

## Breaking recalcitrance: Advances in chilli pepper biotechnology and genome editing

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### Abstract

Chilli peppers (*Capsicum* spp.) are important spices and horticultural crops that are subject to biotic and abiotic stresses. Genetic improvement in these species is challenging due to their inherent recalcitrance to tissue culture-based regeneration and genetic transformation. We comprehensively surveyed strategies to enhance in vitro regeneration in *Capsicum* spp., including the use of morphogenic regulators, plant hormones, explant selections, growth enhancers, and culture medium standardizations. In parallel, highlighting transformation methods that can supplement existing regeneration protocols or entirely bypass them, including *in planta*, biolistic, protoplast transfections, virus and nanoparticle-based gene delivery, and synergistic approaches. Additionally, this review article discusses various genetic engineering tools, such as transgenic overexpression, RNA interference (RNAi), Virus-Induced Gene Silencing (VIGS), Virus-Induced Genome Editing (VIGE), and CRISPR-Cas9 gene editing, which are enriching the *Capsicum* spp. genomes, enabling rapid functional characterization, and producing heritable genetic modifications. By integrating current understandings and novel biotechnological advancements, this review provides a foundation for *Capsicum* spp. genetic improvement, enhancing its stress resilience and productivity.

**Keywords:** *Capsicum* spp., Regeneration, Transformation, Genetic engineering, Genome editing

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## Introduction

The genus *Capsicum* in the Solanaceae family comprises the most significant spice species in the world, generally referred to as chilli peppers or peppers, which are grown for their flavor and pungency and possess nutritional and pharmacological properties. These species grow well in temperate, tropical, and subtropical climates and are widely used in the food, pharmaceutical, and cosmetic industries (Anaya-Esparza et al., 2021; Duranova et al., 2022). The genus *Capsicum* includes thirty species, and among them, six species (*Capsicum annuum*, *Capsicum chinense*, *Capsicum pubescens*, *Capsicum frutescens*, *Capsicum baccatum*, and *Capsicum assamicum*) have been domesticated (Swamy, 2023; Tripodi and Kumar, 2019). *C. chinense* is peculiar for its high capsaicinoid content, while *C. annuum* is the most economically important as it is the most widely grown species worldwide (Kalita et al., 2024).

*Capsicum* spp. contributed around 4.1 million tons, constituting nearly 32% of the world's total spice production, underscoring their crucial role in both household consumption and the food industry (Sundari et al., 2023). The current market value of *Capsicum* spp. is nearly \$1.3 billion USD, and it is estimated to reach \$1.79 billion USD by 2030, at a compound annual growth rate of 6.4% (GlobeNewswire, 2025). India produced 2,909,844 tons of dried chillies in 2024, followed by China (326,171 tons), Thailand (324,004 tons), Bangladesh (301,311 tons), and Ethiopia (298,088 tons), making the world's top five leading producers (FAO, 2025). The rising global demand is driven by a growing preference for spicy food, and projections suggest that the demand for chilli-based commodities will continue to grow, supported by abundant opportunities in culinary innovations and improvements (Ali et al., 2025). Additionally, *Capsicum* spp. play a significant role in the agricultural and rural economies of many developing countries, often supporting subsistence farmers and small-scale industries based on chilli products (Ali et al., 2025).

Beyond their significance as a spice, *Capsicum* spp. are rich in vitamins A and C (ascorbic acid), phenolic compounds, dietary fiber, minerals, and bioactive metabolites such as capsaicinoids, flavonoids, and carotenoids (Duranova et al., 2022). Capsaicin, the compound that gives *Capsicum* spp. their distinctive spiciness, has been found to have anticancer, anti-

inflammatory, and pain-relieving properties (Duranova et al., 2022; Zhang Q et al., 2024).

The five commonly cultivated *Capsicum* species differ in genetic structure and chromosome number from their wild relatives. For instance, domesticated *C. annuum* has  $2n = 24$  chromosomes; in contrast, wild species such as *C. mirabile* and *C. campylopodium* have  $2n = 26$  (Almeida et al., 2022; Scaldaferrero and Moscone, 2019). The genetic diversity reinforces phenotypic variation in fruit color, shape, and pungency. Comparative and pan-genomic studies reveal extensive transposon proliferations that drive structural variation and single-nucleotide polymorphisms, highlighting the evolutionary adaptation of *Capsicum* spp. and providing valuable genetic resources for breeding (Liu F et al., 2023; Lozada et al., 2021, 2022; Zhang K et al., 2025).

Inter-specific crosses between *Capsicum* spp. encounter significant challenges due to genetic barriers, chromosomal differences, and reproductive mechanisms. These differences in basic chromosome numbers between domesticated ( $x=12$ ) and wild species ( $x=13$ ) make it difficult to produce fertile progeny due to lethal aneuploidy (de Souza-Macedo et al., 2017). Variations in flowering patterns and mechanisms that produce sterile gametes further decrease the success of inter-specific crosses (Liu F et al., 2023). Furthermore, conventional breeding in *Capsicum* spp. remains challenging due to long generation times, complex trait inheritance, and the lengthy backcrossing required to remove undesirable genetic linkage, hindering the development of improved cultivars (Lozada et al., 2022; Selvakumar et al., 2022).

These challenges associated with conventional breeding can be addressed by biotechnological approaches, which enable gene transfer from wild relatives, robust functional characterization of genes controlling important traits, and accurate genetic manipulation of target genes in a limited time (Kalita et al., 2024). The genetic background of vital agronomic traits has been elucidated through recent advancements in whole-genome sequencing and forward genetic tools, such as quantitative trait locus (QTL) mapping, genome-wide association studies (GWAS), and positional cloning (Cao et al., 2022; Chunthawodtiporn et al., 2018; Qin et al., 2014; Wu et al., 2019). However, obtaining stable transgenic lines and improvements through biotechnological advancements have been hampered by pepper's notorious resistance to *in vitro* morphogenesis, genetic

transformation, and genotype-specific tissue culture methods, as well as challenges in generating stable transgenic lines (Kothari et al., 2010; Tang et al., 2025).

Advancements have been made in genetic transformation protocols and genome editing in *Capsicum* spp. in recent years, despite challenges in tissue culture and regeneration. Recent progress in *Capsicum* spp. has focused on optimizing *in vitro* tissue culture protocols, including selecting appropriate genotypes and explants and standardization of culture media. While improvements in transformation strategies have involved *Agrobacterium*-mediated techniques, direct transformation methods that bypass *Agrobacterium*, such as biolistic approaches (Bulle et al., 2024a), and innovative technologies that circumvent tissue culture procedures, such as *in planta* transformations, have evolved over time (Naeem et al., 2025b). Novel inventions such as nanotechnology have opened a new pathway for genetic transformation (Zhao et al., 2017). The gene modification techniques such as VIGS, and RNAi allows silencing of negative regulators of favourable traits while allowing quick screening before taking on stable transformation approaches (Abhary and Rezk, 2015; Giudice et al., 2021). CRISPR/Cas9 tools, like prime editor and base editor have enabled precise and heritable modifications without the integration of foreign DNA (Bulle et al., 2025b; Mishra et al., 2021). The current focus has been on developing tissue culture free, transgene-free, stable gene edited lines in *Capsicum* spp. These technologies facilitate the development of *Capsicum* spp. cultivars with improved stress resistance, disease tolerance, and nutritional quality traits, which are increasingly essential in light of global climate challenges and ever-changing consumer preferences. Herein, we encapsulate recent progress in *Capsicum* spp. *in vitro* culture, transformation, and genome editing, comparing genetic advancements across studies, and discuss current challenges and future pathways for advancing *Capsicum* spp. genetics and molecular breeding.

### **Recalcitrance to the *in vitro* regeneration and approaches to overcome this barrier**

In stark contrast to their relatives in the Solanaceae family, genetic improvement and functional genomics for *Capsicum* spp. lag, despite their economic significance (Zhao et al., 2024). The biggest challenge in *Capsicum* spp. transformation is their resistance to

*in vitro* regeneration and low transformation efficiency. *Capsicum* spp. exhibit a low tendency for shoot organogenesis (Kothari et al., 2010). The inability to generate transformed plants can partly be attributed to the prevalence of transposable elements in the *Capsicum* spp. genome, which has been shown to contain 81% transposons (Yañez-Santos et al., 2021).

To increase the efficacy of transgenic *Capsicum* spp. regenerations, various modifications, including explant selection, media optimization through hormone addition, and the use of morphogenic or developmental regulators, are significant developments. Figure 1 summarizes these key factors and their contributions towards increasing regeneration capacity in *Capsicum* spp.

### **Explant selection**

A suitable selection of explants primarily determines the success of organogenesis and plant tissue growth under *in vitro* conditions (Kothari et al., 2010). So far, cotyledons have been proven to be the best choice as explants; however, other studies reported that, for the understudy cultivars, hypocotyls were a superior option to cotyledons (Gammoudi et al., 2018; Mahto et al., 2018). Another protocol used excised shoot-tip explants (1.5 cm) obtained aseptically from 20- to 30-day-old *in vitro* established seedlings (Hernández-Amasifuen et al., 2024).

### **Culture media optimizations**

The composition of the different growth regulators/hormones in the media is equally important. Various combinations and doses of plant growth regulators have been extensively studied. The experimental evidence suggests that particular combinations of hormones significantly enhance the *Capsicum* spp. somatic embryogenesis and organogenesis, which are crucial for regeneration. Among growth regulators, the ratios and concentrations of cytokinin and auxin are vital factors in determining the effectiveness of regeneration. Commonly used cytokinins for *Capsicum* spp. regeneration include 6-benzylaminopurine (BAP), benzyladenine (BA), Zeatin (Kim et al., 2023), Thidiazuron (TDZ), and Kinetin (Naeem et al., 2025b). On the other hand, 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-butyric acid (IBA), Naphthaleneacetic acid (NAA), and Indole-3-acetic acid (IAA) are commonly used auxins for the same purpose. *Capsicum* spp. regeneration

typically involves a two-stage process. During shoot induction, explants are cultured on media containing both cytokinins and auxins, with a relatively high cytokinin:auxin ratios, such as 5:1 to 10:1, to promote shoot development. Subsequently, the cytokinin level is drastically reduced during the second stage to facilitate root induction (Zhao et al., 2008).

### Shoot induction

Studies reported that 5 mg/L BAP + 1 mg/L IAA (Mahto et al., 2018; Shams et al., 2024), 5 mg/L BAP + 0.5 mg/L IAA (Martínez-López et al., 2021), 5 mg/L BA + 1 mg/L IAA (Gammoudi et al., 2018), and 8 mg/L BAP + 0.5 mg/L IAA + 0.02 mg/L NAA (Nadim et al., 2024), 1.0 mg/L BAP + 2.0 mg/L Kinetin (Hernández-Amasifuen et al., 2024) were optimum for shoot regeneration in understudied *Capsicum* spp. cultivars.

### Root induction

For root induction, a combination of two auxin supplements in MS media, such as 0.5-2 mg/L IBA and NAA, was effective for *C. pubescens* (Hernández-Amasifuen et al., 2024). Another report suggested adding a single auxin in basal MS media can serve the same purpose, such as 1 mg/L IBA was suffice for root induction (Shams et al., 2024).

### Callus induction

A more balanced ratio of cytokinins to auxins is needed for callus induction. For instance, combinations of 4 mg/L 2,4-D + 1.5 mg/L BAP and 2 mg/L 2,4-D + 1.86 mg/L BAP were optimal for callus induction (Bora et al., 2018; David et al., 2021).

### Regeneration enhancers

Alongside gibberellic acid (GA<sub>3</sub>) (Tang et al., 2025), coconut water (Zhao et al., 2024), and activated carbon (100 mg/L) (Tang et al., 2025) are also used to enhance shoot elongation. The development of rosette shoots or poorly shaped leafy shoots that either resist or fail to elongate well is one of the most limiting factors in culturing *Capsicum* spp. on regeneration media (Kothari et al., 2010). In contrast, the measure of regeneration success is based on the production of lateral branches (Tang et al., 2025). Various reports have suggested that the addition of adjuvants, such as silver nitrate, to the Murashige and Skoog (MS) media enhanced the shoot development (Gammoudi et al., 2018; Martínez-López et al., 2021; Naeem et al.,

2025a; Shams et al., 2024). Furthermore, the combined effect of silver and copper salts (0.47 mg/L CuSO<sub>4</sub>), along with other growth regulators, resulted in higher regeneration efficiency during direct organogenesis in *Capsicum* spp. (Martínez-López et al., 2021). Similarly, the inclusion of the CaREF1 peptide into culture media increased shoot regeneration, elongation, and rooting efficiency (Naeem et al., 2025a).

### Environmental factors

Through interaction between medium constituents and genotypes, culturing conditions can play a significant part. For example, four genotypes from *C. annuum* anther culture required 48 hours of cold pre-treatment for successful embryogenesis. In contrast, certain media required cold treatment for embryo formation, indicating the importance of environmental variables and media components in altering developmental mechanisms towards regeneration (Popova et al., 2016). The beneficial effects of adding nanoparticles to *Capsicum* spp. *in vitro* culture have also been reported. For example, copper, zinc, and silver oxide nanoparticles can influence cellular processes and may help address problems such as cadmium toxicity in the medium (Díaz-Parra et al., 2025; Karmous et al., 2023). Certain nanoparticle types and doses can cause phytotoxicity, leading to growth inhibition in *in vitro* cultures (Asgari-Targhi et al., 2018), therefore it requires stringent dose optimization before widespread usage.

### Morphogenic regulators

A highly promising strategy to combat regeneration recalcitrance involves the ectopic expression of key morphogenic transcription factors, such as *BABY BOOM* (*BBM*) (Horstman et al., 2017) and *WUSCHEL* (*WUS*) (Wang K et al., 2022), which regulate cellular totipotency. The individual or synergistic expression of both genes promotes callus tissue development and the transgenic plants regeneration from transformed explants, thereby improving the genetic transformation capability of hard-to-transform plant species (Lowe et al., 2016). Recalcitrancy of sweet pepper cultivars was overcome by transient expression of *Brassica napus* *AP2/ERF* transcription factor *BBM*, which effectively generated transgenic seedlings (Heidmann et al., 2011). Ectopic expression of *BBM* in progeny plants resulted in robust cellular regeneration, yielding an abundance of somatic embryos that conveniently grown into seedlings.

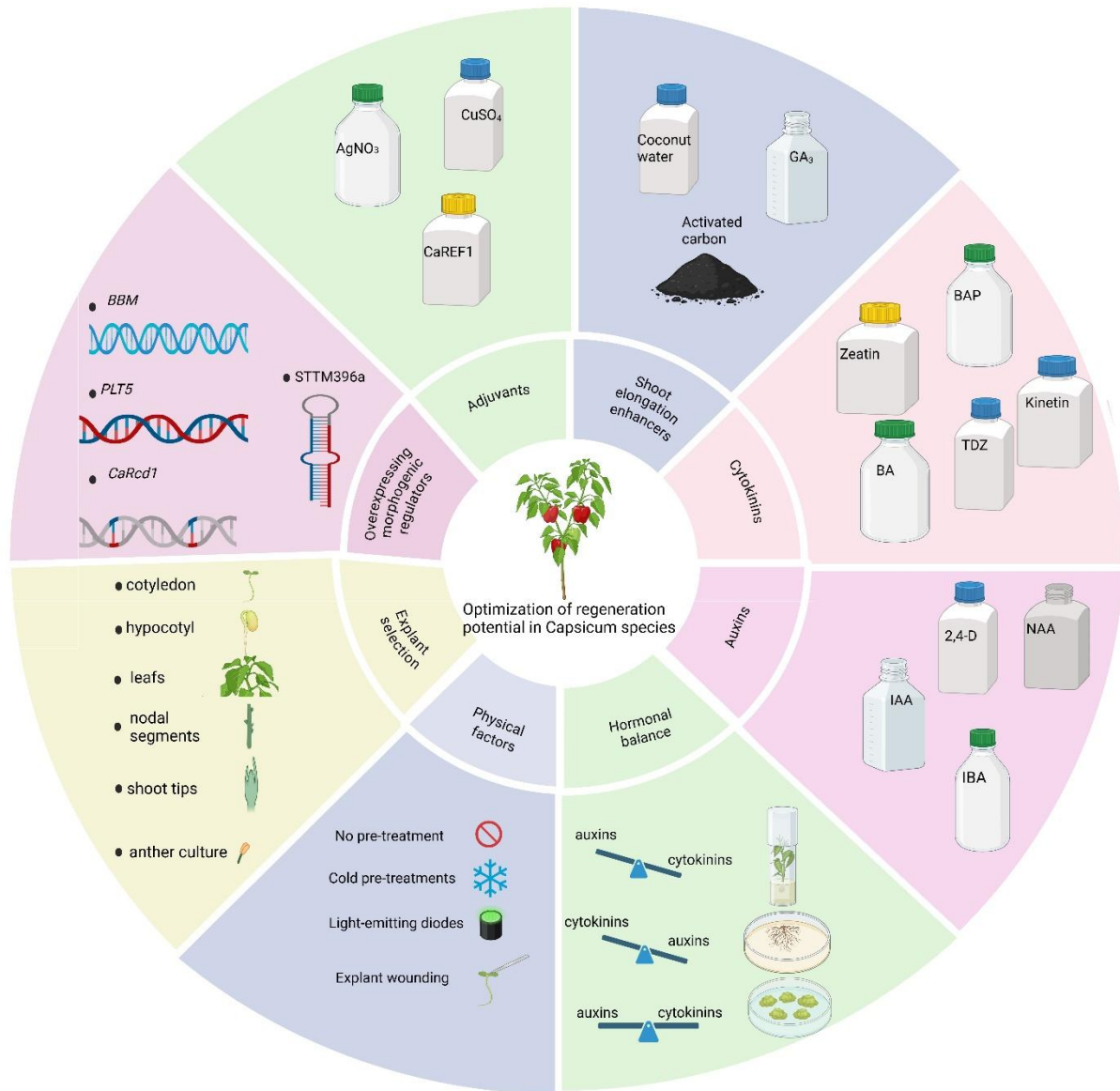
Similarly, *WOX5* functions as a basal morphogenic regulator that enhances cellular differentiation through regulating root apical meristem activity by recruiting PLETHORA (PLT) factors (Ding and Friml, 2010). One of the *PLTs*, namely *PLT5* in sweet pepper, facilitates the formation of transgenic calli and somatic embryogenesis in tissue culture (Lian et al., 2022). However, attempts to deliver *PLT5* directly *in vivo* in *C. annuum* failed, perhaps due to the accumulation of lignin and suberin at the injury site.

In addition to the morphogenic transcription factors, transgenic overexpression of *CaRcd1* (*Required for cell differentiation 1*) enhanced the regeneration capacity of the pepper R60 cultivar to double the shoot differentiation (Qiao et al., 2025). *CaRcd1* protein-protein interaction with *CaGATA26* inhibits the transcriptional activation of the *CaGATA26* transcription factor on the *Transmembrane Kinase-Like 1* (*CaTMKLI*) encoding an auxin-binding protein. Reduced expression of *CaTMKLI* impairs auxin transport in callus, thereby enhancing pepper regeneration capacity.

Plant organ growth and development are regulated by plant-specific transcription factors called Growth Regulation Factors (GRFs), which promote cell proliferation (Khatun et al., 2017). Organ size and cell number are increased through transcriptional activation of cell cycle genes by GRF and GRF's INTERACTING FACTORS (GIFs) complex (Wang W et al., 2022). Across several plant species, including monocots and dicots, shoot regeneration capability has

been enhanced by either GRF-GIF chimera (Swinnen et al., 2025) or *GRF* overexpression alone (Kong et al., 2020). Post-transcriptional suppression of *GRF* expression is mediated by microRNA396 (miR396) through complementary base pairing with *GRF* mRNA (Rodriguez et al., 2010). To inhibit microRNA396 activity in pepper, Shin et al. (2026) employed an artificial RNA called short tandem target-mimic (STTM396a). The study reported that callus induction increased to 45% in T<sub>0</sub>, and a nearly 4.1-fold higher rooting rate was observed for regenerated shoots under ectopic expression of STTM396a, suggesting that STTM396a promotes viable shoot regeneration.

During the early phases of tobacco shoot regeneration, the *plant callus highly expresses genes* (*PCE*) encoding calreticulin-like protein (CRT), display an exceptionally high expression level. These gene promoters are enriched with *Cis*-regulatory elements linked to hormone, wound responses, and meristem expression (Li B et al., 2025). Calreticulin-like proteins are highly conserved among plant species; therefore, it can be speculated that this class of promoters and CRT proteins might be highly effective in *Capsicum* spp. regeneration as well. Integration of the above-mentioned elements into current culture protocols can greatly enhance *Capsicum* spp. regenerative capacity, thereby overcoming the recalcitrancy barrier in these species (Figure 1).



**Figure-1.** Essential components and strategies for efficient regeneration of post-transformed *Capsicum* genotypes.

Explant sources such as cotyledons, hypocotyles, nodal segments, leaves, shoot tips, and anthers provide starting materials for regeneration. Auxins and cytokinins are key components of culture media, and their relative concentrations determine the direction of micropropagation. Enhancers like GA<sub>3</sub>, activated carbon, and coconut water promote shoot elongation, whereas adjuvants, such as CaREF1 peptide, AgNO<sub>3</sub>, and CuSO<sub>4</sub>, increase shoot regeneration capacity. Ectopic expression of developmental regulators such as *BBM*, *PLT5*, *CaRcd1*, and *STTM396a* proposes novel solutions to enhance the regeneration of

transformed cells. Stresses such as explant wounding, light, and cold treatments increase the regenerative capacity. The conglomeration of versatile approaches warrants an efficient system for *Capsicum* spp. regeneration.

### Genetic transformation strategies in *Capsicum* spp.

For understanding the gene function and genetic advancements, both transient and stable transformations play a pivotal role. Stable transformation refers to the integration of the

transgene into the host plant genome and its successful inheritance by the progeny plants (Hwang et al., 2017). Contrarily, in transient transformation, the transferred DNA can be transcribed to synthesize the desired gene product while temporarily remaining in the host plant cell nucleus without incorporating into the host genome. *Agrobacterium* can be used to deliver foreign DNA for both transformation types, whereas PEG-mediated transfection of protoplasts induces transient transformation (Liu H et al., 2024). Figure 2 provides a general view of various transformation approaches employed in *Capsicum* spp.

### Conventional *Agrobacterium*-mediated explant co-culture-based transformations

The most common vector delivery method in *Capsicum* spp. is *Agrobacterium*-mediated transformation. This method is favoured because it relies on *Agrobacterium*'s innate capacity to transfer a portion of its DNA to the host genome, making it easy to use and cost-effective (Kim et al., 2020). Similar to regeneration, various factors affect the efficiency of *Agrobacterium*-mediated transformation as discussed below.

### Genotype and explant selection

The optimization starts with selecting a highly transformable genotype, as it is the most critical factor influencing *Agrobacterium* susceptibility (Li et al., 2019). Following genotype selection, cotyledonary explants (Bulle et al., 2024a; Kumar et al., 2025; Shams et al., 2024; Tang et al., 2025) and hypocotyl explants (Mahto et al., 2018; Mishra et al., 2021) are prominently used for transformations.

### *Agrobacterium* species and strains

*A. tumefaciens* strains LBA4404 (Bulle et al., 2016; Mahto et al., 2018), EHA105 (Kumar et al., 2025), and GV1301 (Kang et al., 2025; Park et al., 2021) have demonstrated specific utility across different *Capsicum* spp. genotypes.

### Co-culture components: *Agrobacterium* concentration, co-culture period, transformation enhancers and selection agents

Research has indicated *A. tumefaciens* optical density (OD<sub>600</sub>) of 0.1 (Shams et al., 2024), 0.2–0.5 (Mahto et al., 2018), 0.5 (Mishra et al., 2021), and 0.6 (Kang et al., 2025; Tang et al., 2025), followed by 48 hours (Tang et al., 2025) to 72 hours (Mishra et al., 2021;

Shams et al., 2024) co-cultivation period on solid MS media with 100 µM acetosyringone (Mishra et al., 2021) yield maximized transformations. For the selection of transformant calli or explants, optimal Kanamycin concentrations of 30 mg/L (Mahto et al., 2018; Mishra et al., 2021), 100 mg/L (Kumar et al., 2025), and cefotaxime (Shams et al., 2024)/Augmentin (Mishra et al., 2021) 300 mg/L for bacterial control have been reported. The transformation efficiency of cotyledonary explants increased significantly when a combination of non-pre-culture and vacuum infiltration was used (Tang et al., 2025).

### *In planta* transformations

*In planta* transformations have emerged as a groundbreaking substitute to circumvent the regeneration and transformation bottlenecks. This general term refers to an array of techniques where entire plant tissues or particular tissues are immediately exposed to the foreign DNA encompassing *Agrobacterium tumefaciens* or *A. rhizogenes*, without the need for an *in vitro* regeneration phase.

### Agroinfiltration

Agroinfiltration involves using a needleless syringe to inject the *Agrobacterium* suspension into the abaxial leaf surface. Vacuum infiltration can also be used, especially for larger leaf areas or whole plants, to force the bacteria into the tissue (Chincinska, 2021). It is widely used in research to rapidly screen gene functions, such as protein-protein interactions and localization, or for the synthesis of recombinant proteins, as results can be obtained within a few days without the need for expensive instruments or complicated procedures (Nosaki et al., 2021; Zhang et al., 2020). It can also be used to test disease resistance functions of genes before establishing stable genetic transformation protocols (Bagga et al., 2019). Various *A. tumefaciens* strains, such as GV3101 and LBA4404, are used, along with derived strains (AGL1) or variants of *Rhizobium rhizogenes*, including A4, which exhibit hyper-transient expression in solanaceous plants, such as hot peppers (Lopez-Agudelo et al., 2025). High-expression systems, such as those incorporating a geminiviral replication system, e.g., the "Tsukuba system", can significantly boost protein expression levels in *Nicotiana benthamiana* (Nosaki et al., 2021). The efficiency of agroinfiltration can be highly reliant on

the variety or genotype and the age of the plant, with younger leaves or plantlets often showing better results (Lee and Yang, 2006).

### Stable *in planta* transformations

This approach typically involves direct infection with *A. tumefaciens* at early-stage plant material, such as young seedlings, allowing the bacteria to transfer the gene of interest directly into the plant's cells and incorporate into the host plant genome, bypassing the need for plant regeneration and hormonal supplementations (Bélanger et al., 2024; Saifi et al., 2020). *In planta* transformations can be a valuable alternative or supplement traditional *in vitro* tissue culture based *Agrobacterium* transformations. It is less prone to somaclonal variation and offers a quick, straightforward, economical, and efficient method for gene transformations. The two common *in planta* delivery approaches are regenerative activity-dependent *in planta* injection delivery (RAPID) and cut-dip-budding (CDB). RAPID implies direct gene delivery into meristematic regions (Mei et al., 2024) and is commonly employed in *Capsicum* spp. It includes seedlings, often at the two-leaf stage, that are co-cultivated, imbibed directly in an *Agrobacterium* suspension, and subsequently grown to set seeds. Other approaches of *in planta* transformation include fruit injection, floral dip, vacuum infiltration, pollen tube, apical meristem injury/injection, and sonication (Kharb et al., 2022). Bélanger et al. (2024) classified more than 30 kinds of *in planta* methods developed for 139 plant species in 105 genera, using multiple types of explants, including adventitious meristems, embryos, gametes (ovules or pollen), shoot apical meristems, and other vegetative tissues.

CDB represents a significant step forward in the approach for crops with root suckering ability. It eliminates the need for *in vitro* culture by delivering gene-editing cassettes and transgenes via hairy root induction, followed by shoot formation from transformed hairy roots (Cao et al., 2023; Lu et al., 2024a, 2024b). Hairy root generation via *A. rhizogenes*-mediated transformation offers a robust alternative to routine *A. tumefaciens* transformations for studying gene function (Aarouf et al., 2012; Toth et al., 2021). However, regeneration of plants carrying genetic constructs from transformed roots has not yet been established in *Capsicum* spp.

### Standardization of *in planta* protocols in *Capsicum* spp.

*In planta* transformation techniques have also undergone significant improvement in *Capsicum* spp., since the early report of *Agrobacterium*-mediated tissue culture-independent transformation of two *C. annuum* cultivars (Kumar et al., 2009). For example, wounded tobacco leave sap (Arthikala et al., 2014; Shivakumara et al., 2017), and acetosyringone are used as a source of phenolic compounds to activate the *Vir* (virulence) genes of *Agrobacterium*. Zhang et al. (2020) reported a highly efficient protocol for *Capsicum* spp. transient transformations involving hyper-virulent *A. tumefaciens* strains AGL1, washing solution (10mM MgCl<sub>2</sub> + 100 μM acetosyringone), infiltration solution (1.1g MS + 1% sucrose + 100 μM acetosyringone + 0.001% Silwet L-77), and targeting young, fully developed leaves from six-week-old plants. Similarly, a robust *in planta* method for red chillies (*C. annuum* L.) has also been established (Hamdani et al., 2024). The young seedlings of two varieties, Tanjung 2 and Ciko, were submerged in transformation medium containing the *A. tumefaciens* strain GV3101 carrying the binary vector pCAMBIA1301, encoding the β-glucuronidase (*GUS*) (Hamdani et al., 2024). *GUS* staining of the primary transformants showed 50% of the seedlings continued to express the *GUS* gene in their shoot after 10 days' post-infection.

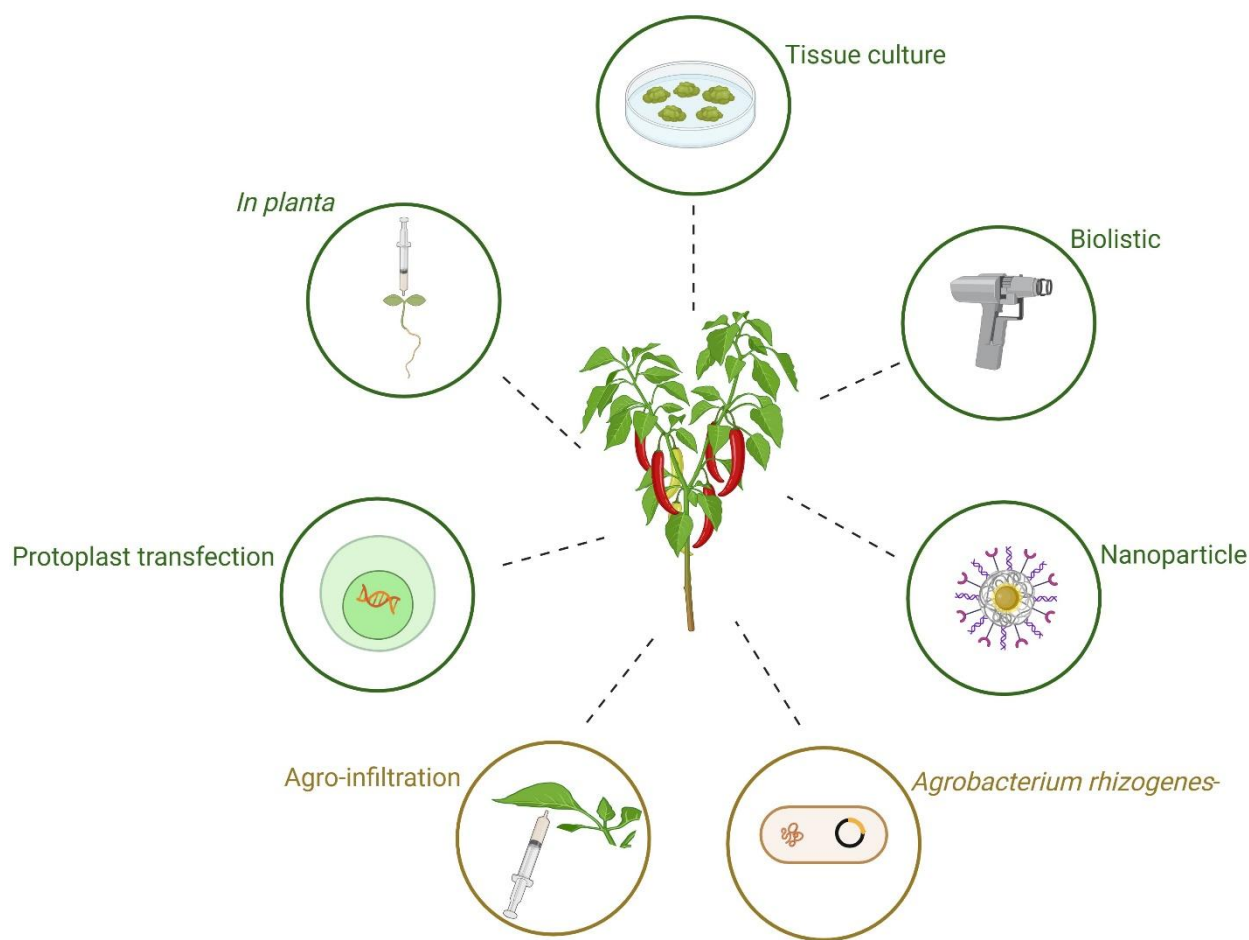
*In planta* transformations have been exploited in *Capsicum* spp. to overexpress the *non-expressor of pathogenesis-related* (*NPRI*) (Arthikala et al., 2014), *RB* gene from *Solanum bulbocastanum* (Bagga et al., 2019), *NDR1/HINI-Like 4* (*CaNHL4*)/*CBL-interacting protein kinase 1* (*CaCIP1*) (Ma et al., 2019), and *pea DNA helicase 45* (*PDH45*) (Shivakumara et al., 2017), respectively, conferring resistance against powdery mildew, *Phytophthora capsici*, and multiple abiotic stresses in the supplementation of enhancing various stress-responsive gene expression.

### *Agrobacterium*-free transformations

Despite *Agrobacterium*'s ability to yield single-copy events and transfer entire T-DNAs, its transformation efficiency varies among species and genotypes, particularly in recalcitrant species such as *Capsicum* spp. (Kim et al., 2020). Additionally, hypocotyl explant *Agrobacterium*-mediated transformations in *Capsicum* spp. produced inconsistent homozygosity at

mutated loci for CRISPR applications (Mishra et al., 2021). Biolistic delivery provides a means to circumvent *Agrobacterium*-mediated transformation and allows direct DNA delivery into the plant cells (Chee et al., 2018). It has been used for plastid transformation with a 20% efficiency (Kota et al., 2019), and proven highly effective for generating multiplex gene editing in the *C. annuum* genome (Bulle et al., 2024a). Nonetheless, this method still involves labour-intensive, time-consuming tissue culture procedures to generate gene-edited plants. Nanoparticle-mediated delivery systems are emerging as novel alternatives to introduce transgenes in plants and *Capsicum* spp. with a high biological

compatibility (Francis et al., 2024). A pioneering study in *Capsicum* spp. reported a highly effective nanoparticle-mediated transformation approach addressing a major bottleneck in *Capsicum* spp. biotechnology by offering a quick, genotype and tissue culture independent tool for gene editing and transformation (Sun et al., 2021). The researchers used magnetofection to deliver magnetic nanoparticles (MNPs) complexed with a CRISPR/Cas9 into pollens to produce genetically modified plants. T<sub>0</sub> plants were produced with an extraordinarily high average transformation efficiency of about 64% in terms of Cas9 presence, and mutations were found to be stably inherited.



**Figure-2.** Transformation strategies used for transgene delivery into *Capsicum* spp.

Agro-infiltration and *Agrobacterium rhizogenes* are used for transient transformations, while the others are used for stable transformations. Tissue culture-based explant co-culture transformation is a traditional method for transformation. *In planta* and nanoparticle-

based transformations circumvent tissue culture-based regeneration, whereas biolistic approaches enable direct plasmid transformation without the use of *Agrobacterium*. Protoplast transfections enable RNP delivery, bypassing the need for a DNA plasmid.

## Innovative hybrid or synergistic strategies to overcome recalcitrance to regeneration and transformations

### Protoplast transfections

The development of an effective protoplast system is highly valuable for both transient and stable transformation of pepper. Protoplast-based transfection and regeneration can become a robust tool for efficient gene delivery in *Capsicum* spp., enabling plasmid-independent CRISPR-Cas9 editing. However, regeneration from protoplasts is much more difficult than from callus. Recently, using soil-grown leaves of two pepper varieties, sweet pepper 'Demsey' and hot pepper 'CM334', successfully induced and maintained pepper-derived calluses (Kim and Lim, 2019). These calluses provide stable protoplasts for establishing cellular functional genetic screens in sweet and hot peppers. Protoplasts derived from CM334 were unstable and difficult to harvest, whereas Dempsey leaf protoplasts were sufficiently stable for CRISPR applications (Kim et al., 2020). Protoplasts are ideal target for CRISPR/Cas9-gRNA nucleoprotein (RNP) delivery as they are cell wall free, enabling efficient delivery with few chances of genetic mosaicism in regenerated plants due to the single-cell origin of the protoplasts (Hsieh et al., 2026). Protoplasts provide a reliable platform for testing the efficiency of gRNA design, measuring indel frequency, and assessing *in vitro* gRNA off-target effects. The *in vitro* on-target activity of five single gRNAs targeting *C. annuum* *PARENTAL ADVICE-1* (*CaPAD1*) was tested by introducing them into the protoplasts generated from leaves of three important Korean cultivars, including C15, Dempsey, and Younggo (Choi et al., 2024).

There are two commonly used methods for protoplast transfection polyethylene glycol (PEG)-mediated and PEG-independent transformations. PEG-mediated transfection increases the likelihood of successful transgene incorporation into the cells (Jeon et al., 2007; Negara et al., 2024) by nearly 50%. However, PEG-mediated protoplast transfection still exhibits reduced and variable efficacy; therefore, for transient transgene expression, a PEG-independent protoplast transformation system was developed to significantly enhancing the gene function studies in *Capsicum* spp. (Chu et al., 2025). Meanwhile, this system needs to be tested for RNP transfections.

## Synergistic genetic regulation and employment of morphological markers

Plant defense systems tend to silence transgenes failing transformations. Therefore, Kumar et al. (2025) devised a strategy to knock down the *SUPPRESSOR OF GENE SILENCING 3* (*SGS3*) via RNAi constructs in a commercial pepper variety (*C. annuum* cv Cayenne), along with an anthocyanin-based phenotypic marker, resulting in the identification of recombinant lines with vigorous foreign gene expression and obtaining a high efficacy of transformations (Kumar et al., 2025).

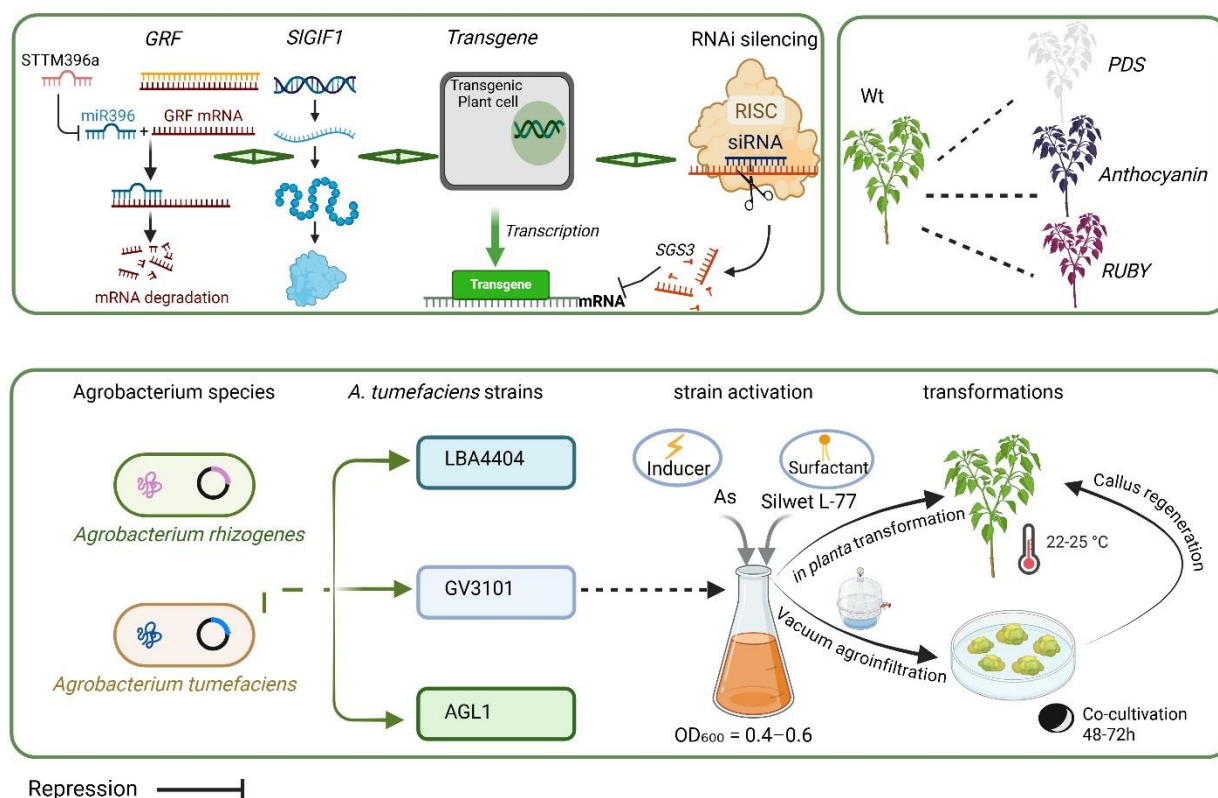
Similar to regeneration enhancement roles of GROWTH REGULATING FACTOR-INTERACTING FACTOR (GRF-GIF) complexes, the overexpression of the *tomato GRF interaction factor* (*SlGIF1*) resulted in a substantial enhancement in transformation efficiency in tomato and *Capsicum* spp. plants (Tang et al., 2025). In another study ectopic expression of STTM396a, which silences the suppressor of GRFs (miR396), led to 2.5 times higher transgenic production in *Capsicum* spp. (Shin et al., 2026).

## Advantages of phenotypic markers compared to traditional markers

Transformation efficiency, in terms of transgene introduction and integration into the host genome, can be measured by reporter gene expression, such as Green Fluorescent Protein (GFP), Yellow Fluorescent Protein (YFP), and  $\beta$ -glucuronidase (GUS) (Bulle et al., 2025a). However, reporter gene expression measurement requires expensive equipment and laboratory reagents, such as a fluorescence microscope for GFP/YFP (Dinant et al., 2008) and X-Gluc for GUS. On the other hand, the use of selection markers, such as antibiotic and herbicide-tolerance genes, may pose biosafety concerns (Afolabi, 2007). Additionally, these genes may interfere with the regeneration of host plants and require an optimized dose of the selection agents (Deo et al., 2011). In contrast, the emergence of markers such as RUBY (Tang et al., 2025), Phytoene Desaturase (PDS) (Kang et al., 2025; Tang et al., 2025), anthocyanin (Kumar et al., 2025), ARGONAUTE7 (Kumar et al., 2025), and fasciculate (Kang et al., 2025) eliminates the aforementioned constraints and yields a visually distinct phenotype, often under the control of a single recessive allele. Loss-of-function mutations in the *PDS* gene lead to a clear photobleached or an albino phenotype by

disrupting carotenoid biosynthesis, making it a perfect target for genome-editing studies (Bulle et al., 2024a; Tang et al., 2025). RUBY (Tang et al., 2025) and anthocyanin (Kumar et al., 2025) gene expressions confer the characteristic reddish and purple colors, respectively, on transformed plants. The integration of diverse biotechnological approaches, phenotypic

markers, and the strategic improvement of *Agrobacterium*-mediated transformation have significant potential to enhance *Capsicum* spp. transformation efficiency. Figure 3 illustrates how these approaches cooperatively contribute towards improving transformation efficacy and transgenic plant recovery.



**Figure-3.** Integrative approaches to enhance the transformation efficiency in chilli peppers.

The top-left panel illustrates the synergistic effect of STTM396a (Shin et al., 2026), *SIGIF1* overexpression (Tang et al., 2025), and *SGS3* RNAi silencing (Kumar et al., 2025) in independent studies, enhancing transgene expression. The top-right panel highlights the importance of selection markers. The bottom panel depicts *Agrobacterium* transformation efficiency enhancements achieved through selection between *Agrobacterium* species and strains, employing activators and surfactants, and using co-cultivation/agroinfiltration methods, as well as controlling temperature and light regimes. Abbreviations: As: Acetosyringone, OD: optical density.

### Genetic engineering tools used in the genetic improvement

Genetic engineering techniques have been used in *Capsicum* spp. biotechnology to transfer a foreign gene from a different species. These approaches have been extensively developed, including gene silencing, overexpression, and editing, to enhance tolerance to abiotic and biotic stresses, metabolic content, and postharvest quality (Rai and Shekhawat, 2015). In the past, genetic improvement of *Capsicum* spp. has primarily been conducted through traditional breeding, including heterosis and mutational breeding combined with molecular markers (Usman et al., 2018). However, these approaches are constrained by biological barriers, such as incompatibility, limited

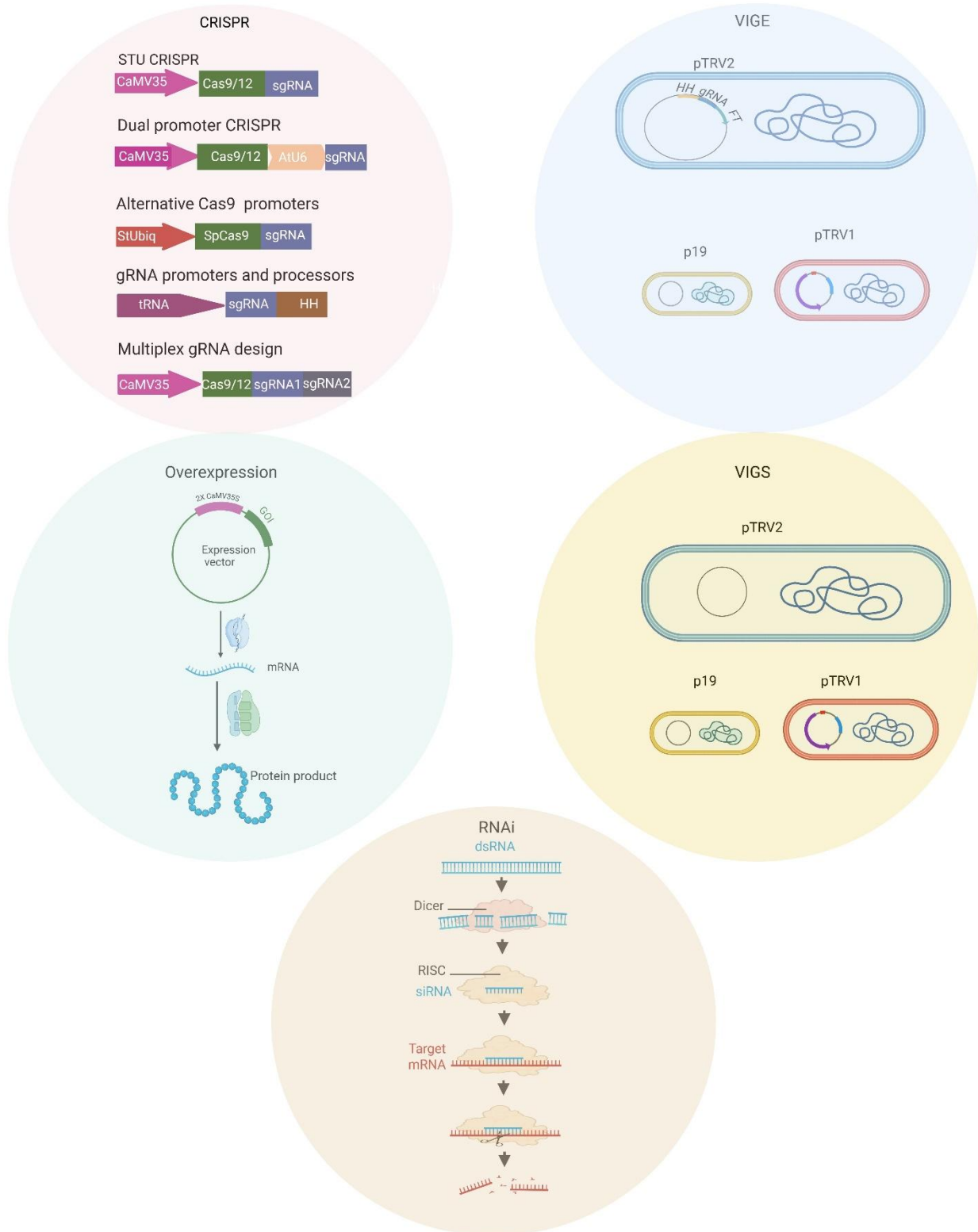
interspecific fertility, and narrow genetic diversity (Martins et al., 2015; Pinar et al., 2025). Therefore, the genetic engineering of *Capsicum* spp. can contribute to the development of noval cultivars by enabling rapid gene modification using either foreign or endogenous genes. Moreover, crucial genes underlying advantageous traits may be discovered in other plant species, and incorporating foreign genes can be valuable in introducing unique traits not naturally occurring in *Capsicum* spp., thereby broadening its diversity and enhancing the quality (Bulle et al., 2016; Bulle et al., 2024b). Additionally, genetic engineering can accelerate breeding by

bypassing traditional barriers and enabling more precise, targeted modifications to achieve desired traits (Rao and Anilkumar, 2020). Figure 4 presents an overview of the major genetic modification tools currently available for *Capsicum* spp., including transgenic overexpression, virus-induced gene silencing (VIGS), RNA interference (RNAi), CRISPR-based genome editing, and virus-induced gene editing (VIGE) approaches. The resultant progress over the last three decades in *Capsicum* spp. through genetic engineering has been summarized in Table 1.

**Table-1.** Genetic advances in *Capsicum* spp. through genetic engineering in the last three decades.

Serial No.	Transgene	Genetic engineering tool	Transformation strategy	Phenotypic outcomes	References
1	<i>CgCOM1</i>	RNAi	Agroinfiltration	Anthracnose tolerance	Mahto et al., 2020
2	<i>ERF</i>	Can-miRn37a	Agroinfiltration	Anthracnose tolerance	Mishra et al., 2018
3	<i>RXLRI, 4</i>	RNAi	Agroinfiltration	Blight resistance	Cheng et al., 2022
4	<i>R gene</i>	vsRNAs	Agroinfiltration	Tobacco mosaic virus	Kim et al., 2021
5	<i>CaALDH7B1</i>	VIGS	Agroinfiltration	Enhanced tolerance to heat and drought	Yang et al., 2025
6	<i>CaCP15</i>	VIGS and transient overexpression	Agroinfiltration	Negative regulator of salt and osmotic stress	Zhou et al., 2023
7	<i>CaSkl1, CaSgt1</i>	VIGS	Agroinfiltration	Enhanced tolerance to heat, drought, and basal disease tolerance	Chung et al., 2006
8	<i>CaCOMT36</i>	VIGS	Agroinfiltration	Capsaicinoid biosynthesis	Li S et al., 2025
9	<i>CaABCG14</i>	VIGS	Agroinfiltration	Capsaicinoid biosynthesis	Fei et al., 2024
10	<i>CaMATE27</i>	VIGS	Agroinfiltration	Capsaicinoid biosynthesis	Fei et al., 2025
11	<i>CaMYB31</i>	VIGS	Agroinfiltration	Capsaicinoid biosynthesis	Arce-Rodríguez and Ochoa-Alejo, 2017
12	<i>CaMLO2</i>	VIGS	Agroinfiltration	Enhanced tolerance against pathogens.	Kim and Hwang, 2012
13	<i>CaMYB113</i>	VIGS and overexpression	Agroinfiltration	Anthocyanin contents	Liu S et al., 2024
14	<i>elicitor</i>	Stable overexpression	Cotyledon <i>Agrobacterium</i> -mediated	Blight resistance	Kwon et al., 2007
15	<i>β-Lcy</i>	Stable overexpression	Cotyledon <i>Agrobacterium</i> -mediated	Enhanced fruit β-carotene content	El nagar, 2018
16	<i>Tsi1</i>	Stable overexpression	Cotyledons and hypocotyl <i>Agrobacterium</i> -mediated	Multiple biotic stress tolerance	Shin et al., 2002
17	<i>PepEST</i>	Stable overexpression	Cotyledons and hypocotyls <i>Agrobacterium</i> -mediated	Anthracnose resistance	Ko et al., 2016
18	<i>TaNHX2</i>	Stable overexpression	Cotyledon, <i>Agrobacterium</i> -mediated	Salt stress tolerance	Bulle et al., 2016

19	<i>AdDjSKI</i>	Stable overexpression	Cotyledon, <i>Agrobacterium</i> -mediated	Drought tolerance	Bulle et al., 2024b
20	<i>Osmotin</i>	Stable overexpression	Leaves, <i>Agrobacterium</i> -mediated	Salt stress tolerance	Subramanyam et al., 2011
21	<i>NPRI</i>	Stable overexpression	<i>In planta</i>	Decreased incidence of powdery mildew by 76.7%	Arthikala et al., 2014
22	<i>PDH45</i>	Stable overexpression	<i>In planta</i>	Tolerance to multiple abiotic stresses	Shivakumara et al., 2017
23	<i>GUS</i>	Transient expression	<i>In planta</i>	GUS expression	Hamdani et al., 2024
24	<i>RB</i>	Stable overexpression	Agroinfiltration and <i>in planta</i>	Blight resistance	Bagga et al., 2019
25	<i>GFP</i>	Transient expression	<i>In planta</i>		Zhang et al., 2020
26	<i>CaCaM1</i>	Transient expression	Agroinfiltration	ROS and nitric oxide production	Choi et al., 2009
27	<i>PDS</i>	CRISPR-Cas9	Biolistic	Albino or mosaic	Bulle et al., 2024a
28	<i>NAC72</i>	CRISPR/Cas9 editing	<i>Agrobacterium</i> -mediated	Endowment of Anthracnose resistance	Joshi (unpublished)
29	<i>INSL, INSR</i>	Cytidine base Plastid transformation	Biolistic		Kota et al., 2019
30	<i>CaERF28</i>	STU-CRISPR-Cas9	Hypocotyl, <i>Agrobacterium</i> -mediated	Enhanced anthracnose resistance	Mishra et al., 2021
31	<i>CaPDS</i>	Dual promoter CRISPR	Cotyledon, <i>Agrobacterium</i> -mediated	Albino or mosaic	Tang et al., 2025
32	<i>CaAGO7, CaPPO, SGS3</i>	CRISPR+RNAi	Cotyledon, <i>Agrobacterium</i> -mediated	Wiry leaves, reduced enzymatic activity	Kumar et al., 2025
33	<i>CaMLO2</i>	CRISPR/Cas9	Callus, <i>Agrobacterium</i> -mediated	Indel frequency, selection of <i>Agrobacterium</i> type	Park et al., 2021
34	<i>CaMLO2</i>	CRISPR/Cas9-RNP	RNP delivery to protoplasts	Higher efficiency of sgRNA1 than sgRNA2 across 6 cultivars	Park and Kim, 2023
35	<i>CaMLO2</i>	CRISPR-Cas9/12-RNP	RNP delivery to protoplasts	Higher efficiency of sgRNA1 than sgRNA2 between 2 cultivars	Kim et al., 2020
36	<i>CaPAD1</i>	CRISPR/Cas9-RNP	RNP delivery to protoplasts	Evaluation of <i>in vivo</i> target efficiency and off-target effects	Choi et al., 2024
37	<i>PDS, FASCICULAT E</i>	TRV-based VIGE	<i>Agrobacterium</i> -mediated, and <i>in planta</i>	Albino and fasciculate phenotype	Kang et al., 2025
38	<i>PDS</i>	TSWV-based VIGE	<i>In planta</i> transformation via frictional inoculation		
39	<i>PDS</i>	TSWV-based VIGE	Virus-inoculated leaf tissue culture	Albino or mosaic	Liu Q et al., 2023
					Zhao et al., 2024



**Figure-4.** Schematic illustration of genetic modification tools in *Capsicum* spp.

The top left circle depicts various CRISPR designs, including STU, dual promoters, processors, and multiplex gRNAs. Transgenic overexpression involves the expression of the gene of interest (GOI) driven by a constitutive promoter, such as 2XCaMV35S (bottom-left circle). VIGE and VIGS vectors contain pTRV2, which encodes gene-editing and silencing cassettes, respectively (right top and bottom circles). The circle at the bottom depicts a pathway for an *in planta*-delivered RNAi assembly that silences a pathogen mRNA depicted in blue and red colors, respectively.

### Transgenic overexpression

Most genetic enhancement in *Capsicum* spp., aimed at increasing resistance to biotic and abiotic stresses, has been achieved through this approach (Figure 4). Transgenic overexpression of *tobacco stress-induced 1 (Tsi1)* (Shin et al., 2002), *pepper esterase (PepEST)* (Ko et al., 2016) enhanced stress-responsiveness and transcription of *pathogenesis-related (PR)* genes, and *elicitor* produced blight resistance in transgenic peppers (Kwon et al., 2007). Acquired resistance against *Xanthomonas campestris* pv. *Vesicatoria* was observed when transient expression of *calmodulin 1 (CaCaM1)* via agroinfiltration induced nitric oxide (NO) and reactive oxygen species (ROS) production and a hypersensitive response (HR)-like cell mortality in *C. annuum* leaves (Choi et al., 2009). Overexpression of the tobacco *osmotin* (Subramanyam et al., 2011) and *wheat antiporter (TaNHX2)* (Bulle et al., 2016) improved salt stress tolerance in the transgenic *Capsicum* spp. plants. Similarly, overexpression of the wild peanut (*Arachis diogeni*) *AdDjSKI* in *Capsicum* spp. enhanced drought stress tolerance and levels of ROS-scavenging antioxidant enzymes (Bulle et al., 2024b).

Ectopic expression has also been employed to improve nutritional quality in peppers. The introduction of the *lycopene  $\beta$ -cyclase ( $\beta$ -Lcy)* gene from *Arabidopsis* into transgenic *Capsicum* resulted in a substantial increase in fruit  $\beta$ -carotene content, reaching 7-10 times that of the wild type (El Nagar, 2018). Stable overexpression of *C. annuum* MYB Transcription Factor 113 (*CaMYB113*) resulted in increased anthocyanin contents in the cotyledons, suggesting it is a candidate gene for purple cotyledons in *C. annuum* (Liu S et al., 2024). Nevertheless, significant commercial, sociological, and regulatory constraints still prevent the widespread use of genetically modified crops (Domingo, 2025).

### Viral-induced gene silencing (VIGS)

A significant paradigm shift in *Capsicum* spp. functional genomics and genetic improvement techniques have been the evolution of virus-based vectors, beginning from transient gene silencing to heritable gene editing (Figure 4). VIGS has set a critical precedent of employing viral vectors to control plant gene expression. Tobacco rattle virus (TRV), which has a wide host range of more than 400 plant species, has emerged as a benchmark for VIGS applications in *Capsicum* spp. and other solanaceous plant species (Shingaliev et al., 2025). VIGS vectors based on the tobacco rattle virus (TRV) have been widely used to study many plant species, including *Capsicum* spp., for gene silencing to characterise gene functions and test the suitability of *Agrobacterium* strains (Zhou et al., 2021). The initial high-frequency VIGS protocol in pepper reported by Chung et al. (2004) unlocked a transient, transformation-free, and rapid method for gene silencing. The VIGS system employs cloning a portion of the coding sequence of the gene of interest into the pTRV2 plasmid to generate a silencing vector, which is co-transformed with the pTRV1 plasmid in a 1:1 ratio using *Agrobacterium* (Li S et al., 2025). During the cotyledon-flattening seedling growth phase, the mixed suspension is applied to the underside of the abaxial leaf surface using a syringe. The spread of viral components is systemic, eliciting RNA interference of the targeted endogenous genes.

VIGS has been widely used to elucidate the complex metabolic pathways underlying pungency in *Capsicum* spp. For instance, *Caffeic Acid O-Methyltransferase 36 (CaCOMT36)* knockdown led to a decrease in capsaicinoid biosynthesis (Li S et al., 2025), whereas silencing of *Phytoene Synthase 1 (CaPSY1)* decreased zeaxanthin and increased lutein level, suggesting its crucial role in the carotenoid biosynthesis pathway at the fruit ripening stage (Wei et al., 2020). Similarly, transcriptional suppression of transporter genes *ATP-Binding Cassette G14 (CaABCG14)* and *Multidrug and Toxic Compound Extrusion 27 (CaMATE27)* in the CA59 cultivar resulted in decreased levels of capsaicin (CAP) and dihydrocapsaicin (DhCAP) in the septum and placenta tissues, respectively (Fei et al., 2024, 2025). *CaMYB31* silencing significantly reduced capsaicinoid content and expression of capsaicinoid biosynthesis pathway genes (Arce-Rodríguez and Ochoa-Alejo, 2017). While gene silencing of *CaMYB113* comprehensively reduced anthocyanin

contents in the cotyledons (Liu S et al., 2024). Functional characterisation of *S-phase Kinase-associated Protein 1* (*CaSkp1*) and *CaSgt1* suggested their critical roles in pepper growth and development, and basal disease tolerance was provided by TRV2-based gene silencing (Chung et al., 2006). *Mildew Locus O 2* (*CaMLO2*) VIGS-silenced plants exhibited enhanced resistance when infected with *Xanthomonas campestris* pv. *Vesicatoria* (Kim and Hwang, 2012). Along with capsaicinoid biosynthesis and pathogenic defence, abiotic stress-related genes have been studied using VIGS. For instance, a conserved gene *C. annuum* *Aldehyde Dehydrogenase 7B1* (*CaALDH7B1*), was found to increase heat and drought stress resistance by promoting antioxidant enzyme activity (POD, APX, CAT, SOD) and limiting ROS accumulation (Yang et al., 2025). In contrast, transgenic overexpression and VIGS experiments reported *Cysteine Protease 15* (*CaCP15*) acts as a negative modulator of salt and osmotic stress (Zhou et al., 2023).

Additionally, the introduction of ectopic protein expression through virus-based vectors has emerged as a new development in crop genetics and improvement (Wang et al., 2023). A broad bean wilt virus 2 (BBWV2)-based vector tool was developed for transient loss- and gain-of-function studies in *C. Annuum* (Choi et al., 2019). To express two recombinant proteins simultaneously, the BBWV2 was designed as a dual gene expression vector. Coexpressing an ectopic viral suppressor of RNA silencing, the researchers were able to attain an improved and steady expression of the recombinant proteins. Additionally, a BBWV2-based VIGS vector was developed and validated by silencing the *PDS* gene.

### RNA interference (RNAi)

RNA Interference (RNAi) in eukaryotes, a conserved gene-regulation mechanism, has been extensively used to silence the expression of genes of interest (Baulcombe, 2004; Ketting, 2011) (Figure 4). Although VIGS has been the method of choice for numerous gene-silencing studies in *Capsicum* spp., RNAi-based silencing approaches have been used in a few exceptions. RNAi can serve as a complementary, highly versatile regulatory tool to enhance stress resilience against fungal and viral infections in *Capsicum* spp. (Borah et al., 2018; Mahto et al., 2020). Studies under varying elevated-temperature conditions in *C. annuum* reveal a temperature-

mediated switch from R-gene-based immune responses to RNAi-dependent antiviral defence mechanisms (Tsai et al., 2022), driven by the substitution of heat-sensitive L-protein resistance by a significant increase in virus-derived small interfering RNAs (vsiRNAs). The mechanism of these vsiRNAs is mainly generated by RNA-Dependent RNA Polymerase 6–Dicer-Like 2–Argonaute 7 (RDR6–DCL2–AGO7) activity. The vsiRNA functions have hotspots on viral genomic templates and include temperature-specific species that are preferentially incorporated into AGO protein complexes, providing redundant resistance to temperature-stress-induced infections (Kim et al., 2021; Zhang B et al., 2025). Exogenous application of synthetic vsiRNAs in *C. annuum* provides strong biological validation of vsiRNA efficacy in suppressing the accumulation of Tobacco mosaic virus pathotype P0 (TMV-P0) through precision-targeted RNAi-mediated therapy for climate warming, regardless of temperature-related physiological conditions (Kim et al., 2021).

In parallel, experiments on tospovirus-infected *Capsicum* show that the plant engages in two biologically independent modes of host-mediated RNA-directed interference defence responses, namely vsiRNAs and RDR1-dependent virus-activated siRNAs (vasiRNAs) (Fletcher et al., 2022). These vasiRNAs target endogenous transcripts, particularly those associated with ribosomal and endoplasmic reticulum protein-processing pathways, underscoring the cell-wide role of RNAi engagement during infection. However, the biological significance of vasiRNA-mediated transcript regulation remains poorly understood.

An increasing pool of studies indicates that host-induced or spray-induced gene silencing (HIGS or SIGS) of virulent or critical pathogen genes may offer an effective and environmentally viable approach to disease control (Cai et al., 2018; Wang and Dean, 2020; Wang and Jin, 2017). SIGS denotes spraying small interfering or double-stranded RNA (siRNA or dsRNA) molecules on plant leaves to silence pathogen genes (Koch et al., 2016; Wang M et al., 2016), while HIGS represents *in planta* expressed RNAi assemblies that knock down pathogen mRNAs (Nowara et al., 2010; Nunes and Dean, 2012). HIGS demonstrated *Phytophthora capsici* (pathogen) growth was suppressed after exogenous application of siRNAs targeting *Receptor-like Lyophilic Cytoplasmic Kinases* (*RXLR4* and *RXLR1*) expressions (Cheng et al., 2022). *RXLR* genes act as effectors in *P. capsici*

and are upregulated during pathogen infection. Similarly, HIGS in chilli targeting the *Colletotrichum gloeosporioides conidial morphology 1* (*CgCOM1*) conferred enhanced tolerance to anthracnose (Mahto et al., 2020). In another study, Can-miRn37a-mediated suppression of *ethylene response factor (ERF)* expression increased resistance to the *Colletotrichum truncatum* pathogen, which causes anthracnose in *Capsicum* spp. (Mishra et al., 2018). Overall, these reports highlight the key regulatory role of RNA interference in *Capsicum* spp. research, offering immediate avenues for basic and applied research on genetic improvement. These include the development of dsRNA-based antiviral interventions and integration of RNAi with transcription-factor engineering for multilayered climate-resilient *Capsicum* spp. improvement.

### Gene editing in *Capsicum* spp.: From concept to practice

The most effective approach in New Breeding Techniques (NBT) for improving the crop trait is genome editing (Gao, 2021). This technique aims to generate plants that can withstand the mounting challenges of global warming, including increasing abiotic and biotic stresses (Younas et al., 2024). Through DNA repair processes, gene editing generates genetically identical plants to the parent plant, except for the targeted trait. It leads to precise, stable, and heritable mutations in the targeted loci, with minimal off-target effects and without the incorporation of alien DNA (Ansori et al., 2023; Zhang and Gao, 2017). Gene editing toolkits enable the accurate alteration of genes using single or multiple-guide RNAs (sgRNAs or gRNAs) (Wang H et al., 2016). It has great potential to improve *Capsicum* spp. breeding, particularly in terms of increasing disease resistance (Nerkar et al., 2022). In comparison to other genetic manipulation systems, such as VIGS and RNAi, CRISPR/Cas9 can perform complete gene knockout of endogenous genes (Chen et al., 2019).

Several challenges still hinder the widespread use of this technology in *Capsicum* spp. to realize its full potential, including plasmid architecture (Figure 4). The expression of gRNA in the CRISPR vector is driven by either the U3 or U6 small nucleolar RNA (snoRNA) promoter, while Cas9 expression is mostly driven by either *UBIQUITIN* or *2XCAMV35S* promoters, respectively, in monocots and dicots (Ma et al., 2023; Xie et al., 2015). Tailoring the U3 or U6 promoter specific to each target species can increase

editing efficiency (Massel et al., 2022; Ren et al., 2021; Riu et al., 2023; Zhang et al., 2022). A high editing efficacy of 62.5 per cent was reported for U6-26 small nuclear RNA (snRNA) gene from *Arabidopsis thaliana* (*AtU6-26*) for gRNA expression in chilli peppers (Bulle et al., 2024a). Kang et al. (2025) reported the use of *U6-26* and a subgenomic viral promoter, respectively, for tissue culture-based and *in planta* gene editing protocols in the pTRV-based system. However, one of the main limitations of U3- and U6-promoter usage is their dependence on protospacer adjacent motif (PAM): A(N)<sub>20</sub>GG and G(N)<sub>20</sub>GG target sequences, respectively, to guide Cas9. The employment of self-cleaving Hammerhead (HH) type ribozyme, instead of U3 or U6 promoters, eliminates the requirement of A or G for transcription initiation and broadens the range of Cas9 target to any PAM with NGG motif (Gao and Zhao, 2014). Similar improvements have been achieved by optimising the codon of the Cas protein used in the vector (Zhang et al., 2022). Various Cas proteins are also currently available. The most commonly used Cas protein in genome editing is Cas9, which recognises the NGG PAM (Anders et al., 2014). While it is sufficient for most knock-out usages, using a different Cas protein could widen the choice for PAM, increasing the target range, and facilitating various editing purposes (Csörgő et al., 2020; Hibshman et al., 2024; Kulcsár et al., 2022).

In most studies on CRISPR-Cas9 systems in *Capsicum* spp., Cauliflower Mosaic Virus 35S (2XCAMV35S/35S) has been used to drive Cas9 expression (Bulle et al., 2024a; Kang et al., 2025). An alternative gene-editing method in plants, single transcript unit (STU) CRISPR 2.0, has been developed that uses a single promoter to express both Cas9 and gRNA, eliminating the need for separate promoters for each (Tang et al., 2019). Mishra et al. (2021) reported 74.24% gene-editing efficiency using STU-CRISPR. On the other hand, Kumar et al. (2025) used a dicot-specific promoter from *Solanum tuberosum* *UBIQUITIN10* (*StUbiq10*) to mediate the expression of SpCas9 and two gRNAs, generating heritable gene edits in a commercial pepper variety. Elsewhere, Li B et al. (2025) reported that deployment of calreticulin promoters (*PCE8pro*) outpaced conventional 2X35S promoters for SpCas9 expression in tobacco calluses, and achieved comparatively higher editing efficiency for generating mutations in dicot species such as tobacco, lettuce, and tomato. There is a necessity to modify or test the relative efficiency of the current

gene-editing system in *Capsicum* spp. by incorporating *2X35S/StUbiq10/CaUbiq10/PCE8pro* for Cas9 expression. Similarly, *Capsicum* endogenous U6 promoters (*CaU6*) can substitute Arabidopsis *AtU6-26/29* to drive sgRNA expression in conjunction or in substitution for processors such as mobile gRNAs or ribozyme hammerhead components, to achieve greater specificity and increased gene-editing efficiency.

CRISPR-Cas9 gene editing has been successfully employed in *C. Annuum* to confer resistance against key pathogens. CRISPR/ Cas9-fused cytidine base editing (CBE) system, followed by *Agrobacterium*-mediated transformation approaches, with a base editing rate of 69%, accurately knocked out *NAC72*, endowing anthracnose resistance in chilli peppers (Joshi, unpublished results). Knockout mutants of *proliferating cell nuclear antigen (PCNA)* in two chilli cultivars generated through *in planta* delivery are resistant to geminiviruses (Rahmatika et al., 2020). In a subsequent study, gene-edited mutants of *C. annuum Ethylene Response Factor 28 (CaERF28)* in susceptible cultivars demonstrated enhanced resistance to the anthracnose pathogen (*Colletotrichum truncatum*) via a STU CRISPR construct {Pol(II)promoter+sgRNA+HH} (Figure 4) (Mishra et al., 2021). Furthermore, gene editing efficiency increased from 72.5% to 85.7% when multiple sites within *CaERF28* were targeted, suggesting that STU CRISPR/Cas9-based gene knockout targeting multiple sites is a versatile, robust, and safe method for *Capsicum* spp. and other solanaceous species. Using biolistic transformation CRISPR-Cas9 gene-editing cassettes were used to introduce mutations in chilli pepper cultivar G4's *PDS* by simultaneously targeting exons 14 and 15, achieving 62.5% gene editing efficiency (Bulle et al., 2024a). Kumar et al. (2025) reported successful gene editing of *POLYPHENOL OXIDASE (CaPPO)* and *ARGONAUTE7 (CaAGO7)*, evidenced by reduced enzyme content and abnormal wiry-leaf phenotypes, respectively. Going further, CRISPR/Cas has the potential to enable organelle genome editing in chilli pepper plastids, promoting the development of superior genotypes that are resilient to various environmental stresses and rich in a range of nutrients (Bulle et al., 2025b).

### Viral-induced gene editing (VIGE)

The integration and expression of CRISPR components in viral genome-based plasmids have led

to the evolution of VIGE tools (Biswas et al., 2021; Oh et al., 2021). VIGE induces highly effective, heritable, and systemic mutations, offering a convenient method for significant genome alterations (Uranga and Daròs, 2023). This method enables genetic changes without requiring consecutive transformations, as distinct target genes can be knocked out by injecting plants with virus-based carriers expressing the desired sgRNAs. This approach is increasingly favoured in many plant species, where conventional methods are challenging (Hasan et al., 2024).

The VIGE proof-of-concept was first demonstrated when an sgRNA was introduced into *Nicotiana benthamiana* plants overexpressing Cas9 using a TRV-based vector (Ali et al., 2015). A major technical obstacle for achieving heritable gene edits was the limited mobility of the viral construct. Mobile sgRNAs have been generated through the fusion of mobile RNAs, such as transfer RNA (tRNA) or *FLOWERING LOCUS T (FT)*, with sgRNAs, which significantly enhances the mobility of the gRNA cassette within shoot apical meristems from the site of inoculation to improve editing efficiency. The mobile sgRNAs delivered through viral vectors such as barley stripe mosaic virus, TRV, and cotton leaf crumple virus, and enhanced the effectiveness of heritable and systemic gene editing in wheat, Arabidopsis, *N. benthamiana*, and cotton, respectively (Chen et al., 2022; Ellison et al., 2020; Lei et al., 2021; Nagalakshmi et al., 2022).

The introduction of a hammerhead ribozyme (HH) sequence with self-cleaving function in the middle of the sgRNA sequence and subgenomic viral promoter further increased systemic editing and decreased sgRNA expression variation in VIGE applications by creating an sgRNA free of viral promoter overhangs (Gao and Zhao, 2014; Oh et al., 2021). By integrating all the above-mentioned features, an optimal TRV2-based construct was synthesized, overcoming the constraints of limited regenerations and conventional *Agrobacterium*-mediated genetic transformation (Kang et al., 2025). The gene-editing efficiency was further validated by knocking out the *FASCICULATE* gene using the same approach. The standardization approaches, including plasmid design and culturing conditions, led to an editing efficiency of 36.3%, as indicated by phtbleaching phenotypes, and 8.5% of the inoculated plant progeny inherited the *PDS* mutations.

Similar to the TRV, highly effective DNA-free genome modification in pepper has been achieved

using Tomato spotted wilt virus (TSWV) carrying a CRISPR/Cas9 construct (Liu Q et al., 2023; Lou et al., 2024). Liu F et al. (2023) and Lou et al. (2024) developed a novel *in planta* approach for gene editing in different plant species, including chilli peppers. Tobacco (*N. benthamiana*) plants were first agro-inoculated to extract specialised TSWV-based plasmids from leaf sap as inocula, which were rubbed onto young leaves of sweet, habanero, and chilli pepper among *Capsicum* spp. Although these studies demonstrated that heritable editing could be reproduced *in vitro* by culturing infected tomato and tobacco leaf tissues with remdesivir, favipiravir, or ribavirin, the practical efficiency of this method in *Capsicum* spp. remained unknown. The subsequent research by Zhao et al. (2024) established a transformation-free protocol using the frictional inoculation technique to facilitate the succession of TSWV-based gene editing through *in vitro* culture of two *Capsicum* spp. cultivars (Zhao et al., 2024). Interestingly, heritable edits were recovered in up to 77.9% of regenerated plants. However, a significant bottleneck to achieving stable transgene overexpression in *Capsicum* spp. persisted despite the progress.

### Challenges and prospects in *Capsicum* spp. biotechnology

Recent advances in genetic engineering have significantly improved the capabilities for genetic transformation and genome editing in *Capsicum* spp. However, there are several constraints in biological and technical factors, which still limit the application of genetic engineering as a breeding tool in *Capsicum* spp.

Although constitutive expression of morphogenic regulators such as BBM, WUS, and GRF-GIF chimaeras significantly increases regeneration efficiency, it also carries risks of pleiotropic effects, long-term phenotypic instability, and genotype specificity (Gordon-Kamm et al., 2019). To separate regeneration signals from post-transformational developments, future approaches should aim to develop self-eliminating, tissue-specific, and inducible expression systems (Borghini, 2010). Research in *Zea mays* has indicated the desiccation-induced CRE/lox recombinase system efficiently excises *WUSCHEL2* and BBM transcription factors, followed by plant regeneration (Mookkan et al., 2017). Simultaneously, the use of phenotypic markers such as PDS, RUBY, and anthocyanins underscores the

effectiveness of antibiotic-free, non-destructive selection procedures. Real-time monitoring of transformation efficiency under controlled and field conditions can be enhanced by ongoing advances in hormone-responsive, spectrally unique, and high-contrast markers (Yang et al., 2023).

*Agrobacterium*-mediated transformation has become the gold standard for plant genetic engineering. While it is relatively easy to use and has low operational costs, it is by no means without its flaws. Initial optimization is necessary to accommodate various plant species, explants, and *Agrobacterium* strains, to achieve optimal transformation efficiency. Although there is a growing impetus to use *in planta* transformation approaches, the majority of earlier studies in *Capsicum* spp. focused on transient overexpression (Bagga et al., 2019; Hamdani et al., 2024) with a few exceptions (Arthikala et al., 2014; Shivakumara et al., 2017), targeting stable transgene expression in offspring (T1 or T2). Furthermore, the large-scale implementation of such techniques is limited by the lack of robust markers under non-sterile conditions, variations in methods across species, cross-species differences in transformation success rates, and the absence of credible information on transgene stability in field settings (Ma et al., 2025). Protoplast-based transformation is another approach for delivering genetic material in *Capsicum* spp. transformations. The biggest obstacle to applying this technique to *Capsicum* spp. is the isolation and regeneration of protoplasts. While protoplast isolation is relatively straightforward, the same cannot be said for protoplast regeneration. Although considerable success has been achieved in protoplast transfection regeneration of whole plants from protoplast culture in *Capsicum* spp. has been reported in only a few studies (Díaz et al., 1988; Prakash et al., 1997), albeit in untransformed protoplasts. Systematic research for *Capsicum* spp. is necessary for successful transformed protoplast regeneration. Viral infection has also been developed as a genetic engineering strategy for plants (Wu et al., 2024). While virus-based genetic transformation is promising, currently it is still eclipsed by *Agrobacterium*-based methods. Virus-based constructs have significant shortcomings despite their speed and flexibility. Potential environmental hazards, insert size limitations, and host-range limitations constrain the usefulness of viral vectors (Ma et al., 2025). The involvement of a virus, or at least viral genetic material, makes the method harder for a wide range of laboratories to adopt. Not all

laboratories that can work with non-virus-related genetic engineering technology can easily adopt a virus-based transformation method, often due to regulatory requirements. Virus-based delivery systems can infect other organisms, including humans, in addition to their intended target, raising biosafety concerns (Sami et al., 2024).

Nanoparticles are emerging as advanced materials that can enhance the *Capsicum* spp. regeneration and transformation capabilities. These materials can be applied to multiple species, offering cross-genotype application. With the evolution of plant-based green nanomaterials, environmental safety concerns can be addressed. However, the high cost of manufacture, non-targeted gene transfer, varying tissue dissemination, long-term ecological effects, and unpredictable biological interactions of nanomaterials remain concerns for their broader applicability, as the technology is still in its infancy (Aydin, 2024; Ma et al., 2025).

Aside from the delivery method, genetic engineering techniques have advanced rapidly over the last decade. Genome editing is gaining the most attention, and various tools are being developed. Since its development in 2012, CRISPR-Cas9 has been the current mainstream tool for plant genome editing. It is a versatile genome-editing system with multiple applications, including knockouts, knock-ins, and base editing. Further discovery of other Cas proteins and improvements have enriched the tool's versatility. Cas12a and Cas13 are other Cas proteins that are as popular as Cas9 but used for different purposes. Compared to Cas9, those two Cas proteins in plants remain limited. Cas12a, which has a similar mode of action to Cas9, has been gaining more traction recently and has already been useful to introduce genome editing to tobacco, rice, maize, tomato, cotton, Arabidopsis, and orchid (Bernabé-Orts et al., 2019; Eini et al., 2022; Hui et al., 2024; Malzahn et al., 2019; Xia et al., 2022; Zheng et al., 2025). CRISPR/Cas12 (Cpf1) has been successfully tested for cleaving *CaMLO2* in CM334 and Dempsey protoplasts (Kim et al., 2020). Meanwhile, Cas13, which targets RNA, is still lagging behind the other two in plant applications, with most applications focused on improving virus resistance (Noureen et al., 2022).

The CRISPR components can be removed after gene editing to obtain transgene-free genome-edited mutants more easily by integrating a site-specific recombinase system (Nishizawa-Yokoi and Toki, 2021). Site-specific recombination has been used to

remove selection marker genes, using tools such as Cre/lox recombination (Wang et al., 2005; Zhang et al., 2003). Another site-specific recombination tool, like PiggyBac, has also been adapted for use in plants (Nishizawa-Yokoi and Toki, 2021, 2023). Site-specific recombinase technology has been used in animal genetic engineering for many years to generate mutants. However, the adoption of such technology in plant genetic engineering is relatively slow. Using these site-specific recombination tools would enable rapid development of transgene-free mutants. While some recombinase technologies may yield a less "clean," transgene-free mutant than the RNP method, they are faster than more traditional approaches and introduce only minimal modifications to commonly used workflows. Furthermore, co-transformation of CRISPR-Cas9 plasmids with overexpression vectors for STTM396a (Park et al., 2024) and co-expression of GRF-GIF chimeras (Swinnen et al., 2025) increased gene-edited plant regeneration and decreased the time required for shoot regeneration in tomato. Such innovative cross-genra insights can be applied to enhance *Capsicum* spp. genome editing (Bulle et al., 2025b).

Recently, additional RNA-guided nuclease systems for genome editing, such as Fanzor, CASTs (CRISPR-associated transposons), and TnpB, have been discovered and developed (Saito et al., 2023; Song and Bae, 2024). These three systems are closely related to Cas and have recently been used in plant species such as rice, Arabidopsis, soybean, mung bean, and adzuki bean (Cao et al., 2025; Li et al., 2024; Zhang R et al., 2024). These new tools are enriching our genome-editing toolbox and offer opportunities for more precise, robust genome editing in *Capsicum* spp.

## Future framework and Conclusions

The genetic engineering techniques offer a multidimensional framework for overcoming *Capsicum* spp. long-standing recalcitrance to regeneration and transformations. While VIGS and RNAi offer transient, robust, and genotype-independent functional characterization, conventional transgenic overexpression provides novel trait introgression and stable inheritance for cultivar development. The advent of CRISPR-Cas made it possible to perform accurate, heritable, and transgene-free genetic modifications. However, the effectiveness of CRISPR-Cas systems in *Capsicum* spp. is restricted by limited regenerations, genotypic reliance, and

poor transformation efficiency. STU CRISPR systems, endogenous promoter optimization, mobile sgRNAs, ribozyme-assisted constructs, RNP delivery, and VIGE are recent innovations that can collectively address these bottlenecks by decreasing reliance on tissue culture, increasing editing efficiency, and facilitating *Agrobacterium* / transformation-independent genome engineering. Additionally, the incorporation of transformant regeneration enhancer elements, such as GRF-GIF chimeras and STTM-mediated development coordinators, and small peptides (CeREF1) offers a promising strategy to enhance shoot induction and the recovery of gene-edited plants. Future research should therefore focus on merging efficient delivery systems, regeneration-enhancing morphogenic transcription factors, genotype-independent editing systems, and transgene-free approaches into an interconnected pipeline for scalable *Capsicum* spp. improvement. By overcoming the main biological and technological obstacles that have historically hindered *Capsicum* spp. biotechnology, such a framework can unleash the production of nutritionally enhanced, climate-resilient, and disease-resistant cultivars.

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### Contribution of Authors

Wahyuni Y & Zainuddin IM: Conceived the original idea and arranged software resources.

Shad MA, Supatmi S, Kusumanegara K, Lawrie MD, Azizah NFN, Wahyuni Y, Polosoro A & Mudzaffar SN: Wrote initial manuscript draft.

Shad MA, Zainuddin IM, Hatta ANNL, Riyanti EI, Listanto E, Fitriah N & Wahyuni Y: Edited, proofread and revised the manuscript.

Shad MA: Generated the figures and table.

Shad MA, Zainuddin IM & Wahyuni Y: Cross checked the references and made final corrections.

All authors read and approved the final draft of the manuscript.

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