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CRISPR-Cas9 Mediated Genome Editing in Groundnut (*A. hypogaea* L.)

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Groundnut (*Arachis hypogaea* L.), commonly known as peanut, is an important oilseed and food legume belonging to the family Fabaceae, subfamily Papilionoidea, and genus Arachis. It is the 2nd most important oilseed crop in world and most important oilseed crop in India. Being originated from South America (mostly Central Brazil) peanut is used as a source of edible oil, protein-rich food, and animal feed. 100 g of kernels provide 567 kcal of energy and 8.5 g of dietary fiber (Murali T *et al*, 2017). The peanut oil contains several fatty acids like palmitic (7–12%), oleic (40–50%) and linoleic (25–35%) acid (Murali T *et al*, 2017). Conventional breeding becomes difficult due to its narrow genetic base, allotetraploid genome, and low levels of natural variability. Long breeding cycles and linkage drag further limit the development of superior cultivars with resistance to diseases, drought, and aflatoxin contamination. Recent advances in genome-editing technologies provide new opportunities to overcome these constraints. Tools such as CRISPR/Cas systems enable precise modification of target genes, allowing rapid improvement of yield, quality, and stress tolerance. Integration of genome editing offers sustainable improvement in groundnut.

Constrains In Conventional Breeding

Though conventional breeding depends has produced many successful varieties, it has several biological, technical, and practical limitations which are as follows:

a) **Limited availability of resistant sources**: Resistance genes may not exist in cultivated germplasm. They are often found in wild relatives, which are difficult to use, sometimes no effective resistance is available against new pests/pathogens.
b) **Crossability Barrier:** Groundnut is a segmental allotetraploid crop, so it has crossability barrier with other wild sp.
c) **Linkage Drag:** Along with resistance genes, undesirable traits are also transferred from donors: low yield, poor seed quality, late maturity, undesirable plant type.
d) **Time-consuming process**: It requires 6–12 years or more multiple generations of: Crossing, Selection, Screening, Yield trials etc.
e) **Strong environmental pressure:** Disease expression depends on: temperature, humidity, inoculum pressure. Which leads to: unreliable phenotyping genotype × environment interaction.
f) **Breakdown of Resistance**: Pathogens and pests evolve rapidly. Varieties with single major genes often become susceptible within few years.
g) **Low precision:** Selection is based on visible symptoms. It Cannot detect carrier plants, early generation resistant genotypes, hidden resistance genes.

By considering this constrains scientists are moving on to advanced breeding methods (e.g.- **Genome Editing)** conventional breeding.

Genome Editing: A powerful novel plant breeding tool which allows addition, substitution, and alternation of genetic material at specific location within the genome. These changes are

induced by specially engineered DNA or protein regents such as site-directed nucleases (SDN), base editors (Bes) and chimeric oligonucleotides (COs). In simple terms these reagents either introduce doble strand breaks (DSBs) or nicks or base mismatches at the targeted genomic site that are repaired by cell's own DNA repair system. These DSB or nicks are induced in target sequence by SDNs. Zinc-finger nucleases were first SDN developed during 1990s. Others SDNs are TALEN (Transcription Activator Like Effector Nucleases), Meganucleases, CRISPER-Cas9 (Singh B D, 2022).

CRISPR-Cas9: Clustered Regularly Interspaced Palindromic Repeats- CRISPR associated protein9- SDN system entered in the genome editing arena in the year of 2013 (Singh B D, 2022). This made up of 2 main parts- CRISPR sequence and Cas protein. Short regions of the unique DNA (spacers) are separated from each other by short palindromic repeats are known as CRISPR sequence (Redman M *et al*,2016). These sequences are present near Cas gene which encode for Cas proteins.

The designing of CRISPR-Cas9 for new genomic sites involves redesigning of only 20 nucleotide long region of single-guide RNAs, while the rest of sgRNA and the Cas9 protein remain the same for all genomic targets. The designing of this RNA is very simple and several robust computer programmes is there for this.

Bacterial defence mechanism: Before understanding mechanism of Genome editing by CRISPR-Cas9 in eukaryotic cell lets under how it helps bacteria from attack of phage or plasmids. This involves following steps (Fuguo Jiang, Jennifer A. Doudna, 2017) -

- When foreign DNA from phage or plasmid enters the cell, small fragments of it are inserted as new spacers into the CRISPR array in the host genome. The spacers act as a genetic memory of previous infections.
- The CRISPR array is transcribed into a long precursor RNA (pre-crRNA). This transcript is processed by Cas proteins into short mature CRISPR RNAs (crRNAs). Each crRNA contains a spacer sequence that matches a specific foreign DNA target.
- crRNAs guide Cas proteins to complementary sequences in invading DNA or RNA. Binding between crRNA and target sequence triggers cleavage of the foreign genetic material. A short DNA motif called the PAM (Protospacer Adjacent Motif) is essential for accurate target recognition.
- Cas9 requires two RNAs- crRNA and tracrRNA, which form a dual-RNA structure. This complex guides Cas9 to a complementary 20-nt DNA target next to a PAM sequence. Cas9 creates a double-strand break (DSB) using HNH and RuvC nuclease domains.

In this way they cut the DNA at target site and protect itself from foreign DNA of plasmid or phage.

Mechanism of Genome Editing

A. Target search and recognition: Requires Complementary base pairing between the 20-nt spacer sequence of sgRNA and a protospacer in the target DNA and presence of PAM sequence. Ribonucleoprotein complex scans DNA by 3D collision technique (rapidly dissociating from non-PAM sites). For Cas9 PAM sequence: 3'-NGG-5' on non-target strand.

B. Unwinding and target DNA binding: Recognition triggers destabilization of local DNA duplex. A kink is observed in target DNA strand near PAM sequence. Kink triggers unwinding of DNA double strand. sgRNA binds with the target sequence. RNA-DNA duplex & R loop structure forms

C. Cleavage: R loop stabilizes HNH domain near target strand and RuvC domain near non target. RNAase III enzyme cleaves DNA strands accordingly. Cleaved DNA strands are repaired by cell's own DNA repair system

D. Repair: DNA cleavage triggers cell's own DNA repairing mechanism- 2 pathways-i) NHEJ, ii) HDR

- **NHEJ:** Non-Homologous End Joining. Dominates in all cell types. Error prone ligation at DSB end by ligase IV. Introduction of small incantations/delectations(indel) or

substitutions at 3bp upstream of PAM in the absence of donor template. Frameshift mutation, disruption of gene function (Fuguo Jiang, Jennifer A Doudna, 2017)

- **HDR:** Homology Directed Repair. Adding of artificially synthesized DNA- a small piece of artificial template introduced in the target locus. 2 types of templates (Nemudryi et. al., 2014)-

✓ Single strand oligonucleotides- opt length 90nt
✓ Plasmid vectors- sufficiently long arms, 500-1000bp, can flank reporter genes, disease resistance genes. Can alter genome by replacing original nucleotide sequence

Genome Editing in Groundnut

A. Increase in Oleic acid content: Fatty acid desaturase (FAD2) converts oleic acid to linoleic acid: ahFAD2A and ahFAD2B gene. Mutagens (rays, gamma rays, sodium azide and EMS) had been employed previously along with naturally existing mutations- but they induce undesirable mutations. So, genome editing has been employed by some scientists-

✓ Yuan *et al*, 2023: Induced mutagenesis in hot spots on the gene- 3 mutation: 2 existing (G448A and 441_442insA) and 1 new(G451T) containing high oleic acid.
✓ Han *et al*, 2023: applied genome editing in Huayu 23, a popular cultivar dysfunctional FAD2A and functional FAD2B. Inserted 442A in FAD2B-inactivation- increase oleic acid by 80%.

B. Reduction of Palmitic acid: Principal approach to minimize the content of SFAs in peanut is to reduce the content of palmitic acid, linked to cardiovascular disease. GE in AhFatB gene reduce PA and increase OA (Tang Y *et al*, 2022).

C. Reduction of Allergen: Out of 12 proteins responsible for allergens in groundnut Ara h 1, Ara h 2 and Arah 3 are major. Ara h 2 cause 90% allergic reaction. Previously RNAi tech reduces 25% content. CRISPR-Cas9: disrupts this gene (Biswas et al, 2022)

D. Other applications: Han *et al*, 2025

Genes edited	Methods	Results
1. AhALS2-A and AhALS2-B	Base editing	Create herbicide resistance
2. AhAftr1	Genetic Transformation	Aflatoxin resistance
3. AhNFR1 and AhNFR5	Indels	Functional Validation of nodulation genes
4. gfp	Prime editing	Stop codon is inserted in gfp region: prime editing validation.

Future Prospect

➢ Biotic & Abiotic Stress management: Knock out susceptible genes or genes responsible for infection- development of wide range of biotic stress resistant and abiotic stress tolerant variety.
➢ Precision Nutrition & Biofortification: Beyond fatty acids, genome editing can be used to enhance the micronutrient profile of the kernels.
➢ Enhancing Symbiotic Nitrogen Fixation: Targeting genes that regulate nodule formation or the "autoregulation of nodulation" (AON) pathway
➢ De novo Domestication of wild relatives: Using CRISPR to "domesticate" wild *Arachis* species by editing a few key orthologs of domestication genes (e.g., genes controlling seed shattering, seed size, and plant architecture).
➢ Speed Breeding 2.0: Integrating CRISPR with "Speed Breeding" (controlled environment growth with extended photoperiods) can drastically reduce the time needed to stabilize edited lines

Conclusion

The advent of Genome editing has paved the way for efficient genetic enhancement of crops but in polyploid species like peanuts even more precision is required. The recent reports on different attributes of peanut improvement through GE provide promising insights into exploring new areas of editing in peanut which has great potential to contribute to nutritional security. Furthermore, the major challenges faced by the peanut crop like abiotic and biotic stress, fresh seed dormancy, impaired nutrient bioavailability need to be addressed more to have better knowledge of the genes and the pathways that would be fundamental for studies aimed for genome editing for that particular trait. In addition to the above-mentioned prospective traits, there are many other potential traits in peanut like resistant starch, raffinose family oligosaccharide (RFOs), resveratrol and Vitamin E content in seeds which could be the future research thrusts for nutritional enhancement in peanut through genome editing. The regulatory policies also need much more refinement and amendments in different nations worldwide to have a harmonized state of enforcements to foster the trade market for genome edited products.

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