glyphosate sensitive and resistant Palmer amaranth from geographically distant populations. PLoS ONE. 2022;17(9):e0260906.
21. Li R, Wang Y, Li J, Zhou X. Extrachromosomal circular DNA (eccDNA): an emerging star in cancer. Biomark Res. 2022;10(1):53.
22. Prada-Luengo I, Krogh A, Maretty L, Regenberg B. Sensitive detection of circular DNAs at single-nucleotide resolution using guided realignment of partially aligned reads. BMC Bioinformatics. 2019;20(1):663.
23. Kumar P, Dillon LW, Shibata Y, Jazaeri AA, Jones DR, Dutta A. Normal and cancerous tissues release extrachromosomal circular DNA (eccDNA) into the circulation. Mol Cancer Res. 2017;15(9):1197–205.
24. Mann L, Seibt KM, Weber B, Heitkam T. ECCsplorer: a pipeline to detect extrachromosomal circular DNA (eccDNA) from next-generation sequencing data. BMC Bioinformatics. 2022;23(1):40.
25. Song Y, Yu K, Guo Y, Cao Y, Xu C, Ma L, et al. Integration analysis of circle-sequencing and transcriptome reveals extrachromosomal circular DNA is involved in the regulation of vascular cambium annual cycle in chinese pine. Plant Cell Environ. 2025;48(7):5545–58.
26. Gao X, Liu K, Luo S, Tang M, Liu N, Jiang C, et al. Comparative analysis of methodologies for detecting extrachromosomal circular DNA. Nat Commun. 2024;15(1):9208.
27. Ling X, Han Y, Meng J, Zhong B, Chen J, Zhang H, et al. Small extrachromosomal circular DNA (eccDNA): major functions in evolution and cancer. Mol Cancer. 2021;20(1):113.
28. Yang H, He J, Huang S, Yang H, Yi Q, Tao Y, et al. Identification and characterization of extrachromosomal circular DNA in human placentas with fetal growth restriction. Front Immunol. 2021;12:780779.
29. Sun Z, Ji N, Zhao R, Liang J, Jiang J, Tian H. Extrachromosomal circular DNAs are common and functional in esophageal squamous cell carcinoma. Ann Transl Med. 2021;9(18):1464.
30. Zaidi SS-e-A, Shakir S, De Kort H, Mehta D, Nguyen V, Gutzat R, et al. A comprehensive atlas of full-length Arabidopsis eccDNA populations identifies their genomic origins and epigenetic regulation. PLoS Biol. 2025;23(7):e3003275.
31. Koo DH, Molin WT, Saski CA, Jiang J, Putta K, Jugulam M, et al. Extrachromosomal circular DNA-based amplification and transmission of herbicide resistance in crop weed Amaranthus palmeri. Proc Natl Acad Sci U S A. 2018;115(13):3332–7.

32. Koo DH, Sathishraj R, Nakka S, Ju Y, Nandula VK, Jugulam M, et al. Extrachromosomal circular DNA-mediated spread of herbicide resistance in interspecific hybrids of pigweed. Plant Physiol. 2023;193(1):229–33.
33. Dillon LW, Kumar P, Shibata Y, Wang YH, Willcox S, Griffith JD, et al. Production of extrachromosomal microDNAs is linked to mismatch repair pathways and transcriptional activity. Cell Rep. 2015;11(11):1749–59.
34. Paulsen T, Kumar P, Koseoglu MM, Dutta A. Discoveries of extrachromosomal circles of DNA in normal and tumor cells. Trends Genet. 2018;34(4):270–8.
35. Yang F, Su W, Chung OW, Tracy L, Wang L, Ramsden DA, et al. Retrotransposons hijack alt-EJ for DNA replication and eccDNA biogenesis. Nature. 2023;620(7972):218–25.
36. Møller HD, Parsons L, Jørgensen TS, Botstein D, Regenberg B. Extrachromosomal circular DNA is common in yeast. Proc Natl Acad Sci U S A. 2015;112(24):E3114–3122.
37. Mehanna P, Gagné V, Lajoie M, Spinella JF, St-Onge P, Sinnett D, et al. Characterization of the microDNA through the response to chemotherapeutics in lymphoblastoid cell lines. PLoS ONE. 2017;12(9):e0184365.
38. Sin STK, Jiang P, Deng J, Ji L, Cheng SH, Dutta A, et al. Identification and characterization of extrachromosomal circular DNA in maternal plasma. Proc Natl Acad Sci U S A. 2020;117(3):1658–65.
39. Zhu M, Tong X, Qiu Q, Pan J, Wei S, Ding Y, et al. Identification and characterization of extrachromosomal circular DNA in the silk gland of Bombyx mori. Insect Sci. 2023;30(6):1565–78.
40. Bode J, Kohwi Y, Dickinson L, Joh T, Klehr D, Mielke C, et al. Biological significance of unwinding capability of nuclear matrix-associating DNAs. Science. 1992;255(5041):195–7.
41. Mielke C, Kohwi Y, Kohwi-Shigematsu T, Bode J. Hierarchical binding of DNA fragments derived from scaffold-attached regions: correlation of properties in vitro and function in vivo. Biochemistry. 1990;29(32):7475–85.
42. Narwade N, Patel S, Alam A, Chattopadhyay S, Mittal S, Kulkarni A. Mapping of scaffold/matrix attachment regions in human genome: a data mining exercise. Nucleic Acids Res. 2019;47(14):7247–61.
43. Rudd S, Frisch M, Grote K, Meyers BC, Mayer K, Werner T. Genome-wide in silico mapping of scaffold/matrix attachment regions in Arabidopsis suggests correlation of intragenic scaffold/matrix attachment regions with gene expression. Plant Physiol. 2004;135(2):715–22.
44. Zhang P, Mbodj A, Soundiramourtty A, Llauro C, Ghesquière A, Ingouff M, et al. Extrachromosomal circular DNA and structural variants highlight genome instability in Arabidopsis epigenetic mutants. Nat Commun. 2023;14(1):5236.
45. Esposito S, Barteri F, Casacuberta J, Mirouze M, Carputo D, Aversano R. LTR-TEs abundance, timing and mobility in Solanum commersonii and S. tuberosum genomes following cold-stress conditions. Planta. 2019;250(5):1781–7.

46. Hu J, Zhang Z, Xiao S, Cao Y, Chen Y, Weng J et al. Microhomology-mediated circular DNA formation from oligonucleosomal fragments during spermatogenesis. Elife. 2023; 12.
47. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al. Initial sequencing and analysis of the human genome. Nature. 2001;409(6822):860–921.
48. Arensburger P, Hice RH, Zhou L, Smith RC, Tom AC, Wright JA, et al. Phylogenetic and functional characterization of the hAT transposon superfamily. Genetics. 2011;188(1):45–57.
49. Shi L, Song J, Guo C, Wang B, Guan Z, Yang P, et al. A CACTA-like transposable element in the upstream region of BnaA9.CYP78A9 acts as an enhancer to increase silique length and seed weight in rapeseed. Plant J. 2019;98(3):524–39.
50. Huang C, Sun H, Xu D, Chen Q, Liang Y, Wang X et al. ZmCCT9 enhances maize adaptation to higher latitudes. Proceedings of the National Academy of Sciences. 2018; 115(2):E334-E341.
51. Wang X, Xu Y, Zhang S, Cao L, Huang Y, Cheng J, et al. Genomic analyses of primitive, wild and cultivated citrus provide insights into asexual reproduction. Nat Genet. 2017;49(5):765–72.
52. Gao Y, Xu Z, Zhang L, Li S, Wang S, Yang H, et al. MYB61 is regulated by GRF4 and promotes nitrogen utilization and biomass production in rice. Nat Commun. 2020;11(1):5219.

Figures

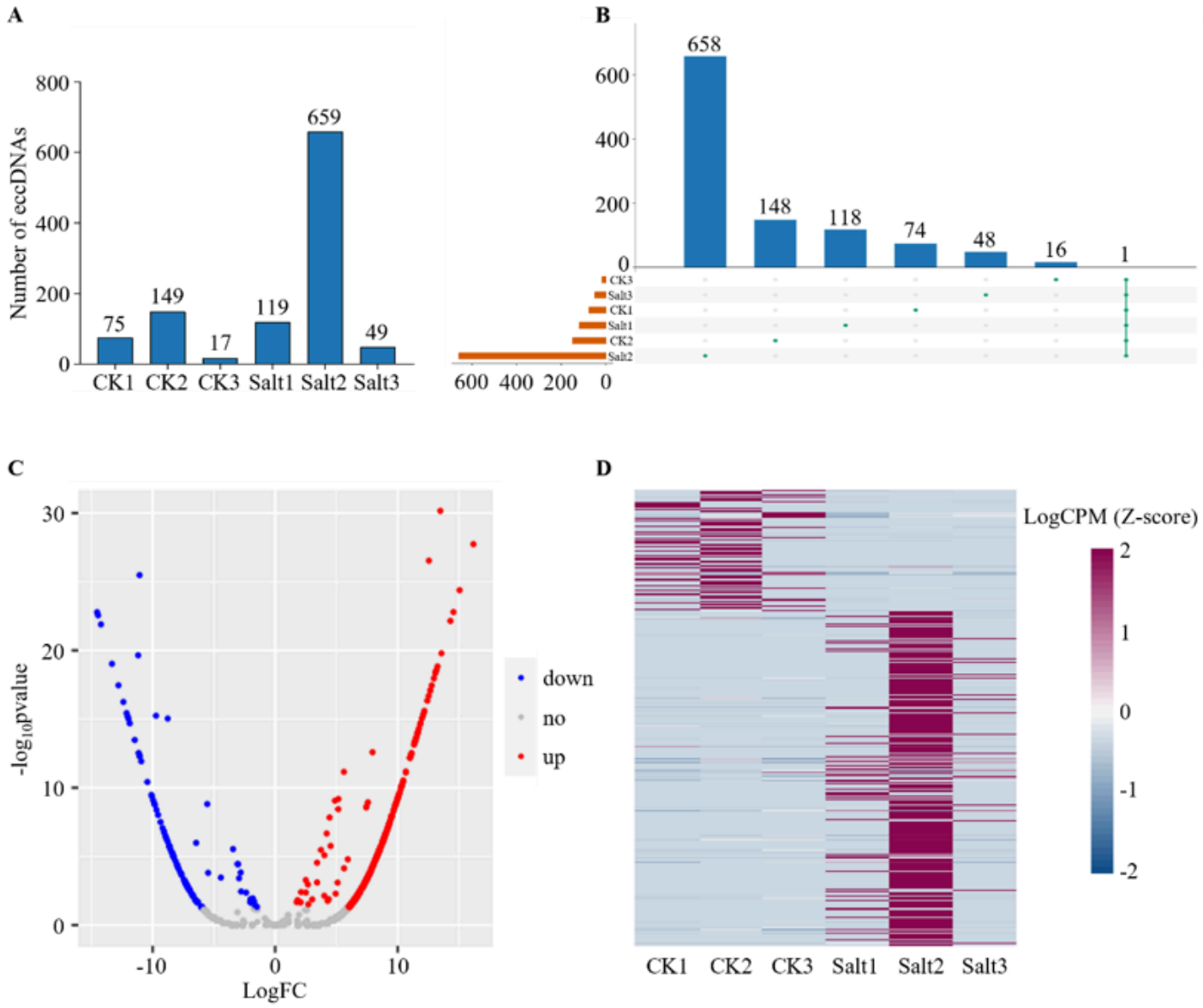

Figure 1

Identification of the eccDNA in bermudagrass. A: Statistics on the number of eccDNA in each group. **B:** The UP-set diagram shows the eccDNAs of each group. **C:** Volcano plot and **D:** cluster analysis of DE-eccDNAs. CPM: Counts per million.

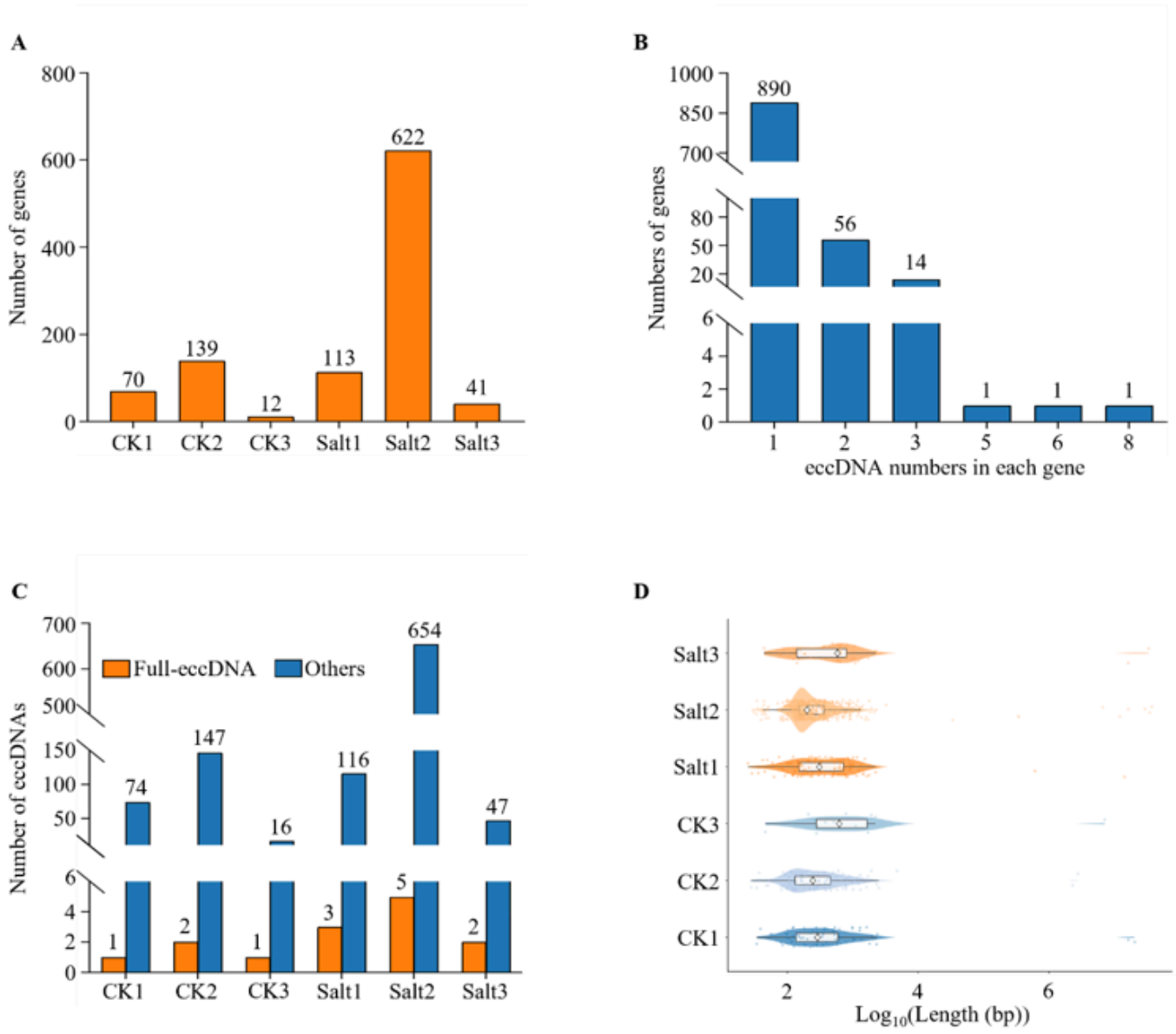

Figure 2

Analysis of eccDNA characteristics. A: Gene annotation of eccDNA identified at CK and Salt. **B:** Statistics on the amount of eccDNA produced by a gene. **C:** Distribution and characterization of Full-eccDNAs. **D:** Length distribution of eccDNA.

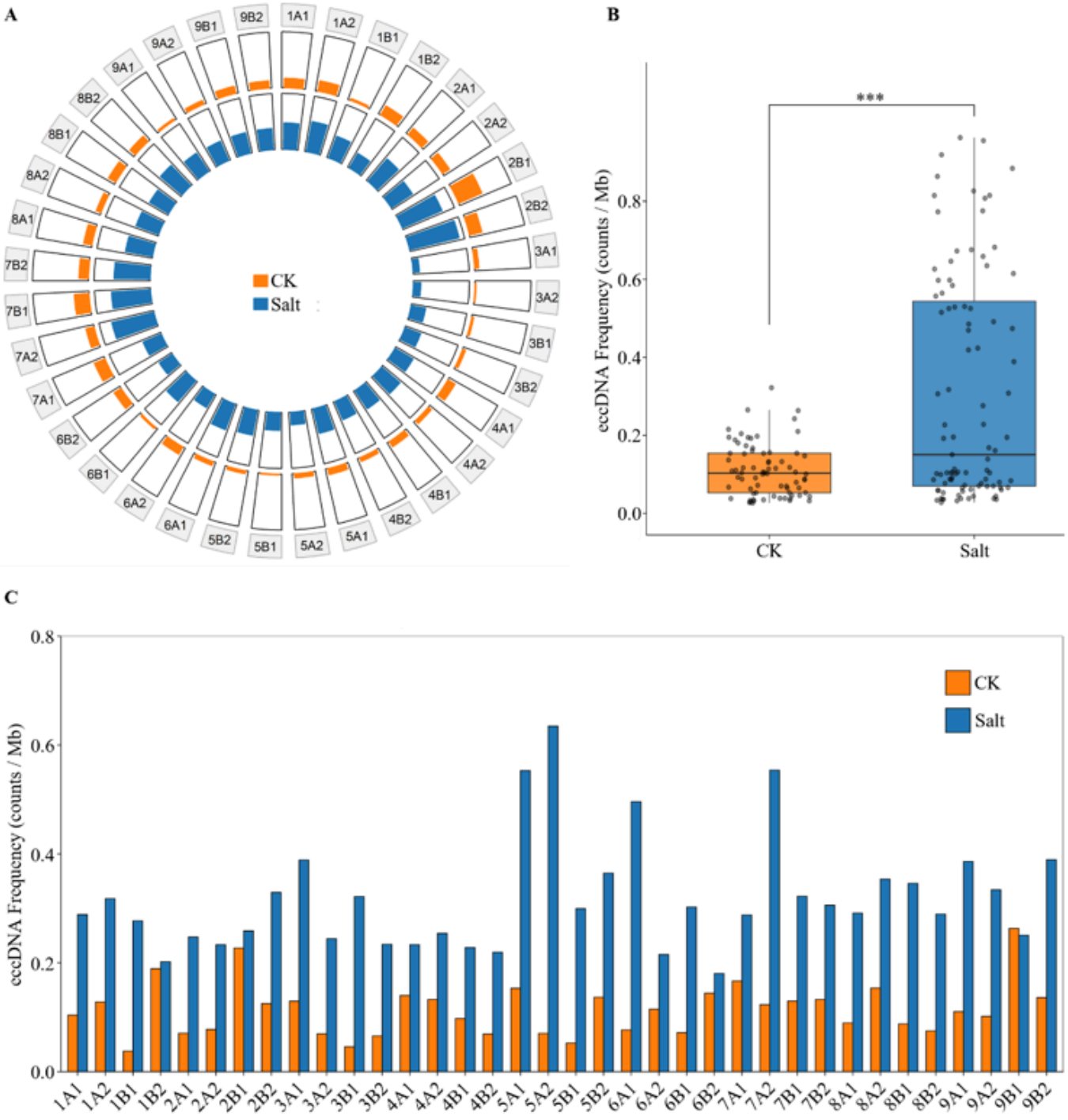

Figure 3

Genomic distribution and generation frequency of eccDNA in bermudagrass. **A:** The number of eccDNA molecules per chromosome in Bermuda grass, and the area of each graph represents the quantity of eccDNA. **B:** Global eccDNA frequency per megabase on CK and Salt. The *** indicated an extremely significant difference ($p < 0.001$). **C:** eccDNA frequency per megabase on each chromosome.

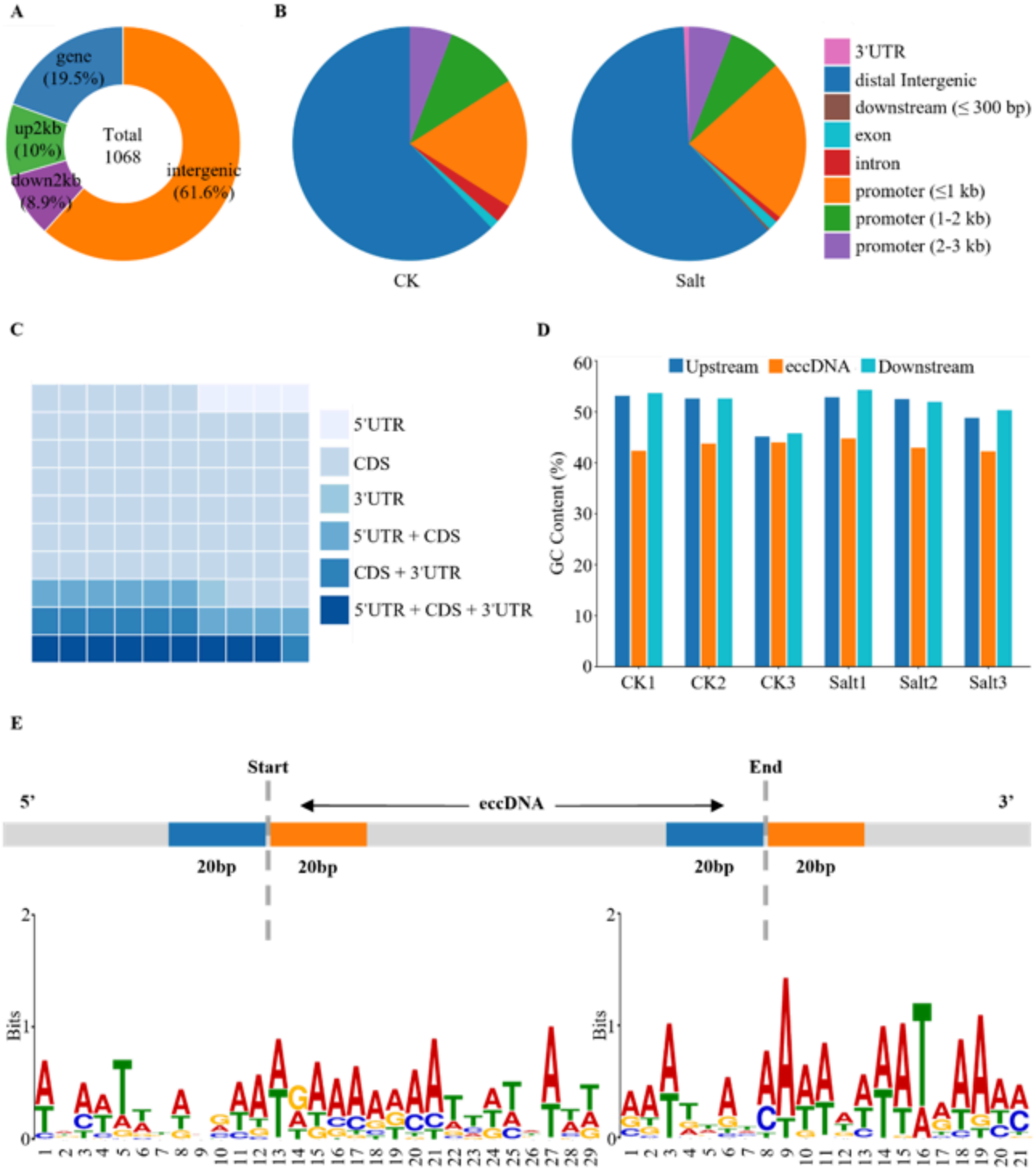

Figure 4

Mechanistic analysis of eccDNA formation. A: Proportion of overall eccDNAs overlapping with genes, upstream 2 kb regions (up2kb), downstream 2 kb regions (down2kb), and intergenic regions**. B:** Distribution of gene elements in eccDNAs. **C:** The waffle plot illustrates the proportion of overlap in the following regions among all eccDNAs overlapping with genes: unique 5'UTR, unique CDS, unique 3'UTR, combined 5'UTR and CDS region (5'UTR + CDS), combined CDS and 3'UTR region (CDS + 3'UTR), and the total sum of all regions with 5'UTR, CDS, and 3'UTR regions. Each square represents a 1% share. **D:**GC

content of eccDNA and its upstream and downstream sequences. **E:** Analysis of motif sequences on both sides of eccDNA junction sites.

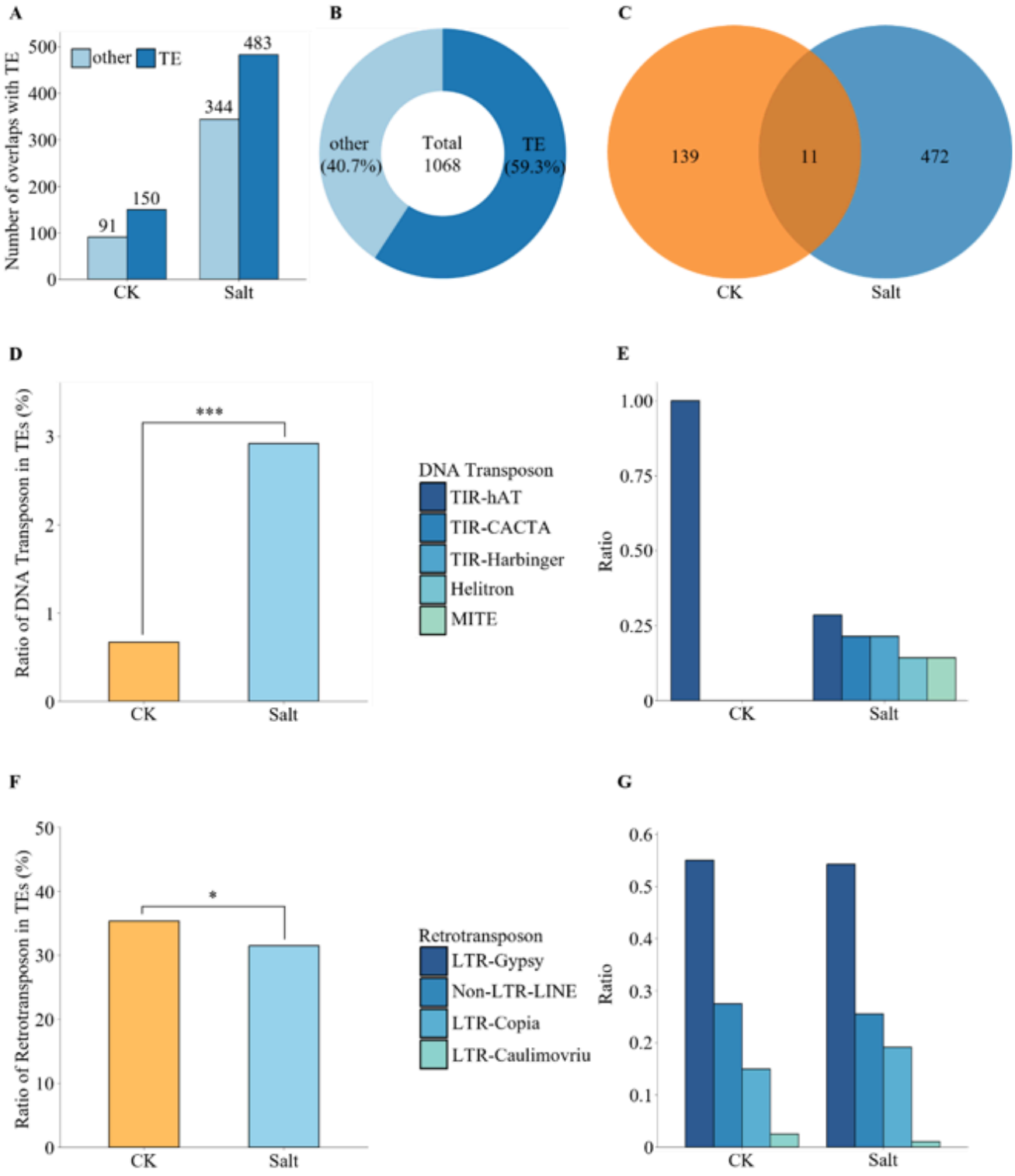

Figure 5

A: Counts of eccTEs between CK and Salt. **B:** Proportion of overall eccDNAs overlapping with transposable elements (eccTEs). **C:** Venn diagram showing the specific and shared eccTEs identified

between CK and Salt. **D:** Proportion of DNA transposon in eccTEs between CK and Salt. **E:** Ratio distribution on DNA transposon classification in eccTEs between CK and Salt. **F:** Proportion of retrotransposon in eccTEs between CK and Salt. **G:** Ratio distribution on retrotransposon classification in eccTEs between CK and Salt. The * and ***indicated, respectively, a significant difference ($p < 0.05$) and an extremely significant difference ($p < 0.001$).

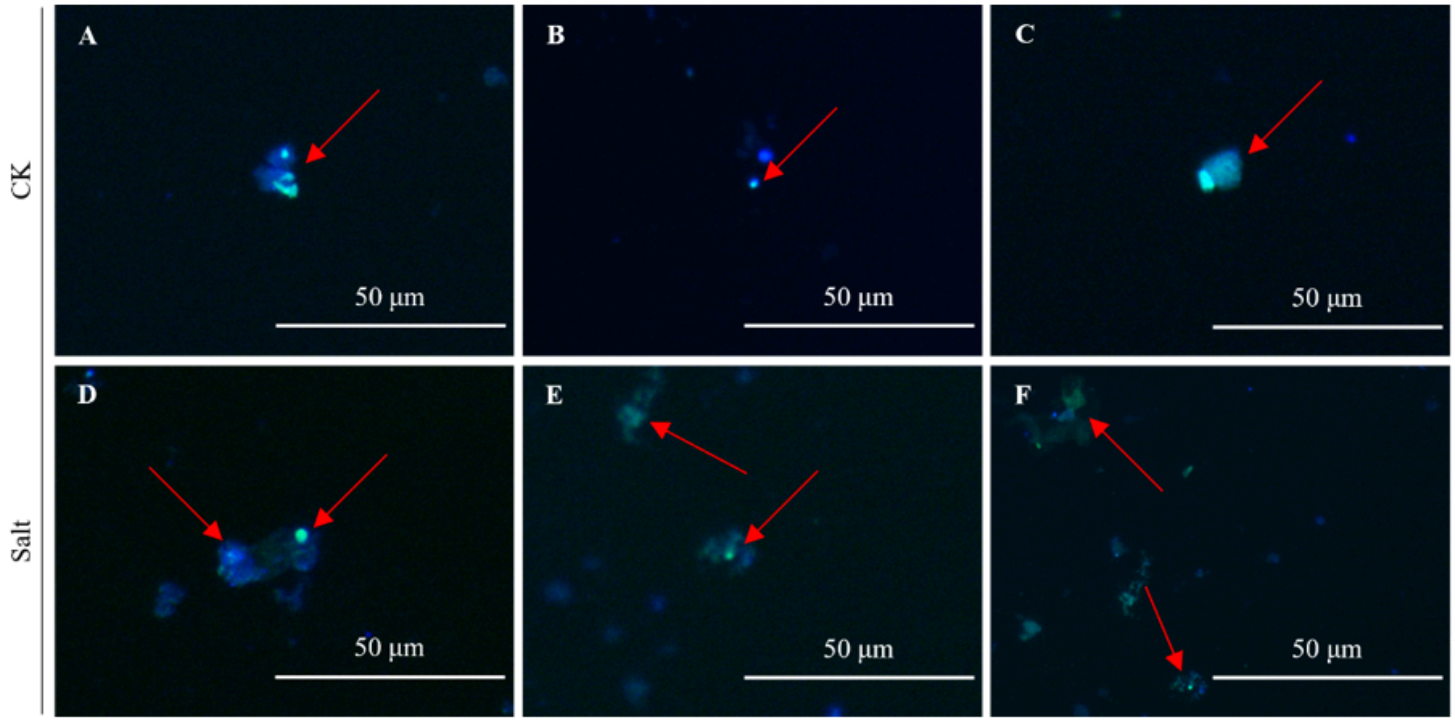

Figure 6

Fluorescence in situ hybridization of eccDNA in bermudagrass. A-C for CK and **D-F** for Salt. Blue signals represent DAPI-stained nuclei, and green signals represent the selected eccDNA probes. Bar = 50 μm.

Supplementary Files

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- Supplementary.docx