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SOYBEAN'S GENETIC ODYSSEY: UNVEILING DIVERSE TRANSFORMATION STRATEGIES

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Abstract

Soybean (Glycine max) is a globally pivotal crop, serving as a major source of protein and oil for food, feed, and industrial applications. With production exceeding 350 million metric tons annually and cultivation spanning over 125 million hectares worldwide, soybean underpins food security and rural economies, especially in major producers like Brazil, the USA, and India. Despite its importance, yield is often constrained by biotic and abiotic stresses, with up to 50% losses attributed to "yield limiters." To address these challenges, researchers have advanced genetic transformation techniques—including Agrobacterium-mediated, biolistic (particle bombardment), and emerging genome editing technologies such as CRISPR/Cas9. These methods have enabled the rapid development of soybean varieties with enhanced yield, stress tolerance, pest and disease resistance, and improved nutritional quality. Recent innovations, such as the use of split-seed explants and high-throughput automated transformation systems, have increased transformation efficiency to over 20% in some protocols, significantly reducing breeding cycles. Genome editing tools now allow precise, targeted modifications, offering the potential for "non-GMO" solutions and improved public acceptance. Additionally, advances in functional genomics and omics technologies have accelerated the discovery of key genes for yield and resilience, while sustainable practices—such as optimizing symbiosis with beneficial microbes—further boost productivity. Looking ahead, integrating synthetic biology, advanced gene delivery systems, and machine learning promises to further optimize transformation strategies and trait stacking. These innovations will enable the development of climate-resilient, nutritionally enhanced, and sustainable soybean varieties, reinforcing soybean's critical role in global food systems and its capacity to meet the demands of a growing population under changing environmental conditions.

INTRODUCTION

Soybean holds immense global agricultural significance due to its multifaceted uses in food, animal feed, and industrial applications, making it a cornerstone crop in global agriculture and trade. Soybeans are a vital source of protein and essential nutrients for human consumption worldwide. They are used directly in various food products such as tofu, soy milk, soy sauce, and meat substitutes, catering to diverse dietary needs including vegetarian and vegan diets. Soybean protein is highly valued for its quality and digestibility, often surpassing other legumes, and contains beneficial compounds like isoflavones and phytosterols which contribute to human health by lowering LDL cholesterol and blood pressure, and potentially reducing risks of heart attack and stroke (Rotundo et al., 2024). Soybean, originating in China and spreading via trade, is a global "Miracle Crop" due to its diverse food and industrial applications. Brazil and the USA lead global production (353 million MT). India ranks fourth in cultivation area (11.25 million hectares), producing 9.30 million MT, significantly impacting its economy and farmers' livelihoods (Kumari et al., 2025). Soybean is a vital global oilseed and protein source, increasingly used in food and diverse industrial products. Though 348.7 million tons were harvested in 2018 across 125 million hectares, "yield limiters" reduce production by 50%. Researchers employ advanced techniques like cross hybridization, molecular breeding, and genome editing to overcome these challenges, aiming to secure soybean's role in global food security (Dilawari et al., 2022). Soybean production globally is crucial for food, protein, and oil. Research is vital to increase its yield under various conditions, including stress. This involves understanding its symbiosis with beneficial soil microbes like arbuscular mycorrhizal fungi and rhizobia, and the impact of abiotic factors. Optimal inoculum use and knowledge of diverse environmental parameters are key to enhancing soybean growth and global yield (Pagano & Miransari, 2016). Soybean, a vital global crop for oil and protein, has seen surging production since the 1970s. Research focuses on increasing yield under stress, with soil bacteria and farming techniques playing a significant role. Key producers are the USA, Brazil, Argentina, China, and India (Hamza et al., 2024).

Soybeans (Glycine max) are versatile legumes with edible seeds, typically green but also yellow, brown, or black. They are a valuable alternative to meat, forming the basis of soy milk, tofu, and other products. Rich in protein, vitamins, minerals, and insoluble fiber, soybeans are considered a complete protein source and are crucial for nutrition and potential global food security (Saha & Mandal, 2019). Soybean, originating in Asia, is a globally valued crop due to its health benefits. Rich in isoflavonoids, folic acid, proteins, and saponins, it offers antioxidant, anti-diabetic, anti-cancer, anti-obesity, and anti-inflammatory properties. Its consumption may reduce chronic illnesses like heart disease, diabetes, and certain cancers, making it promising for functional foods and pharmaceuticals (Dukariya et al., 2020). A 2012 study in southern Brazil compared the sustainability of conventional (GM/non-GM) and organic soybean farming. Organic systems showed higher probability of lower global warming potential (77%), lower energy use, higher land occupation, higher profitability (60%), and higher employment. This highlights the varied environmental, economic, and social performance of different farming systems (Kamali et al., 2017).

B. Rationale for Genetic Transformation in Soybean:

Recent advancements in soybean genetic improvement, focusing on enhancing stress resistance, nutritional value, and yield. It covers breeding techniques, including traditional methods, marker-assisted selection, and biotechnological innovations like genetic engineering (Agrobacterium-mediated, biolistic) and genome editing. Challenges like genotype recalcitrance and regulatory hurdles persist, but integrating these strategies with sustainable practices is crucial for global food security (Vargas-Almendra et al., 2024). Soybean is a vital global crop, valuable for food, feed, and fuel, and enhances soil fertility. Beyond conventional breeding, genetic engineering (GE) has improved traits like herbicide and pest resistance. Newer plant breeding technologies (NPBTs) like CRISPR/Cas9 offer precise genome editing for trait improvement, addressing ethical concerns and boosting public acceptance, crucial for meeting rising global food demands (Rahman et al., 2023). Tissue culture and

genetic transformation are crucial for improving soybean, creating resistant transgenics and enhancing beneficial traits. Efficient plant regeneration from explants is essential, influenced by various factors like genotype, explant age, and media composition. Both *Agrobacterium*-mediated transformation and particle bombardment are employed. This review covers advancements, constraints, and future strategies for biotechnological soybean improvement globally and in India (Tiwari et al., 2021).

Table 1: Rationale for Genetic Transformation in Soybean.

Objective	Description	Examples & References
Improving Agronomic Traits	Genetic engineering enhances yield and stress resistance, including drought, heat, and photosynthesis efficiency.	Transgenic soybeans with engineered photosynthesis proteins (*AtVDE*, *AtPsbS*, *AtZEP*) showed up to 21.7% yield increase (De Souza et al., 2022). Heat tolerance improved by overexpressing *GmHsp90A2* and editing heat shock protein genes via CRISPR/Cas9 (Jianing et al., 2022). Drought tolerance conferred by TF *HaHB4* transgene (Jianing et al., 2022).
Enhancing Nutritional Quality	Genetic modification reduces antinutritional factors and improves protein quality, increasing digestibility and value.	CRISPR/Cas9 and RNAi used to reduce trypsin inhibitors and allergenic β-conglycinin, boosting soybean meal nutritional value (Krishnan, 2025). Breeding for higher 11S globulin content improves sulfur amino acid profile, enhancing protein quality (Guo et al., 2022).
Developing Pest and Disease Resistance	Insertion of genes conferring resistance to insect pests and diseases reduces crop losses and pesticide use.	Bt gene (Cry1Ac) combined with native QTLs (229-H, 229-M) confers resistance to multiple lepidopteran pests (Grossi-de-Sa et al., 2011). Pyramiding strategies combining synthetic Bt toxin and QTLs enhance durability of insect resistance (Grossi-de-Sa et al., 2011).
Producing Novel Compounds	Genetic engineering enables production of industrially valuable or health-promoting compounds in soybean.	Soybean oil genetically engineered for improved fatty acid profiles for industrial uses (not detailed in search but known in literature). Production of bio-based products like adhesives and bioplastics from modified soybean components (general knowledge).

To meet global demand and address climate change, soybean breeding needs acceleration. This review summarizes a decade of progress in soybean functional genomics, leveraging the 2010 reference genome. It covers advancements in omics (genomics, transcriptomics, epigenomics, proteomics), germplasm, gene discovery for traits like yield and stress resistance, and transformation technology, also discussing future challenges (Zhang et al., 2022). Researchers seek to optimize soybean architecture for higher yields. Overexpressing GmmiR156b significantly increased long branches, nodes, pods, and 100-seed weight, boosting yield by 46-63% without affecting plant height, but increasing stem thickness. This gene primarily acts by cleaving SPL transcripts. GmSPL9d, interacting with WUS, regulates branching. GmmiR156b is a promising target for soybean yield improvement (Sun et al., 2019). A soybean NAM population of 5,600 lines, evaluated across 22 environments using SNP markers, identified numerous significant associations for yield (23), maturity (19), plant height (15), lodging (17), and seed mass (29). Elite parents contributed more positive yield alleles, but exotic founders offered unique desirable ones, underscoring the need to broaden the US soybean genetic base (Diers et al., 2018).

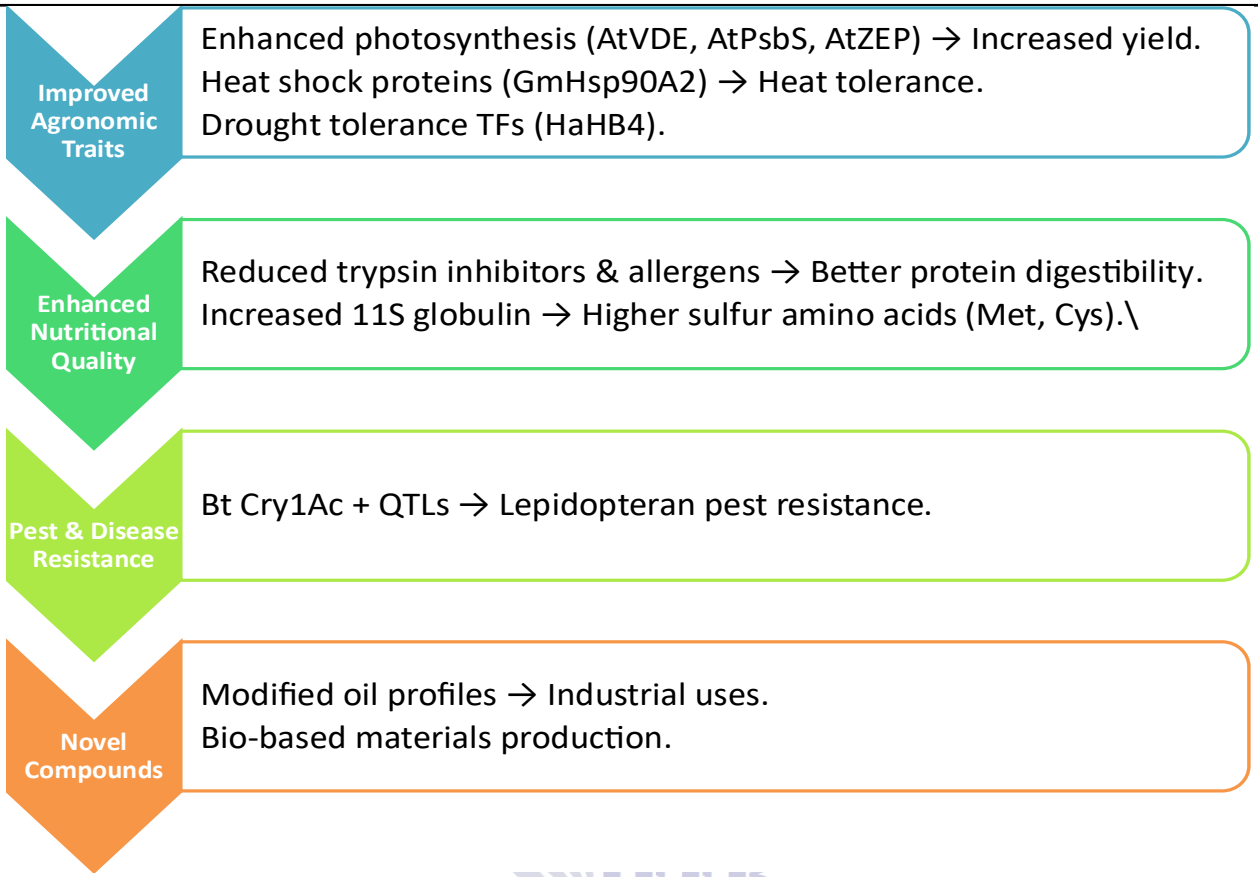

Figure 1: Schematic Overview of Genetic Transformation Benefits in Soybean.

Table 2: Examples of Genetic Transformation Targets and Outcomes in Soybean.

Trait Category	Target Gene(s) / Protein(s)	Methodology	Outcome / Benefit	Reference
Yield Improvement	AtVDE, AtPsbS, AtZEP	Transgenic overexpression	Up to 21.7% increase in seed yield	(De Souza et al., 2022)
Heat Stress Tolerance	GmHsp90A2, GmTIP2;6, GmDREB1	CRISPR/Cas9, transgenic lines	Enhanced heat tolerance, reduced oxidative stress	(Jianing et al., 2022)
Nutritional Quality	Trypsin inhibitors (KTi, BBi), β-conglycinin	CRISPR/Cas9, RNAi	Reduced antinutritional factors, improved digestibility	(Krishnan, 2025)
Pest Resistance	Bt gene (Cry1Ac), QTLs 229-H, 229-M, QTL-G	Transgenic + marker-assisted selection	Resistance to multiple lepidopteran pests	(Grossi-de-Sa et al., 2011)

I. Overview of Genetic Transformation:

Genetic transformation in plants is the process of introducing foreign DNA into a plant's genome to alter its genetic makeup, enabling the expression of new traits. This technology is widely used to improve crop traits such as yield, stress tolerance, disease resistance, and nutritional quality, as well as to produce novel compounds for industrial and pharmaceutical uses (John Innes Centre, 2017). Soybean, an ancient crop from Asia, is now a global

oilseed leader, with 80-85% grown in the Western Hemisphere. US breeding has shifted to private investment, utilizing diverse tools from classical to genome editing. Efforts focus on improving yield, seed quality, and stress resistance through advanced breeding and agricultural practices to meet rising global demand for protein and oil (Anderson et al., 2019). Plant regeneration, the process of tissue repair and replacement, is vital for global agricultural sustainability, especially with climate change. This review synthesizes advanced genetic transformation technologies across diverse plant and algal species, examining organellar modifications, crucial regeneration factors for *Agrobacterium*-mediated transformations, hormonal networks regulating regeneration, and comparative analyses of transient transformation and marker genes. It also identifies bottlenecks and suggests future research directions (Wang et al., 2025). Plant genetic transformation, vital for crop improvement and fundamental insights, still faces challenges in efficient transformation and regeneration for many crops despite decades of development. While FokI endonuclease-based genome editing offers exciting possibilities, its implementation is hindered by these same roadblocks. Future progress requires innovations in tissue culture simplification, discovery of genes controlling developmental reprogramming and homologous recombination, and the development of new, low-cost universal DNA/RNA/protein delivery systems. Advances in synthetic biology and novel vectors for precision genome editing could revolutionize crop potential, emphasizing the critical need for standardized, efficient plant transformation systems across species (Ramkumar et al., 2020).

Table 3. Brief Historical Context in Plant Biotechnology

Year / Period	Milestone	Significance
Late 19th Century	Discovery of crown gall disease caused by Agrobacterium tumefaciens	Identification of natural gene transfer mechanism from bacteria to plants
1973	Development of genetic engineering by Boyer and Cohen inserting DNA into bacteria	Foundation for recombinant DNA technology
1977-1983	Demonstration of Agrobacterium-mediated plant transformation and creation of first transgenic plants	Enabled stable integration of foreign genes into plant genomes
1985	Introduction of microinjection and biolistic (gene gun) methods	Alternative transformation methods allowing DNA delivery into plant cells
1990s	Commercial release of first GM crops including soybean	Start of modern GMO agriculture with traits like herbicide tolerance and insect resistance
2010s-2020s	Advances in genome editing (CRISPR/Cas9) and improved transformation protocols in soybean	Increased precision and efficiency in trait development and soybean genetic transformation

Source: (Somssich, 2019).

Trends in GM food perception research, finding that government decisions significantly sway consumer response. Public support for GM foods increases with clear communication of benefits, price discounts, and greater trust in science and government, bolstered by positive media. Europe and the USA lead in research output and citations. The study suggests policies for industry, research, and society to enhance GM food safety, consumer studies, and public awareness (Sendhil et al., 2022).

Table 4. Challenges and Opportunities in Soybean Transformation

Aspect	Challenges	Opportunities / Advances
Transformation Efficiency	Low transformation frequency; genotype dependency limits use across diverse soybean varieties	Protocol improvements in explant selection, culture media, and Agrobacterium strains have increased efficiency (>10%)
Genotype Flexibility	Many soybean genotypes are recalcitrant to transformation and regeneration	Development of genotype-flexible protocols and transient transformation methods
Protocol Complexity	Long, laborious, and technically demanding tissue culture and regeneration steps	Automation and optimization of tissue culture conditions; biolistic and Agrobacterium rhizogenic-mediated transient assays
Regulatory and Public Acceptance	GMO regulatory hurdles and consumer concerns	Genome editing offers non-transgenic alternatives; improved safety assessment frameworks
Trait Development	Need for multiple traits stacking (yield, stress tolerance, pest resistance)	Use of marker-assisted selection and gene pyramiding strategies; CRISPR/Cas9 multiplex editing

Source: (Homrich et al., 2012; Xu et al., 2022).

II. *Agrobacterium*-Mediated Transformation

Agrobacterium tumefaciens is a remarkable bacterium often referred to as nature's natural genetic engineer due to its unique ability to transfer a segment of its own DNA into plant cells and integrate it into the host plant's genome. This natural process is the basis of its pathogenicity, leading to the formation of crown gall tumors on infected plants. *Agrobacterium tumefaciens* transfers T-DNA and virulence (Vir) proteins into plant cells. While bacterial Vir protein roles are somewhat understood, their precise functions within plant cells and the involvement of host-encoded proteins in this genetic transformation remain largely unknown (Gelvin, 2000).

Agrobacterium tumefaciens has been transformed from a plant pathogen into a key tool for genetic engineering since the 1980s. While some bacterial modifications extended its host range to vital crops, improvements largely stemmed from optimizing plant tissue culture. Understanding both bacterial and host biology remains crucial for advancing *Agrobacterium*-mediated plant transformation (Gelvin, 2003). *Agrobacterium*, identified over a century ago as crown gall's cause, uses a Ti-plasmid's T-DNA as its "tumor inducing principle." This T-DNA, delivered by a Type IV secretion system, integrates into the plant genome, expressing genes for hormones and opines. This process, initiated by plant signals, explains *Agrobacterium*'s role as nature's genetic engineer (Nester, 2015). Gene transformation introduces and expresses foreign genes in a host. While various methods exist, plant gene transformation, often for transgenesis, is crucial. *Agrobacterium tumefaciens*, a natural genetic engineer, inserts its T-DNA into host plant nuclei, integrating or transiently replicating. Originally for dicots, protocols now enable monocot and fruit plant transformation, with new methods continually emerging for diverse applications (Shreni Agrawal, 2022).

Table 5: Principles of *Agrobacterium tumefaciens* as a Natural Genetic Engineer.

Key Principle	Description
Natural DNA Transfer Mechanism	Transfers T-DNA (Transfer DNA) from its Ti (Tumor-inducing) plasmid to plant cells, causing crown gall disease.
Ti Plasmid Components	Contains:

Plant Signal Recognition	Wounded plant cells release phenolic compounds (e.g., acetosyringone), activating vir genes via a two-component system (VirA/VirG).
Host Range	Naturally infects dicotyledonous plants but engineered to transform monocots, fungi, and even human cells.
Biotechnology Tool	Engineered Ti plasmids (binary vectors) replace oncogenes with foreign genes, enabling safe plant genetic modification.

Source: (Nester, 2015; Otten, 2018; Weir & Dalzell, 2020).

Agrobacterium tumefaciens-mediated transformation is the dominant plant genetic engineering technique, enabling stable/transient gene transfer, random/targeted integration, and genome editing. Its advantages include cost-effectiveness, high reproducibility, and capacity for large DNA fragments, including CRISPR/Cas systems. Ongoing research aims to optimize its effectiveness for diverse plant species (Azizi-Dargahlou & Pouresmaeil, 2024). The most common method for introducing new genes into plants uses *Agrobacterium tumefaciens*' natural DNA transfer. Research shows this mechanism evolved from ancient processes like bacterial conjugation, with T-DNA integration in plant chromosomes aided by bacterial proteins. This chapter details *Agrobacterium*'s molecular basis, focusing on virulence proteins and T-DNA characteristics, critically assessing its use for transgenic plant production (Gheysen et al., 2022). Gene transformation, altering a cell's genes by introducing exogenous DNA, revolutionizes crop improvement and functional genetics. Recombinant DNA technology now allows tailored genes for enhanced crop traits. Key methods include *Agrobacterium*-mediated transformation (first isolated 1897) and biolistics (gene gun, invented 1983-1986), facilitating the production of transgenic plants with desirable agronomic characteristics (Saeed & Shahzad, 2016).

Table 6: T-DNA Transfer Mechanism: Step-by-Step Process

Step	Mechanism	References
1. Bacterial Attachment	Agrobacterium binds to wounded plant cells via cellulose fibrils and surface proteins (e.g., VirB2).	(de la Riva et al., 1998; Lacroix & Citovsky, 2019)
2. Vir Gene Activation	Plant phenolic compounds activate VirA/VirG two-component system, inducing expression of vir genes.	(Lacroix & Citovsky, 2019) (Nutter et al., 1981)
3. T-DNA Processing	VirD1/VirD2 endonuclease excises T-DNA as a single-stranded molecule (T-strand) bound to VirD2.	(Nester, 2015) (ThompsonMitchell et al., 2020)
4. T-Complex Formation	T-strand + VirD2 + VirE2 (single-strand DNA-binding protein) form a protected T-complex.	(Nester, 2015) (Lacroix & Citovsky, 2019) (Dafny-Yelin et al., 2015)
5. Transfer via Type IV Secretion	T-complex is transported into plant cells through a VirB/D4-encoded pilus (type IV secretion system).	(Nester, 2015) (Costa et al., 2024) (Wen et al., 2024)
6. Nuclear Targeting	VirD2 and VirE2 contain nuclear localization signals (NLS), guiding T-complex to the plant nucleus.	(Nester, 2015) (Mendel & Hänsch, 2017) (Frangedakis et al., 2021)
7. T-DNA Integration	T-DNA integrates randomly into plant genome via non-homologous end joining (NHEJ), aided by plant repair machinery.	(Livitsanos, 2022) (Sunaryo, 2021) (Zaccaron et al., 2018)

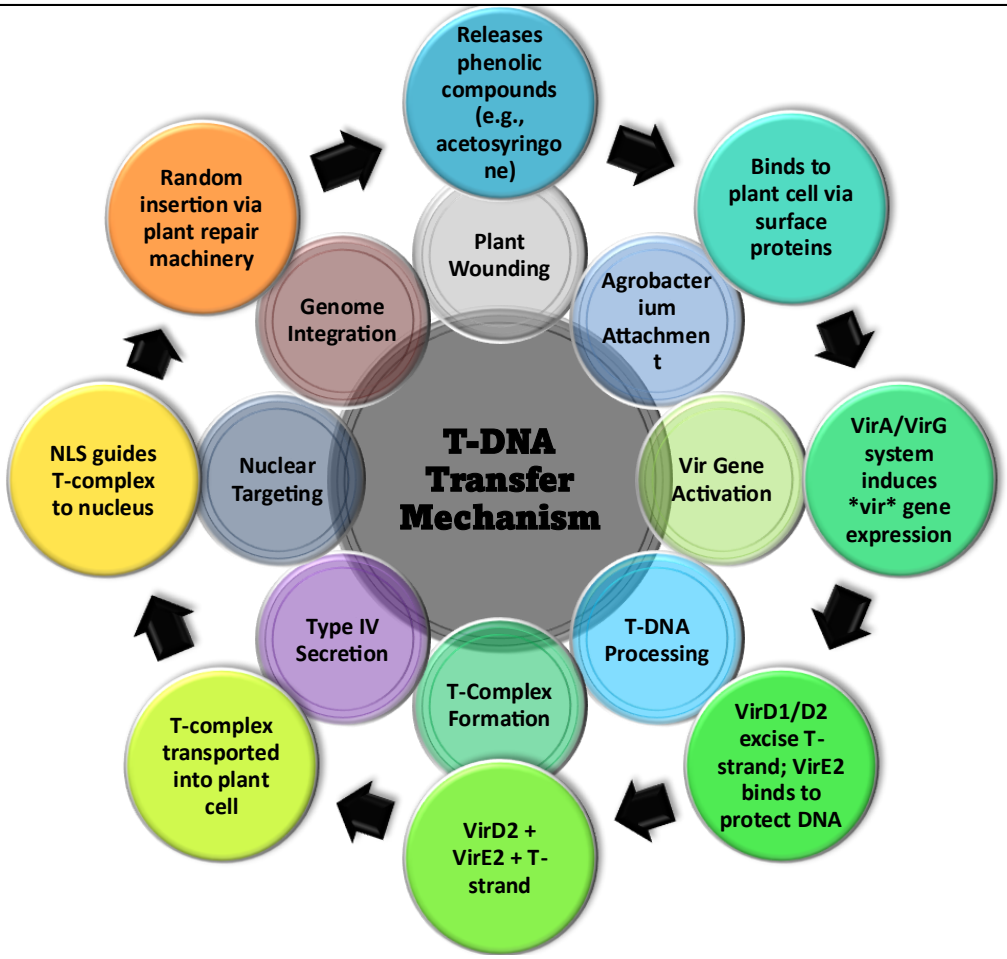

Figure 2: T-DNA Transfer Mechanism Flowchart

III. Biolistic (Particle Bombardment) Transformation

The biolistic method, also known as particle bombardment or gene gun transformation, offers a direct and versatile approach to introduce foreign DNA into a wide range of cells, particularly plant cells, overcoming some limitations of *Agrobacterium*-mediated transformation. The core principle involves direct DNA delivery into target cells. This is achieved by coating microscopic particles, typically made of gold or tungsten, with the desired DNA (Sanford et al., 1993). These DNA-coated micro-projectiles are then accelerated to high velocities using a device known as a "gene gun" or biolistic particle delivery system, employing a burst of high-pressure propulsion (Klein et al., 1987). The force of this propulsion allows the micro-projectiles to penetrate the cell wall and plasma membrane, delivering the DNA directly into the cytoplasm or even the nucleus of the target cells. This physical method of DNA delivery bypasses the biological host range limitations of *Agrobacterium*, making it effective for transforming monocots, organelles, and a broader array of plant species (Gordon-Kamm et al., 1990).

Plant genetic engineering is crucial for sustainable agriculture. While *Agrobacterium*, biolistics, electroporation, and PEG methods are widely used, they face limitations like species dependency, tissue damage, and low efficiency. Nanotechnology-based gene delivery offers promising alternatives, showing high efficiency, biocompatibility, and nucleic acid protection. Though nascent, this nano-strategy, combined with CRISPR-Cas, holds potential to revolutionize plant genetic transformation (Yan et al., 2022). Particle bombardment (biolistics) offers a versatile alternative to *Agrobacterium* for plant transformation, overcoming biological constraints and enabling organelle transformation. Its key

advantage is facilitating DNA-free gene editing by directly delivering proteins, RNAs, and RNPs. This broad applicability ensures particle bombardment remains a crucial tool for future plant research and biotechnology (Ozyigit & Yucebilgili Kurtoglu, 2020). A new MOF-Jet system enables controlled, needle-free delivery of stable protein, DNA, and RNA drugs encapsulated in ZIF-8 powder into tissues. Using CO2 as carrier gas provides burst release via ZIF-8 dissolution, while air leads to slow release over a week. This innovative biolistic approach offers a versatile tool for research and applications (Wijesundara et al., 2022).

Principles of Biolistic Transformation:

a) Direct DNA Delivery: Biolistic transformation bypasses biological vectors by physically delivering DNA into cells using high-velocity microprojectiles. This method is genotype-independent and avoids limitations of bacterial-mediated systems.

b) Micro-projectiles (Gold/Tungsten) Coated with DNA: DNA is bound to dense, inert metal particles (0.5–1.5 µm diameter) that penetrate cell walls and membranes. Gold is preferred for higher transformation efficiency and reduced cytotoxicity, while tungsten binds DNA more effectively but may inhibit cell growth (Ozyigit & Yucebilgili Kurtoglu, 2020).

c) High-Pressure Propulsion: A helium-driven gene gun accelerates DNA-coated particles into target cells. Adjustable pressure (450–2,200 psi) and particle size optimize penetration for different cell types (Uchida et al., 2009).

Table 7: Key Components of Biolistic Transformation.

Component	Description	Key Elements
Micro-projectiles	DNA-coated gold/tungsten particles (0.5–1.5 µm)	- Gold: Higher transformation efficiency, chemically inert
DNA Coating	DNA adhered to particles using spermidine, $CaCl_2$, or PEG	- Requires 1–10 µg DNA per bombardment
Propulsion System	Helium-driven gene gun (e.g., PDS-1000/He, Helios)	- Rupture disks control pressure (450–2,200 psi)
Target Tissues	Embryogenic callus, meristems, or cultured cells	- Vacuum applied (~28 inHg) to reduce air resistance during bombardment

Sources: Fribourg et al., 2009; (Wang et al., 2004).

Plant transformation, crucial for plant biology and crop development, utilizes various verified methods for stable gene introduction. While efficiency improvements are sought, many published techniques are impractical. Particle bombardment and *Agrobacterium*-mediated transformation remain the predominant and reliable methods for routine transgenic plant production across diverse species (Keshavareddy et al., 2018). An efficient biolistics method was developed for hornworts, enabling rapid transient expression (average 569 cells/bombardment, 48-72h for GFP) and stable transformation in *Anthoceros agrestis* (81 lines across 3 experiments, averaging 6 lines/bombardment). This versatile technique was also used for transient transformation in nine other hornwort species and confirmed Rubisco localization, offering a key genetic tool for this unique land plant lineage (Lafferty et al., 2024). A biolistics protocol successfully transformed immature Carrizo citrange epicotyls, achieving 18.4% stable transformation with GFP and nptII. Fluorescing tissues regenerated into shoots, confirmed as transgenic by PCR and Southern blot, displaying multiple/single gene copies. Optimizing tissue culture and sampling can enhance throughput for this system, enabling transformation with minimal gene cassettes (Wu et al., 2016). This protocol details wheat genetic transformation using the BioRad PDS/1000-He system. Immature wheat embryos (12-16 days post-anthesis) are precultured. Gold particles coated with desired DNA and a selectable marker (*bar* gene for bialaphos resistance) are delivered via bombardment. Early selection with PPT (phosphinothricin tripeptide, active in glufosinate-ammonium herbicides) during tissue culture enables

faster plantlet recovery with high efficiency (Sparks & Doherty, 2020).

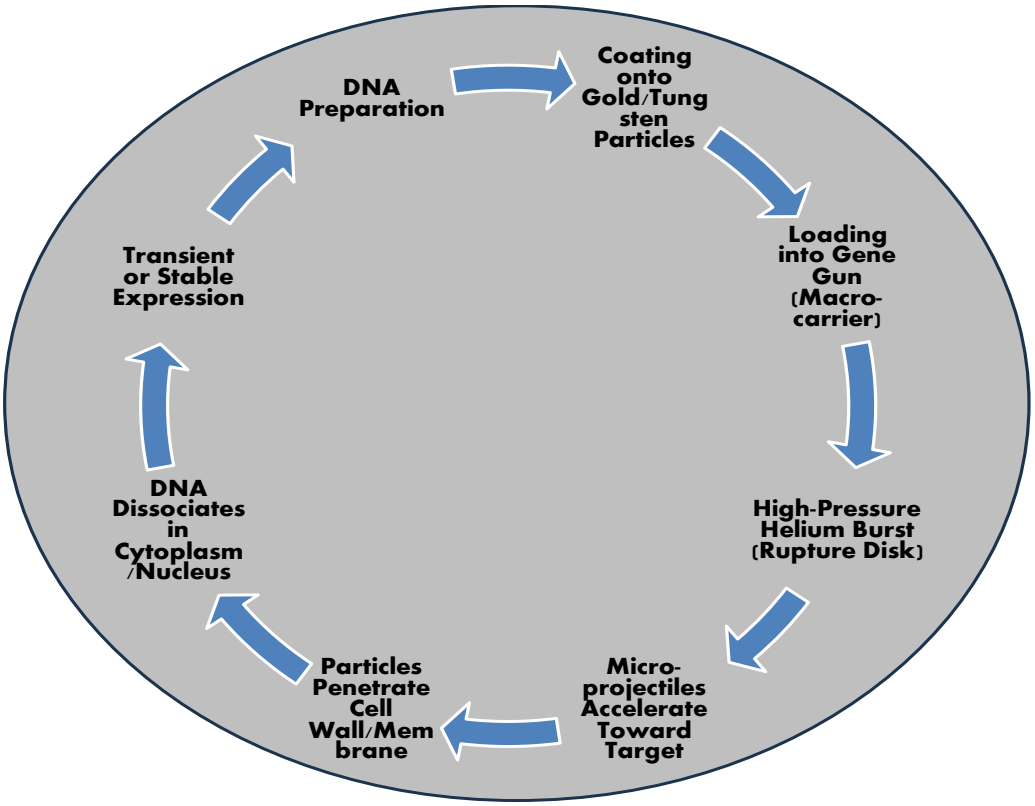

Figure 3: Biolistic Transformation Process.

Table 8: Gold vs. Tungsten Micro-projectiles

Parameter	Gold	Tungsten
DNA Binding	Moderate	High (due to surface charge)
Transformation Efficiency	Higher (plant cells: 10–20% transient)	Lower (often <5% stable)
Cytotoxicity	Low	High (inhibits cell growth post-bombardment)
Cost	Expensive	Affordable
Common Use	Stable transformation in plants/mammals	Transient assays, fungal transformation

Source: Fribourg et al., 2009; (Carter & Shieh, 2015) (Bulle et al., 2025).

IV. Emerging and Alternative Transformation Techniques

A. Protoplast-Mediated Transformation:

Protoplast-mediated transformation is a direct gene delivery technique that leverages the unique properties of isolated plant cells lacking cell walls. The principle hinges on the fact that the rigid plant cell wall normally acts as a significant barrier to the uptake of foreign DNA. By enzymatically removing this barrier, typically using enzymes like cellulase and pectinase, researchers can generate protoplasts – individual plant cells enclosed only by their plasma membrane (Davey et al., 2005). Once isolated, these "naked" protoplasts become highly susceptible to the direct DNA uptake from their surrounding environment. This uptake is often facilitated by chemical agents such as polyethylene glycol (PEG) and/or divalent cations like calcium ions. PEG is thought to induce transient pores in the plasma membrane and promote the association between the

DNA and the protoplast surface, thereby enhancing DNA entry (Negrutiu et al., 1987).

Another study optimized a protocol for isolating high-quality protoplasts and intact lytic vacuoles from mature sugarcane stalks. Optimal enzymatic treatment (2.5% Cellulase R-10, 0.6% Macerozyme R-10), 0.4 M mannitol, and 5 hours enzymolysis at 28°C yielded 11.36×104 protoplasts/g FW with high viability. Vacuoles, purified via Ficoll density gradient centrifugation and validated by neutral red/MDY-64 staining and a 43-fold α-mannosidase enrichment, showed minimal contamination and distinct metabolic roles compared to protoplasts (Thangavel et al., 2025). Plant genetic transformation, involving adding or deleting DNA segments, revolutionizes crop improvement by introducing transgenes for nutritional enhancement and stress resilience. This non-conventional approach transcends species barriers, greatly expanding possibilities for plant breeders to improve efficiency and protection (Quezada et al., 2024). Soybean, a vital pulse and oilseed, faces threats from pests and drought. Recombinant DNA technology, particularly *Agrobacterium tumefaciens*-mediated transformation, offers solutions to enhance nutrition and stress resistance. This book details soybean transformation, including *Agrobacterium* methods, *in vitro* culture, and emerging techniques, acknowledging *Agrobacterium*'s affordability and efficiency, while noting the potential of CRISPR/Cas despite cost and public acceptance challenges (Mangena, 2022).

Study developed an efficient protocol for isolating and transfecting soybean protoplasts from hypocotyls of the Williams 82 cultivar. Optimal conditions for protoplast isolation were 0.4 M mannitol, 1.5% cellulase, 0.4% macerozyme, and 8 hours digestion, yielding >3.0×106 protoplasts/g FW with high viability. PEG-Ca$^ (2+) $ mediated transfection parameters were evaluated. This robust system facilitates genetic trait analysis and understanding cell-to-cell interactions in soybean (Kim et al., 2025). A review advocated for in-planta *Agrobacterium tumefaciens* transformation as a simplified alternative for soybean genetic improvement, bypassing complex tissue culture. Despite fewer transgenic shoots, it significantly increases glufosinate-resistant plants. This method, though less reported, offers advantages over in-vitro approaches by reducing lengthy steps and common challenges like contamination and genotype specificity (Mangena, 2019).

Table 9: Protoplast-Mediated Transformation Principles and Challenges in Soybean.

Aspect	Description	References
DNA Uptake Method	PEG-mediated fusion of naked DNA with protoplast membrane	(Mathur & Koncz, 1998) (Yang et al., 2022)
Transformation Efficiency	High transient expression in many species (up to 60% in broccoli)	(Yang et al., 2022)
Regeneration Capacity	Low in soybean; regeneration from protoplasts not yet reported	(Xu et al., 2022)
Genotype Dependency	Regeneration efficiency varies widely among genotypes and tissue sources	(Reed & Bargmann, 2021)
Tissue Source Impact	Embryogenic callus and young tissues yield better regeneration than mature leaves	(Reed & Bargmann, 2021)
Main Limitation in Soybean	Difficulty in regenerating whole plants from protoplasts limits stable transformation use	(Paes de Melo et al., 2020)

Gene-editing offers immense potential for crop improvement, but delivering tools to the host genome and recovering edited plants are major bottlenecks. Method suitability varies by species and desired changes. Protoplast-mediated transient transformation and subsequent regeneration offer a unique strategy to overcome these challenges, providing a valuable approach for new plant breeding technologies despite its own specific advantages and complexities (Reed & Bargmann, 2021). Protoplasts are versatile tools for plant molecular biology and genome editing, enabling direct delivery of DNA,

RNA, or proteins and high-throughput validation of gene-editing reagents, including homology-directed repair templates for precise edits. This chapter details improved protocols for isolating and transforming soybean protoplasts from immature seeds, achieving 44% transfection efficiency (validated by GFP), and outlines a method for gene editing in soybean protoplasts using single guide RNA (Patil et al., 2022). CRISPR/Cas technology has revolutionized soybean genome editing, proving more precise and cost-effective than earlier methods like ZFN and TALENs for enhancing agronomic traits, nutrition, and stress tolerance. However, challenges persist in optimizing CRISPR cassette design, improving transformation frequency, increasing editing efficiency in target cells, and boosting overall crop production. This review addresses these bottlenecks, offering insights and practical suggestions for more effective genome editing in elite soybean cultivars, predominantly relying on *Agrobacterium*-mediated and particle bombardment methods for stable transformation (Freitas-Alves et al., 2024). Soybean, a vital, cost-effective protein and oil source, faces production limits due to cultivation area and climate. Plant tissue culture techniques like embryo culture, somatic embryogenesis, organo-genic differentiation, and protoplast culture offer solutions for mass propagation of high-quality, high-yield varieties in limited spaces, free from seasonal constraints, benefiting growers and consumers (Singh et al., 2020).

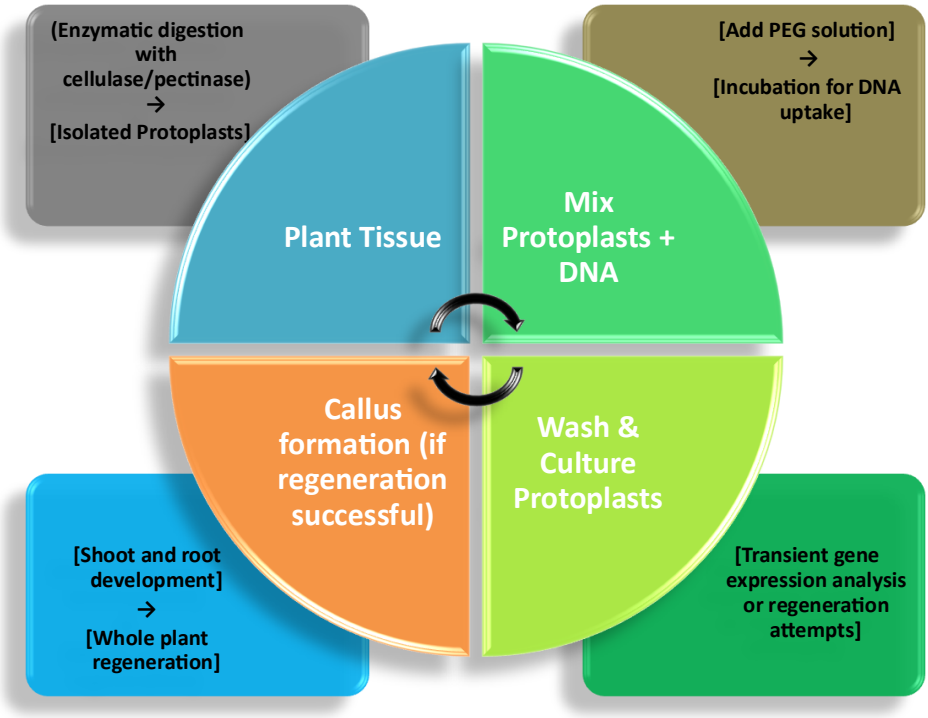

Figure 4: PEG-Mediated Protoplast Transformation Workflow.

Table 10: Challenges in Soybean Protoplast Regeneration.

Stage	Status in Soybean	Notes
Protoplast Isolation	Efficient isolation protocols available	Enzymatic digestion yields viable protoplasts
DNA Uptake (Transformation)	High transient expression possible	PEG-mediated transformation effective for transient assays
Callus Induction	Very low or no callus formation from protoplasts	Major bottleneck; soybean protoplasts fail to dedifferentiate into regenerable callus

Shoot Regeneration	Not reported from protoplast-derived callus	Regeneration protocols from explants exist but not from protoplasts
Whole Plant Regeneration	Not achieved from protoplasts	Limits stable transformation and breeding applications

Sources: (Xu et al., 2022); (Reed & Bargmann, 2021).

B. Electroporation: Principles and Application in Soybean.

Electroporation, also known as electro-permeabilization, is a physical method for gene delivery that relies on the application of brief, high-voltage electric pulses to a suspension of cells or tissues (Kandušer & Miklavčič, 2008). The fundamental principle is that these electrical pulses induce a temporary destabilization and increase in the permeability of the cell's plasma membrane, leading to the formation of nanoscale, transient pores (Microbe Notes, 2023). This phenomenon is often explained by the "transient aqueous pore model," where hydrophilic pores form in the lipid bilayer of the cell membrane when subjected to an external electric field (LBK Electroporation, 2009).

When cells are suspended in a solution containing the desired DNA (or other macromolecules like RNA, proteins, or RNP complexes) and then subjected to these electric pulses, the DNA can move through these newly formed pores and enter the cell's cytoplasm (Microbe Notes, 2023; Thermo Fisher Scientific, n.d.). Once inside, the DNA can be transiently expressed or, in the case of stable transformation, integrate into the host cell's genome (Microbe Notes, 2023). The parameters of the electric pulse, such as field strength, pulse length, and number of pulses, are crucial for optimizing transformation efficiency and cell viability, as excessive or prolonged pulses can lead to irreversible membrane damage and cell death (Microbe Notes, 2023; Thermo Fisher Scientific, n.d.). When cells are suspended in a solution containing the desired DNA (or other macromolecules like RNA, proteins, or RNP complexes) and then subjected to these electric pulses, the DNA can move through these newly formed pores and enter the cell's cytoplasm (Microbe Notes, 2023; Thermo Fisher Scientific, n.d.). Once inside, the DNA can be transiently expressed or, in the case of stable transformation, integrate into the host cell's genome (Microbe Notes, 2023). The parameters of the electric pulse, such as field strength, pulse length, and number of pulses, are crucial for optimizing transformation efficiency and cell viability, as excessive or prolonged pulses can lead to irreversible membrane damage and cell death (Microbe Notes, 2023; Thermo Fisher Scientific, n.d.).

In plant genetic engineering, electroporation has found its most common and effective application with protoplasts – plant cells from which the rigid cell wall has been enzymatically removed (News-Medical.Net). The absence of the cell wall makes protoplasts particularly amenable to membrane permeabilization by electric pulses, allowing for efficient direct DNA uptake. This approach is highly valuable for studying gene function, subcellular localization, and performing high-throughput screens, including CRISPR/Cas9-mediated genome editing (Frontiers, 2023; ScienceDirect, 2021). For soybean specifically, electroporation has been extensively used for the transient expression of introduced DNA in protoplasts derived from various tissues, including cotyledons and trifoliate leaves (Shivashakarappa et al., 2025). Recent advancements include the standardization of protocols for delivering CRISPR/Cas9 ribonucleoprotein (RNP) complexes into soybean protoplasts via electroporation for efficient DNA-free gene editing (Subburaj & Agapito-Tenfen, 2023).

Feature	Description
Electric Pulse	Short high-voltage pulses (e.g., 300–400 V/cm, <1 ms) create transient membrane pores
DNA Uptake Mechanism	DNA electro-phoretically moves to membrane, adsorbs, then enters cytosol via pores or endocytosis

Pore Dynamics	Small pores form quickly and may flicker; larger pores allow DNA entry; pores reseal within minutes
DNA Presence Timing	DNA must be present before or during pulse; post-pulse addition ineffective
Intracellular Trafficking	DNA interacts with cytoplasmic proteins and microtubules for nuclear transport

Sources: (Young & Dean, 2015); Microbe Notes; Electroporation – Wikipedia; (Kandušer & Miklavčič, 2008).

C. Silicon Carbide Whisker-Mediated Transformation: Principles and Status in Soybean

Silicon carbide (SiC) whisker-mediated transformation is a physical method for direct gene delivery into plant cells that offers an alternative to biological vectors like Agrobacterium and other physical methods such as particle bombardment (Asad & Arshad, 2011). The fundamental principle relies on the use of sharp, needle-like silicon carbide whiskers to create microscopic punctures in the plant cell walls and membranes (Ortiz-Matamoros et al., 2018).

The process typically involves mixing plant cells or explants (such as embryogenic callus or cell suspensions) with plasmid DNA and a suspension of SiC whiskers in a small tube (Que et al., 2014). This mixture is then subjected to vortexing or oscillation (Kaeppler et al., 1992). During this agitation, the abrasive, negatively charged SiC whiskers physically penetrate the cell walls and plasma membranes, acting like numerous fine needles (Asad & Arshad, 2011). This transient permeabilization allows the exogenous DNA, present in the surrounding medium, to enter the plant cells. While the exact mechanism of DNA entry is still debated, it is understood that the whiskers create channels through which the DNA can pass into the cytoplasm, where it can then be stably integrated into the host genome or transiently expressed (Su et al., 2023). This method is considered relatively simple, inexpensive, and does not require specialized equipment like a gene gun.

Table 12: Comparison of Silicon Carbide Whisker-Mediated Transformation Principles and Status in Soybean.

Aspect	Description
Physical Mechanism	Micro-puncturing of cells by sharp silicon carbide whiskers during vortexing or sonication
DNA Binding	Whiskers do not bind DNA; DNA enters through micro-wounds created by whiskers
Transformation Efficiency	Depends on whisker amount, vortexing/sonication intensity, and tissue type; balanced with cell viability
Tissue Culture Requirements	Requires specific callus types with favorable morphology for regeneration and DNA uptake
Application in Soybean	Explored but less widely adopted; limited stable transformation reports; regeneration challenges
Advantages	Simple, cost-effective, potentially single-copy insertions, no need for sophisticated equipment
Limitations	Cell damage risk, low reproducibility, genotype and tissue dependency, lower adoption than other methods

Source: (Akram et al., 2016); (Asad et al., 2008); (Terakawa et al., 2005).

Silicon carbide whisker-mediated transformation relies on the physical puncturing of plant cells by sharp whiskers to facilitate DNA uptake. While it is a simple and cost-effective method, its use in soybean has been limited by challenges in regeneration and inconsistent transformation efficiency. Compared to *Agrobacterium* and biolistic methods, SCW transformation is less widely adopted but remains a valuable alternative, especially where other methods face limitations.

Table 13: Key Parameters Affecting Silicon Carbide Whisker Transformation Efficiency

Parameter	Effect on Transformation	Notes
Whisker Quantity	Higher whisker amounts increase DNA delivery but reduce cell survival	Balance needed for optimal efficiency
Vortexing/Sonication Time	Longer exposure increases transformation but damages cells	Sonication combined with centrifugation improves efficiency
Tissue Age	Younger callus tissues show higher transformation rates	Optimal subculture days vary by species
Centrifugation Treatment	Increases physical contact between whiskers and cells, enhancing DNA uptake	Combined with sonication for best results

Sources: (Asad et al., 2008); (Terakawa et al., 2005)

D. Viral Vector Systems (e.g., Geminiviruses): Principles and Potential in Soybean

Viral vector systems harness the natural ability of plant viruses to infect host cells, replicate, and express their genetic material, by modifying them to deliver and express foreign genes (Zaidi & Mansoor, 2017). These systems exploit various types of plant viruses, including both RNA viruses (e.g., Tobacco rattle virus, TRV) and DNA viruses (e.g., Geminiviruses) (Mahmood et al., 2023).

The core principle involves replacing or inserting target genes into the viral genome while removing or inactivating the viral pathogenicity genes (Mahmood et al., 2025). The modified viral vectors are then introduced into plant cells, often via Agrobacterium-mediated delivery (known as agroinfiltration or magnification) or biolistics (Gleba et al., 2013). Once inside the host cell, the viral machinery takes over, leading to high-level replication of the viral genome (now containing the foreign gene) and subsequent robust expression of the foreign gene (Mahmood et al., 2025). This can result in rapid and abundant production of the desired protein or RNA within the plant. A key advantage of many viral vectors is their ability to move systemically throughout the plant, leading to widespread gene expression without requiring stable integration into the plant genome (Mahmood et al., 2023).

In soybean (Glycine max), viral vector systems, particularly those derived from Geminiviruses (like Bean yellow dwarf virus, BeYDV), have shown significant potential, primarily for transient expression and gene silencing studies (Fuhrmann-Aoyagi et al., 2024). For transient expression, modified geminiviruses can deliver genes that are quickly expressed at high levels throughout the infected plant, allowing for rapid functional genomics studies, protein production, and even the evaluation of genome editing reagents like CRISPR/Cas9 (Subburaj & Agapito-Tenfen, 2023). This circumvents the lengthy tissue culture and regeneration steps required for stable transformation, dramatically reducing experimental timelines (Mahmood et al., 2025).

For gene silencing studies, such as virus-induced gene silencing (VIGS), viral vectors (e.g., those derived from Bean pod mottle virus, BPMV, a comovirus, or Geminiviruses) are engineered to carry a fragment of a host gene (Rustgi et al., 2022). When the recombinant virus infects the plant, the host's natural antiviral defense mechanisms (RNA interference) target the viral RNA, leading to the degradation of both the viral RNA and the homologous endogenous host gene mRNA, effectively "silencing" the gene. This allows researchers to infer gene function by observing the resulting phenotypes (Kachroo & Ghabrial, 2012).

However, a significant challenge in stable integration remains a limiting factor for widespread application of viral vectors in generating commercially viable transgenic soybean lines. Most plant viruses, including Geminiviruses, typically replicate episomally (as circular DNA or RNA in the cytoplasm or nucleus) and do not integrate their genetic material into the host plant's chromosome (Zaidi & Mansoor, 2017). While transient expression is invaluable for research, stable integration is crucial for heritable traits in crop improvement. Overcoming this hurdle by developing strategies for efficient, targeted integration of viral-delivered DNA into the plant genome is an active area of research (Rustgi et al., 2022). Despite these

challenges, ongoing innovations in viral vector design and delivery methods are continually expanding their utility in plant biotechnology (Mahmood et al., 2025).

Table 14: Overview of Gemini-virus-Based Vector Systems

Feature	Description	References
Virus Type	Geminiviruses (e.g., Mastrevirus, Begomovirus)	(Mahmood et al., 2025)
Genome	Single-stranded circular DNA (~2.5–3 kb)	(Bhattacharjee & Hallan, 2022)
Delivery Methods	Agrobacterium-mediated agroinfiltration, biolistic bombardment	(Mach, 2014)
Expression Type	Transient high-level expression of foreign genes; gene silencing (VIGS)	(Bhattacharjee & Hallan, 2022)
Insert Size Limit	Small inserts only (~<3 kb)	(Stanley, 1993)
Stable Integration	Rare; vectors replicate episomally; stable transgenics require additional integration methods	(Mach, 2014)
Applications	Functional genomics, protein production, gene silencing, genome editing (HDR)	(Mahmood et al., 2025) (Bhattacharjee & Hallan, 2022)
Advantages	Rapid expression, high yield, cost-effective, avoids lengthy stable transformation	(Regnard et al., 2010)
Limitations	Insert size, lack of stable integration, delivery method dependence	(Mahmood et al., 2025) (Regnard et al., 2010)

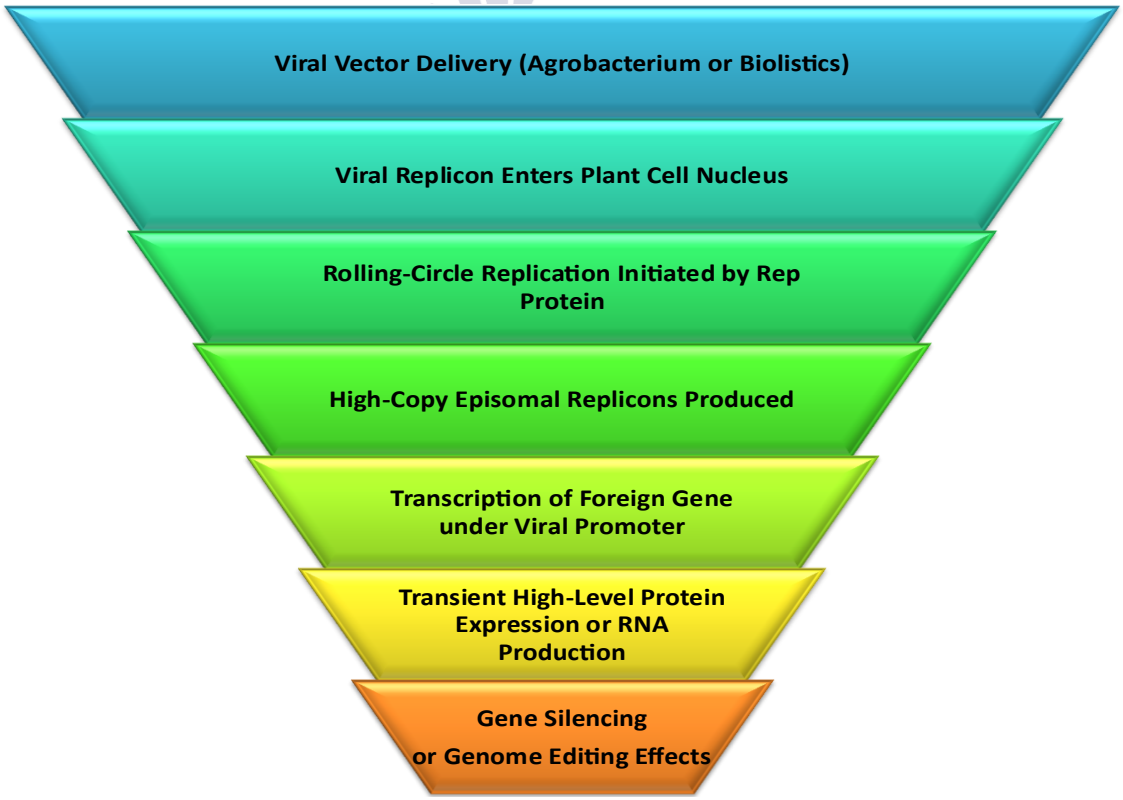

Figure 4: Gemini-virus Vector Replication and Expression Cycle.

VI. Future Directions and Impact

A. Advancements in Gene Editing Technologies (CRISPR-Cas9):

The advent of CRISPR-Cas9 technology has revolutionized plant genetic engineering by enabling precise and targeted gene modification, moving beyond the often-random insertion of foreign DNA associated with traditional transgenic approaches (Erdoğan et al., 2023). Unlike older methods that could insert multiple gene copies at unpredictable locations in the genome, leading to potential unintended effects, CRISPR-Cas9 utilizes a guide RNA (gRNA) to direct the Cas9 nuclease to a highly specific DNA sequence (Erdoğan et al., 2023). This precise targeting allows for the introduction of specific edits, such as gene knockouts (disrupting a gene's function), single-base substitutions, or even the precise insertion of new genetic material at a pre-determined site through homology-directed repair (Chen et al., 2024). This specificity significantly reduces the risk of off-target mutations and undesirable phenotypic changes, making genome editing a more controlled and predictable process for crop improvement (Ahmad et al., 2023).

One of the most significant impacts of CRISPR-Cas9 technology, particularly for public acceptance and regulatory frameworks, is its potential to create "non-GMO" solutions in plant breeding (Ahmad, 2023). This concept arises from the ability of CRISPR-Cas9 to induce precise genetic changes without necessarily introducing foreign DNA from other species (NCSU, 2020). When CRISPR-Cas9 is used to make small edits, such as deletions, insertions of a few nucleotides, or changes to existing DNA sequences (e.g., base editing or prime editing), the resulting plant may contain only minor modifications that could theoretically occur through conventional breeding or natural mutation (Chen et al., 2024). Furthermore, methods for delivering the CRISPR components (Cas9 protein and guide RNA) directly into plant cells without integrating the Cas9 gene itself into the host genome (e.g., via protein delivery to protoplasts, or transient expression systems) result in "transgene-free" edited plants (Erdoğan et al., 2023).

Since the mid-1990s, GMOs (first generation) have been cultivated. Newer genetic engineering, like genome editing (CRISPR, ZFN, TALEN), creates novel traits and broader genetic combinations, but faces risk assessment challenges (e.g., RNAi-based crops lacking EFSA guidance). While precise, genome editing struggles with complex, polygenic traits like drought tolerance, where conventional breeding, encompassing whole-genome regulation, remains more suitable (Cotter et al., 2020). CRISPR/Cas and RNAi technologies are revolutionizing plant breeding by enabling precise gene modification for improved yield, nutrition, and stress resistance. However, their widespread adoption faces hurdles like inherent technical limitations, public perception, and regulatory complexities. Scientists are actively researching ways to enhance specificity, uptake, and stability of these tools while minimizing off-target effects and environmental risks. This includes exploring strategies to increase on-target precision, leveraging nanotechnology, and addressing public concerns and diverse global regulatory frameworks (Touzdjian Pinheiro Kohlrausch Távora et al., 2022). Modern breeding aims for food security by developing resilient crops against stressors. While traditional genetic engineering is laborious, OMICS and advanced tools like TALENs, ZFNs, and MNs allow targeted mutagenesis. However, CRISPR/Cas9 stands out as a cost-effective, easy-to-design, and versatile tool for precise plant genome editing, including multiplex editing and knockouts, despite nascent regulatory frameworks for its products (Razzaq et al., 2019).

Table 15. Targeted Gene Modification Without Random Integration.

Technology	Mechanism	Applications in Plants	References
DNA-Free RNP Delivery	Cas9 protein + guide RNA complexes (RNPs) delivered directly into cells, bypassing DNA vectors.	Soybean protoplast editing with 4.2–18.1% mutation frequency.	(Subburaj et al., 2022)
Transient Transformation	CRISPR components delivered via Agrobacterium T-DNA without genomic integration.	Birch plants edited without foreign DNA integration.	(Sun et al., 2024)

Base Editing	Catalytically impaired Cas9 fused with deaminases for single-nucleotide changes.	Precise C-to-T or A-to-G substitutions without double-strand breaks.	(Azeez et al., 2024)
Prime Editing	Cas9 nickase + reverse transcriptase for precise insertions/deletions.	Complex edits (e.g., 30 bp deletions) in soybean protoplasts.	(Khoshandam et al., 2024)

Table 16. Potential for "Non-GMO" Solutions.

Approach	Description	Advantages	References
RNP-Based Editing	No foreign DNA introduced; transient Cas9-gRNA complexes degrade naturally.	Avoids GMO classification; reduces off-target risks.	(Kanchiswamy et al., 2015)
Agroinfiltration	Transient delivery of CRISPR components via Agrobacterium without T-DNA integration.	Edits germline cells for heritable mutations without transgenes.	(Liu et al., 2019) (Sun et al., 2024)
Viral Vector Delivery	Engineered plant viruses deliver CRISPR components transiently.	Rapid editing without genomic integration; suitable for field crops.	(Mollanoori & Teimourian, 2018) (Bhattacharjee et al., 2022)

CRISPR-Cas9 advancements enable targeted gene modification without random integration, leveraging DNA-free RNP delivery, transient transformation, and precision tools like base/prime editing. These methods offer non-GMO solutions by avoiding foreign DNA integration, with applications in soybean and other crops. Challenges remain in delivery efficiency and regulatory alignment, but ongoing innovations promise to expand the scope of genome editing in agriculture.

Table 17: Comparison of Traditional vs. Non-GMO CRISPR Strategies

Parameter	Traditional CRISPR (DNA-Based)	Non-GMO CRISPR (DNA-Free)
Integration Risk	Random T-DNA or vector integration possible.	No foreign DNA integration.
Regulatory Status	Often classified as GMO.	May avoid GMO regulations in some jurisdictions.
Editing Window	Limited by delivery efficiency and tissue culture requirements.	Broader applicability in recalcitrant species (e.g., soybean).
Key Tools	Binary vectors, antibiotic selection.	RNPs, viral vectors, transient transformation.

B. High-Throughput Transformation Systems:
High-throughput transformation systems in soybean are evolving rapidly to overcome the plant's historical recalcitrance to genetic manipulation and meet the demands of modern breeding and functional genomics. These systems aim to accelerate the process of introducing and expressing foreign genes, or making precise edits, in a large number of soybean lines (Zhong et al., 2024).

Key recent advancements and approaches for high-throughput transformation in soybean include:

- **Genotype-Independent Fast Transformation (GiFT):** A novel method that significantly improves operational efficiency and reduces costs by minimizing the need for extensive tissue culture. GiFT utilizes germinated seeds as explants, followed by *Agrobacterium* infection of wounded explants and direct transplantation into soil

with herbicide selection (GUO et al., 2018). This method can generate greenhouse-ready T0 transgenic plants in about 35 days, is applicable to diverse genotypes, and can be used with both conventional binary vectors and CRISPR-Cas12a for genome editing. T1 progeny show high inheritance rates, making it suitable for industry-scale applications (Wang et al., 2025).

- **Optimized *Agrobacterium*-mediated Transformation:** While *Agrobacterium* remains the most widely used method due to its low copy number and stable transgene integration, continuous efforts focus on optimizing its efficiency and throughput in soybean (Paes de Melo et al., 2020). Recent improvements include the use of specific explant types (like split-seed explants with partial embryonic axis), optimized culture media components, and enhanced *Agrobacterium* strains. For example, some protocols have achieved transformation frequencies of up to 18.7% for certain genotypes (Riaz et al., 2025).
- **Protoplast-based Systems for Transient Expression and Genome Editing:** Soybean protoplasts provide a versatile platform for high-throughput transient gene expression, functional gene analysis, and rapid validation of genome editing reagents like CRISPR/Cas9 ribonucleoproteins (RNPs) (Zhong et al., 2024). Automation of protoplast isolation and transformation, including robotic systems for promoter screening and gene validation, has been demonstrated, enabling high-throughput analysis relevant to shoot tissues in whole plants (Sultana et al., 2019).
- **Integration with Robotics and Automation:** The future of high-throughput transformation involves increasing automation of labor-intensive steps in plant tissue culture and transformation (Chennareddy et al., 2018). Robotic systems are being developed for precise explant preparation, transfer to media, subculturing, and quality control, aiming to increase throughput and consistency while minimizing contamination risks (Plant Cell Technology, 2025; ORNL, 2021). While still in development, these technologies have the potential to revolutionize soybean transformation.
- **Improved CRISPR/Cas Systems and Delivery:** The efficiency of CRISPR/Cas9-mediated genome editing in soybean heavily relies on efficient transformation systems. Advances in CRISPR cassette design, gRNA efficacy, and delivery methods (primarily *Agrobacterium* and particle bombardment) are continuously refined to increase editing efficiency and target multiple genes simultaneously (Liang et al., 2023).

Table 18. Automation and Optimization Strategies.

Component	Advancements	Impact on Throughput	References
Split-Seed Explant	Use of imbibed split seeds with partial embryonic axis for Agrobacterium-mediated transformation	Simplified protocol; achieved 18.7–20.3% transformation efficiency	(Pareddy et al., 2020) (Xu et al., 2022)
Agrobacterium Strain	EHA105 strain outperforms EHA101, producing higher low-copy transgene events	Reduced screening effort; improved quality of transgenic lines	(Pareddy et al., 2020) (Xu et al., 2022)
Selection Markers	Phosphinothricin acetyl transferase (PAT) for glufosinate tolerance	Efficient selection with lower escape rates; compatible with automation	(Pareddy et al., 2020)
Culture Medium	Antioxidant-rich media (e.g., cysteine, silver nitrate) to reduce oxidative stress	Enhanced regeneration rates; reduced tissue necrosis	(Xu et al., 2022) (Homrich et al., 2012)
Automated Tissue Culture	Robotic systems for explant preparation, infection, and transfer	Scalable workflows; reduced human error and labor costs	(Xu et al., 2022) (Zhao et al., 2024)

Table 19. Key High-Throughput Protocols.

Method	Features	Transformation Efficiency	Genotype Flexibility
Split-Seed Explant	Uses imbibed seeds split along hilum; retains partial embryonic axis for regeneration	Up to 20.3%	Limited to select genotypes
GRF3-GIF1 Chimera	Overexpression of growth-regulating factors (GRF3) and GRF-interacting factor 1 (GIF1)	2–4x improvement in regeneration	Broad applicability across genotypes
Ochrobactrum haywardense	Novel bacterial vector with higher single-copy insertion rates than Agrobacterium	Up to 35% in some genotypes	Compatible with diverse cultivars
Biolistic Optimization	Gold microparticles coated with DNA; automated bombardment systems	~5–10% (stable transformation)	Limited by tissue damage

High-throughput soybean transformation systems leverage split-seed explants, optimized Agrobacterium strains, and automation to achieve efficiencies exceeding 20%. Innovations like the GRF3-GIF1 chimera and novel bacterial vectors (e.g., Ochrobactrum) address genotype limitations and improve regeneration. Integration with machine learning and robotic platforms further accelerates large-scale studies, enabling rapid development of transgenic and gene-edited soybean lines for research and breeding.

Table 20: Comparison of Traditional vs. High-Throughput Soybean Transformation.

Parameter	Traditional Methods	High-Throughput Systems
Transformation Efficiency	0.3–8.7%	10–35% (depending on protocol)
Time per Cycle	6–12 months	3–6 months with automated workflows
Labor Intensity	Manual explant preparation and selection	Robotic handling and automated imaging
Genotype Flexibility	Limited to a few amenable cultivars	Expanded to recalcitrant genotypes (e.g., via GRF3-GIF1)
Cost per Event	High (due to low efficiency and manual labor)	Reduced by scalability and automation

C. Impact on Soybean Breeding and Agriculture:

The rapid advancements in plant genetic transformation, particularly in soybean, are profoundly impacting breeding programs and agriculture by enabling accelerated trait development, supporting sustainable practices, and directly addressing global food security (Homrich et al., 2012).

Accelerated Trait Development: Modern breeding strategies, including advanced genetic engineering techniques like CRISPR-Cas9, are significantly accelerating the development of desirable traits in soybean (Vargas-Almendra et al., 2024). Unlike traditional breeding, which can take decades, new approaches allow for precise and rapid genetic modifications. For instance, the Genotype-independent Fast Transformation (GiFT) method can produce greenhouse-ready T0 transgenic soybean plants in approximately 35 days, greatly reducing the breeding cycle time (Zhong et al., 2024). This acceleration is further enhanced by integrating speed breeding, genomic selection, high-throughput phenotyping, and marker-assisted selection (Liang et al., 2023). CRISPR-Cas9, being cost-effective and easy to design, facilitates targeted gene modification, including single-base substitutions and multiplex gene editing, leading to improved yield, quality, and resistance to biotic and abiotic stresses (Naveed et al., 2022). This rapid genetic gain allows breeders to quickly respond to emerging challenges and consumer demands.

Sustainable Agriculture Practices: Genetic engineering in soybean contributes to sustainable agriculture by developing varieties that reduce the environmental footprint of farming. Traits such as drought tolerance, pest resistance, and herbicide tolerance in genetically modified (GM) soybeans significantly reduce the need for external inputs like water, pesticides, and herbicides (Vargas-Almendra et al., 2024). For example, herbicide-resistant soybeans (e.g., Roundup Ready) facilitate no-till or strip-till farming, which reduces soil erosion, conserves soil moisture, increases soil organic matter, and lowers fuel consumption (Soy Connection, n.d.). Drought-tolerant varieties, like HB4 soybean, improve water use efficiency, making soybean cultivation more resilient to climate change and reducing reliance on irrigation (Wikipedia, 2025; Genetic Literacy Project, 2024). These practices contribute to better soil health, reduced greenhouse gas emissions, and overall resource efficiency, promoting more environmentally friendly farming systems (Soy Connection, n.d.).

Addressing Global Food Security: Soybean is a critical crop for global food security, serving as a primary source of protein and oil for human consumption, animal feed, and industrial products (Guo et al., 2022). With a rapidly growing global population, increasing agricultural productivity and resilience is paramount. Genetic engineering, including GM and gene-edited soybeans, plays a vital role by:

a) **Increasing Yield and Nutritional Value:** By enhancing traits like yield potential, protein content, and oil composition, these technologies directly contribute to increasing the available food supply. For instance, breeding efforts focus on improving oil composition, raising total protein content, and enhancing protein quality (Tyagi et al., 2025).

b) **Enhancing Stress Tolerance:** Developing soybean varieties resilient to changing climatic conditions (e.g., drought, heat, flood) and resistant to pests and diseases ensures more stable and reliable harvests, even in challenging environments (Genetic Literacy Project, 2024). This is particularly important for regions facing increasing environmental stressors (NDSU, 2025).

c) **Reducing Production Costs:** Traits like insect and herbicide resistance can lower farmers' input costs, making soybean production more economically viable and accessible, especially for smallholder farmers, thereby contributing to local food security and poverty reduction (The Borgen Project, 2014).

d) **Diversifying Production:** The development of varieties adapted to diverse agroecological zones promotes global accessibility and ensures tolerance to varying climates and soil conditions, further bolstering food supply resilience (NDSU, 2025).

Table 21. Accelerated Trait Development.

Trait Category	Examples	Mechanism	Impact
Yield Improvement	Enhanced photosynthesis efficiency via CRISPR-edited promoters	Increased light and water use efficiency; up to 21.7% yield gains in trials	Reduces breeding cycles from 10+ years to <3 years
Stress Resistance	Drought-tolerant soybeans with HaHB4 transgene	Overexpression of stress-responsive transcription factors	Maintains yield under water-limited conditions
Nutritional Quality	High-oleic acid soybeans (CRISPR-edited FAD2 genes)	Reduced polyunsaturated fats; improved oil stability	Eliminates trans fats in food products; extends shelf life
Pest/Disease Resistance	Bt soybean varieties with Cry1Ac + native QTLs	Pyramided insect resistance genes	Reduces pesticide use by 37–60% in field trials

Source: (Yao et al., 2023)

While challenges like public perception and regulatory frameworks persist, the continuous advancements in soybean genetic modification are undeniable in their capacity to accelerate breeding, foster sustainable practices, and address the pressing demands of global food security (Singh & Nanda) (Vargas-Almendra et al., 2024). Biotechnology and precision agriculture offer a synergistic solution for global food security. Precision agriculture optimizes resources and minimizes environmental impact, while

biotechnology creates resilient, nutritious crops. Their integration tackles climate change effects and reduces agriculture's footprint. Challenges like data management and public acceptance need addressing for equitable, sustainable food production {Hayat et al., 2025).

CONCLUSIONS

Soybean stands as a cornerstone of global agriculture, valued for its protein-rich seeds, oil content, and versatility in food, feed, and industrial applications. Over recent decades, significant advances in genetic transformation—such as Agrobacterium-mediated, biolistic, and emerging genome editing techniques—have revolutionized soybean improvement. These technologies have enabled the rapid development of varieties with enhanced yield, stress resistance, pest and disease tolerance, and improved nutritional quality. The integration of molecular breeding, marker-assisted selection, and precise genome editing tools like CRISPR/Cas9 has accelerated trait development, reduced breeding cycles, and addressed challenges posed by environmental stresses and climate change. Despite these advancements, challenges remain, including genotype recalcitrance, tissue culture bottlenecks, and regulatory hurdles. However, the adoption of high-throughput and automated transformation systems, coupled with a deeper understanding of plant-microbe interactions and the optimization of regeneration protocols, is steadily overcoming these barriers. The shift toward non-GMO gene editing approaches is also enhancing public acceptance and regulatory feasibility. Looking forward, future prospects for soybean biotechnology are promising. The integration of synthetic biology, advanced delivery systems, and machine learning is expected to further optimize transformation efficiency and trait stacking. Continued research into stress resilience, nutritional biofortification, and sustainable agricultural practices will ensure soybean's pivotal role in addressing global food security and environmental sustainability. As transformation technologies become more efficient and accessible, soybean breeding will continue to evolve, supporting the development of climate-smart, high-yielding, and nutritionally superior cultivars for a rapidly growing world population.

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