Plant Cell Reports (2024) 43:273
https://doi.org/10.1007/s00299-024-03359-9

REVIEW

# Advancements in plant transformation: from traditional methods to cutting-edge techniques and emerging model species

**Hannah Levengood[1] · Yun Zhou[2] · Cankui Zhang[1]**

Received: 30 July 2024 / Accepted: 7 October 2024 / Published online: 29 October 2024


## Abstract

The ability to efficiently genetically modify plant species is crucial, driving the need for innovative technologies in plant biotechnology. Existing plant genetic transformation systems include *Agrobacterium*-mediated transformation, biolistics, protoplast-based methods, and nanoparticle techniques. Despite these diverse methods, many species exhibit resistance to transformation, limiting the applicability of most published methods to specific species or genotypes. Tissue culture remains a significant barrier for most species, although other barriers exist. These include the infection and regeneration stages in *Agrobacterium,* cell death and genomic instability in biolistics, the creation and regeneration of protoplasts for protoplast-based methods, and the difficulty of achieving stable transformation with nanoparticles. To develop species-independent transformation methods, it is essential to address these transformation bottlenecks. This review examines recent advancements in plant biotechnology, highlighting both new and existing techniques that have improved the success rates of plant transformations. Additionally, several newly emerged plant model systems that have benefited from these technological advancements are also discussed.

**Keywords** Plant biotechnology · *Agrobacterium* · Biolistic · Protoplast · Nanoparticle · Model species

## Introduction

The finding that species in the plant kingdom evolved the ability to regenerate into whole plants without fertilization (Steward et al. 1958a, b) marked the first of several significant breakthroughs in plant cell theory. Skoog and Miller demonstrated that this ability could be induced by exogenously applying the plant hormones auxin and cytokinin in a specific ratio (Skoog and Miller 1957). Other researchers showed that whole plants can be regenerated from single cell lines, and can be manipulated to induce somatic embryogenesis. (Guha and Maheshwari 1966; Steward et al. 1958a, b; Steward and Pollard 1958), these findings laid the foundation for plant tissue culture, a technique that has significantly advanced various fields of study over traditional methods of plant propagation (Bennur et al. 2024). More importantly, plant tissue culture has proved to be a crucial tool for plant biotechnology, as the totipotency of plant cells could easily be manipulated to produce whole plants (Ramkumar et al. 2020), thereby widening the potential for plant genetic manipulation.

As plant tissue culture is rooted in plant cell theory, plant biotechnology gained its framework from studies in bacterial genetic transformation. The discovery of *Agrobacterium tumefaciens*, a naturally occurring soil bacterium with the native ability to transfer its DNA into the plant nuclear genome, revolutionized the field of plant genetic engineering by providing a powerful tool for plant genetic manipulation (Victor M. Loyola-Vargas and Ochoa-Alejo 2018; Ramkumar et al. 2020). Genetic experiments show that *Agrobacterium* contains a specialized, tumor-inducing (Ti) plasmid that is responsible for mediating the transfer of genetic material (Chilton et al. 1977; Larebeke et al. 1974) to the plant nuclear genome. Therefore, it was theorized that *Agrobacterium* could be utilized as an effective tool for plant genetic manipulation and was engineered to become adaptable for easy laboratory use.

Communicated by Wusheng Liu.

✉ Cankui Zhang
ckzhang@purdue.edu

[1] Department of Agronomy, Center for Plant Biology, Purdue University, West Lafayette, IN 47907, USA

[2] Department of Botany and Plant Pathology, Center for Plant Biology, Purdue University, West Lafayette, IN 47907, USA

The ability to genetically manipulate a species of interest is important, as it enables comprehensive investigations into the relationships between gene function and their associated phenotype, and allows for faster advancements in crop improvement. However, techniques of gene delivery are not universally applicable to all plant species. *Agrobacterium* transformation systems typically exhibit species and even accession specificity, limiting which plants can be effectively transformed. The use of alternative strains of *Agrobacterium,* e.g., *A. rhizogenes* or *A. vitus,* combined with alternative methods of gene delivery such as particle bombardment, PEG and other techniques, has significantly increased the range of plants that can be transformed. However, many plants remain recalcitrant to plant transformation. The development of a single method to transform plants that can be broadly applied to many species is a common goal in the field of plant biotechnology but has yet to be developed. Therefore, the improvement and development of new and existing transformation technologies is important to achieving the successful transformation of previously recalcitrant species.

This review highlights advancements in plant biotechnology, focusing on new and existing techniques that have enhanced the success of plant transformation. In addition, several plant model systems that have recently emerged are also discussed.

## *Agrobacterium* mediated transformation

*Agrobacterium* mediated plant transformation is the most commonly used method for introducing foreign genetic material to plants. Two of the most popular methods to introduce a gene of interest to a plant using *Agrobacterium* as the vector include the floral dip technique for *Arabidopsis* (X. Zhang et al. 2006) or transformation methods involving plant tissue culture (Niazian et al. 2017). However, even with widespread adoption of *Agrobacterium* as a vector, relatively few plants can be transformed. While the exact mechanisms behind plant-specific recalcitrance to transformation are yet to be fully understood, it is generally recognized that there are three barriers to plant transformation: tissue culture, infection and regeneration. Each of these barriers, along with strategies used to overcome them, are detailed below.

### Optimizing tissue culture

Although tissue culture is often overlooked as a primary barrier to plant transformation (Benson 2000), its importance should not be disregarded, as it significantly contributes to the success of most protocols. Almost all current methods of plant transformation require a tissue culture step (Bekalu et al. 2023; Altpeter et al. 2005), which is especially important for monocot transformation. For many monocots, transformation is only feasible when callus is used as an explant (Hofmann 2016; Tripathi et al. 2023; Grogg et al. 2022), and callus induction is limited to a select few cultivars or species in a genus. In addition, many non-*Agrobacterium* based protocols also require a tissue-culture step. Thus, it is important to identify the factors responsible for tissue culture recalcitrance to broaden the scope of transformable plant species. The mechanisms involved in plant-tissue specific recalcitrance to *in-vitro* culture have been previously described (Bekalu et al. 2023). Researchers have achieved success in reducing tissue culture recalcitrance by modifying external factors to make the *in-vivo* environment more conducive to plant tissue. These factors include the type and age of the explant, the culture media, and the use of hormones and additives to promote cell division or by altering the light conditions to prevent oxidative browning (Long et al. 2022). Often, a combination of these factors needs to be optimized to achieve tissue culture success.

In addition to tissue recalcitrance, some transformation protocols are limited by the long incubation steps required for plant tissue culture. To circumvent this, researchers have developed methods that eliminate the tissue culture steps by transforming plants *in-vivo.* The most popular method of this is floral dip transformation, which involves immersing developing floral organs in a solution of *Agrobacterium.* This protocol has been most effective for *Arabidopsis thaliana* but can be adapted to other species by altering the concentrations of surfactants like silwet to aid bacterial binding or by adding a vacuum infiltration step to the protocol. Some researchers have generated transgenic plants by direct injection of *Agrobacterium* to plant tissues (Luo et al. 2020). The most successful injection sites have been meristematic (Benson 2000; Bekalu et al. 2023) or reproductive tissues (Luo et al. 2020; Sharada et al. 2017; Bahari et al. 2020), although leaf and stem tissues have also been used as successful injection sites (Bahari et al. 2020; Meng et al. 2019). In one example, Maher et al (2020) found that adding an overexpressed morphogenic gene to their construct resulted in the regeneration of transgenic shoots from injected meristematic embryos. In the same paper, a second method showed that these morphogenic genes could be used to induce the development of transgenic shoots on the surface of wild type leaf tissue, using whole seedlings as the explant. Both methods eliminate the need for an in vitro tissue culture step, which could be especially useful when applied to species which exhibit tissue-culture specific recalcitrance.

Another recently developed method of *in-vivo* transformation is the cut-dip-budding (CDB) gene delivery system (Cao et al. 2022). *A. rhizogenes* delivers genetic material to the cut root sites of seedlings, where transgenic hairy roots form. These hairy roots are then transferred to soil, where the native plant root-suckering ability induces the

development of shoots into whole plants, thus providing a transformation and regeneration method without the use of tissue culture. The successful transformation of a wide variety of plant species, including herbaceous, woody (Cao et al. 2022), and succulent plants (Lu et al. 2024), suggests that CDB is much less genotype and species dependent compared to other *in-vivo* methods. Although it is limited to species that can be transformed with *A. rhizogenes* and can sucker, this represents significant progress in developing species independent transformation systems.

## Overcoming the infection barrier

Optimizing the infection step is crucial for efficient plant transformation. Several factors impact the ability of *Agrobacterium* to effectively deliver T-DNA to plants, including explant type, *Agrobacterium* strain and concentration, co-culture media and culture conditions (Zhao et al. 2020). Researchers have enhanced *Agrobacterium* virulence by adding phenolic compounds like acetosyringone or spermidine to co-culture media, which stimulate the activity of *Agrobacterium* virulence genes. Methods have also been developed to aid the delivery of *Agrobacterium* to plant cells. These include physical techniques, such as wounding, agroinfiltration (Kaur et al. 2021), and sonification (Santarém et al. 1998; Vasudevan et al. 2020). Additionally, plant host genes important for transformation have also been manipulated to increase infection rates (Gelvin 2010), although this technique requires a reproducible method for plant transformation and would not be viable for recalcitrant species.

The current successful strategies for overcoming the infection steps of plant transformation mirror early efforts to establish *Agrobacterium* as a usable vector. In nature, *Agrobacterium* species infect a broad range of dicots, causing tumorigenesis that is typically presented as crown galls or hairy roots. Scientists enhanced native *Agrobacterium* to be an effective tool for gene transfer by implementing two main modifications, removing the tumorigenesis genes and creating the binary vector system. These modifications required an understanding of *Agrobacterium* biology, which have been extensively covered in several excellent reviews (Gelvin 2003; Azizi-Dargahlou and Pouresmaeil 2023). Current strategies focus on the identification or enhancement of different strains to reduce host plant defenses.

The strain of *Agrobacterium* used for transformation significantly impacts infection efficiency (Gelvin 2003; Torregrosa, Locco, and Thomas 2002; Chetty et al. 2013) and is often genotype and species dependent. Therefore, screening various strains or species is necessary to optimize transformation success. Reviews have detailed genotype differences among popular *Agrobacterium* strains (Gelvin 2003; De Saeger et al. 2021). Briefly, commonly used strains for *Agrobacterium tumefaciens* include *GV3101*, *EHA105*, *LBA4404,* and *AGL1*. GV3101 is generally used for dicotylous plants, while EHA105, a hyper-virulent strain, is favored for monocot transformation. Other *Agrobacterium* species can also enhance transformation success. Woody and leguminous species, often resistant to *A. tumefaciens,* show improved transformation rates with modified strains of *A. rhizogenes* (Gong et al. 2024), which induce hairy roots. Most common strains of *Agrobacterium* have been isolated from one or two lines, which likely contributes to the small number of plant species that are able to be transformed. Therefore, improving or isolating genes from other wild type *Agrobacterium* strains, like *A. vitis* (Torregrosa et al. 2002) and *A. rubi* (Ondřej et al. 1987), shows promise in increasing the number of transformable plants. For example, 'shooty' genes isolated from wild-type *Agrobacterium* strains, such as gene *6B,* have been identified as significantly improving plant infection and transformation (M. Wang et al. 2011).

Determining which strain of *Agrobacterium* is most suitable for infecting a plant of interest involves the use of a reporter gene to evaluate infection capabilities. GFP and GUS (β-glucuronidase) are particularly useful as reporters for monitoring both transient and stable transformation but can be limited in their application since they are either invasive or require expensive equipment to visualize. In 2020, a new reporter named *RUBY* was developed (He et al. 2020). It is both non-invasive and allows for visualization without other equipment or assays. *RUBY* converts tyrosine to betalain, which is vividly red, and visible to the naked eye. While it has primarily been used for visualizing gene expression (Yu et al. 2023) and screening explants for transformation (Tripathi et al. 2023) capabilities, it could also be an excellent method for screening the infection capability of *Agrobacterium* strains. In addition, evidence for *RUBY* expression appears quickly, suggesting a rapid screening method.

Even when the optimal strains of *Agrobacterium* can be identified, it is common for many plants to have a relatively low infection rate. A major contribution to plant recalcitrance during the infection stage is native plant resistance. Past studies have worked to address this issue by altering plant growth conditions to make them more favorable for *Agrobacterium* binding. However, the newer methods for reducing plant defenses have involved further modifying strains of *Agrobacterium* to include additional genes that aid in transformation. Early efforts included the creation of a super binary vector, which built on the binary vector system by incorporating extra virulence genes to aid transformation (Anderson and Birch 2012; Komari et al. 2006). However, the larger size of these vectors made them difficult to work with, necessitating alternative methods. The invention of ternary vector systems addressed this issue by placing additional virulence genes on a third plasmid, known as the helper plasmid, thus benefiting from virulence genes without impacting the Ti-plasmid. These systems have been shown to

enhance transformation efficiency in several studies (Anand et al. 2018; Yu Zhang et al. 2020). In a similar approach, Raman (2022) and colleagues recently engineered *Agrobacterium* to express a type III secretion system, through the transport of Pseudomonas effector proteins. By individually delivering effector proteins that work to suppress native plant resistance, they achieved a significant increase in transformation efficiency in *Arabidopsis*, alfalfa, switchgrass, and a recalcitrant form of wheat (Raman et al. 2022). These results show that the native plant defenses against transformation can be effectively disarmed through the use of engineered *Agrobacterium* strains, and reviews have cited this as a promising method improving plant transformation efficiencies in the field (Lee and Wang 2023).

## Enhancing regeneration

Another significant barrier to successful plant transformation is the regeneration of whole plants from transformed cells. In many cases, this challenge arises from the explants' low or in-ability to regenerate into whole plants, even when hormonal conditions in the culture media are optimized. In addition, determining the correct hormonal ratio to promote regeneration can be tedious, necessitating a faster method to promote regeneration.

To enhance the regeneration stage of plant transformation, several studies have shown improved transformation efficiencies by co-expressing developmental regulators/morphogenic transcription factors such as *BBM*, *Wus2*, and *GRF4*. Overexpression of *BBM*and *Wus2* resulted in increased plant transformation efficiency in several recalcitrant varieties of maize, rice and sorghum (Lowe et al. 2016). The overexpression of the *Arabidopsis*-derived, wound induced transcription factor *PLT5*, was also shown to enhance transformation efficiencies by promoting the development of transgenic callus and shoots at wounding sites in multiple species (Lian et al. 2022).

Newly identified morphogenic genes, either discovered from pathways involved in meristem formation or homologs of existing genes is another strategy that can be successful in promoting enhanced regeneration efficiencies. For example, wheat *TaWOX5,* a homologous gene of Arabidopsis *WUS,* was shown to reduce the genotype dependence of regeneration in several varieties of wheat (Wang et al. 2022). In addition, *WOX5* also improved the regeneration rates of several other cereal crops. Both *TaDOF* (Liu et al. 2023), and *ZmWIND1* (Jiang et al. 2024) were identified by examining the regeneration related pathways in wheat and maize, and were successfully applied to increase regeneration in recalcitrant varieties in their respective species.

However, several problems exist when using morphogenic genes to improve regeneration. The first is that most of these genes do not promote increased regeneration across all species. For example, the overexpression of *WUS* promoted regeneration in tomato, but had little to no effect in snapdragon (Lian et al. 2022)**.** The fusion protein GRF4/GIF1, developed by Debernardi et al. seems to have a higher capability of increasing regeneration across multiple species, compared to other morphogenic genes. These include wheat, rice, citrus (Debernardi et al. 2020), lettuce (Bull et al. 2023) and, significantly, a recalcitrant variety of sorghum, which is notoriously difficult to transform (Silva et al. 2022). Another difficulty with using developmental regulators is that their overexpression can cause mild developmental changes, including curled leaves and shorter flowering times, limiting their use for some studies (Gordon-Kamm et al. 2019). To circumvent this, researchers have used conditional or transient methods of expressing the morphogenic genes. Conditional expression methods include inducible or excisable expression systems. Inducible promoters allow morphogenic genes to be expressed only under specific conditions, such as the presence of a particular chemical (Kyo et al. 2018) or steroid (Heidmann et al. 2011; Lutz et al. 2015; Shires et al. 2017) in the media, while excisable promoters utilize the Cre recombinase and LoxP (Cre-Lox) or similar systems to allow the genes to be expressed until the explants are exposed to a specific stress (Lowe et al. 2016; Mookkan et al. 2017). Efforts have also been made to ensure that morphogenic genes are expressed transiently, through the use of *Agrobacterium* strains with lower integration efficiencies (Dhir et al. 1998; Hoerster et al. 2020; Canto 2016). However, many *Agrobacterium* based transient expression systems report a small amount of stable transgene integration, which can be difficult to distinguish from plants in which the genes are transiently expressed. Therefore, inducible or excisable systems are more ideal for the conditional expression of morphogenic genes.

Gene editing strategies have also been used to enhance the regeneration stage of plant transformation. Clustered regularly interspaced short palindromic repeats (CRISPR) was recently repurposed to work on the transcriptional level, using a deactivated Cas9 that still retains single guide RNA-mediated binding activity (Ding et al. 2022), called CRISPR-activation (CRISPR-a). By designing the gRNA to target morphogenic gene regulators, studies have shown that this system is capable of improving regeneration efficiency in plants (C. Zhang et al. 2024a, b). In another study, this principle was applied to create CRISPR systems capable of simultaneously making gene edits and activating morphogenic genes within a single system, demonstrating the broad applicability of this method for enhancing plant improvement (Debernardi and Rowan 2022; Pan et al. 2022). Altering the transcriptome of genes through CRISPR-a technology has the potential to address other bottlenecks in plant transformation. Several reports have shown that the system can be repurposed for transcriptional repression (Debernardi

and Rowan 2022; Lowder et al. 2018; Karlson et al. 2021). Although not yet reported in the literature, gRNA could be designed to repress genes involved in plant native defenses, therefore improving the infection step without causing deleterious effects to the gene. The repression or activation of these genes in both strategies can be temporally controlled by placing the CRISPR system under a conditional promoter, enabling gene expression or repression at the desired transformation stage. Overall, these advancements in CRISPR technology hold significant promise for overcoming the various challenges associated with plant transformation.

Future strategies for improving *Agrobacterium* transformation will likely involve a combination of the approaches outlined above, which are highlighted in Fig. 1. For recalcitrant species, it will be necessary to identify where the transformation barrier exists. First, the optimal tissue culture conditions will need to be determined to produce healthy and transformable tissue. Appropriate strains of *Agrobacterium,* including ternary vectors, can be quickly screened against different explants using RUBY as a reporter. Once infection is determined, regeneration can be enhanced through a combination of exogenously applied hormones and the overexpression of morphogenic genes.

## Biolistic plant transformation

For many plant species that are recalcitrant to *Agrobacterium* mediated transformation, the biolistic method is often preferred. This method is also known as 'particle bombardment' or the 'gene gun method', with well described mechanisms (Altpeter et al. 2005; Ozyigit and Yucebilgili Kurtoglu 2020), summarized in Fig. 2. Genetic material is coated onto a gold or tungsten microcarrier, which is then loaded onto a macro-carrier and then propelled through an acceleration tube to penetrate plant tissue. A barrier prevents the macro-carriers from entering plant cells, allowing for only the microcarriers containing the genetic material to become integrated. Originally popular for the transformation of monocots, this method has recently been used for dicots and seed-free plants that are recalcitrant to other transformation methods. Beyond enabling successful transformation in challenging species, biolistic transformation offers several advantages. Besides DNA, it can easily be adapted to introduce protein and mRNA, offering a method for transferring a wide range of genetic material without the use of a binary vector. Additionally, the absence of a biological host removes restrictions imposed by host-plant defenses, allowing for a wider range of plant material that can be transformed.

Despite these advantages, not all plants are able to be transformed by the biolistic system. The efficiency of the instrument, size and material of the microcarrier and the type of explant chosen all significantly influence the success of a biolistic transformation. Achieving stable transformation and regeneration in transformed cells can be challenging due to high transgene copy numbers or genomic damage from the integration process. Although the basic steps of biolistic transformation have remained unchanged since its inception (Ismagul et al. 2018), current studies aim to improve one or more of these factors to allow for reproducible and highly efficient protocols.

### Explant and culture conditions

Choice of the correct explant is arguably the most critical factor in biolistic transformation. Despite the wider range of explant types available for use compared to other types of transformation, studies show that certain tissues have distinct advantages. For example, potato transformation success varies depending on the explant or genotype used (Malakhova et al. 2021; Altpeter et al. 2005; Ozyigit and Yucebilgili Kurtoglu 2020). Studies have also shown that the stage of tissue chosen also has an influence on successful DNA delivery. In their experiment with banana meristems, Mahdavi and colleagues showed that targeting tissues with actively dividing cells leads to a significant improvement in transformation rates when applying the biolistic method (Mahdavi et al. 2014). This discovery has been reflected in the literature, where callus, immature embryos, and embryotic meristems (Y. Liu et al. 2024) are among the more popular targets for transformation. In addition, it has also been shown that treatment of tissues before and after transformation has a positive influence on transformation success. Pretreatment of *Camellia sinensis* (tea plant) somatic embryos with hormone free media, followed by a period of darkness after bombardment was shown to increase the frequency of secondary embryo production (Furukawa et al. 2020)**.** Wheat transformation was also improved by introducing a low temperature pre-treatment step, combined with high levels of maltose in the media (Abe et al. 2020). Orchid tissue experienced the highest transformation rates when post-bombarded tissue was incubated for two days on antibiotic-free media before transferring to a selection medium (Men et al. 2003). These examples highlight the importance of optimizing tissue culture conditions to achieve transformation success.

Although the optimal explant type and tissue culture conditions will likely need to be tailored to the plant and genotype used, there are several tissue types that should be avoided for a biolistic transformation. Tissues with thicker or waxy cuticles, or those with a hairy surface, have been shown to be universally incompatible with biolistic transformation processes, due to the difficulty of penetrating the outer layers of the tissue (Lacroix and Citovsky 2020). As previously mentioned, some plant species are incompatible with tissue culture methods. To bypass this issue, methods of

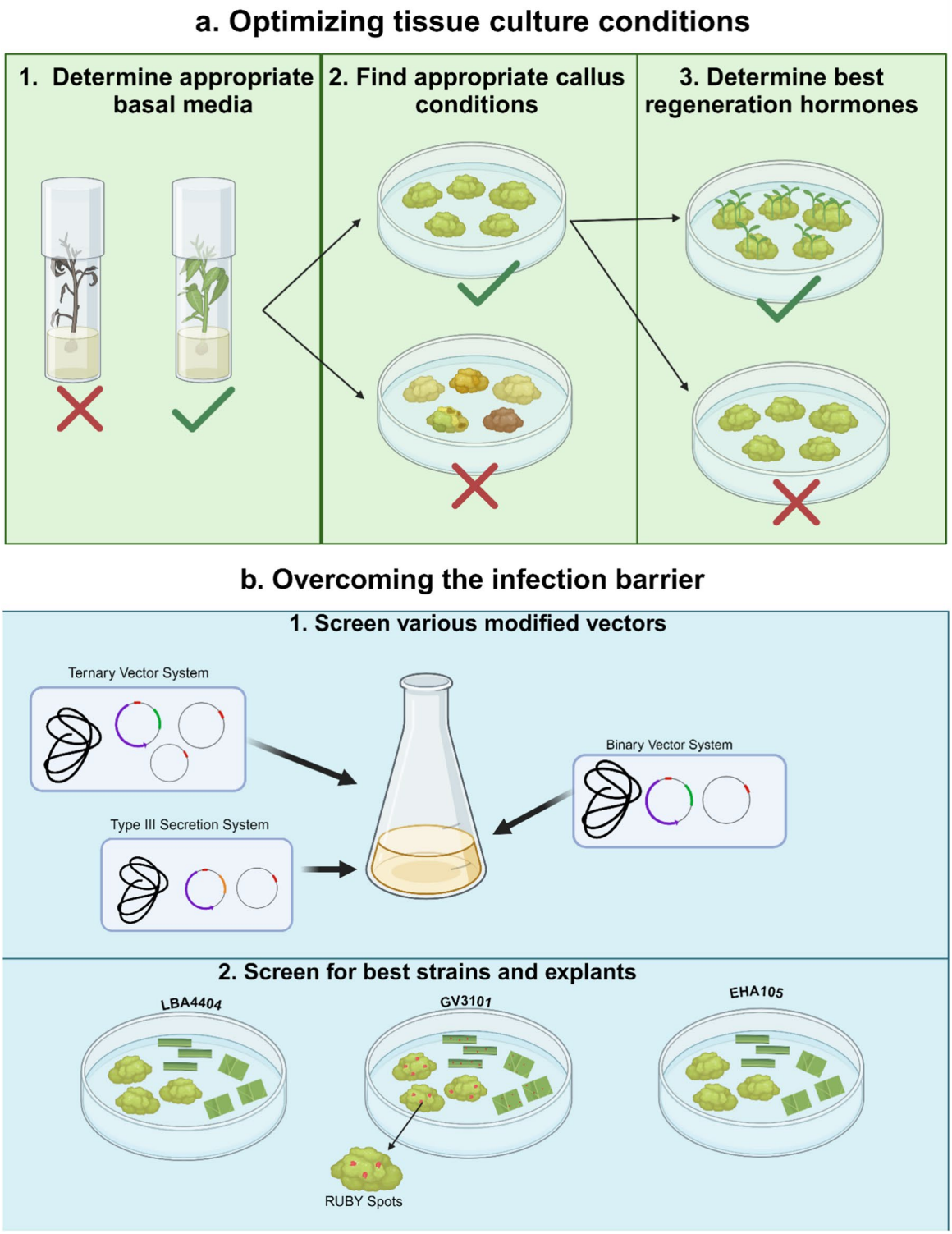


**Fig. 1** Optimizing *Agrobacterium* mediated transformation: strategies to improve *Agrobacterium* transformation involve a combination of approaches to optimize protocols. **a** Optimizing tissue culture conditions consists of: **1** Determining the appropriate basal media, **2** Finding appropriate conditions conducive to callus growth, and **3** Screening media to determine the best regeneration hormones. **b** Overcoming infection barriers. To determine which strains of *Agrobacterium* are best for infecting a species of interest, various strains, including modified strains, need to be screened. The reporter gene *RUBY* is well-suited for screening infection efficiency, as vibrant red spots appear relatively quickly. **c** Enhancing regeneration. Once tissue culture and infection conditions have been optimized, morphogenic genes can be used to improve regeneration efficiency. This can be done by screening various genes which contain morphogenic genes under an overexpression promoter, or through using the recently developed CRISPR-a system to activate the expression of native morphogenic genes in the plant of interest. This image was generated using biorender.com

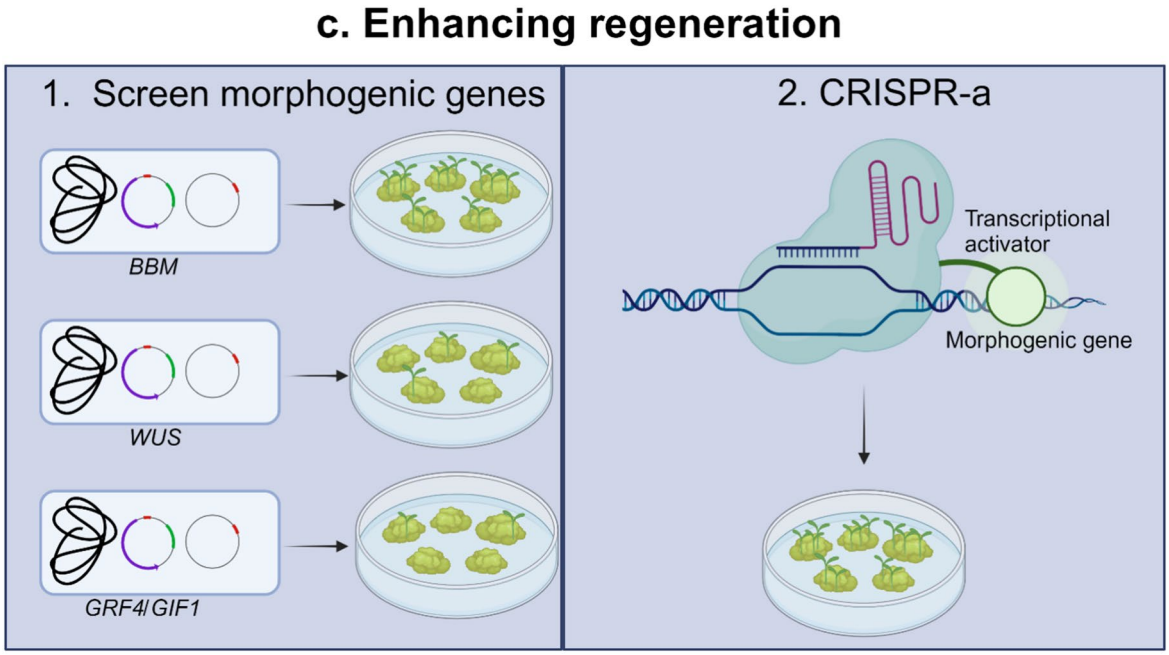


**Fig. 1** (continued)

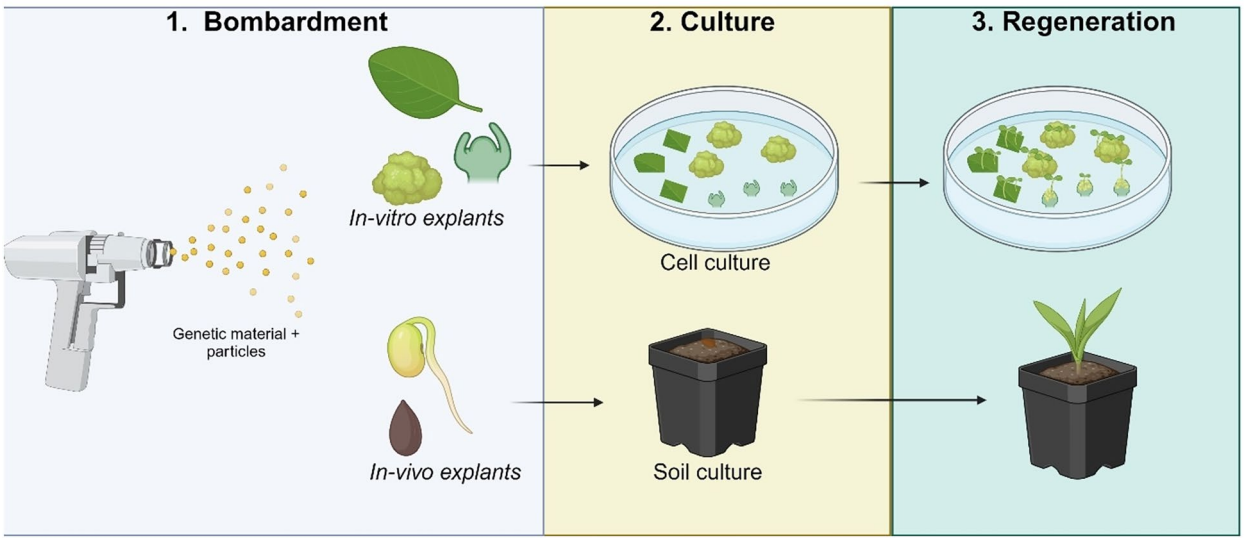


**Fig. 2** Biolistic Plant Transformation. **1** Genetic material-coated particles are propelled through an acceleration tube to penetrate plant tissue. Leaves, calli, and meristems can be used for *in-vitro* bombardments, although seeds and developing embryos have shown success in *in-vivo* methods. **2** In the *in-vitro* method, bombarded tissue is cultured on selective media, until callus develops. In *in-vivo* methods, explants can be directly transferred to soil. **3** Plants are regenerated through growth hormones (*in-vitro)* or are grown on soil (in vivo). This image was generated using biorender.com

*in-planta* particle bombardment have been developed. Imai et al (2020) developed an *in-planta* strategy for the transient transformation of wheat, by targeting embryotic shoot apical meristems. Shoot apical meristems are exposed on embryos using a needle, bombarded, then transferred to soil for further analysis, showing a biolistic method that does not require a tissue culture step. The same group also showed that the same method could be used to achieve stable transformation (Hamada et al. 2017).

## Instruments and microparticle materials

Another common consideration with biolistic transformation is genomic damage caused by the highly pressurized

particles necessary for the integration process. The extent of this damage was largely unknown for many years, until Liu and colleagues (2019) investigated the effects in their paper in 2019, showing that biolistic transformation has the potential to result in extreme genomic damage in transgenic rice and maize. This damage includes chromosome truncations, large deletions, and other damage, which would generally be undesirable in the transgenic progeny. In addition, plant cells are often transformed inconsistently, which can cause problems, especially for the analysis of transient expression. Recent studies have worked to address these challenges, either by implementing new devices or modifying microcarriers.

Several devices have been implemented to mediate biolistic transformation. The instrument Biolistic® PDS-1000/He is one popular device, and applies vacuum infiltration to the traditional bombardment method to achieve reproducible results in multiple species (Men et al. 2003; K. Wang, Zhu, and McCaw 2020; Davlekamova et al. 2023; Martin-Ortigosa and Wang 2020; Geng et al. 2022). Another commonly used tool is the Helios® Gene Gun, which is favored for greenhouse and field studies. Its handheld design allows it to be used on plants of various sizes, and has been used for both transient and stable transformation in several species (Acanda, Wang, and Levy 2019; Kuriakose et al. 2012; Joshi et al. 2015). Recently, such devices have been modified to reduce the negative effects associated with genomic integration, such as the implementation of double-barrel devices. Double-barrel devices reduce tissue damage following bombardment, improve reproducibility, and allow for the addition of a control, as multiple constructs can be delivered simultaneously (Miller et al. 2021; Kale and Tyler 2011). Controls in the double-barrel method have the additional benefit of allowing for easier assessment of transformation efficiency. It can be theorized that similar modifications of previously developed biolistic devices will also result in improved transformation procedures, especially those focused on reducing tissue or genomic damage.

The size and type of microcarrier also significantly influences delivery efficiency. Gold and tungsten are the most popular microcarriers, due to their ability to effectively bind genetic material while causing minimal damage to plant cells or tissues (Ozyigit and Kurtoglu 2020). However, each has their disadvantages: tungsten can cause DNA damage or inhibit cell growth, whereas gold does not bind DNA as effectively. The size of the microcarrier also significantly impacts transformation success, with several studies showing that optimizing this size is crucial for achieving successful transformation (Mahdavi et al. 2014; Davlekamova et al. 2023; Aesaert et al. 2022). Nanoparticles have also recently been of interest as alternative microcarriers, as they offer several advantages over their micro-sized counterparts.

Studies have shown that nanoparticles can reduce genomic damage, achieve similar levels of transgene integration (Mortazavi and Zohrabi 2018), and increased transformation efficiency (Rajkumari et al. 2021). Additionally, nanoparticles can penetrate biological barriers that traditional microcarriers cannot access (Cunningham et al. 2020). Theoretically, this should allow for more efficient transformation of organelles. While successful transformation of chloroplast and mitochondrial genomes using the biolistic method has been reported (Elghabi et al. 2011; Tanwar et al. 2024; Y. Wu et al. 2021), such achievements have not yet been demonstrated with nanocarriers in plants. Continued development of new nano-carrier based techniques may allow for more efficient transformation methods with minimal genomic damage.

## Other factors impacting biolistic transformation

Although instrumentation, microcarrier type, and tissue culture conditions are major factors influencing the success of a biolistic transformation, several other aspects can be further optimized. The quantity of genetic material loaded onto the microcarrier significantly impacts transformation success. Higher amounts of DNA can increase transformation efficiencies but may lead to tissue damage and high copy numbers of transgene integration (Ozyigit and Yucebilgili Kurtoglu 2020). For example, a study on wheat transformation found that the high copy number effect could be mitigated by reducing vector DNA to nanogram amounts (Ismagul et al. 2018), demonstrating that lower amounts of starting material can more positively impact the experiment.

The bombardment distance and pressure also should be refined. Increasing the distances reduces transformation efficiency due to decreased pressure, which allows less genomic material to enter the cell. Conversely, shorter distances can cause cell death (Davlekamova et al. 2023). Pressure adjustments are also crucial; higher pressures can cause excessive tissue damage and reduced germination rates, while lower pressures decrease transformation efficiency. Bio-Rad, the developer of the PDS-1000/He biolistic gun recommends an optimal pressure of 1100 psi for their device. Notably, multiple studies have found that this pressure is best when optimizing their experiments, including those with banana (Mahdavi et al. 2014), and sorghum and lettuce (Ruhlman 2021). While this pressure may not be universally optimal for all species and explants, it serves as a good starting point when developing new biolistic procedures.

Like any transformation method, biolistic transformation methods must be individually tailored to each species or explant type. First, the appropriate explants and tissue culture conditions for pre- and post- bombardment need to be determined to promote cell division and plant regeneration. Next, optimizing the microcarrier size and type is crucial

to achieve the highest transformation efficiency minimizing cell death. To mitigate genomic damage and increase consistency, consider reducing the amount of DNA per microcarrier, implementing a double-barrel instrument or adapting nanomaterials as alternative carriers. Finally, fine-tuning bombardment factors, such as bombardment distance and pressure, can further increase the efficiency of the transformation process. Incorporating a combination of these strategies will improve the success of new biolistic transformation procedures.

## Protoplast transformation

In comparison to plants, mammalian genetic transformation is well characterized. Exogeneous genetic material, including RNA or protein, can be delivered directly to cells through biological, chemical and physical means in a process known as transfection (Fus-Kujawa et al. 2021). These processes are well refined, especially for studies of human disease. However, transfection-based techniques are difficult to apply to plants due to the presence of the cell wall, which acts as a physical barrier to the delivery of exogeneous biomolecules (Cunningham et al. 2018).

The development of plant protoplasts has enabled the adaptation of some common mammalian cell transfection techniques for use in plants. Protoplasts are plant cells with their cell walls removed by chemical, physical or enzymatic means. Although not as widely used as *Agrobacterium* or biolistic transformation, protoplast-based transfection methods offer several advantages over traditional transformation techniques. These advantages include rapid gene functional analysis, transient expression, and the introduction of genetic material without the need for a biological vector. The ability to manipulate transient gene expression provides a straightforward method for transgene-free gene editing, which has emerged as a potential tool for future breeding. Additionally, some plants can only be transformed through protoplasts, showing the importance of considering these methods when developing new plants transformation strategies.

Protoplast transfection faces bottlenecks in creating undamaged protoplasts from plant tissue and regenerating them into whole plants. As emphasized in several reviews, optimizing culture conditions is crucial for overcoming these challenges (Duarte et al. 2016; Reed and Bargmann 2021). Like *Agrobacterium* and biolistic transformation, steps of protoplast isolation systems and regeneration are highly species dependent and must be individually tailored to achieve the most efficient and reproducible results. In addition, the method used for transfection, which delivers exogeneous genomic material, also plays a role in the success of the protocol. Some of the most common protoplast-based systems are summarized in Fig. 3. However, for plant species with established protoplast isolation and regeneration systems, protoplast transfection remains a valuable tool for genetic modification. Some of the most common protoplast-based systems, along with modifications made to enhance their success, are discussed.

### Polyethylene glycol

Polyethylene glycol (PEG) mediated transformation is often the preferred method for protoplast transformation due to it being simple, relatively inexpensive, and applicable to many species where a protoplast isolation protocol is available. PEG causes DNA to precipitate in a solution, protecting it from nuclease degradation and allowing easier entry into the cell (Maas and Werr 1989). It also increases the permeability of the plasma membrane. After PEG transformation, transformed protoplasts are spread on media to encourage cell division and eventual regeneration into whole plants. This transformation method is useful for plants with seeds that have low germination rates or are not compatible with other transformation methods.

The importance of maintaining protoplast viability and regenerative capability is emphasized in several studies (Ahmed et al. 2021; Kharel et al. 2024). Although PEG transfection can be highly efficient, it often causes damage to protoplasts, limiting the viability of transformed cells after transfection. Several studies have investigated methods to mitigate this damage to transfected protoplasts. Earlier studies reported that the addition of divalent cations such as $Ca^{2+}$, $Mn^{2+}$, and $Mg^{2+}$, to the PEG-infection solution increases DNA precipitation efficiency (Maas and Werr 1989). Recently, one group reported that when added to the media, phytosulfokine alpha (PSK-α) increases the regeneration of transformed protoplasts. PSK- α, a chemical that acts as an intercellular signal peptide and growth factor, can greatly simulate protoplast cell division, micro callus formation and shoot regeneration after transfection (Vogrinčič, Kastelec, and Murovec 2024). In addition to optimizing the transfection conditions, several protocols report higher optimization when a reporter gene is used in the construct. Reporters, like GFP, allow for visualization of successful genetic material delivery before proceeding to subsequent culture steps of the protocols.

An advantage of PEG-mediated transfection is that it, along with biolistic transformation, is one of the few methods of plant transformation that allow for genetic modification of plant organelles. The biolistic system is often preferred for this method, since it is relatively less labor intensive and easier to induce regeneration compared to the protoplast method (Ruf and Bock 2021). However, due to species and tissue specific constraints associated with biolistic transformation, the PEG method should be considered as an alternative for species that are recalcitrant to

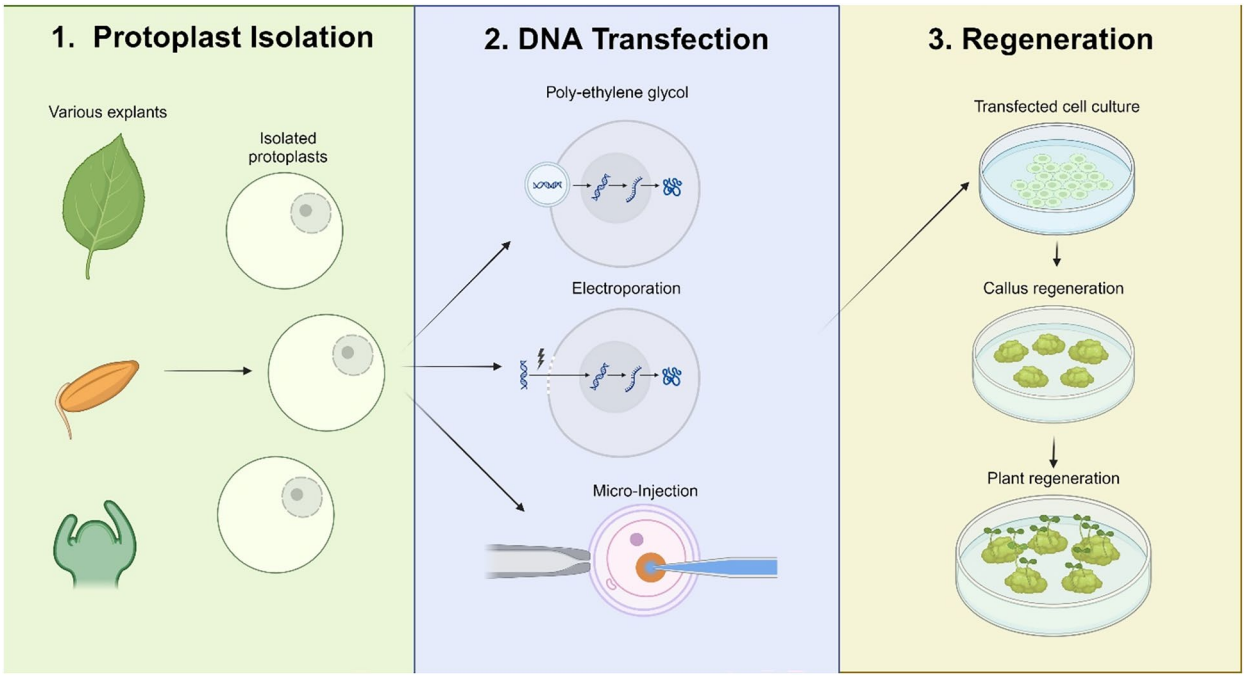


**Fig. 3** Protoplast transfection**:** three methods of protoplast transfection are highlighted. **1** Protoplasts are isolated from various explant types, through chemical, physical or enzymatic methods. **2** Transfection can take place in one of three methods; poly-ethylene glycol, electroporation or micro-injection. **3** Regeneration. After transfection, cells are transferred to a cell culture, until micro-callus colonies form. These colonies are then cultured into larger callus, then transferred to a regeneration media for regeneration. This image was generated using biorender.com

the biolistic method (G. D. Nugent et al. 2005; Sugiura and Sugita 2004; Gregory D. Nugent et al. 2006).

Another advantage of PEG-mediated transfection is the potential for producing transgene-free gene edited plants. PEG facilitates the direct delivery of assembled CRISPR-Cas proteins to protoplasts, without unintended consequences caused by random DNA integration of the CRISPR construct. Gene edits were successfully made to maize protoplasts by transfecting cells with a ribonucleoprotein complex containing CRISPR-Cas9 (Sant'Ana et al. 2020). Similar methods have also been applied to protoplasts of other species, including *Arabidopsis,* tobacco, lettuce, rice (Woo et al. 2015), grapevine, apple (Malnoy et al. 2016), petunia (Subburaj et al. 2016), and potato (Andersson et al. 2018). Generally, the success rates of CRISPR-RNP delivery through PEG mediated transformation are consistent across species and genotype. While this technique has been primarily utilized for gene knockout/knock-in studies, the recent focus on expanding CRISPR's functionality can expand the application of CRISPR-RNPs. For example, protoplast regeneration could be improved by applying a CRISPR-a system to activate genes involved in protoplast regeneration, similar to what was previously described using *Agrobacterium* (C. Zhang et al. 2024a, b). Although this method is limited to species with established protoplast extraction and regeneration systems, this could be a powerful tool for future genetic modification and breeding studies.

## Electroporation

Gene transfer using electroporation is commonly employed in plant biotechnology, particularly for transforming plasmids into *Agrobacterium.* Cells are co-incubated in a solution with a gene of interest and subjected to a series of electrical pulses, creating small transient pores in the cell membrane, through which genetic material can enter. Afterward, cells are grown in propagation media to multiply the number of transformed cells. When applied to plant cells, electroporation methods are efficient, due to their low cost, rapid application, and have the ability to stably transform a large amount of cells (Ozyigit 2020). In addition, electroporation primarily introduces single gene copies into the cells, providing an alternative method to bypass some of the negative effects associated with biolistic transformation (Su et al. 2023). However, electroporation is not universally applicable to all plant cell types, primarily due to the increased impermeability of the plant cell wall. Additionally, the electrical pulses required for genetic material incorporation can cause significant cellular damage, especially to protoplasts, which necessitates the use of a large initial number of cells. To address these challenges, optimizing electroporation

conditions such as voltage and capacitance, plays a significant role in minimizing tissue damage and increasing transfection efficiency (Ozyigit 2020). Current applications of plant electroporation transfection focus on modifying plant material to further improve the success of this method.

Modifications to plant material used for electroporation transfection have expanded the range of species that can be manipulated. By using protoplast suspensions, barriers associated with cell wall impermeability have been reduced, enabling the publication of electroporation protocols for multiple species, including both model (Chupeau, Pautot, and Chupeau 1994; Guerche et al. 1987) and crop plants (H. M. Zhang et al. 1988; Díaz 1994; Tonnies et al. 2023). However, regeneration from protoplasts remains a challenge for electroporation-based methods, as electrical pulses can damage the fragile protoplast cells. When intact cells are used as the explant material there is significant improvement in regenerating after electroporation. This is likely due to more optimized electroporation conditions that have recently been developed, allowing for resealable pores that bypass the cell wall. *Cre* recombinases were successfully delivered to intact *Arabidopsis* cell suspensions (Furuhata et al. 2019), and the application of callus as an explant has made electroporation the most efficient method of transfection for several species of algae (Wałpuski et al. 2024). Whole plant tissues can also be used; soybean embryos (S. Li et al. 2000), sugarcane meristems (Arencibia et al. 1995), and carnation stems (Mori et al. 2024) from greenhouse grown plants have all been successfully transfected using electroporation.

In comparison to other methods discussed, few methods of *in-vivo* electroporation transfection have been developed. Nodal auxiliary buds of several legumes were exposed and then transfected through electroporation, resulting in chimeric branches that could produce transgenic progeny (Chowrira et al. 1996). Wheat pollen was also successfully transfected, allowing for transgenic progeny to be produced through pollination, with transgenes being inheritable to the T2 generation (Zhang et al. 2013). Although neither of these methods have been widely adopted in research due to the availability of other transformation/transfection systems, they offer alternative, tissue-culture-free approaches that could be applicable to traditionally hard-to-transform species, such as wheat.

### Microinjection

Compared to other methods discussed, microinjection is significantly less popular for plant transfection, despite having multiple advantages. The microinjection technique involves transfixing a single cell on a medium and using a glass microinjection capillary pipette to directly inject genetic material into the cell. This allows for targeted DNA delivery to specific cells or organelles at a high transformation efficiency, which is advantageous in studies involving single cell biological reactions. It also provides an alternative technique for genetic manipulation in species that are difficult to transform using other methods, like date palm (Yasha Zhang et al. 2024a, b). However, despite these advantages, the technique can be inefficient, requiring significant technical skill to transform a small number of cells. In addition, plant cells contain a cell wall, which acts as a significant barrier to microinjection methods, while protoplasts can be delicate and hard to regenerate after transfection. Current improvements in microinjection techniques have focused on altering culture conditions to allow for damage-free injections or optimizing regeneration conditions of transfected cells.

The structure of the plant cell wall makes it relatively difficult for microinjection pipettes to penetrate the cell (Shruti, Tripathi, and Shukla 2024). One study concluded that the success of microinjection into intact cells may depend on cellular turgor pressure, as transfection was achieved by the reduction of pressure in carrot cultures (Nomura and Komamine 1986). More recently, CRISPR/Cas9 proteins have been successfully injected into intact wheat microspore cells by first making the cells competent via a heat shock procedure (Szabala et al. 2024). Generally, protoplasts are preferred for microinjection studies, because their lack of barrier, combined with the available protocols and commercially made equipment, significantly enhanced their efficiency for transfection (Quezada, Ijaz, and Malik 2024). The regeneration capability of microinjected protoplasts or intact cells is influenced from the explants from which they are isolated from. Cells isolated from regenerating tissue positively influence capability of transfected cells to regenerate into whole plants, with embryonic tissue or microspores often being preferred for these experiments (Jones-Villeneuve et al. 1995).

## Nanotechnology

Regardless of the carrier used for gene transfer into plant cells (*Agrobacterium*, biolistic, etc.), the success of most published transformation/transfection systems heavily depends on the species and explant type used. Generally, most *in-planta* methods are considered genotype independent, although some *in-vitro* genotype independent methods, such as those developed for tomato, have also been established (Sandhya et al. 2022). Methods like the CDB system, discussed above, represent significant advancements in creating species-independent transformation systems (Cao et al. 2022). However, they are constrained by the need for unique developmental features,

such as budding. Therefore, developing a transformation system that is species, genotype, and even tissue independent is of considerable interest and importance to plant biotechnology.

Nanotechnology has recently gained interest for the development of such methods (Yan et al. 2023). Due to their small size, ability to passively enter cellular membranes and capacity to protect naked genetic material, nanoparticles are thought to be well-suited as delivery vectors in plant biotechnology. Studies applying nanoparticles to transformation systems have shown that they can overcome some species-specific barriers associated with conventional methods of plant transformation. The recent application of nanotechnology to plant transformation and its potential to further advance plant biotechnological research is discussed.

Nanoparticles are artificially or naturally made particles on the nanoscale (1-100 nm) with customizable properties that allow for a wide range of targeted biological interactions. They can be produced from various materials, including metal, lipids, enzymes, carbon, silica, and clay, typically through chemical means (K. Wu et al. 2023). Recently, 'green' nanoparticles synthesized from plant materials have emerged as a low-energy, cost-effective alternative (Ying et al. 2022). The mechanisms behind the application of nanoparticles as delivery vectors for plant transformation are well described (Cunningham et al. 2018; Squire et al. 2023). These nanoparticles are synthesized, bound to genetic material, then introduced to plant tissues, typically through incubation or biolistic delivery (Fig. 4). Most nanoparticles can mediate genetic transfer because they are synthesized to be below the size exclusion limits to biological membranes, allowing for passive entry through the cell wall and nuclear membrane. Investigations into stable nanoparticle plasmid delivery into plant cells suggest that, after passing through the nuclear pores, the genetic material is likely able to integrate into the nucleus through local homologous end joining (Hajiahmadi et al. 2020).

Despite the attractive qualities of nanoparticles as delivery vectors, the majority of current nano-transformation methods published do not result in stable transformation (Lv et al. 2020), which is the most significant bottleneck of this method. While transient methods can be useful for gene functional analysis or for integration-free gene editing, stable transformation methods are necessary to maximize the potential of nanoparticles in plant genetic engineering. Several strategies using nanoparticles to achieve stable transformation have been developed, as summarized in Table 1.

Few studies have investigated the factors that affect the stability of transformation through nanoparticles. Mesoporous silica (Hajiahmadi et al. 2020; Z. Wang et al. 2017) and chitosan nanoparticles (Yasha Zhang et al. 2024a,

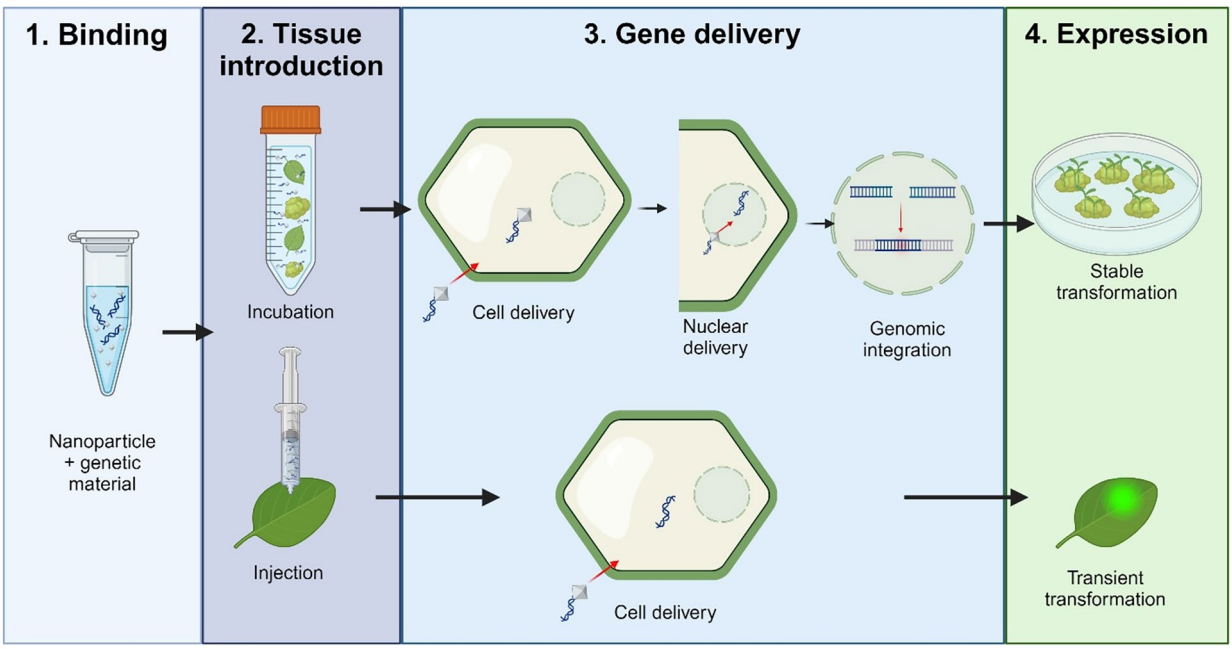


**Fig. 4** Nanoparticle Transformation**.** An overview of the process of nanoparticle transformation. **1** Binding: nanoparticles and genetic material are bound together through an incubation process. **2** Tissue introduction: nanoparticle-genetic material delivery vectors are introduced to tissues, typically through incubation or injection. **3**–**4** Gene delivery and Expression: nanoparticle vectors are able to enter the cell passively, due to being under the size restriction limits of the cell wall. DNA is either deposited into the cell for transient transformation, or carried into the nucleus, where it integrates into the genome through local homologous end joining. This image was generated using biorender.com

**Table 1** An overview of stable nanoparticle methods in plants

| Species | Nanoparticle used | Plant material | Genetic material | DNA:NP ratio | Delivery method | Citation |
|---|---|---|---|---|---|---|
| *Brassica juncea* | Calcium phosphate | Hypocotyl | Plasmid DNA | N/A | Incubation | (Naqvi et al. 2012) |
| Cotton | Polyethyleneimine-modified Fe3O4 MNP | Pollen | Plasmid DNA | 1:1 ug/uL | Magnetofection | (X. Zhao et al. 2017) |
| *Date Palm (Phoenix dactulifera)* | Chitosan nanoparticles | Plumule/seedling | Plasmid DNA | N/A | Injection | (Allah et al. 2023) |
| Okra | FeCl3 | Seed embryo | Plasmid DNA | 1:20 | Incubation | (Farooq et al. 2022) |
| Onion (*Allium cepa*) | Chitosan nanoparticles | Seedlings | Plasmid DNA | N/A | Injection | (Tawfik et al. 2022) |
| *Paulownia tomentosa* | Chitosan nanoparticles | Nodal segments | Plasmid DNA | 1:1 | Incubation | (Hussien 2020) |
| Tobacco | Mesoporous silica nanoparticles (MSN) | Callus | DNA | 1:4 | Sonication/Ultrasonic | (Z. Wang et al. 2017) |
| Tobacco | Silica nanoparticles (SiNP) | Young tobacco leaves | Plasmid DNA | 1:3 | Ultrasound-assisted | (Y. Fu et al. 2015) |
| Tobacco | Carbon nanotubes | Callus | Plasmid DNA | N/A | Incubation | (Burlaka et al. 2015) |
| Tomato (*Solanum lycopersicum*) | Mesoporous silica nanoparticles (MSN) | Tomato fruit/developing seeds | Plasmid DNA | 1:100 (ug/uL) | Injection | (Hajiahmadi et al. 2020) |

Methods of nanoparticle-based transformation which have resulted in stable plant transformation are highlighted

b; Hussien 2020; Tawfik et al. 2022) have been successful in promoting stability in some methods but also promote transient transformation in other protocols (Routier et al. 2023; Hajiahmadi et al. 2019). Additionally, several types of nanoparticles have been used to promote stable transformation in tobacco (Burlaka et al. 2015; Z. Wang et al. 2017; Y. Q. Fu et al. 2012), suggesting that transformation stability is unlikely to be significantly influenced by the nanoparticle material itself. Studies in transient transformation show that nanoparticles usually do not have much difficulty in delivering genetic cargo to cells (M. Liu et al. 2019a, b; Yan et al. 2023), although delivery efficiency can be dependent on having a smaller particle size (H. Zhang et al. 2022). This means that the bottleneck likely exists in the ability of the DNA to integrate into the genome. Interestingly, all current methods of stable nanoparticle transformation involve young, actively dividing cells as explants (Table 1). This mirrors the results of other transformation methods, where similarly young tissues positively influence the success of transformation. This suggests that actively dividing cells facilitate easier genomic integration, although this mechanism has yet to be proven. Nevertheless, this highlights the importance of using younger tissue in nanoparticle-transformation, which should be considered in the development of future transformation studies.

Like most transformation methods, tissue culture also can be a barrier to the efficiency or success of regenerating plants in nanoparticle systems, necessitating the development of *in-vivo* methods that achieve stable transformation. A few *in-vivo* systems have been developed for stable nanoparticle transformation. One such method is pollen magnetofection, where cotton pollen was transformed by magnetic nanoparticles after being subjected to a magnetic field, and then used to generate transgenic plants through pollination (X. Zhao et al. 2017). Although controversy has surrounded this method due to low reproducibility, it has been applied to various species in the *Lillium* genus for transient delivery (M. Zhang et al. 2023), suggesting potential application in multiple species. Another method achieved stable *in-vivo* transformation by directly injecting nanoparticles into the developing fruit of tomato. The resulting seeds were transgenic, and genes could be inherited by the next generation (Hajiahmadi et al. 2020). In addition, many transient transformation protocols involving nanoparticles can also be performed *in-vivo,* such as the foliar spray on method, which mediates transient expression in wheat (Doyle et al. 2019).

The success of future nanoparticle transformations will likely depend on using smaller nanoparticle vectors, targeting young tissue, and developing more *in-vivo* transformation systems that facilitate more efficient, genotype-independent transformations.

## Emerging model species

As biotechnological approaches to plant transformation improve, more plants can be efficiently transformed, increasing the potential for new species to be used as research

models. The importance of plant science's most used model, *Arabidopsis thaliana*, is well understood *Arabidopsis,* and other common model systems have significantly expanded knowledge in their respective fields of study. However, not all questions can be answered using these systems alone, necessitating the development of new models to further advance plant science research.

A summary of currently emerging models is included in Table 2, along with the methods used for transformation. Three of these species, i.e., morning glory, *Plantago,* and sugarcane, are highlighted in this paper. Each has unique features which have resulted in them garnering interest as a species of interest across several research areas.

## Plantago

Species in the genus *Plantago* (Plantaginaceae) are generally small, rosette-forming herbs with a wide geographical distribution. They are generally short-lived perennials and can be either diploid or polyploid, depending on the species (Penczykowski and Sieg 2021). *Plantago spp*. are indigenous to Europe, Asia and the Americas, with evidence suggesting they have existed alongside agriculture for over 4000 years (Samuelsen 2000). The significance of the *Plantago* genus in various areas of plant biology is noteworthy. *Plantago* species have been used as model organisms in anthropology, ecology, genetics, plant development and evolution, medicinal chemistry, and vascular biology, making them valuable for research.

**Table 2** Recently emerged plant model systems

| Species | Field(s) of study | Sequenced? | Transformation method | Explants |
|---|---|---|---|---|
| *Plantago* | Plant vascular biology, plant pathology, medicinal biology, etc | *Select species: P. major, P. ovata, P. astica* | *P. major, (Agrobacterium* Floral Dip) *P. lanceolata (Agrobacterium* Root Transformation) | Flowers/roots |
| Morning Glory (*Ipomoea*) | Ecology, genetics, plant reproduction, evolution | Multiple Species | *Ipomeoea nil (Agrobacterium rhizogenes* root transformation, *Agrobacterium tumefaciens* | Roots, immature embryo |
| Sugarcane | Perennial grass biology, grassland conservation, plant breeding | Yes | *Agrobacterium* tumefaciens (*In-vivo* and tissue culture based), electroporation | Callus, setts, seeds |
| Dandelions | Rubber, food, medicinal plant biology | *Select Species: Taraxacum kok-saghyz Rodin* | *Agrobacterium tumefaciens (Tissue Culture, Infiltration). Agrobacterium rhizogenes* | Leaf, intact whole plants/stem |
| Foxtail millet (*Setaria italica*) | Grass evolutionary genomics, physiology, abiotic stress tolerance, C4 photosynthesis | Yes | *Agrobacterium tumefaciens* | Embryotic callus |
| Duckweed | Human microbial parthogenesis, ecotoxicity, | Yes | *Agrobacterium tumefaciens (Stable, transient),* Biolistic | Fronds, calli |
| *Physcomitrella patens* | Plant evolutionary biology, developmental biology, plant biology, cell biology | Yes | Biolistic (Stable, transient), PEG | Cell culture, protoplast |
| *Marchantia polymorpha* | Evolutionary biology, plant physiology | Yes | *Agrobacterium tumefaciens* (Stable) | Thalli |
| *Zostera marina* | Ecology, physiology, genetics | Yes | None | None |
| *Echinacea purposa* | Medicinal biology | No | *Agrobacterium rhizogenes* | Seedlings |
| *Salvia miltiorrhiza* | Medicinal biology | Yes | *Agrobacterium tumefaciens, Agrobacterium rhizogenes, PEG* | Leaf disks, protoplast |
| *Kalanchoe daigremontiana* | Asexual reproduction, CAM studies | Yes | *Agrobacterium tumefaciens* | Leaf |
| Sunflower (*Helianthus annuus*) | Climate change adaption | Yes | *Agrobacterium tumefaciens, Particle bombardment* | Mature embryos, immature embryos, cotyledons, shoot apices |

Several recently emerged plant model systems are highlighted

Unlike in other species, pure vascular tissue can easily be obtained from *Plantago spp,* due to the presence of endodermal tissue that completely surrounds the vascular bundle (Pommerrenig et al. 2006 and Fig. 5). This unique feature has allowed them to be used extensively in plant vascular studies, where the identification of genes and pathways from vascular tissue has traditionally been difficult due to the challenges of accurate tissue collection. Studies have used this property to identify and localize the phloem-specific carbohydrate transporters in *P. major* (Nadwodnik and Lohaus 2008; Gahrtz et al. 1994). For instance, it has been found that sorbitol, a secondary carbohydrate transported in *Plantago,* is differentially regulated in response to salt stress (Pommerrenig et al. 2007). Other important biological discoveries have been made using *Plantago* vascular tissue. Due to this feature, and the fact that they have already been used as species of interest in plant vascular biology, several papers have proposed *Plantago* as a new model organism for this field (Pommerrenig et al. 2006; Huang et al. 2019; Levengood et al. 2023).

Several species in the *Plantago* genus have desirable features that make them suitable for model organisms. *Plantago* species generally have small genomes and short life cycles. Reference genomes have been published for *Plantago major* (Lyu et al. 2023)*, Plantago ovata* (Herliana et al. 2023)*,* and *Plantago asiatica* (Si et al. 2022). Both *Plantago lanceolata* (Levengood et al. 2023) and *Plantago major* (Pommerrenig et al. 2007) have been successfully transformed using either the *Agrobacterium* floral dip or tissue-culture method.

## Morning glory

Species in the genus *Ipomoea* are generally classified as ornamental due to their flowers, which are typically bisexual, bell-shaped, and are available in a variety of colors, enhancing their value as a garden plant. However, the relative diversity of phenotypes that exist among species in the genus give them significant value as species of interest for comparative analysis in multiple areas of research. Significant discoveries have been made in ecological genetics, molecular evolution, and medicinal biology studies on through species in the *Ipomoea* genus (Noraini et al. 2021). *Ipomoea nil* and *Ipomoea purpurea* are the most studied species in the genus (Clegg and Durbin 2003). They have also been used to study interactions between plants and herbivores, insects, and agricultural crops. In addition, their rapid generation times and ease of adaptation to greenhouse or field environments make them especially suitable for laboratory studies. As a result, there has been increased interest in establishing *Ipomoea* as a new model species. Efforts to develop multiple species of morning glory within the *Ipomoea* genus as model systems are highlighted.

Several advances in genomics have enabled for more complex investigations into functional genomics of *Ipomoea* species. Complete sequences are available for *Ipomoea nil* (Hoshino et al. 2016)*,* and the complete chloroplast genomes of *I. triloba, I. lacunosa, I. hederacea,* and *I. hederacea var. integriuscula* have also been published (Park et al. 2018). In addition, the transcriptomics of several species are available (Baucom et al. 2011; Solis et al. 2016). Among the studied species in the genus, genetic transformation systems have been developed for *Ipomoea nil,* and *Ipomoea purpurea* (Tan, Tao, and Li 2007)*,* enhanced by efficient micropropagation systems. Early methods of *Ipomoea nil* transformation targeted immature embryo tissue to achieve transformation at a low efficiency (Ono et al. 2000). Further studies significantly improved transformation efficiency by using modified *Agrobacterium* strains that include a ternary vector system with additional *vir* genes (Kikuchi et al. 2005). More recently, CRISPR-Cas9 was used to knock-out several flower color related genes in *Ipomoea nil,* demonstrating


**Fig. 5** *Plantago* is a good model species for plant vascular biology studies. **a** *Plantago major*. **b** *Plantago lanceolata*. **c** Vascular tissue is exposed when petiole is broken. **d** Pure vascular tissues extracted from *Plantago* petiole

the compatibility of gene editing to this species (Watanabe et al. 2017).

It is likely that more transformation systems will be developed for morning glory species within the *Ipomoea* genus. However, *Ipomoea nil* stands out as the ideal model species due to its efficient transformation system, compatibility with CRISPR/Cas9, and fully sequenced genome. Additionally, its rapid generation times, established micropropagation systems, and adaptability to both greenhouse and field conditions make it a valuable tool for various research areas.

### Sugarcane

Very recently, sugarcane has been proposed as a model species for perennial grass biology (Thirugnanasambandam, Hoang, and Henry 2018; Healey et al. 2024; C. Li and Iqbal 2024). However, sugarcane has a large, relatively complex genome. Interestingly, it is this large, polyploid nature of the sugarcane genome that makes it particularly valuable as a model species, with the potential to serve as a model for other plant species with polyploid genomes (Thirugnanasambandam, Hoang, and Henry 2018). Most polyploid species, especially grasses, are often challenging to study due to the lack of genomic resources. In contrast, the role of sugarcane as an important crop for energy and sucrose production in many countries means that there have been increased efforts to generate new genetic tools for this species.

Curiously, the complexity of the sugarcane genome has not prevented the development of its genetic tools. The use of bacterial artificial chromosome libraries has enabled the publication of the first fully sequenced genome of sugarcane (Okura et al. 2016), as the result of efforts funded by the sugarcane genome sequencing initiative. Mitochondrial (Evans et al. 2019) and chromosomal (Asano et al. 2004; Calsa Júnior et al. 2004; Hoang et al. 2015) genomes for several species in the genus have also been sequenced. Multiple methods of plant transformation have been developed for sugarcane species, including *Agrobacterium* mediated transformation (Anderson and Birch 2012; Nawaz et al. 2021; Basso et al. 2017), and electroporation (Arencibia et al. 1995). However, issues such as low transformation efficiency and the development of chimeric plants in tissue culture have necessitated the development of *in-vivo* transformation method. Two methods have been established for sugarcane, an *Agrobacterium* based seed method that seems to be genotype independent (Mayavan et al. 2013), and a nodal cutting method that is particularly effective for commercial hybrids (Mayavan et al. 2015). In addition, gene-editing methods have also been developed, and have been used in accelerating sugarcane breeding efforts (C. Li and Iqbal 2024).

The emergence of sugarcane as a model will be highly beneficial for studying plants, especially grasses, with polyploid genomes. In addition, multiple genomic resources have been developed for sugarcane, including those for genetic transformation, gene editing and sequencing. Taken together, these advantages make sugarcane a unique model with broad applicability for future research.

## Conclusions and future perspectives

Advancements of tissue culture and biotechnological systems for genetic delivery have significantly expanded the range of transformable plant species. *Agrobacterium* based delivery systems have improved in efficiency by incorporating new technologies that address specific barriers to efficient transformation. Biolistic delivery systems have been enhanced by adjusting machine parameters, reducing the amount of bombarded material to minimize genomic damage and applying treatments before or after bombardment to ensure the survival of transformed tissue. Protoplast systems can be remarkably effective at genomic delivery and can be strengthened by the development of new methods to create and regenerate undamaged protoplasts from plant tissue. Among all these methods, nanoparticle delivery systems have the highest potential for achieving a species-independent transformation method. However, most published protocols have only succeeded in transient transformation, necessitating the development of more stable methods before a species-independent method can be achieved.

One common theme across all plant gene delivery methods is the importance of tissue culture. Effective micropropagation systems are essential for maintaining healthy, transformable tissue for most *Agrobacterium,* biolistic, and nanotechnology methods. Similarly, the success of most protoplast-based systems is highly dependent on techniques for generating and regenerating protoplasts in media. Effective regeneration of transformed tissue also remains a challenge for most methods of plant biotechnology. However, regeneration can be increased by overexpressing or activating 'shooty' morphogenic-promoting genes or by using young, actively dividing cells as starting explant material in the future studies. Young tissue generally seems more amenable to infection and regeneration in most plant species, and, as seen in nanoparticle transformations, this is likely to contribute to the success of stable transformations.

For plant species that are particularly recalcitrant to tissue culture or regeneration, even with the application of young tissue or morphogenic genes, *in-vivo* techniques will be preferable in future attempts. In transformations using *Agrobacterium,* biolistic, nanoparticle or electroporation, tissue-culture free gene delivery methods

will likely offer advantages like increased transformation efficiency and shorter transformation timelines. In addition, published *in-vivo* methods often lack the genotype-dependence seen in other transformation methods, which could be a significant advantage for species where future studies are hindered by this issue.

The recent emergence of new plant models highlights the advancements in plant biotechnology. However, several problems still exist in plant transformation. A major challenge is the relative difficulty in identifying where certain transformation barriers exist. For example, if there is a lack of regeneration in *Agrobacterium* mediated transformation, then it can be difficult to determine if the bottleneck exists due to lack of infection or inability of transformed cells to regenerate. Likewise, it can be difficult to discern if tissue death in the biolistic method is due to cellular damage caused by bombardment, lack of optimal culture conditions, or excessive genomic damage. In addition, other than in *Agrobacterium* mediated transformation, few of the other techniques have been applied genetics-based techniques to overcome these barriers, once they have been identified. Therefore, future studies should explore the use of tools which help to further increase the efficiency of transformation techniques.

One strategy is to combine techniques which have allowed for increased transformation success within systems. *Agrobacterium*-based transformation can be optimized by using RUBY to quickly screen the most successful strains of bacteria, including modified strains like ternary vectors or type III secretion systems. After the best strains have been determined, regeneration efficiency can be improved by combining regeneration hormones with overexpressed or activated morphogenic genes. Biolistic-transformation methods can be improved by using nanogram amounts of DNA loaded onto nanoparticles through a device with a double barrel attachment to reduce genomic damage and increase infection consistency. Explants from areas of actively dividing cells should also be targeted, to increase the success of regeneration.

Another approach is to learn from the success of overcoming barriers in and in different transformation techniques and apply these approaches to new and existing transformation systems. Current examples of this have been shown to be widely successful, such as the example of nanoparticle-based biolistic techniques, which often help to decrease many of the negative effects associated with biolistic transformation. Combining the advantages of biolistics and RUBY could result in a rapid screening method for transformation stability in various bombardment conditions. It would also allow for a large amount of easily identifiable transgenic cells that could be used for downstream applications, such as the testing of regeneration media. The overexpression or activation of morphogenic genes could also be applied to other methods of transformation to increase regeneration efficiency. This could be especially useful in biolistic or protoplast-based methods, where limited cell regeneration is often a major problem of the technique. Advances in gene editing will also allow for increased efforts of plant transformation. Regeneration efficiencies have been improved through *Agrobacterium* mediated CRISPR-combo systems, and the delivery of RNP-CRISPR complexes will continue to facilitate genetic modifications in systems where stable transformation is difficult or undesirable.

Altogether, all methods of gene delivery have massively benefited from recent advancements and the application of new technologies. Future approaches that build on the success of existing techniques and incorporate emerging technologies will be crucial in overcoming barriers associated with recalcitrance.

**Author contributions** HL wrote the paper. HL, CZ and YZ revised the paper.

**Funding** This work was supported by the National Science Foundation (EDGE IOS-1923557 to C.Z. and Y.Z.).

**Data availability** No datasets were generated or used for this review paper.

## Declarations

**Conflict of interest** The authors declare that they have no competing interests.

## References

Abe F, Nakamura S, Mori M, Ashikawa I (2020) Low-temperature pretreatment of explants and high maltose concentration during callus culture improves particle-bombardment-mediated stable transgene expression in common wheat. Plant Biotechnol 37(2):177–184. https://doi.org/10.5511/plantbiotechnology.19.1216d

Acanda Y, Chunxia W, Amit L (2019) Gene Expression in citrus plant cells using Helios® gene gun system for particle bombardment. In: Catara AF, BarJoseph M, Licciardello G (eds) Citrus tristeza virus: methods and protocols. Totowa: Humana Press Inc. pp. 219–28

Aesaert S, Impens L, Coussens G, Van Lerberge E, Vanderhaeghen R, Desmet L, Vanhevel Y et al (2022) Optimized transformation and gene editing of the B104 public maize inbred by improved tissue culture and use of morphogenic regulators. Front Plant Sci 13:883847. https://doi.org/10.3389/fpls.2022.883847

Ahmed MAA, Miao M, Pratsinakis ED, Zhang H, Wang W, Yuan Y, Lyu M et al (2021) Protoplast isolation, fusion, culture and transformation in the woody plant *Jasminum* Spp. Agric Basel 11(8):699. https://doi.org/10.3390/agriculture11080699

Allah KWA, Ezz EDGHA, Kasem ZA, Eman TH, Ashraf BAR (2023) Phoenix dactylifera in vitro culture and transformation of Thio-60 antifungal gene via chitosan nanoparticle. Plant Cell Tissue Organ Cult (PCTOC). 155(2): 603–12. https://doi.org/10.1007/s11240-023-02505-7

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H et al (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Mol Breed 15(3):305–327. https://doi.org/10.1007/s11032-004-8001-y

Anand A, Bass SH, Emily Wu, Wang N, McBride KE, Annaluru N, Miller M, Hua Mo, Jones TJ (2018) An improved ternary vector system for agrobacterium-mediated rapid maize transformation. Plant Mol Biol 97(1–2):187–200. https://doi.org/10.1007/s11103-018-0732-y

Anderson DJ, Birch RG (2012) Minimal handling and super-binary vectors facilitate efficient, agrobacterium-mediated, transformation of sugarcane (*Saccharum* Spp. hybrid). Trop Plant Biol 5(2):183–192. https://doi.org/10.1007/s12042-012-9101-1

Andersson M, Turesson H, Olsson N, Fält A-S, Ohlsson P, Gonzalez MN, Samuelsson M, Hofvander P (2018) Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. Physiol Plant 164(4):378–384. https://doi.org/10.1111/ppl.12731

Arencibia A, Molina PR, de la Riva G, Selman-Housein G (1995) Production of transgenic sugarcane (*Saccharum officinarum* L.) plants by intact cell electroporation. Plant Cell Rep 14(5):305–309. https://doi.org/10.1007/BF00232033

Asano T, Tsudzuki T, Takahashi S, Shimada H, Kadowaki K-I (2004) Complete nucleotide sequence of the sugarcane (*Saccharum officinarum*) chloroplast genome: a comparative analysis of four monocot chloroplast genomes. DNA Res 11(2):93–99. https://doi.org/10.1093/dnares/11.2.93

Azizi-Dargahlou S, Mahin P (2023) *Agrobacterium tumefaciens*-mediated plant transformation: a review. Mol Biotechnol. https://doi.org/10.1007/s12033-023-00788-x

Bahari Z, Sima S, Ali N, Alireza A (2020) The application of an agrobacterium-mediated in planta transformation system in a catharanthus roseus medicinal plant. Czech J Genet Plant Breed. 56(1): 34–41. https://doi.org/10.17221/153/2018-CJGPB

Basso MF, Brito BAD, da Cunha A, Ribeiro P, Martins PK, Rodrigo W, de Souza N, de Oliveira G, Nakayama TJ et al (2017) Improved genetic transformation of sugarcane (*Saccharum* Spp.) embryogenic callus mediated by *Agrobacterium tumefaciens*. Curr Protoc Plant Biol 2(3):221–239. https://doi.org/10.1002/cppb.20055

Baucom RS, Chang S-M, Kniskern JM, Rausher MD, Stinchcombe JR (2011) Morning glory as a powerful model in ecological genomics: tracing adaptation through both natural and artificial selection. Heredity 107(5):377–385. https://doi.org/10.1038/hdy.2011.25

Bekalu ZE, Panting M, Holme IB, Brinch-Pedersen H (2023) Opportunities and challenges of in vitro tissue culture systems in the era of crop genome editing. Int J Mol Sci 24(15):11920. https://doi.org/10.3390/ijms241511920

Bennur PL, Martin OB, Shyama CF, Monika SD (2024) Improving transformation and regeneration efficiency in medicinal plants: insights from other recalcitrant species. J Exp Bot. https://doi.org/10.1093/jxb/erae189

Benson EE (2000) Special symposium. In vitro plant recalcitrance in vitro plant recalcitrance: an introduction. In Vitro Cell Dev Biol Plant 36(3):141–148. https://doi.org/10.1007/s11627-000-0029-z

Bull T, Debernardi J, Reeves M, Hill T, Bertier L, Van Deynze A, Michelmore R (2023) GRF–GIF chimeric proteins enhance in vitro regeneration and agrobacterium-mediated transformation efficiencies of lettuce (*Lactuca* Spp.). Plant Cell Rep 42(3):629–643. https://doi.org/10.1007/s00299-023-02980-4

Burlaka OM, Pirko YV, Yemets AI, Blume YB (2015) Plant genetic transformation using carbon nanotubes for DNA delivery. Tsitol Genet 49(6):3–12

Calsa TJ, Carraro DM, Benatti MR, Barbosa AC, Kitajima JP, Carrer H (2004) Structural features and transcript-editing analysis of sugarcane (*Saccharum officinarum* L.) chloroplast genome. Curr Genet 46(6):366–373. https://doi.org/10.1007/s00294-004-0542-4

Canto T (2016) Transient expression systems in plants: potentialities and constraints. In: Vega MC (eds) Advanced technologies for protein complex production and characterization. Cham: Springer International Publishing Ag. pp. 287–301

Cao X, Xie H, Song M, Jinghua Lu, Ma P, Huang B, Wang M et al (2022) Cut–dip–budding delivery system enables genetic modifications in plants without tissue culture. Innov 4(1):100345. https://doi.org/10.1016/j.xinn.2022.100345

Chetty VJ, Ceballos N, Garcia D, Narváez-Vásquez J, Lopez W, Orozco-Cárdenas ML (2013) Evaluation of four agrobacterium tumefaciens strains for the genetic transformation of tomato (*Solanum lycopersicum* L.) cultivar micro-tom. Plant Cell Rep 32(2):239–247. https://doi.org/10.1007/s00299-012-1358-1

Chilton MD, Drummond MH, Merio DJ, Sciaky D, Montoya AL, Gordon MP, Nester EW (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 11(2):263–271. https://doi.org/10.1016/0092-8674(77)90043-5

Chowrira GM, Akella V, Fuerst PE, Lurquin PF (1996) Transgenic grain legumes obtained byin planta electroporation-mediated gene transfer. Mol Biotechnol 5(2):85–96. https://doi.org/10.1007/BF02789058

Chupeau M-C, Pautot V, Chupeau Y (1994) Recovery of transgenic trees after electroporation of poplar protoplasts. Transgenic Res 3(1):13–19. https://doi.org/10.1007/BF01976022

Clegg MT, Durbin ML (2003) Tracing floral adaptations from ecology to molecules. Nat Rev Genet 4(3):206–215. https://doi.org/10.1038/nrg1023

Cunningham FJ, Goh NS, Demirer GS, Matos JL, Landry MP (2018) Nanoparticle-mediated delivery towards advancing plant genetic engineering. Trends Biotechnol 36(9):882–897. https://doi.org/10.1016/j.tibtech.2018.03.009

Cunningham FJ, Gozde SD, Natalie SG, Huan Z, Markita PL (2020) Nanobiolistics: an emerging genetic transformation approach. In: Sachin R, Hong L (eds) Biolistic DNA delivery in plants. New York: Springer US. pp. 141–59

Davlekamova AA, Zubritsky AV, Timofeeva TA, Yakovleva IV, Kamionskaya AM (2023) Optimization of biolistic transformation parameters for *Nicotiana tabacum*. Appl Biochem Microbiol 59(3):368–372. https://doi.org/10.1134/S0003683823030055

Debernardi JM, Rowan BA (2022) Make it a combo. Nat Plants 8(5):457–458. https://doi.org/10.1038/s41477-022-01155-5

Debernardi JM, Tricoli DM, Ercoli MF, Hayta S, Ronald P, Palatnik JF, Dubcovsky J (2020) A GRF–GIF chimeric protein improves the regeneration efficiency of transgenic plants. Nat Biotechnol 38(11):1274–1279. https://doi.org/10.1038/s41587-020-0703-0

Dhir SK, Oglesby J, Bhagsari AS (1998) Plant regeneration via somatic embryogenesis, and transient gene expression in sweet potato protoplasts. Plant Cell Rep 17(9):665–669. https://doi.org/10.1007/s002990050462

Díaz I (1994) Optimization of conditions for DNA uptake and transient GUS expression in protoplasts for different tissues of wheat and barley. Plant Sci 96(1–2):179–187. https://doi.org/10.1016/0168-9452(94)90235-6

Ding X, Lu Yu, Chen L, Li Y, Zhang J, Sheng H, Ren Z et al (2022) Recent progress and future prospect of CRISPR/Cas-derived transcription activation (CRISPRa) system in plants. Cells 11(19):3045. https://doi.org/10.3390/cells11193045

Doyle C, Katie H, Thomas AS, Mark W, Christopher B, David BA, Taryn F, Carmen GM, Keith E, Heather MW (2019) A simple method for spray-on gene editing in planta. bioRxiv. https://doi.org/10.1101/805036

Duarte P, Diana R, Ines C, Sara B, Mariana S (2016) Protoplast transformation as a plant-transferable transient expression system." In: FettNeto AG (eds) Biotechnology of plant secondary metabolism: methods and protocols. Totowa: Humana Press Inc. pp. 137–48

Elghabi Z, Ruf S, Bock R (2011) Biolistic co-transformation of the nuclear and plastid genomes. Plant J 67(5):941–948. https://doi.org/10.1111/j.1365-313X.2011.04631.x

Evans DL, Hlongwane TT, Joshi S, Riano DM, Pachon. (2019) The sugarcane mitochondrial genome: assembly, phylogenetics and transcriptomics. PeerJ 7:e7558. https://doi.org/10.7717/peerj.7558

Farooq N, Ather L, Shafiq M, Nawaz-ul-Rehman MS, Haseeb M, Anjum T, Abbas Q et al (2022) Magnetofection approach for the transformation of okra using green iron nanoparticles. Sci Rep 12(1):16568. https://doi.org/10.1038/s41598-022-20569-x

Fu YQ, Li L, Wang PW, Qu J, Fu YP, Wang H, Sun JR, Lu CL (2012) Delivering DNA into plant cell by gene carriers of ZnS nanoparticles. 28(4): 672–76

Fu Y, Li L, Wang H, Jiang Y, Liu H, Cui X, Wang P, Changli Lu (2015) Silica nanoparticles-mediated stable genetic transformation in *Nicotiana tabacum*. Chem Res Chin Univ 31(6):976–981. https://doi.org/10.1007/s40242-015-5088-0

Furuhata Y, Sakai A, Murakami T, Morikawa M, Nakamura C, Yoshizumi T, Fujikura U, Nishida K, Kato Y (2019) A method using electroporation for the protein delivery of cre recombinase into cultured arabidopsis cells with an intact cell wall. Sci Rep 9(1):2163. https://doi.org/10.1038/s41598-018-38119-9

Furukawa K, Koizumi M, Hayashi W, Mochizuki H, Yamaki K (2020) Pretreatment and posttreatment in the biolistic transformation of tea plant (*Camellia sinensis*) somatic embryos. Plant Biotechnol 37(2):195–203. https://doi.org/10.5511/plantbiotechnology.20.0404a

Fus-Kujawa A, Prus P, Bajdak-Rusinek K, Teper P, Gawron K, Kowalczuk A, Sieron AL (2021) An overview of methods and tools for transfection of eukaryotic cells in vitro. Front Bioeng Biotechnol 9:701031. https://doi.org/10.3389/fbioe.2021.701031

Gahrtz M, Stolz J, Sauer N (1994) A phloem-specific sucrose-H $^+$ symporter from *Plantago major* L. supports the model of apoplastic phloem loading. Plant J 6(5):697–706. https://doi.org/10.1046/j.1365-313X.1994.6050697.x

Gelvin SB (2003) *Agrobacterium* -mediated plant transformation: the biology behind the 'gene-jockeying' tool. Microbiol Mol Biol Rev 67(1):16–37. https://doi.org/10.1128/MMBR.67.1.16-37.2003

Gelvin SB (2010) Manipulating plant histone genes to improve agrobacterium- and direct DNA-mediated plant genetic transformation

Geng Y, Yan An, Zhou Y (2022) Positional cues and cell division dynamics drive meristem development and archegonium formation in ceratopteris gametophytes. Commun Biol 5(1):1–14. https://doi.org/10.1038/s42003-022-03627-y

Gong J, Yishan C, Yanna X, Miaofeng G, Haijie M, Xiaoli H, Xiaolong L, Chen J, Xuepeng S (2024) Tracking organelle activities through efficient and stable root genetic transformation system in woody plants. Hortic Res. 11(1): uhad262. https://doi.org/10.1093/hr/uhad262

Gordon-Kamm B, Sardesai N, Arling M, Lowe K, Hoerster G, Betts S, Jones T (2019) Using morphogenic genes to improve recovery and regeneration of transgenic plants. Plants 8(2):38. https://doi.org/10.3390/plants8020038

Grogg D, Rohner M, Yates S, Manzanares C, Bull SE, Dalton S, Bosch M, Studer B, Broggini GAL (2022) Callus induction from diverse explants and genotypes enables robust transformation of perennial ryegrass (*Lolium perenne* L.). Plants 11(15):2054. https://doi.org/10.3390/plants11152054

Guerche P, Bellini C, Le Moullec J-M, Caboche M (1987) Use of a transient expression assay for the optimization of direct gene transfer into tobacco mesophyll protoplasts by electroporation. Biochimie 69(6–7):621–628. https://doi.org/10.1016/0300-9084(87)90181-7

Guha S, Maheshwari SC (1966) Cell division and differentiation of embryos in the pollen grains of datura in vitro. Nature 212(5057):97–98. https://doi.org/10.1038/212097a0

Hajiahmadi Z, Shirzadian-Khorramabad R, Kazemzad M, Sohani MM (2019) Enhancement of tomato resistance to *Tuta absoluta* using a new efficient mesoporous silica nanoparticle-mediated plant transient gene expression approach. Sci Hortic 243:367–375. https://doi.org/10.1016/j.scienta.2018.08.040

Hajiahmadi Z, Reza SK, Mahmood K, Mohammad MS, Jahangir K (2020) A novel, simple, and stable mesoporous silica nanoparticle-based gene transformation approach in *Solanum lycopersicum*. Biotech. 10(8): 370. https://doi.org/10.1007/s13205-020-02359-2

Hamada H, Linghu Q, Nagira Y, Miki R, Taoka N, Imai R (2017) An in planta biolistic method for stable wheat transformation. Sci Rep 7(1):11443. https://doi.org/10.1038/s41598-017-11936-0

He Y, Zhang T, Sun H, Zhan H, Zhao Y (2020) A reporter for noninvasively monitoring gene expression and plant transformation. Hortic Res 7(1):152. https://doi.org/10.1038/s41438-020-00390-1

Healey AL, Garsmeur O, Lovell JT, Shengquiang S, Sreedasyam A, Jenkins J, Plott CB et al (2024) The complex polyploid genome architecture of sugarcane. Nature 628(8009):804–810. https://doi.org/10.1038/s41586-024-07231-4

Heidmann I, de Lange B, Lambalk J, Angenent GC, Boutilier K (2011) Efficient sweet pepper transformation mediated by the BABY BOOM transcription factor. Plant Cell Rep 30(6):1107–1115. https://doi.org/10.1007/s00299-011-1018-x

Herliana L, Schwerdt JG, Neumann TR, Severn-Ellis A, Phan JL, Cowley JM, Shirley NJ et al (2023) A chromosome-level genome assembly of plantago ovata. Sci Rep 13(1):1528. https://doi.org/10.1038/s41598-022-25078-5

Hoang NV, Furtado A, McQualter RB, Henry RJ (2015) Next generation sequencing of total DNA from sugarcane provides no evidence for chloroplast heteroplasmy. New Negat in Plant Sci 1–2:33–45. https://doi.org/10.1016/j.neps.2015.10.001

Hoerster G, Wang N, Ryan L, Emily Wu, Anand A, McBride K, Lowe K, Jones T, Gordon-Kamm B (2020) Use of non-integrating Zm-Wus2 vectors to enhance maize transformation. In Vitro Cell Dev Biol Plant 56(3):265–279. https://doi.org/10.1007/s11627-019-10042-2

Hofmann NR (2016) A breakthrough in monocot transformation methods. Plant Cell 28(9):1989–1989. https://doi.org/10.1105/tpc.16.00696

Hoshino A, Jayakumar V, Nitasaka E, Toyoda A, Noguchi H, Itoh T, Shin-I T et al (2016) Genome sequence and analysis of the Japanese morning glory ipomoea nil. Nat Commun 7(1):13295. https://doi.org/10.1038/ncomms13295

Huang J, Huang Z, Zhou X, Xia C, Imran M, Wang S, Congshan Xu, Zha M, Liu Y, Zhang C (2019) Tissue-specific transcriptomic profiling of plantago major provides insights for the involvement of vasculature in phosphate deficiency responses. Mol Genet Genom 294(1):159–175. https://doi.org/10.1007/s00438-018-1496-4

Hussien ET (2020) Production of transgenic paulownia tomentosa (Thunb.) Steud. using chitosan nanoparticles to express antimicrobial genes resistant to bacterial infection. Mol Biol Res Commun. 9(2): 55–62. https://doi.org/10.22099/mbrc.2019.35331.1454

Imai R, Hamada H, Liu Y, Linghu Q, Kumagai Y, Nagira Y, Miki R, Taoka N (2020) In planta particle bombardment (iPB): a new method for plant transformation and genome editing. Plant

Biotechnol 37(2):171–176. https://doi.org/10.5511/plantbiotechnology.20.0206a
Ismagul A, Yang N, Maltseva E, Iskakova G, Mazonka I, Skiba Y, Bi H et al (2018) A biolistic method for high-throughput production of transgenic wheat plants with single gene insertions. BMC Plant Biol 18:135. https://doi.org/10.1186/s12870-018-1326-1
Jiang Y, Wei X, Zhu M, Zhang X, Jiang Q, Wang ZiXiao, Cao Y, An X, Wan X (2024) Developmental regulators in promoting genetic transformation efficiency in maize and other plants. Curr Plant Biol 40:100383. https://doi.org/10.1016/j.cpb.2024.100383
Jones-Villeneuve E, Huang B, Prudhomme I, Bird S, Kemble R, Hattori J, Miki B (1995) Assessment of microinjection for introducing DNA into uninuclear microspores of rapeseed. Plant Cell Tissue Organ Cult 40(1):97–100. https://doi.org/10.1007/BF00041124
Joshi J, Beslin SG, Birla Singh KK, Kumar E, Kokiladevi L, Arul PB, Sudhakar D (2015) A maize α-zein promoter drives an endosperm-specific expression of transgene in rice. Physiol Mol Biol Plants 21(1):35–42. https://doi.org/10.1007/s12298-014-0268-9
Kale SD, Brett MT (2011) Assaying effector function in planta using double-barreled particle bombardment. In: McDowell JM (eds) Plant immunity: methods and protocols. Totowa: Humana Press Inc. pp. 712:153–72
Karlson CK, Soong SN, Mohd-Noor NN, Tan BC (2021) CRISPR/dCas9-based systems: mechanisms and applications in plant sciences. Plants 10(10):2055. https://doi.org/10.3390/plants10102055
Kaur M, Manchanda P, Kalia A, Ahmed FK, Nepovimova E, Kuca K, Abd-Elsalam KA (2021) Agroinfiltration mediated scalable transient gene expression in genome edited crop plants. Int J Mol Sci 22(19):10882. https://doi.org/10.3390/ijms221910882
Kharel A, Rookes J, Ziemann M, Cahill D (2024) Viable protoplast isolation, organelle visualization and transformation of the globally distributed plant pathogen *Phytophthora cinnamomi*. Protoplasma. https://doi.org/10.1007/s00709-024-01953-y
Kikuchi R, Sage-Ono K, Kamada H, Ono M (2005) Efficient transformation mediated by agrobacterium tumefaciens with a ternary plasmid in pharbitis nil. Plant Biotechnol 22(4):295–302. https://doi.org/10.5511/plantbiotechnology.22.295
Komari T, Yoshimitsu T, Jun U, Norio K, Yuji I, Yukoh H (2006) Binary vectors and super-binary vectors. In: Kan W (eds) Agrobacterium protocols. Totowa: Humana Press. pp. 15–42
Kuriakose B, Du Toit ES, Jordaan A (2012) Transient gene expression assays in rose tissues using a bio-rad Helios® hand-held gene gun. S Afr J Bot 78:307–311. https://doi.org/10.1016/j.sajb.2011.06.002
Kyo M, Maida K, Nishioka Y, Matsui K (2018) Coexpression of WUSCHEL related homeobox (WOX) 2 with WOX8 or WOX9 promotes regeneration from leaf segments and free cells in *Nicotiana tabacum* L. Plant Biotechnol 35(1):23–30. https://doi.org/10.5511/plantbiotechnology.18.0126a
Lacroix B, Vitaly C (2020) Biolistic approach for transient gene expression studies in plants. Methods Mol Biol (Clifton, N.J.). 2124:125–39. https://doi.org/10.1007/978-1-0716-0356-7_6
Lee K, Wang K (2023) Strategies for genotype-flexible plant transformation. Curr Opin Biotechnol 79:102848. https://doi.org/10.1016/j.copbio.2022.102848
Levengood H, Dou Y, Fan J, Bajszar A, Huang J, Abbas SM, Zhou Y, Zhang C (2023) Agrobacterium tumefaciens-mediated genetic transformation of narrowleaf plantain. J vis Exp 193:64777. https://doi.org/10.3791/64777
Li S, Wang S, Yang H (2000) Transformation of plant young proembryos by electroporation. Chin Sci Bull 45(13):1202–1206. https://doi.org/10.1007/BF02886080
Li C, Muhammad AI (2024) Leveraging the sugarcane CRISPR/Cas9 technique for genetic improvement of non-cultivated grasses. Front Plant Sci. https://doi.org/10.3389/fpls.2024.1369416
Lian Z, Nguyen CD, Liu Li, Wang G, Chen J, Wang S, Yi G et al (2022) Application of developmental regulators to improve in planta or in vitro transformation in plants. Plant Biotechnol J 20(8):1622–1635. https://doi.org/10.1111/pbi.13837
Liu J, Nannas NJ, Fang-fang Fu, Shi J, Aspinwall B, Parrott WA, Kelly Dawe R (2019a) Genome-scale sequence disruption following biolistic transformation in rice and maize. Plant Cell 31(2):368–383. https://doi.org/10.1105/tpc.18.00613
Liu M, Feng S, Ma Y, Xie C, He X, Ding Y, Zhang J et al (2019b) Influence of surface charge on the phytotoxicity, transformation, and translocation of $CeO_2$ nanoparticles in cucumber plants. ACS Appl Mater Interf 11(18):16905–16913. https://doi.org/10.1021/acsami.9b01627
Liu X, Xiao MB, Xuelei L, Menglu L, Hongzhe W, Xiaoyu Z, Yiman Y, Chunyan Z, Xian SZ, Jun X (2023) Uncovering the transcriptional regulatory network involved in boosting wheat regeneration and transformation. Nature
Liu Y, Zhang S, Zhang S, Zhang H, Li G, Sun R, Li F (2024) Efficient transformation of the isolated microspores of Chinese cabbage (*Brassica Rapa* L. Ssp. *Pekinensis*) by particle bombardment. Plant Methods 20(1):17. https://doi.org/10.1186/s13007-024-01134-1
Long Y, Yang Y, Pan G, Shen Y (2022) New insights into tissue culture plant-regeneration mechanisms. Front Plant Sci 13:926752. https://doi.org/10.3389/fpls.2022.926752
Lowder LG, Zhou J, Zhang Y, Malzahn A, Zhong Z, Hsieh T-F, Voytas DF, Zhang Y, Qi Y (2018) Robust transcriptional activation in plants using multiplexed CRISPR-Act2.0 and mTALE-Act systems. Mol Plant 11(2):245–256. https://doi.org/10.1016/j.molp.2017.11.010
Lowe K, Emily Wu, Wang N, Hoerster G, Hastings C, Cho M-J, Scelonge C et al (2016) Morphogenic regulators *Baby Boom* and *Wuschel* improve monocot transformation. Plant Cell 28(9):1998–2015. https://doi.org/10.1105/tpc.16.00124
Loyola-Vargas VM, Neftalí OA (2018) An introduction to plant tissue culture: advances and perspectives. In: Víctor MLV, Neftalí OA (eds) Plant cell culture protocols. New York: Springer. pp. 3–13
Lu J, Li S, Deng S, Wang M, Yinghuang Wu, Li M, Dong J et al (2024) A Method of genetic transformation and gene editing of succulents without tissue culture. Plant Biotechnol J 22(7):1981–1988. https://doi.org/10.1111/pbi.14318
Luo B-X, Zhang Li, Zheng F, Kun-Lin Wu, Li L, Zhang X-H, Ma G-H, Teixeira JA, Silva Da, Fang L, Zeng S-J (2020) Ovule development and in planta transformation of paphiopedilum maudiae by agrobacterium-mediated ovary-injection. Int J Mol Sci 22(1):84. https://doi.org/10.3390/ijms22010084
Lutz KA, Martin C, Khairzada S, Maliga P (2015) Steroid-inducible BABY BOOM system for development of fertile arabidopsis thaliana plants after prolonged tissue culture. Plant Cell Rep 34(10):1849–1856. https://doi.org/10.1007/s00299-015-1832-7
Lv Z, Jiang R, Chen J, Chen W (2020) Nanoparticle-mediated gene transformation strategies for plant genetic engineering. Plant J 104(4):880–891. https://doi.org/10.1111/tpj.14973
Lyu S, Qiming M, Hui L, Baosheng W, Jun W, Hans L, Zhengfeng W, Bin D, Zhanfeng L, Shulin D (2023) Genome assembly of the pioneer species *Plantago major* L. (Plantaginaceae) provides insight into its global distribution and adaptation to metal-contaminated soil. DNA Res. 30(4): dsad013. https://doi.org/10.1093/dnares/dsad013
Maas C, Werr W (1989) Mechanism and optimized conditions for PEG mediated DNA transfection into plant protoplasts. Plant Cell Rep 8(3):148–151. https://doi.org/10.1007/BF00716828

Mahdavi F, Mahmood M, Noor NM (2014) Optimization of particle bombardment parameters for DNA delivery into the male flowers of banana. Biologia 69(7):888–894. https://doi.org/10.2478/s11756-014-0391-7

Maher MF, Nasti RA, Vollbrecht M, Starker CG, Clark MD, Voytas DF (2020) Plant gene editing through de novo induction of meristems. Nat Biotechnol 38(1):84–89. https://doi.org/10.1038/s41587-019-0337-2

Malakhova NP, Skiba YA, Iskakova GA, Naizabayeva DA, Tezekbaeva BK, Ismagulova GA, Maltseva ER (2021) A positive experience in applying the biolistic approach to potato varieties aksor and nevskiy. Vavilov J Genet Breed. 25(2): 157–63. https://doi.org/10.18699/VJ21.019

Malnoy M, Roberto V, Min-Hee J, Ok-Jae K, Seokjoong K, Jin-Soo K, Riccardo V, Chidananda NK (2016) DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front Plant Sci. https://doi.org/10.3389/fpls.2016.01904

Martin-Ortigosa S, Kan W (2020) Proteolistics: a protein delivery method. In: Rustgi S, Luo H (eds) Biolistic DNA delivery in plants: methods and protocols. Totowa: Humana Press Inc. pp. 295–307

Mayavan S, Subramanyam K, Arun M, Rajesh M, Dev GK, Sivanandhan G, Jaganath B, Manickavasagam M, Selvaraj N, Ganapathi A (2013) *Agrobacterium tumefaciens*-mediated in planta seed transformation strategy in sugarcane. Plant Cell Rep 32(10):1557–1574. https://doi.org/10.1007/s00299-013-1467-5

Mayavan S, Subramanyam K, Jaganath B, Sathish D, Manickavasagam M, Ganapathi A (2015) Agrobacterium-mediated in planta genetic transformation of sugarcane setts. Plant Cell Rep 34(10):1835–1848. https://doi.org/10.1007/s00299-015-1831-8

Men S, Ming X, Wang Y, Liu R, Wei C, Li Y, Wang Y, Liu R (2003) Genetic transformation of two species of orchid by biolistic bombardment. Plant Cell Rep 21(6):592–598. https://doi.org/10.1007/s00299-002-0559-4

Meng D, Yang Q, Dong B, Song Z, Niu L, Wang L, Cao H, Li H, Yujie Fu (2019) Development of an efficient root transgenic system for pigeon pea and its application to other important economically plants. Plant Biotechnol J 17(9):1804–1813. https://doi.org/10.1111/pbi.13101

Miller K, Eggenberger AL, Lee K, Liu F, Kang M, Drent M, Ruba A, Kirscht T, Wang K, Jiang S (2021) An improved biolistic delivery and analysis method for evaluation of DNA and CRISPR-Cas delivery efficacy in plant tissue. Sci Rep 11(1):7695. https://doi.org/10.1038/s41598-021-86549-9

Mookkan M, Nelson-Vasilchik K, Hague J, Zhang ZJ, Kausch AP (2017) Selectable marker independent transformation of recalcitrant maize inbred B73 and sorghum P898012 mediated by morphogenic regulators BABY BOOM and WUSCHEL2. Plant Cell Rep 36(9):1477–1491. https://doi.org/10.1007/s00299-017-2169-1

Mori K, Tanase K, Sasaki K (2024) Novel electroporation-based genome editing of carnation plant tissues using RNPs targeting the anthocyanidin synthase gene. Planta 259(4):1–11. https://doi.org/10.1007/s00425-024-04358-6

Mortazavi SE, Zohrabi Z (2018) Biolistic co-transformation of rice using gold nanoparticles

Nadwodnik J, Lohaus G (2008) Subcellular concentrations of sugar alcohols and sugars in relation to phloem translocation in *Plantago major*, *Plantago maritima*, *Prunus persica*, and *Apium graveolens*. Planta 227(5):1079–1089. https://doi.org/10.1007/s00425-007-0682-0

Naqvi S, Maitra AN, Abdin MZ, Akmal Md, Arora I, Samim Md (2012) Calcium phosphate nanoparticle mediated genetic transformation in plants. J Mater Chem 22(8):3500–3507. https://doi.org/10.1039/c2jm11739h

Nawaz M, Naeem I, Rabia H, Mehwish M, Shakra J (2021) Agrobacterium mediated transformation optimizations for sugarcane (*Saccharum officinarum* L.) cultivar SPF-234 with direct organogenesis. J Bioresour Manag. 8(2): 61–71. https://doi.org/10.35691/JBM.1202.0181

Niazian M, Seyed ASN, Petr G, Mahdi M (2017) Tissue culture-based agrobacterium-mediated and in planta transformation methods. Czech J Genet Plant Breed. 53: 133–43. https://doi.org/10.17221/177/2016-CJGPB

Nomura K, Komamine A (1986) Embryogenesis from microinjected single cells in a carrot cell suspension culture. Plant Sci 44(1):53–58. https://doi.org/10.1016/0168-9452(86)90168-8

Noraini T, Amirul-Aiman AJ, Ruzi AR, Bunawan H, Nurdiana SF (2021) Adaptation and taxonomic value of leaf anatomical characteristics of selected ipomoea L. species." J Environ Biol. 42: 872–78. https://doi.org/10.22438/jeb42/3(SI)/JEB-20

Nugent GD, ten Have M, van der Gulik A, Dix PJ, Uijtewaal BA, Mordhorst AP (2005) Plastid transformants of tomato selected using mutations affecting ribosome structure. Plant Cell Rep 24(6):341–349. https://doi.org/10.1007/s00299-005-0930-3

Nugent GD, Coyne S, Nguyen TT, Kavanagh TA, Dix PJ (2006) Nuclear and plastid transformation of *Brassica oleracea* Var. *Botrytis* (cauliflower) using PEG-mediated uptake of DNA into protoplasts. Plant Sci 170(1):135–142. https://doi.org/10.1016/j.plantsci.2005.08.020

Okura VK, Rafael SCS de, Susely FST de, Paulo A (2016) BAC-pool sequencing and assembly of 19 mb of the complex sugarcane genome. Front Plant Sci. https://doi.org/10.3389/fpls.2016.00342

Ondřej M, Matoušek J, Vlasák J (1987) Differentiation of transformed plants from tumors induced by *Agrobacterium rubi* ATCC 13335. J Plant Physiol 126(4):397–407. https://doi.org/10.1016/S0176-1617(87)80025-1

Ono M, Sage-Ono K, Kawakami M, Hasebe M, Ueda K, Masuda K, Inoue M, Kamada H (2000) Agrobacterium-mediated transformation and regeneration of pharbitis nil. Plant Biotechnol 17(3):211–216. https://doi.org/10.5511/plantbiotechnology.17.211

Ozyigit II (2020) Gene transfer to plants by electroporation: methods and applications. Mol Biol Rep 47(4):3195–3210. https://doi.org/10.1007/s11033-020-05343-4

Ozyigit II, Kurtoglu KY (2020) Particle bombardment technology and its applications in plants. Mol Biol Rep 47(12):9831–9847. https://doi.org/10.1007/s11033-020-06001-5

Pan C, Li G, Malzahn AA, Cheng Y, Leyson B, Sretenovic S, Gurel F, Coleman GD, Qi Y (2022) Boosting plant genome editing with a versatile CRISPR-combo system. Nat Plants 8(5):513–525. https://doi.org/10.1038/s41477-022-01151-9

Park I, Sungyu Y, Wook JK, Pureum N, Hyun OL, Byeong CM (2018) The complete chloroplast genomes of six ipomoea species and indel marker development for the discrimination of authentic pharbitidis semen (Seeds of I. Nil or I. Purpurea). Front Plant Sci. https://doi.org/10.3389/fpls.2018.00965

Penczykowski RM, Drew Sieg R (2021) *Plantago* Spp. as models for studying the ecology and evolution of species interactions across environmental gradients. Am Nat 198(1):158–176. https://doi.org/10.1086/714589

Pommerrenig B, Barth I, Niedermeier M, Kopp S, Schmid J, Dwyer RA, McNair RJ, Klebl F, Sauer N (2006) Common plantain. A collection of expressed sequence tags from vascular tissue and a simple and efficient transformation method. Plant Physiol 142(4):1427–1441. https://doi.org/10.1104/pp.106.089169

Pommerrenig B, Papini-Terzi FS, Sauer N (2007) Differential regulation of sorbitol and sucrose loading into the phloem of *Plantago major* in response to salt stress. Plant Physiol 144(2):1029–1038. https://doi.org/10.1104/pp.106.089151

Quezada GDÁ, Siddra I, Riffat M (2024) Genetic transformation in plants: methods and applications. In: Siddra I, Imran UH, Hayssam MA (eds) Trends in plant biotechnology. Singapore: Springer Nature. pp. 23–55

Rajkumari N, Swapna A, Soni KB, Anith KN, Viji MM, Kiran AG (2021) Silver nanoparticles for biolistic transformation in *Nicotiana* tabacum L. Biotech. 11(12): 497. https://doi.org/10.1007/s13205-021-03043-9

Raman V, Rojas CM, Vasudevan B, Dunning K, Kolape J, Sunhee Oh, Yun J et al (2022) Agrobacterium expressing a type III secretion system delivers pseudomonas effectors into plant cells to enhance transformation. Nat Commun 13:2581. https://doi.org/10.1038/s41467-022-30180-3

Ramkumar TR, Sangram KL, Sagar SA, Kailash CB (2020) A short history and perspectives on plant genetic transformation. In: Sachin R, Hong L (eds) Biolistic DNA delivery in plants: methods and protocols. New York: Springer US. pp. 39–68

Reed KM, Bastiaan ORB (2021) Protoplast regeneration and its use in new plant breeding technologies. Front Genome Ed. https://doi.org/10.3389/fgeed.2021.734951

Routier C, Vallan L, Daguerre Y, Juvany M, Istif E, Mantione D, Brochon C et al (2023) Chitosan-modified polyethyleneimine nanoparticles for enhancing the carboxylation reaction and plants' CO2 uptake. ACS Nano 17(4):3430–3441. https://doi.org/10.1021/acsnano.2c09255

Ruf S, Ralph B (2021) Plastid transformation in tomato: a vegetable crop and model species. In: Pal M (eds) Chloroplast biotechnology: methods and protocols, 2nd edn. Totowa: Humana Press Inc. pp. 217–28

Ruhlman TA (2021) Biolistic plastid transformation in lettuce (*Lactuca sativa*) for oral delivery of biopharmaceuticals. In: Pal M (eds) Chloroplast biotechnology: methods and protocols. New York: Springer US. pp. 267–81

Saeger De, Jonas JP, Chung HS, Hernalsteens J-P, Van Lijsebettens M, Inzé D, Van Montagu M, Depuydt S (2021) *Agrobacterium* strains and strain improvement: present and outlook. Biotechnol Adv 53:107677. https://doi.org/10.1016/j.biotechadv.2020.107677

Samuelsen AB (2000) The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. J Ethnopharmacol 71(1–2):1–21. https://doi.org/10.1016/S0378-8741(00)00212-9

Sandhya D, Jogam P, Venkatapuram AK, Savitikadi P, Peddaboina V, Allini VR, Abbagani S (2022) Highly efficient agrobacterium-mediated transformation and plant regeneration system for genome engineering in tomato. Saudi J Biol Sci 29(6):103292. https://doi.org/10.1016/j.sjbs.2022.103292

Sant'Ana RRA, Clarissa AC, Rubens ON, Sarah ZAT (2020) PEG-delivered CRISPR-Cas9 ribonucleoproteins system for gene-editing screening of maize protoplasts. Genes 11(9):1029. https://doi.org/10.3390/genes11091029

Santarém ER, Trick HN, Essig JS, Finer JJ (1998) Sonication-assisted agrobacterium-mediated transformation of soybean immature cotyledons: optimization of transient expression. Plant Cell Rep 17(10):752–759. https://doi.org/10.1007/s002990050478

Sharada MS, Kumari A, Pandey AK, Sharma S, Sharma P, Sreelakshmi Y, Sharma R (2017) Generation of genetically stable transformants by *Agrobacterium* using tomato floral buds. Plant Cell Tissue Organ Cult 129(2):299–312. https://doi.org/10.1007/s11240-017-1178-7

Shires ME, Florez SL, Lai TS, Curtis WR (2017) Inducible somatic embryogenesis in theobroma cacao achieved using the DEX-activatable transcription factor-glucocorticoid receptor fusion. Biotech Lett 39(11):1747–1755. https://doi.org/10.1007/s10529-017-2404-4

Shruti AT, Susmita S (2024) Methods of genetic transformation: major emphasis to crop plants. J Microbiol Biotechnol Food Sci. 13(4): e10276–e10276. https://doi.org/10.55251/jmbfs.10276

Si H, Li R, Zhang Q, Liu L (2022) Complete chloroplast genome of *Plantago asiatica* and its phylogenetic position in plantaginaceae. Mitochondrial DNA Part B 7(5):819–821. https://doi.org/10.1080/23802359.2022.2073838

Silva TN, Jason BT, Jeff D, Seung YR, Jenny CM (2022) Progress and challenges in sorghum biotechnology, a multipurpose feedstock for the bioeconomy. J Exp Bot. 73(3): 646–64. https://doi.org/10.1093/jxb/erab450

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp Soc Exp Biol 11:118–130

Solis J, Baisakh N, Brandt SR, Villordon A, La Bonte D (2016) Transcriptome profiling of beach morning glory (Ipomoea imperati) under salinity and its comparative analysis with sweetpotato. PLoS ONE 11(2):e0147398. https://doi.org/10.1371/journal.pone.0147398

Squire HJ, Tomatz S, Voke E, González-Grandío E, Landry M (2023) The emerging role of nanotechnology in plant genetic engineering. Nat Rev Bioeng 1(5):314–328. https://doi.org/10.1038/s44222-023-00037-5

Steward FC, Pollard JK (1958) 14C-proline and hydroxyproline in the protein metabolism of plants; an episode in the relation of metabolism to cell growth and morphogenesis. Nature 182(4639):828–832. https://doi.org/10.1038/182828a0

Steward FC, Mapes MO, Mears K (1958a) Growth and organized development of cultured cells. II. Organization in cultures grown from freely suspended cell. Am J Bot 45(10):705–708. https://doi.org/10.1002/j.1537-2197.1958.tb10599.x

Steward FC, Mapes MO, Smith J (1958b) Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. Am J Bot 45(9):693–703. https://doi.org/10.2307/2439507

Su W, Mingyue Xu, Radani Y, Yang L (2023) Technological development and application of plant genetic transformation. Int J Mol Sci 24(13):10646. https://doi.org/10.3390/ijms241310646

Subburaj S, Chung SJ, Lee C, Ryu S-M, Kim DH, Kim J-S, Bae S, Lee G-J (2016) Site-directed mutagenesis in petunia × hybrida protoplast system using direct delivery of purified recombinant Cas9 ribonucleoproteins. Plant Cell Rep 35(7):1535–1544. https://doi.org/10.1007/s00299-016-1937-7

Sugiura C, Sugita M (2004) Plastid transformation reveals that moss tRNA(Arg)-CCG is not essential for plastid function. Plant J Cell Mol Biol 40(2):314–321. https://doi.org/10.1111/j.1365-313X.2004.02202.x

Szabala BM, Swiecicka M, Lyznik LA (2024) Microinjection of the CRISPR/Cas9 editing system through the germ pore of a wheat microspore induces mutations in the target *Ms2* gene. Mol Biol Rep 51(1):706. https://doi.org/10.1007/s11033-024-09644-w

Tan RF, Jun T, Ling L (2007) Genetic transformation of ipomoea purpurea mediated by agrobacterium rhizogenes

Tanwar N, Binod KM, James ER, David MC, Kailash CB, Sangram KL (2024) Chloroplast transformation in new cultivars of tomato through particle bombardment. Biotech. 14(4): 120. https://doi.org/10.1007/s13205-024-03954-3

Tawfik E, Hammad I, Bakry A (2022) Production of transgenic allium cepa by nanoparticles to resist aspergillus niger infection. Mol Biol Rep 49(3):1783–1790. https://doi.org/10.1007/s11033-021-06988-5

Thirugnanasambandam PP, Nam VH, Robert JH (2018) The challenge of analyzing the sugarcane genome. Front Plant Sci. https://doi.org/10.3389/fpls.2018.00616

Tonnies J, Mueth NA, Gorjifard S, Chu J, Queitsch C (2023) Scalable transfection of maize mesophyll protoplasts. Jove J vis Exp 196:e64991. https://doi.org/10.3791/64991

Torregrosa L, Iocco P, Thomas MR (2002) Influence of agrobacterium strain, culture medium, and cultivar on the transformation efficiency of vitis vinifera L. Am J Enol Vitic 53(3):183–190. https://doi.org/10.5344/ajev.2002.53.3.183

Tripathi PK, Ayzenshtat D, Kumar M, Zemach H, Yedidia I, Bocobza SE (2023) An efficient and reproducible agrobacterium-mediated genetic transformation method for the ornamental monocotyledonous plant ornithogalum dubium houtt. Plant Growth Regul 101(1):201–214. https://doi.org/10.1007/s10725-023-01013-0

Van Larebeke N, Engler G, Holsters M, Van Den Elsacker SV, Zaenen I, Schilperoort RA, Schell J (1974) Large plasmid in agrobacterium tumefaciens essential for crown gall-inducing ability. Nature 252(5479):169–170. https://doi.org/10.1038/252169a0

Vasudevan V, Siva R, Krishnan V, Manickavasagam M (2020) Polyamines, sonication and vacuum infiltration enhances the *Agrobacterium*-mediated transformation in watermelon (*Citrullus lanatus* thunb.). S Afr J Bot 128:333–338. https://doi.org/10.1016/j.sajb.2019.11.031

Vogrinčič V, Damijana K, Jana M (2024) Phytosulfokine alpha enhances regeneration of transformed and untransformed protoplasts of brassica oleracea. Front Plant Sci. https://doi.org/10.3389/fpls.2024.1379618

Wałpuski G, Kozioł Ł, Gasulla F, Zaborski P, Zienkiewicz M (2024) Stable transformation of the unicellular lichenic green alga coccomyxa solorinae-saccatae via electroporation. Symbiosis 93(1):115–123. https://doi.org/10.1007/s13199-024-00985-5

Wang M, Soyano T, Machida S, Yang J-Y, Jung C, Chua N-H, Adam Yuan Y (2011) Molecular insights into plant cell proliferation disturbance by agrobacterium protein 6b. Genes Dev 25(1):64–76. https://doi.org/10.1101/gad.1985511

Wang Z, Liu H, Li L, Li Q, Wang X, Jiang Y, Yuqin Fu, Lü C (2017) Ultrasonic-assisted mesoporous silica nanoparticle-mediated exogenous gene stable expression in tobacco. Chem Res Chin Univ 33(6):912–916. https://doi.org/10.1007/s40242-017-7240-5

Wang K, Huilan Z, Morgan M (2020) Biolistic DNA delivery in maize immature embryos. In: Rustgi S, Luo H (eds) Biolistic DNA delivery in plants: methods and protocols. Totowa: Humana Press Inc. pp. 177–95

Wang Ke, Shi L, Liang X, Zhao P, Wang W, Liu J, Chang Y et al (2022) The gene TaWOX5 overcomes genotype dependency in wheat genetic transformation. Nat Plants 8(2):110–117. https://doi.org/10.1038/s41477-021-01085-8

Watanabe K, Kobayashi A, Endo M, Sage-Ono K, Toki S, Ono M (2017) CRISPR/Cas9-mediated mutagenesis of the Dihydroflavonol-4-Reductase-B (DFR-B) locus in the Japanese morning glory ipomoea (Pharbitis) nil. Sci Rep 7(1):10028. https://doi.org/10.1038/s41598-017-10715-1

Woo JW, Kim J, Kwon SI, Corvalán C, Cho SW, Kim H, Kim S-G, Kim S-T, Choe S, Kim J-S (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. Nat Biotechnol 33(11):1162–1164. https://doi.org/10.1038/nbt.3389

Wu Y, Ling C, Chunmei J, Letian X, Jiang Z (2021) Plastid transformation in poplar: a model for perennial trees. In: Maliga P (eds) Chloroplast biotechnology: methods and protocols, 2nd edn. Totowa: Humana Press Inc. pp.257–65

Wu K, Changbin Xu, Li T, Ma H, Gong J, Li X, Sun X, Xiaoli Hu (2023) Application of nanotechnology in plant genetic engineering. Int J Mol Sci 24(19):14836. https://doi.org/10.3390/ijms241914836

Yan T, Hou Q, Wei X, Qi Y, Aqing Pu, Suowei Wu, An X, Wan X (2023) Promoting genotype-independent plant transformation by manipulating developmental regulatory genes and/or using nanoparticles. Plant Cell Rep 42(9):1395–1417. https://doi.org/10.1007/s00299-023-03037-2

Ying S, Guan Z, Ofoegbu PC, Clubb P, Rico C, He F, Hong J (2022) Green synthesis of nanoparticles: current developments and limitations. Environ Technol Innov 26:102336. https://doi.org/10.1016/j.eti.2022.102336

Yu J, Deng S, Huang H, Mo J, Zeng-Fu Xu, Wang Yi (2023) Exploring the potential applications of the noninvasive reporter gene RUBY in plant genetic transformation. Forests 14(3):637. https://doi.org/10.3390/f14030637

Zhang HM, Yang H, Rech EL, Golds TJ, Davis AS, Mulligan BJ, Cocking EC, Davey MR (1988) Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Rep 7(6):379–384. https://doi.org/10.1007/BF00269517

Zhang X, Henriques R, Lin S-S, Niu Q-W, Chua N-H (2006) Agrobacterium-mediated transformation of arabidopsis thaliana using the floral dip method. Nat Protoc 1(2):641–646. https://doi.org/10.1038/nprot.2006.97

Zhang XH, Xue-Jing Z, Bo L, Fei-Fei L, Pei-Xuan L, Dong-Hong M (2013) Factors optimization of pollen electroporation transformation and identification of transgenic wheat. 46(12): 2403–11. https://doi.org/10.3864/j.issn.0578-1752.2013.12.001

Zhang Yu, Zhang Q, Chen Q-J (2020) Agrobacterium-mediated delivery of CRISPR/Cas reagents for genome editing in plants enters an era of ternary vector systems. Sci China Life Sci 63(10):1491–1498. https://doi.org/10.1007/s11427-020-1685-9

Zhang H, Goh NS, Wang JW, Pinals RL, González-Grandío E, Demirer GS, Butrus S et al (2022) Nanoparticle cellular internalization is not required for RNA delivery to mature plant leaves. Nat Nanotechnol 17(2):197–205. https://doi.org/10.1038/s41565-021-01018-8

Zhang M, Ma Xu, Jin Ge, Han D, Xue J, Yunpeng Du, Chen X, Yang F, Zhao C, Zhang X (2023) A modified method for transient transformation via pollen magnetofection in *Lilium germplasm*. Int J Mol Sci 24(20):15304. https://doi.org/10.3390/ijms242015304

Zhang C, Tang Y, Tang S, Chen L, Li T, Yuan H, Yujun Xu et al (2024a) An inducible CRISPR activation tool for accelerating plant regeneration. Plant Commun Focus Issue Wheat Biol 5(5):100823. https://doi.org/10.1016/j.xplc.2024.100823

Zhang Y, Patankar H, Aljedaani F, Blilou I (2024b) A framework for date palm (*Phoenix dactylifera* L.) tissue regeneration and stable transformation. Physiol Plant 176(1):e14189. https://doi.org/10.1111/ppl.14189

Zhao X, Meng Z, Wang Y, Chen W, Sun C, Cui Bo, Cui J et al (2017) Pollen magnetofection for genetic modification with magnetic nanoparticles as gene carriers. Nat Plants 3(12):956–964. https://doi.org/10.1038/s41477-017-0063-z

Zhao H, Yaqi J, Yanting C, Yucheng W (2020) Improving T-DNA transfer to tamarix hispida by adding chemical compounds during agrobacterium tumefaciens culture. Front Plant Sci. https://doi.org/10.3389/fpls.2020.501358