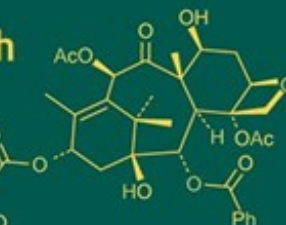


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Tissue culture-independent plant genetic engineering: A review of in planta systems

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ABSTRACT

Conventional crop genetic engineering remains severely constrained by the transformation bottleneck, wherein in vitro tissue culture, callus induction, and organogenesis introduce somaclonal variations, epigenetic instability, and widespread genotype recalcitrance. To overcome these liabilities, tissue culture-independent, in planta transformation strategies have emerged as powerful platforms that introduce foreign DNA directly into differentiated tissues, apical meristems, reproductive organs, and vegetative propagules in situ. This review synthesizes the mechanistic evolution, delivery platforms, and physiological foundations of in planta gene transfer systems. We systematically examine diverse methodologies—including hydrodynamic vacuum infiltration, surfactant-assisted floral dipping and micro-targeted floral drop delivery, embryo axis and juvenile seedling meristem infiltration, the pollen-tube pathway, and the Regenerative Activity-dependent In Planta Injection Delivery (RAPID) platform. Across these approaches, we highlight the central biological trade-off between direct single-cell germline targeting (yielding uniform, non-chimeric progeny) and multicellular shoot apical meristem (SAM) targeting (frequently resulting in sectorial chimerism in the T_0 generation). Critical emphasis is placed on histogenic layer dynamics, demonstrating that stable meiotic inheritance relies strictly on the entrainment of the sub-epidermal L2 cell lineage. Furthermore, we evaluate operational bottlenecks including low baseline transformation frequencies in non-model crops, large-scale progeny screening burdens arising from the lack of early in vitro selective filters, surfactant-induced phytotoxicity, and the diagnostic risks of endophytic bacterial persistence. Finally, we outline rigorous multi-tiered molecular authentication strategies—such as vector-backbone clearance assays—and explore future avenues integrating in planta mechanics with transgene-free genome editing to achieve genotype-independent crop improvement.

Keywords: In planta transformation, Shoot apical meristem (SAM), Floral dip, Tissue culture recalcitrance, Germline transmission, L2 histogenic layer, Somatic chimerism, Hydrodynamic vacuum infiltration, Genotype independence, Crop genetic engineering

1. Introduction: The genetic transformation bottleneck

Recombinant DNA technology and plant genetic engineering have fundamentally expanded the accessible gene pool beyond the biological boundaries imposed by sexual compatibility in conventional breeding systems (Sharma *et al.*, 2005; Kesiraju *et al.*, 2017) [2, 1]. Through direct and vector-mediated gene delivery, specific agronomic traits—ranging from biotic resistance and abiotic stress tolerance to biofortification and accelerated flowering—have been successfully deployed across elite germplasms (Altpeter *et al.*, 2016; Purwantoro *et al.*, 2022) [3, 4].

Historically, genetic transformation has relied predominantly on two platforms: biolistics (particle bombardment using DNA-coated microprojectiles) and *Agrobacterium tumefaciens*-mediated gene transfer (Tzfira *et al.*, 2006) [5]. *Agrobacterium* remains the gold standard owing to its capacity for defined T-DNA integration, lower transgene copy numbers, and predictable, stable intergenerational inheritance (Hu *et al.*, 2003; Shou *et al.*, 2004; Ziemienowicz, 2014) [6, 7, 8].

Despite more than three decades of protocol optimization, genetic transformation remains the primary rate-limiting bottleneck in crop functional genomics and varietal improvement (Altpeter *et al.*, 2016) [3]. Most conventional pipelines require an intervening in vitro tissue culture phase where dedifferentiated callus cells are induced, transformed, and regenerated into whole plantlets. For a substantial number of economically vital species—particularly grain legumes, woody perennials, and elite monocot cultivars—regeneration remains either prohibitively recalcitrant or strictly genotype-dependent (Clough, 2004; Anjanappa *et al.*, 2021) [10, 9]. Consequently, the imperative to bypass cellular dedifferentiation has driven the development of tissue culture-independent, *in planta* transformation strategies (Saifi *et al.*, 2020) [11].

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2. Limitations of tissue culture-based systems vs. the in planta alternative

Tissue culture-dependent transformation is constrained by several systemic liabilities:

- **Somaclonal Variation & Epigenetic Instability:** Extended callus induction and organogenesis cycles frequently introduce undesirable chromosomal rearrangements, point mutations, transposable element activations, and stable DNA methylation changes (Clough, 2004) [10]. These variations can induce pleiotropic abnormalities, reduce fertility, or mask targeted phenotypes.
- **Genotype Dependency & Recalcitrance:** Standardized protocols are rarely transferable across genotypes of the same species. While a

laboratory model cultivar may regenerate efficiently, elite commercial cultivars typically exhibit severe morphogenic recalcitrance (Anjanappa *et al.*, 2021) [9].

- **Resource Intensity & Contamination Vulnerability:** In vitro operations require specialized cleanroom infrastructure, complex hormone cocktails (auxin/cytokinin balancing), labor-intensive subculturing, and prolonged timelines. Additionally, high-sugar media render cultures vulnerable to devastating fungal and bacterial contaminations.

In planta transformation serves as an in vivo bypass of these constraints by targeting foreign DNA directly into differentiated tissues, apical meristems, or reproductive organs in situ (Saifi *et al.*, 2020) [11].

Parameter	Conventional In Vitro Transformation	In Planta Transformation
Culture Medium Requirement	Absolute dependency on sterile, hormone-supplemented synthetic media (e.g., MS/B5)	Completely eliminated or limited to short seed co-cultivation buffers
Somaclonal Variation	High risk (aneuploidy, polyploidy, point mutations, epigenetic silencing)	Negligible; structural genome integrity remains intact
Genotypic Transferability	Highly genotype-specific; recalcitrance is widespread	Broad applicability across elite cultivars, landraces, and wild relatives
Turnaround Time	Prolonged (months to over a year for recalcitrant species)	Significantly shorter; bound strictly to natural plant generation time
Labor & Infrastructure Cost	High (aseptic laminar flow hoods, climate-controlled growth rooms, chemical consumables)	Low to moderate; standard greenhouse or soil-bench compatible
Primary Limitation	Regeneration failure, callogenesis arrest, culture contamination	High incidence of chimeric primary (T_0) tissues; necessitates stringent progeny screening

3. Evolution and mechanistic breadth of in planta systems

The feasibility of *in planta* transformation was firmly established through the *Arabidopsis thaliana* floral dip protocol, where *Agrobacterium* suspended with surfactant directly targets developing female gametophytes within unpollinated ovules, yielding non-chimeric, hemizygous seeds without intermediate callogenesis (Bechtold *et al.*, 1995; Bent, 2000) [12, 13]. While early attempts to duplicate the floral dip in other species met physiological barriers, modified approaches have expanded the taxonomic reach:

- **Reproductive Tissue Adaptations:** Demonstrated feasibility in model legumes such as *Medicago truncatula* confirmed that direct female germline infection could be achieved beyond Brassicaceae with tailored inoculation windows (Bent, 2000) [13].
- **Apical Meristem Targeting:** To overcome floral architecture limitations in larger crops, the apical meristem strategy—utilizing physical micro-wounding techniques such as "clip 'n' squirt" or targeted microinjection—delivers *Agrobacterium* directly to the pluripotent shoot apical meristem (SAM) (Kesiraju *et al.*, 2017) [1]. This methodology has delivered stable, heritable integration in traditionally recalcitrant grain legumes, including chickpea (*Cicer arietinum*).
- **Seed Infiltration & Physical Permeabilization:** Direct inoculation of germinating seeds or excised embryonic axes coupled with physical enhancers—such as vacuum infiltration and ultrasonic cavitation (sonication)—substantially increases *Agrobacterium* adherence and T-DNA delivery. In purslane (*Portulaca oleracea* L.), a dual approach using sonication and vacuum infiltration achieved verifiable integration of the reporter marker β -glucuronidase (*uidA*), validated via Southern hybridization (Sedaghati *et al.*, 2021) [14].
- **Agronomic Trait Transfer:** The paradigm has progressed beyond reporter genes (*gusA*, *gfp*) to field-level agronomic traits. Tissue culture-independent procedures have successfully produced stable herbi-

cide-tolerant (glufosinate-resistant) soybean (*Glycine max*) lines, bypassing micro-shoot regeneration hurdles and proving the method's commercial utility (Mangena, 2019) [15].

3.1. Vacuum infiltration-mediated in planta transformation

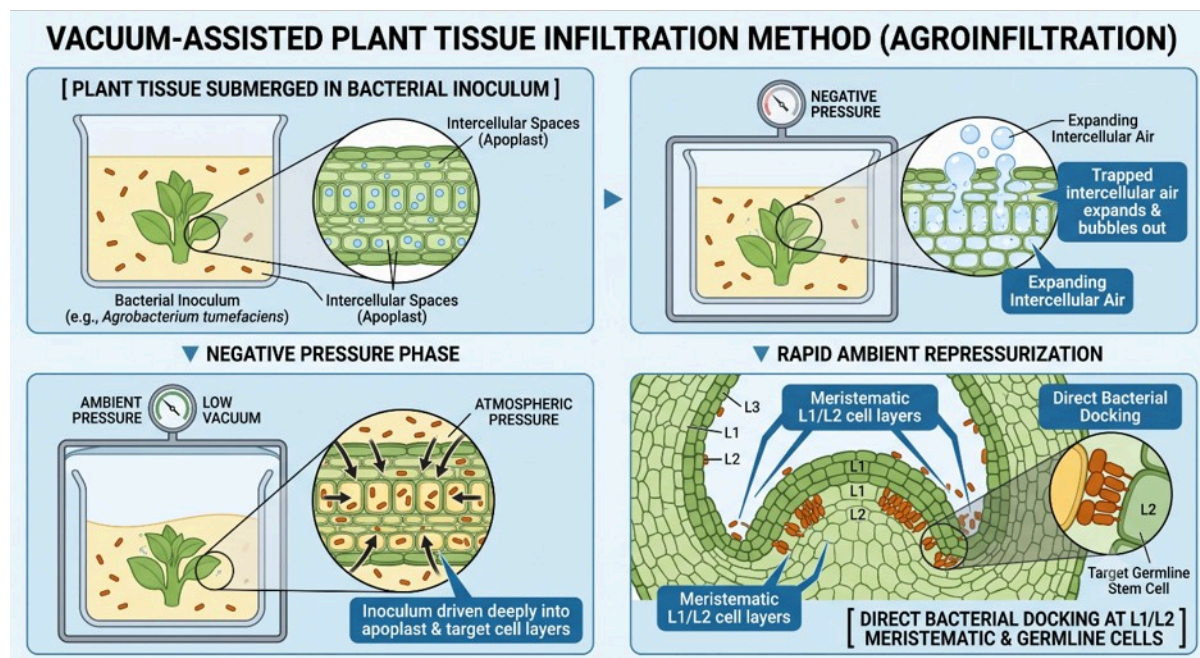
3.1.1. Physical principles and mechanics of hydrodynamic infiltration

Vacuum infiltration functions as a hydrodynamic physical delivery platform designed to overcome the structural, cuticle-bound, and stomatal barriers of intact plant organs. In passive immersion or simple dipping systems, surface tension and narrow stomatal apertures severely restrict bacterial access to deep internal tissues.

The vacuum infiltration mechanism operates via a two-phase pressure differential cycle:

1. **Air Displacement Phase (Negative Atmospheric Pressure):** Submerging intact explants, floral organs, or seeds in an *Agrobacterium* suspension under a controlled partial vacuum draws air out of the apoplast, stomatal cavities, and intercellular air spaces.
2. **Hydrodynamic Inoculation Phase (Rapid Depressurization):** When the vacuum is released, atmospheric pressure returns to ambient conditions. This rapid pressure rebound forces the surrounding liquid inoculum into the evacuated intercellular voids, driving the bacteria into direct contact with sub-epidermal layers, shoot apical meristems (SAM), and internal gametophytic target cells (Hu *et al.*, 2019; Xu *et al.*, 2022) [16, 17].

By mechanically circumventing rigid epidermal cuticles, vacuum infiltration significantly elevates T-DNA delivery efficiency compared to standard surface contact, while avoiding the cellular dedifferentiation, somaclonal aberrations, and long culture periods typical of in vitro regeneration (Kaur *et al.*, 2019; Niazian *et al.*, 2017) [18, 19].



3.1.2. Critical formulation parameters: Surfactants and chemical mediators

The biophysical efficacy of vacuum infiltration relies heavily on media composition. Standard infiltration cocktails integrate specific osmoprotectants and wetting agents:

- Silwet L-77 (Non-ionic Organosilicone Surfactant): Silwet L-77 reduces the surface tension of aqueous inocula, facilitating liquid entry into microscopic stomatal apertures and floral crevices (Bechtold *et al.*, 1995; Tague, 2001) [12, 20].
- Sucrose and Osmotic Regulators: Sucrose (typically 5% w/v) maintains bacterial viability and stabilizes plant cell membranes during pressure shifts.
- Virulence Inducers: Acetosyringone or wounded plant extracts (such as wounded tobacco leaf extracts) trigger the expression of *Agrobacterium vir* genes, enhancing T-complex transfer into target cells (Hasan *et al.*, 2019; Sedaghati *et al.*, 2021) [21, 14].

Because surfactant tolerance and epidermal anatomy vary across taxa, operational concentrations of sucrose and Silwet L-77 must be optimized per species to prevent tissue phytotoxicity and preserve post-treatment seed set (Tague, 2001) [20].

Vacuum infiltration-mediated *in planta* transformation in crops

Vacuum infiltration-mediated *in planta* transformation offers a robust method for bypassing *in vitro* tissue culture across a variety of plant species. Stable germline transmission was achieved in *Arabidopsis thaliana* by treating intact flower buds in a non-sterile pot culture with a medium containing sucrose and Silwet L-77 (Bechtold *et al.*, 1995) [12]. Similar infiltration techniques successfully targeted pollen or stigmas in *Petunia hybrida*, yielding 5–9% Basta® resistant progeny with 1–3 copy integrations (Tjokrokusumo *et al.*, 2000) [22]. In Brassicaceae, applying vacuum infiltration to whole plants transmitted functional herbicide resistance in Pakchoi (Qing *et al.*, 2000) [23] and outperformed passive floral dipping in wild relatives (Tague,

2001) [20]. Additionally, vacuum infiltration proved significantly superior to syringe injection for high-throughput functional genomics in buckwheat (Bratić *et al.*, 2007) [24].

The technique also extends effectively to recalcitrant legumes. In mungbean, a rapid protocol involved 2-minute vacuum infiltration of de-coated half-seed explants (Hasan *et al.*, 2019) [21], while in chickpea, applying it to pre-cultured embryonic axes reduced chimeric shoot frequency and achieved up to 8.6% efficiency (Sadhu *et al.*, 2022) [25].

3.2. Floral dip transformation: Germline-targeted *in planta* gene transfer

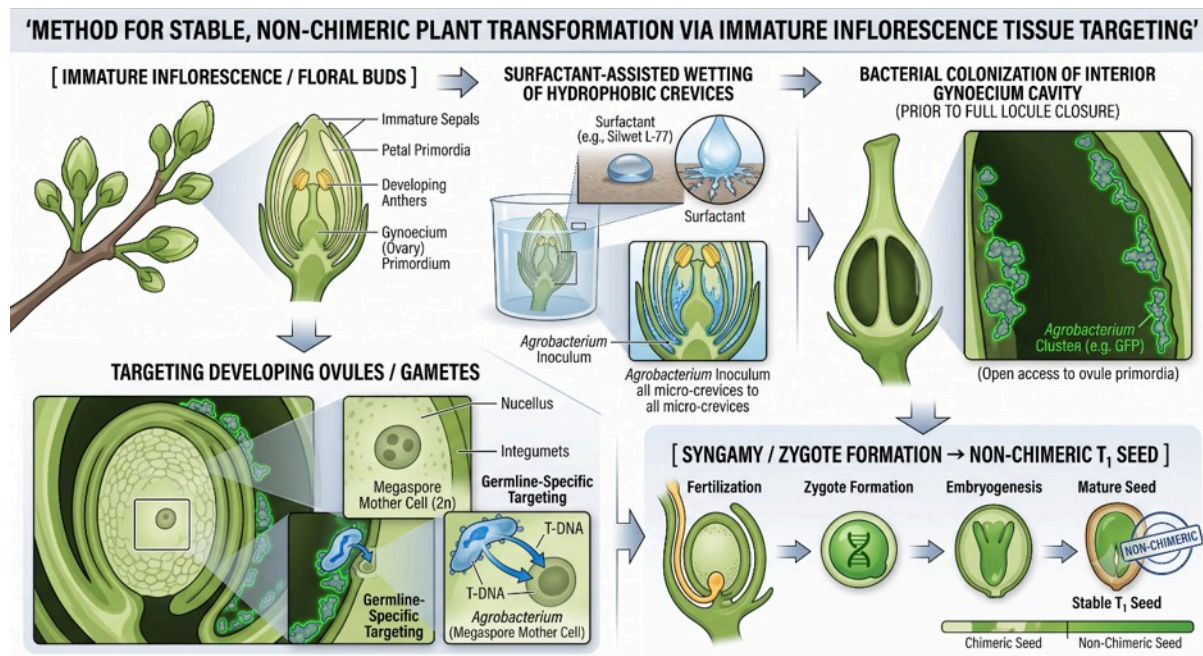
3.2.1. Target site biology and cellular mechanisms

Floral dip transformation represents the standard tissue culture-independent method for producing stable, non-chimeric transgenic plants (Clough *et al.*, 1998; Bent, 2006) [27, 26]. Unlike somatic cell transformation, which requires chemical dedifferentiation into callus followed by organogenic or embryogenic regeneration, floral dipping targets intact reproductive tissues *in situ* (Kaur *et al.*, 2019; Li *et al.*, 2024) [18, 28].

The primary target of *Agrobacterium tumefaciens* during floral dipping is the female reproductive lineage—specifically the developing ovules prior to gynoecium locule closure (Bent, 2006; Hu *et al.*, 2019) [26, 16]. Histological and developmental analyses demonstrate that:

- Inoculated bacteria navigate inner floral crevices and colonize the interior of developing ovaries.
- T-DNA transfer occurs preferentially within accessible female gametophytes before fertilization, or within the single-celled zygote immediately post-fertilization (Li *et al.*, 2024) [28].

Because the integration event precedes embryonic division, the resulting T_1 seeds are hemizygous, non-chimeric individuals that exhibit classical Mendelian segregation in subsequent generations (Hu *et al.*, 2019; Sembiring *et al.*, 2023) [16, 29].



3.2.2. Protocol mechanics and critical optimization parameters

The classical vacuum infiltration process established by Bechtold and Bouchez was streamlined into a simple, high-throughput dipping protocol by Clough *et al.* (1998) [27]. The physical immersion of floral structures avoids tissue damage from vacuum depressurization, but requires strict calibration of biochemical and physiological variables:

- **Surfactant Dynamics (Silwet L-77):** The inclusion of a non-ionic organosilicone surfactant (typically 0.02% to 0.05% v/v, or ~500 $\mu\text{L/L}$) is essential. Silwet L-77 lowers aqueous surface tension, breaking cuticular hydrophobicity and facilitating bacterial entry through tightly appressed sepals and petals into interior floral organs (Clough *et al.*, 1998; Sembiring *et al.*, 2023) [27, 29]. However, surfactant dosage requires tight regulation: supra-optimal concentrations induce floral bud abscission, stigma necrosis, and female sterility (Sembiring *et al.*, 2023) [29].

- **Osmotic Support:** Sucrose (typically 5% w/v) preserves osmotic equilibrium, enhancing bacterial viability and safeguarding floral secretory structures during exposure (Clough *et al.*, 1998) [27].

- **Developmental Synchrony (The Inoculation Window):** Maximum transformation efficiency coincides with plants bearing numerous young, unopened floral buds and few to no mature siliques/pods (Clough *et al.*, 1998; Hu *et al.*, 2019) [27, 16]. Closed buds shield developing ovules while retaining the micro-apertures necessary for bacterial entry. Dipping post-fertilization reduces efficiency, as thickened carpel walls block bacterial migration to the ovule.

- **Microenvironment & Repeated Inoculation:** Maintaining saturated relative humidity (covering plants for 16–24 hours post-treatment) prevents drying of the inoculum film, doubling transformation rates. Repeating the dipping procedure 7 to 10 days later captures secondary floral flushes, improving overall yield of transgenic progeny up to twofold (Clough *et al.*, 1998) [27].

Floral dip transformation: Germline-targeted in planta gene transfer in crops

Floral dip transformation provides a high-throughput, germline-targeted approach. In *Arabidopsis thaliana*, immersing developing floral buds in *A. tumefaciens* with 5% sucrose and 0.05% Silwet L-77 yields typical transformation rates of approximately 1% non-chimeric progeny (Clough *et al.*, 1998; Bent, 2006) [27, 26]. This method is highly effective in Brassicaceae, enabling the expression of a stress-inducible zinc finger gene in *Brassica napus* and *Brassica carinata* (Verma *et al.*, 2008) [30], facilitating stable T_1 recovery in *Brassica*

rapa without tissue culture artifacts (Hu *et al.*, 2019) [16], and overcoming extreme in vitro recalcitrance in *Nocca caerulescens* with up to 0.29% efficiency (Wang *et al.*, 2024) [31].

Beyond brassicas, precise staging is critical. Immersing developing corn silks staged at 5–10 cm in *Zea mays* resulted in a 3.3% positive plantlet recovery (Mu *et al.*, 2012) [32]. In the ornamental *Cosmos sulphureus*, precision calibration of floral bud diameter and surfactant ratio resulted in non-chimeric stable transformants (Sembiring *et al.*, 2023) [29].

3.2.4. Architectural challenges and novel inoculation variations

Applying floral dipping to broader field crops introduces anatomical challenges:

- **Corolla Barrier and Macro-Architecture:** Many non-Brassicaceae crops feature large, tightly wrapped perianths, waxy protective scales, or elongated styles that prevent passive bacterial diffusion to the ovary chamber (Li *et al.*, 2024) [28].

- **Delivery Variations:** To adapt the germline strategy to larger or tightly closed floral architectures without inducing tissue trauma:

- * **Silk Immersion:** Demonstrated in maize (*Zea mays*), where receptive silk surfaces (5–10 cm length) serve as conduits to transport *Agrobacterium* toward the ovule (Mu *et al.*, 2012) [32].

- * **Surfactant-Assisted Micro-Spraying:** Replaces full submersion with targeted high-pressure sprays directly into budding clusters, minimizing floral bud suffocation (Li *et al.*, 2024) [28].

- * **Ovary / Bud Micro-Injection:** Mechanical micro-syringe delivery directly through the perianth base into the locular cavity, circumventing external floral barriers entirely (Li *et al.*, 2024) [28].

3.3. Floral drop transformation: Micro-targeted reproductive delivery

3.3.1. Rationale and mechanical divergence from bulk immersion

While whole-inflorescence floral dipping is highly effective in small-flowered model systems like *Arabidopsis thaliana*, its utility declines when applied to species with complex, dense, or rigid floral morphologies (Hu *et al.*, 2019; Li *et al.*, 2024) [16, 28]. Bulk dipping of species with tightly appressed glumes, protective bracts, or tubular corollas often results in uneven surface coverage, poor inner-cavity penetration, and wasteful runoff of bacterial inoculum (Ratanasut *et al.*, 2017) [33].

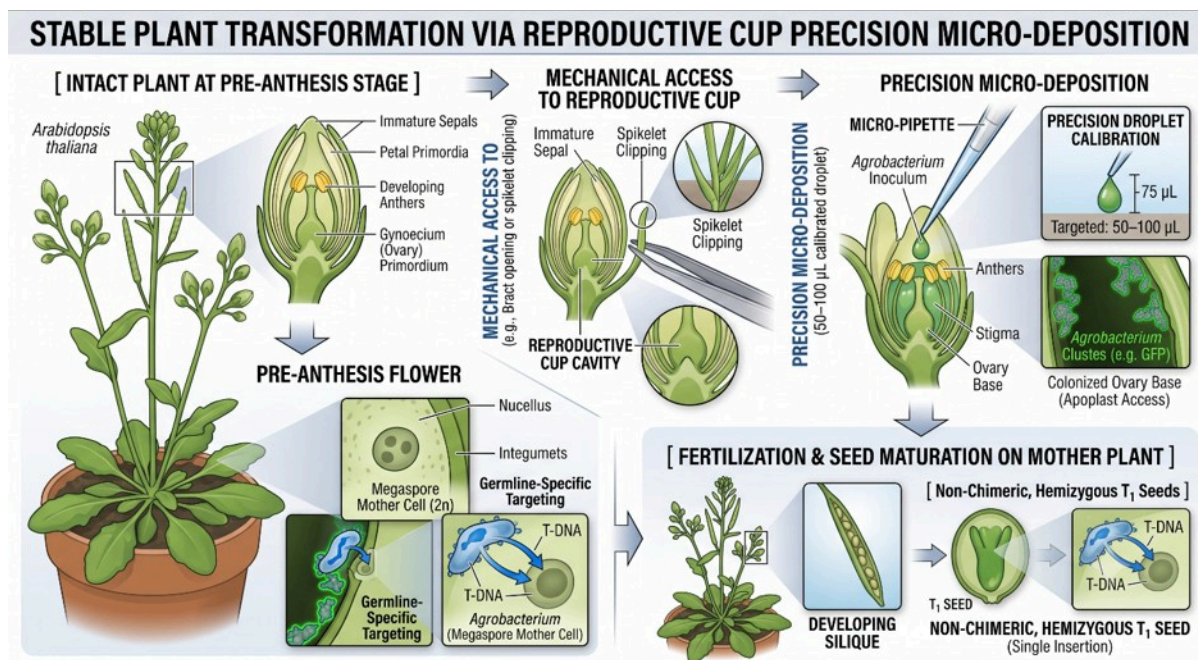
To circumvent these anatomical constraints, floral drop transformation delivers a calibrated micro-droplet of *Agrobacterium tumefaciens*

suspension directly into individual reproductive cavities, clipped spikelet cups, or partially opened bracts (Ratanasut *et al.*, 2017) [33]. By concentrating the inoculum at the target site, this micro-targeted strategy bypasses tissue culture-induced somaclonal variation, hormonal imbalance artifacts, and organogenic regeneration hurdles (Kaur *et al.*, 2019; Li *et al.*, 2024) [18, 28].

3.3.2. Delivery mechanics and optimization parameters

In the floral drop system, small volumes (typically 50–100 μL) are delivered using a micropipette or micro-syringe directly to pre-anthesis floral structures (Ratanasut *et al.*, 2017) [33]. This localizes the

bacterial payload around essential reproductive organs—including anthers, receptive stigmas, and ovules—while minimizing run-off and systemic plant stress (Li *et al.*, 2024; Ratanasut *et al.*, 2017) [28, 33]. Comparative evaluations in monocot models such as rice (*Oryza sativa* L.) demonstrate that localized drop application yields higher transient transformation frequencies in anthers and floral tissues than passive bulk immersion (Ratanasut *et al.*, 2017) [33]. However, high bacterial density and localized retention introduce specific operational constraints:



- Hypersensitive Response (HR) and Necrosis: Prolonged liquid pooling or excessive bacterial cell density ($\text{OD}_{600} > 0.8\text{--}1.0$) within an enclosed floral cup can trigger severe tissue browning, hypersensitive necrosis, and premature bud abscission within 48 to 72 hours post-inoculation (Ratanasut *et al.*, 2017; Sembiring *et al.*, 2023) [33, 29].

- Surfactant and Volume Tuning: Maintaining target-organ viability requires strictly titrating surfactant levels (e.g., Silwet L-77 at 0.01%–0.03% v/v) to promote wetting without causing stigma toxicity or ovule abortion (Sembiring *et al.*, 2023) [29].

- Post-Inoculation Microclimate: Modulating post-treatment relative humidity is critical to facilitate bacterial attachment and T-pilus-mediated T-DNA transfer before the droplet desiccates, all while avoiding fungal proliferation within the wet floral cup (Ratanasut *et al.*, 2017) [33].

3.4. Embryo-targeted *in planta* transformation (meristematic infiltration & seed inoculation)

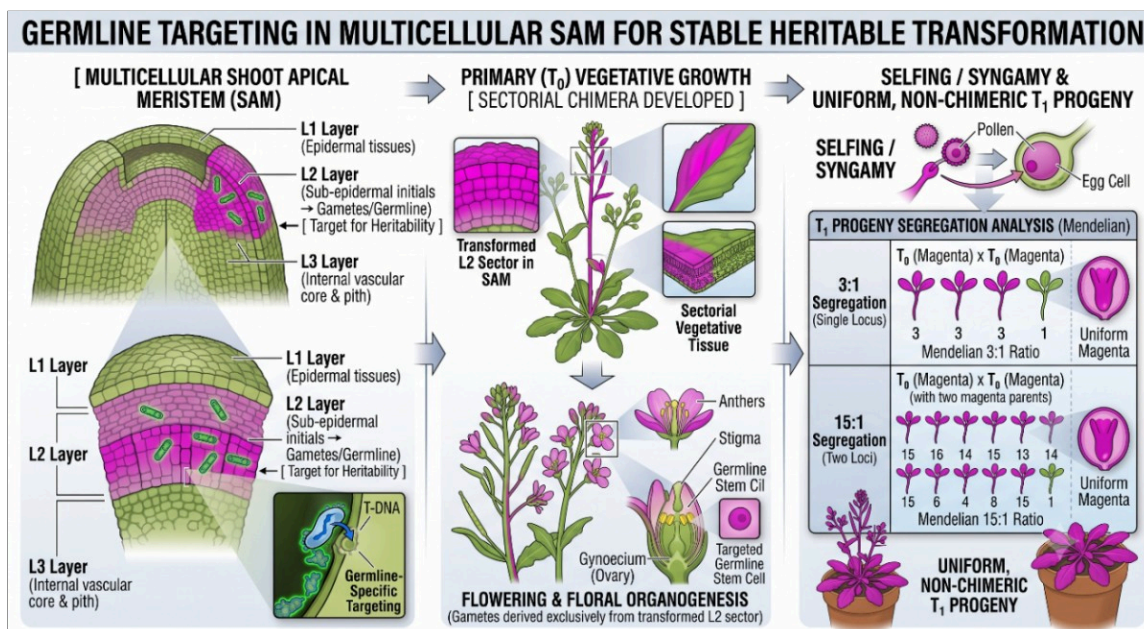
3.4.1. Developmental targeting of the shoot apical meristem (SAM)

For recalcitrant, large-seeded species—particularly grain and forage legumes (Fabaceae), oilseeds, and key monocot cereals (Poaceae)—floral-directed methods are frequently constrained by dense floral

protective bracts, localized necrosis, or low flower-to-seed conversion rates. Embryo-targeted *in planta* transformation addresses this by directly targeting the preformed shoot apical meristem (SAM) or cotyledonary node of mature imbibed seeds or germinating embryonic axes. Because the shoot apical meristem houses the pluripotent stem cell reservoir that directly establishes the primary shoot architecture, vegetative organs, and subsequent floral initials, introducing foreign T-DNA directly into these meristematic cells enables a complete bypass of the *in vitro* dedifferentiation and somatic embryogenesis pathways. Consequently, issues with *in vitro* recalcitrance, hormone-induced epigenetic instability, and prolonged regeneration timelines are substantially mitigated.

3.4.2. Mechanical permeabilization and direct inoculation modes

The primary physiological barrier in embryo transformation is the protective physical envelope surrounding the L1 and L2 meristematic initials, including the seed coat (testa), cotyledons, and embryonic plumular leaf primordia. To facilitate direct access of *Agrobacterium tumefaciens* to the meristematic dome, several mechanical and micro-invasive strategies have been developed:



- Micro-Needle Piercing / Pricking: Imbibed seeds are mechanically pierced in the precise embryonic region overlying the SAM using a fine needle coated with *Agrobacterium*, creating a direct micro-conduit without disrupting the structural viability of the embryo.

- Plumule Decapitation & Cotyledonary Node Wounding: Surgical excision of the plumule or localized micro-pricking of the cotyledonary junction exposes internal regenerative cell layers, maximizing bacterial colonization at regions predisposed to direct shoot organogenesis.

- Cavitation & Vacuum Coupling (SAAT & Hydrodynamic Delivery): Pre-conditioned embryonic axes subjected to ultrasonic cavitation (sonication) and negative atmospheric pressure pull air out of internal cellular voids; rapid pressure release drives the bacterial suspension deep into sub-epidermal cell layers.

3.4.3. Chimerism dynamics and germline transmission mechanics

Unlike floral dip transformations, embryo-targeted delivery transforms cells within a multicellular meristematic population. The primary (T_0) generation is predominantly composed of sectorial chimeras, where transformed cell lineages co-exist alongside wild-type sectors. The heritability of the transgene depends entirely on histogenic layer targeting; when T-DNA integration occurs within initials of the L2 sub-epidermal layer, the transformed cells give rise to the male and female gametophytic lineages. Upon self-pollination, these T_1 progeny are non-chimeric, carry stable nuclear integrations, and exhibit standard Mendelian inheritance ratios.

3.4.4. Applications in crops

In peanut, inoculating the mature seed embryo axis in situ led to single plant transformants with enhanced fungal resistance. Cereal crops also respond well; needle-piercing the embryonic SAM of water-imbibed seeds yielded high T_1 transformation frequencies in both rice and wheat. Legumes benefit significantly: random micro-pricking of the cotyledonary node in sunnhemp established a genotype-independent system, and field bean embryo axes demonstrated a 17% T_1 frequency for a chimeric insecticidal gene. Furthermore, combining plumule excision with SAAT and vacuum infiltration in chickpea embryonic axes eliminated chimeric sectors with up to 8.6% efficiency.

3.5. Seedling-targeted in planta transformation: Post-germinative meristem delivery

3.5.1. Developmental rationale and physiological advantages

While embryo axis transformation operates on dry or freshly imbibed mature seeds, seedling-targeted *in planta* transformation introduces *Agrobacterium tumefaciens* into actively growing juvenile seedlings

(typically 2 to 7 days post-germination) (Kaur *et al.*, 2019; Sen *et al.*, 2020) [18, 34]. At this developmental window, primary shoot and root axes have broken dormancy, and the shoot apical meristem (SAM), axillary buds, and hypocotyl-cotyledonary junctions undergo rapid mitotic cycles (Li *et al.*, 2024; Venkataramaiah *et al.*, 2011) [28, 35].

Directly targeting these active vegetative meristems yields distinct biological advantages over traditional *in vitro* systems:

- Mitigation of Dedifferentiation Hurdles: Bypasses hormone-induced somatic callogenesis, avoiding prolonged exposure to synthetic auxins and cytokinins that frequently drive chromosomal abnormalities, somaclonal variation, and epigenetic transgene silencing (Kaur *et al.*, 2019; Niazian *et al.*, 2017) [18, 19].

- Accelerated Ex Vitro Acclimatization: Because seedlings possess developed vascular tissue and primary root architecture, inoculated plantlets establish rapidly into soil or potting substrates without the protracted acclimatization losses common to delicate agar-regenerated plantlets (Sen *et al.*, 2020) [34].

- Broad Cultivar Amenability: By directing T-DNA into pre-existing, actively dividing stem cell niches rather than coaxing recalcitrant somatic cells into *de novo* organogenesis, the method demonstrates widespread genotype independence across challenging pulses, cereals, and industrial crops (Feldmann *et al.*, 1987; Venkataramaiah *et al.*, 2011) [36, 35].

3.5.2. Mechanical delivery modes and meristem accessibility

The primary physiological obstacle in juvenile seedlings is the dense physical enclosure formed by overlapping plumular leaves, waxy cotyledons, and cuticular barriers that insulate the SAM from external fluid agents (Kaur *et al.*, 2019; Sen *et al.*, 2020) [18, 34]. Successful protocol architectures utilize micro-invasive physical methods to introduce *Agrobacterium* directly to the inner meristematic layers:

- Needle-Pricking and Micro-Puncturing: Directly piercing the meristem dome of 2-day-old germinated seedlings using an *Agrobacterium*-coated micro-needle creates localized channels through leaf primordia. This enables precise infiltration without arresting the continuous developmental drive of the primary apex (Venkataramaiah *et al.*, 2011) [35].

- Plumule/Epicotyl Decapitation: In large-seeded grain legumes, removing the seedling epicotyl triggers dormant meristematic buds at the cotyledonary node. Exposing this wound to bacterial suspension drives multiple *de novo* shoots from transformed axillary initials, generating independent transgenic events per seedling (Sadhu *et al.*, 2022; Sen *et al.*, 2020) [25, 34].

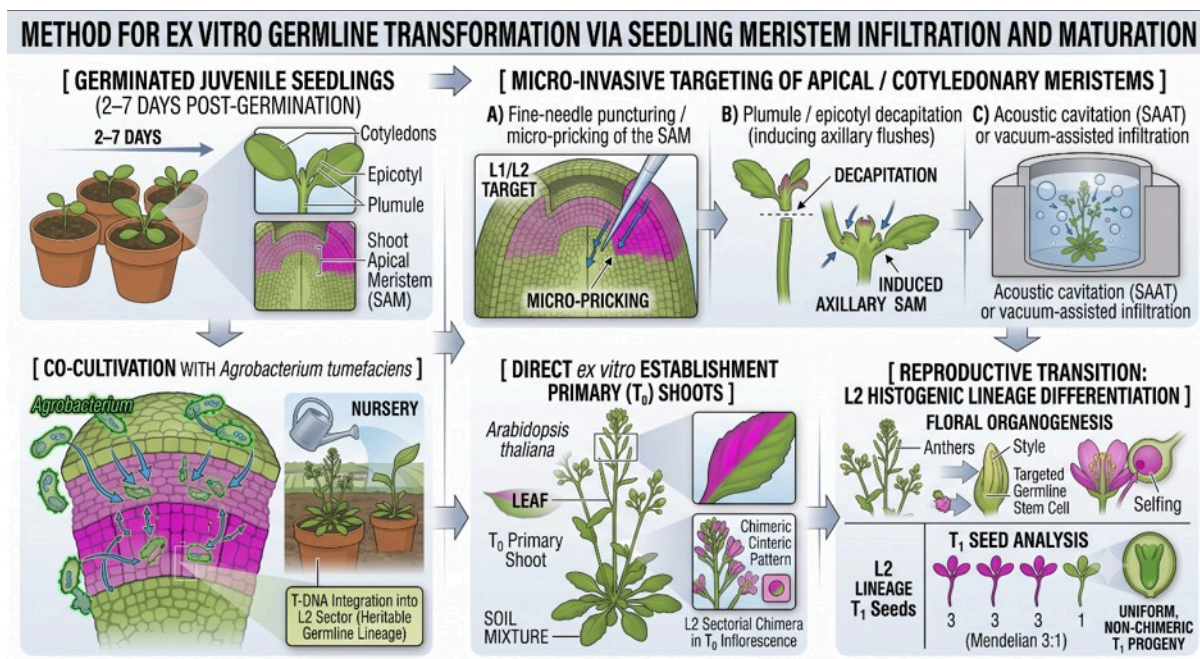
- Imbibition Co-Cultivation: In small-seeded species, early post-germination imbibition cycles combined with prolonged bacterial expo

sure allow natural colonization of emerging apical clefts prior to complete cuticularization (Feldmann *et al.*, 1987) [36].

Seedling-targeted in planta transformation: Post-germinative meristem delivery in crops

Post-germinative meristem delivery allows for effective seedling-targeted transformation. Early foundational work in *Arabidopsis thaliana* exposed imbibed seeds and young germinants to *Agrobacterium*, generating over 200 resistant T_2 seedlings with sta-

ble, predominantly multi-copy T-DNA insertions (Feldmann *et al.*, 1987) [36]. In agricultural crops like blackgram, seedling agro-inoculation of germinated seeds using infectious components of the mungbean yellow mosaic geminivirus established an efficient in vivo viral indexing assay (Mandal *et al.*, 1997) [37]. For cereals, piercing the SAM of 2-day-old germinated rice seedlings enabled the overexpression of *A. thaliana* phytochelatin synthase, conferring tolerance to cadmium stress with single-copy T-DNA integration and stable inheritance (Venkataramaiah *et al.*, 2011) [35].



3.6. The pollen-tube pathway (PTP) and stigmatic inoculation

3.6.1. Biological mechanism and route of entry

The Pollen-Tube Pathway (PTP) is an established, non-tissue culture transformation method designed to introduce foreign genetic material directly into the female germline by exploiting natural fertilization channels. Originally conceptualized in monocot cereals such as cotton and rice, the technique bypasses somatic dedifferentiation and somatic embryogenesis entirely.

The biological premise relies on the physiological sequence following compatible pollination:

1. **Pollination and Tube Elongation:** Pollen grains land on the receptive stigma, hydrate, germinate, and extend a pollen tube through the style and transmitting tract toward the micropyle of the ovule.
2. **Channel Exploitation:** Shortly after the pollen tube penetrates the embryonic sac—or upon surgical excision (decapitation) of the stigma/upper style post-pollination—a micro-conduit remains briefly patent.
3. **Exogenous DNA or Vector Delivery:** Droplets of naked plasmid DNA or dilute *Agrobacterium tumefaciens* suspensions are placed directly onto the cut style surface or pollinated stigmatic tissue. The capillary pull and cytoplasmic streaming within the fading pollen tube path draw the genetic construct directly into the unfertilized or freshly fertilized egg cell (zygote).

Because integration occurs at the single-cell zygote or early pro-embryo stage, progeny plants (T_1) generated via the pollen-tube pathway develop as uniform, non-chimeric transformants that segregate according to standard Mendelian genetics in subsequent generations.

3.6.2. Advantages, optimization, and limitations of PTP

- **Advantages:** Absolute freedom from sterile tissue culture facilities, complete independence from genotype-specific callogenesis protocols, and direct harvesting of mature transformed seeds from field-grown mother plants.
- **Critical Windows:** The success of PTP is temporally narrow. Inoculation must be synchronized with the precise window between pollen tube entry into the embryo sac and the first mitotic division of the zygote. Inoculating too early physically destroys receptive floral tissues; inoculating too late limits exogenous DNA uptake due to rapid cell wall formation around the developing pro-embryo.
- **Technical Constraints:** Delivery efficiency varies widely based on floral size, style length, and endogenous nuclease activity within the style exudates, which can degrade naked plasmid vectors before they reach the micropylar region.

3.7. Specialized and organ-targeted in planta transformation systems

Specialized *in planta* transformation methods bypass traditional tissue culture by targeting alternative sink organs such as developing fruits, intact seeds, and vegetative propagules.

3.7.1. Direct fruit micro-injection (in fructo delivery)

A. tumefaciens is injected directly into the pericarp of developing or ripe fruit, perfusing the locular fluid and coating the seeds in situ. In tomatoes, a 48-hour post-injection incubation period resulted in a 65–74% selective germination rate on kanamycin. Among the surviving seedlings, 87.9–94.9% demonstrated stable GUS expression, though variable reporter intensity indicated some mosaic T-DNA integration.

3.7.2. Intact seed imbibition

This approach offers a high-volume, wounding-free transformation route for solanaceous and vegetable crops. Datta *et al.* (2022) [38] optimized this in tomato cultivars, achieving > 90% GUS expression using an optical density (OD) of 1.1–1.4, a 30-minute infection time,

and a 24-hour co-cultivation period. The resulting transformants expressed vacuolar antiporter genes that conferred strong tolerance to 100 mM NaCl stress in functional bioassays.

3.7.3. Vegetative propagule and tuber-eye micro-injection

This delivery method directly targets dormant vegetative meristems, bypassing tedious regeneration cycles for clonally propagated root and tuber crops. In potatoes, injecting bacterial suspension into dormant vegetative eye buds yielded a 31.75% transformation efficiency based on glyphosate resistance. Transgene expression was successfully confirmed across vegetative clonal generations in harvested daughter tubers via RT-PCR and ELISA.

4. Technical bottlenecks and limitations of in planta transformation systems

- Subdued Frequency: Non-model crops typically yield transformation rates of 0.1% to 2% (Hu et al., 2019; Wang et al., 2024) [16, 31], as only an infinitesimal fraction of applied *Agrobacterium* cells successfully navigate surrounding tissues to reach competent recipient initials (Kaur et al., 2019) [18].
- Sectorial Chimerism: Meristematic T-DNA integration occurs stochastically across distinct cell layers (L1, L2, L3) rather than the whole meristematic corpus (Rech et al., 2020; Sadhu et al., 2022) [39,

- 25]. This often confines transformation events to vegetative lineages, resulting in primary plants with robust somatic expression that fails to transmit to their seed progeny (Kaur et al., 2019; Li et al., 2024) [18, 28].
- Screening Overhead: Bypassing early in vitro antibiotic selection means harvested seed batches consist of thousands of non-transgenic siblings mixed with rare transformants (Kaur et al., 2019; Sen et al., 2020) [18, 34]. Managing this burden requires labor-intensive, high-throughput chemical and genomic screening pipelines (Sen et al., 2020) [34].
- Architectural Barriers: Germline approaches are severely hindered by cleistogamy (self-pollination prior to flower opening) and physical shields like thick bracts, glumes, and waxy cuticles (Hu et al., 2019; Li et al., 2024; Ratanasut et al., 2017) [16, 28, 33]. Shielded vegetative meristems likewise demand mechanical micro-wounding or specialized micro-injection to expose target tissues (Rech et al., 2020; Sadhu et al., 2022) [39, 25].
- Inoculum and Surfactant Toxicity: High surfactant concentrations disrupt lipid bilayers and cause rapid floral abortion (Sembiring et al., 2023) [29]. Furthermore, high bacterial density triggers localized hypersensitive necrosis (Ratanasut et al., 2017; Sembiring et al., 2023) [33, 29], and aggressive vacuum infiltration can disrupt gas exchange, causing fatal tissue vitrification and embryonic death (Xu et al., 2022) [17].

In Planta Method	Predominant Bottleneck	Structural / Biological Cause	Risk of Somatic Escapes / Chimerism	Management Strategy
Floral Dip	Low efficiency in non-Brassicaceae; floral abscission	Closed perianth architecture; hypersensitivity to Silwet L-77	Low (Direct female germline target)	Optimize surfactant titers (0.02–0.03%); synchronize dipping with young buds
Floral Drop	Hypersensitive necrosis; flower shedding	High local bacterial density; prolonged fluid pooling	Moderate (Targeting mix of anther/stigma/ovary)	Calibrate droplet volume (50–100 µL); modulate post-inoculation humidity
Embryo Axis / Seedling Infiltration	High sectorial chimerism; labor-intensive wounding	Multicellular SAM; mechanical shielding by plumular folds	High (Stochastic L1 vs. L2 transformation)	Discard T ₀ screening; systematically screen T ₁ progeny; use SAAT/vacuum combinations
Tuber / Propagule Inoculation	Mosaic vegetative spread; bacterial overgrowth	Dense storage parenchyma; systemic endophytic bacterial persistence	High (Chimeric vegetative tubers)	Post-infection antibiotic clearing (cefotaxime); RT-qPCR/Southern

5. Stable in planta transformation: Targeted germline delivery, vegetative regeneration, and multi-generational inheritance

Recent *in planta* strategies overcome transient expression and sectorial chimerism by precisely targeting the sub-epidermal L2 histogenic layer, utilizing micro-surgery, and exploiting endogenous organogenesis.

- Precision L2 Histogenic Layer Delivery via Biolistics: Targeting the L2 stem-cell layer ensures T-DNA enters the reproductive germline. In wheat, bombarding micro-dissected embryonic shoot apical meristems selectively propelled particles into L2 cell initials without lethal disruption. This achieved stable germline transmission in 62% of primary transformants, circumventing genotype recalcitrance in elite commercial cultivars.
- Severed Meristem Inoculation and De Novo Shoot Induction: Deliberate surgical decapitation of the vegetative shoot apex forces active meristem reorganization (Chang et al., 1994) [40]. In *Arabidopsis thaliana*, inoculating severed stumps yielded de novo secondary shoots. An average of 5.5% of regenerated shoots produced stably transformed progeny exhibiting standard intergenerational Mendelian inheritance.

- Piercing-Incubation Systems for Stress-Inducible Traits: Mechanically piercing the embryonic apex of imbibed tomato seeds enabled the direct delivery of complex stress-inducible cassettes (Shah et al., 2015) [41]. Co-cultivating the pierced meristems with *Agrobacterium* successfully delivered a cold-tolerance transcription factor (*DREB1A*). Stable integration conferred functional cold tolerance, demonstrated by higher stomatal conductance and improved relative water content in stressed progeny.
- The RAPID Platform: The Regenerative Activity–dependent *In Planta* Injection Delivery (RAPID) system targets active endogenous meristems in situ, triggering transformed nascent shoots without a callus phase (Mei et al., 2024) [42]. This system effectively bypasses tissue culture in recalcitrant, clonally propagated crops, delivering stable transgenic propagules in sweet potato, potato, and wild coastal halophytes.
- Molecular Screening to Exclude False Positives: Bypassing sterile media requires rigorous screening to distinguish true nuclear integration from persistent endophytic *Agrobacterium* (Sen et al., 2020; Sultan et al., 2023) [34, 43]. In Egyptian sesame, researchers established a multi-tiered pipeline using dual-target RT-PCR. Positive amplification of the target gene alongside zero amplification of vector-backbone genes (*nptII*) confirmed true T-DNA integration rather than bacterial survival.

6. Conclusion and future perspectives

In planta transformation systems have emerged as a transformative alternative to conventional *in vitro* plant genetic engineering. By directing gene delivery directly into pre-existing reproductive structures, apical meristems, embryonic axes, and vegetative sink tissues *in situ*, these strategies circumvent the protracted cell dedifferentiation, severe genotype recalcitrance, and somaclonal or epigenetic variations inherent to callus-based regeneration platforms. The diversification of delivery platforms—ranging from vacuum infiltration and floral immersion/drop methods to micro-invasive seedling piercing, pollen-tube exploitation, and the RAPID injection platform—has extended the paradigm beyond model plants into traditionally recalcitrant cereals, grain legumes, solanaceous crops, and vegetatively propagated root and tuber species.

Realizing the full potential of *in planta* transformation platforms requires overcoming four core technical bottlenecks. Sectorial chimerism must be resolved through targeted physical delivery directly into germline progenitors to ensure stable inheritance rather than somatic mosaics. Additionally, the heavy burden of progeny screening can be mitigated using high-throughput tools like automated spray selections, ddPCR, and fluorescent optical markers. To eliminate false-positive artifacts caused by surviving endophytic *Agrobacterium*, researchers must implement rigorous, multi-tiered molecular diagnostics to confirm true genomic integration. Finally, physiological optimization—specifically fine-tuning surfactant levels, bacterial titers, and ambient humidity—is essential to prevent tissue damage and preserve recipient organ viability.

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