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REVIEW ARTICLE

Gene cloning: Applications in vegetable crops

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Abstract

Gene cloning is a revolutionary technique in agricultural biotechnology, particularly in vegetable farming, enabling precise genetic modifications to enhance crop traits such as pest resistance, disease tolerance, and nutritional improvement. The process involves inserting DNA fragments into vectors, which replicate within bacterial hosts. This facilitates large-scale gene duplication. Unlike PCR, gene cloning allows for the replication of longer DNA sequences, making it essential for studying gene functions. The first step involves isolating a target gene, typically from a cDNA library, to overcome the challenges of large plant genomes and introns. The Ti plasmid from *Agrobacterium tumefaciens* is widely used in plant genetic engineering, though modified binary vector systems improve its efficiency. Essential enzymes like restriction endonucleases, DNA ligases and polymerases facilitate DNA manipulation. *Escherichia coli* is the preferred host for cloning due to its ease of cultivation and rapid growth, while yeast and plant cell cultures serve as alternatives for eukaryotic gene expression. Different transformation methods are being used to introduce recombinant DNA into host cells. Successful transformants are identified through antibiotic selection, blue-white screening, PCR and DNA sequencing. Gene cloning has significant applications in vegetable science, enabling the development of stress-resistant, nutritionally enhanced crops, thereby contributing to sustainable agriculture and food security.

Keywords: Gene cloning, Recombinant DNA, Vectors, Transformation, Genetic modification..

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Introduction

Gene cloning is an innovative approach in agricultural science that has the potential to revolutionize vegetable farming. It involves the insertion of DNA fragments into vectors to produce genetically modified products. The vector can then be inserted into bacteria, where it replicates along with the host genome. This technique efficiently bypasses the constraints of Polymerase Chain Reaction (PCR) and enables the duplication of lengthier DNA segments. Gene cloning refers to the process of isolating and replicating certain DNA sequences, which allows scientists to decipher the genetic instructions that control different traits. In this article, the process of gene cloning and its applications in various key areas of vegetable science, such as resistance to pests and diseases, nutritional enhancement, tolerance to abiotic stress and precision agriculture has been discussed. Our objective is to demonstrate the transformative potential of gene cloning in vegetable farming by emphasizing accomplishments and current research in the respective field.

Gene Cloning: An overview

The gene to be cloned is included in an extrachromosomal DNA called a vector to create genetic manipulation. The vector transports the gene into the cell, such as bacteria. Within the bacteria, the vector undergoes replication,

resulting in the production of several similar products of both the gene and bacterium. During cell division, the recombinant DNA molecule is replicated and passed on to the progeny. After undergoing multiple cell divisions, a group of cells that are genetically similar forms known as a clone. The clone's cells each contain one or more replicas of the recombinant molecule, suggesting the successful cloning of the gene (Brown, 2016) (Figure 1).

Selection of the target gene

In the beginning step, a specific fragment or target loci that encodes the desired protein has been isolated. When it comes to plants, identifying a gene from a genomic library is not ideal due to two main reasons: Firstly, the large size of plant genomes necessitates screening an enormous number of clones to find the desired gene. Secondly, eukaryotic genes contain introns that complicate the process of gene expression. Utilizing a cDNA library can effectively overcome these issues. The process of constructing a cDNA library commences by extracting total RNA from a particular cell type that synthesizes the desired protein. Next, the extraction of mRNAs from the total RNAs takes place, followed by the conversion of the mRNA molecules into cDNA strands by the enzyme reverse transcriptase. The cDNA library is screened for clones containing the desired gene using DNA hybridization or immunological detection methods. The desired gene can also be extracted by PCR amplification (Wong, 2018).

Selection of a cloning vector

Vectors are DNA molecules that can replicate within the host organism, where a gene can be inserted to create a recombinant DNA molecule. For a vector to succeed, it needs the ability to divide inside bacteria, generating multiple replicas of the inserted gene, should have a comparatively small size (facilitates their easy purification and manipulation), a selectable marker (typically an antibiotic-resistant gene) and must have compatibility with the host organism (Lodge et al., 2007). The primary vectors in rDNA technology are plasmids and bacteriophages, chosen for their high copy number and large carrying capacity (Verma et al., 2022). Plasmids regulate the number of copies per bacterium through their origin of replication. pBR322 is a plasmid with a moderate number of copies, ranging from 12 to 20 copies per bacterial chromosome. On the other hand, pUC18 is a plasmid with a large number of copies, reaching up to 100 copies per chromosome. The choice between these vectors depends on the specific application: for high expression levels, a high copy number plasmid like pUC18 is advantageous, while for minimizing host burden, a medium copy number plasmid similar to pBR322 may be preferred (Lodge et al., 2007).

The Ti plasmid is extensively used to transfer DNA into plant cells. This plasmid is extracted from *Agrobacterium tumefaciens* (bacteria), which attacks crop plants and causes the development of crown gall (tumor tissue). During infection, a 20 kb fragment called T-DNA, located inside the Ti plasmid, is transferred and incorporated into the chromosome of the plant with the assistance of the virulence gene (*Vir*). However, the tumor-inducing genes present in the plasmid cause uncontrolled cell division and the formation of tumors in the infected crop species. Also, the Ti plasmid is very large, ranging from 150 to 200 kilobases (kb) in size, making it extremely difficult to manipulate in a laboratory setting, as large plasmids are harder to isolate, modify, and introduce into host cells compared to smaller, more manageable vectors. To overcome these limitations, modified versions of the Ti plasmid are developed for use as cloning vectors, i.e., the Binary vector system, which consists of a vector that contains the target gene and selectable marker genes and a helper vector (which is a disarmed plasmid, containing virulence (*vir*) genes). By using these modified vectors, the drawbacks associated with the natural Ti plasmid can be removed. Another technique, the cointegration strategy in molecular biology, involves the

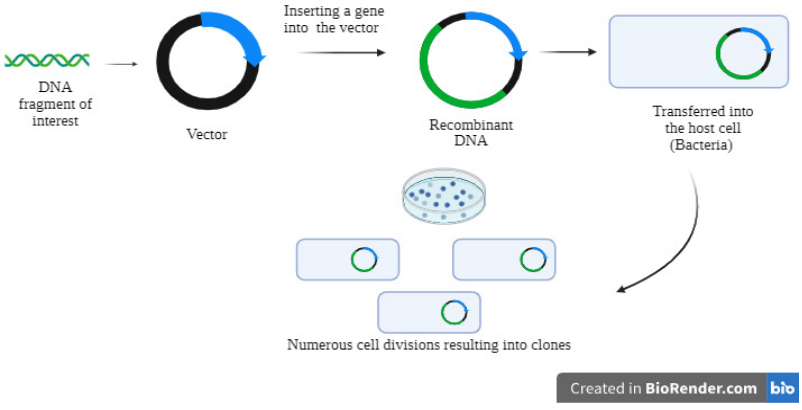

Figure 1: Schematic representation of the gene cloning process

creation of a novel chimeric plasmid that combines elements from both the Ti plasmid of *Agrobacterium tumefaciens* and a bacterial vector such as pBR322 (Table 1). Recent advances in vector design have further improved their utility in vegetable crops. Binary vectors such as pCAMBIA and pBIN19 have been optimized with stronger promoters, multiple cloning sites, and reporter genes for efficient selection in tomato, brinjal, pepper, and cucurbits (Hellens et al., 2000; Lee & Gelvin, 2008). Gateway and Golden Gate cloning systems now enable modular assembly of multiple genes, allowing stacking of resistance or nutritional traits (Engler et al., 2009). Synthetic biology approaches are being applied to design de novo vectors with minimal backbones, higher stability, and compatibility across multiple vegetable species, thus overcoming host-range limitations traditionally seen in Ti-based systems (Patron et al., 2015).

Enzymes required for cloning

Restriction enzymes break down internal phosphodiester bonds at specified sequences, referred to as restriction sites, within a DNA molecule. The insertion of a foreign gene into the vector is facilitated by using these restriction endonucleases. It is common for a restriction site to appear multiple times within a DNA molecule. Therefore, digestion by a restriction enzyme typically produces several DNA fragments with distinct ends (Sticky or blunt). Once the sequence of a DNA molecule is determined, creating a complete restriction map that shows all possible restriction sites for a set of common restriction enzymes is beneficial. This mapping can be easily performed using computer software (Wong, 2018). Type II enzymes cleave their substrates with great precision, a characteristic that is crucial for gene cloning (Brown et al., 2016). The widely used DNA ligase is derived from a bacteriophage known as T4, which infects bacteria. Restriction enzymes and DNA ligase can be combined to precisely cleave DNA at specified locations and connect different DNA segments. (Lodges et al., 2007). DNA polymerases are a class of enzymes frequently employed in gene cloning. Among them, the DNA-dependent DNA polymerases include *E. coli* T4 & T7 DNA polymerase, DNA polymerase I, and Taq polymerase; The RNA-dependent polymerase, i.e., reverse transcriptase. Dephosphorylation is a frequently employed technique in cloning to prevent the vector DNA from undergoing self-ligation, or recircularization, during ligation procedures. Alkaline phosphatase, derived from either *E. coli* or calf gut, catalyzes the removal of phosphate residues from the 5′ terminus. The enzyme is utilized to decrease the level of interference in ligation assays, in which a DNA fragment is joined to a plasmid vector (Wong, 2018).

Selection of a host organism

Escherichia coli serves as the preferred host for cloning purposes due to its multitude of advantages. Firstly, its ease of cultivation and manipulation with basic laboratory equipment simplifies experimental procedures. Secondly, a wide array of vectors and host strains has been tailored to maximize gene expression efficiency. Thirdly, extensive knowledge regarding the genetics and physiology of *E. coli* enhances experimental design and troubleshooting capabilities. Moreover, the rapid attainment of protein expression from eukaryotic cDNA clones, followed by efficient purification yielding milligram quantities within a fortnight, underscores its efficiency. Additionally, well-established fermentation techniques further streamline production processes. Furthermore, the potential for generating abundant quantities of recombinant proteins economically renders *E. coli* the best option for biotechnological innovations (Fakruddin et al., 2013). The most commonly used *E. coli* strains for molecular cloning are: BL21(DE3), DB3.1, JM110, Stbl2, etc. (Bertero et al., 2017). *E. coli*, a prokaryotic organism, lacks the cellular machinery required for conducting posttranslational modifications, a process characteristic of eukaryotic cells (Fakruddin et al., 2013). Yeast cells are frequently used as host organisms for studying eukaryotic gene expression. Plant cell cultures, including hairy root cultures and suspension cultures, are used as host systems for producing plant recombinant proteins. Other eukaryotic systems, such as nematodes (e.g., *Caenorhabditis elegans*) and protozoa (e.g., *Trypanosoma brucei*), are also used as host organisms in specific research areas.

Table 1: Most commonly used vectors

Vector	*Insert size*	*Purpose*	*Examples*
Plasmid	10–20 kb	Protein expression, DNA manipulation	pBR322, pUC18, pCMV
Cosmid	~45 kb	Genomic libraries	pHM, pLAFR1, pJB8
Bacterial Artificial Chromosome (BAC)	130–150 kb	Genomic libraries	pBeloBAC11, pBACe3.6
Yeast Artificial Chromosome (YAC)	1000–2000 kb	Genomic libraries	pYAC4
Phage (λ, insertion)	~10 kb	cDNA libraries	λgt11
Phage (λ, replacement)	~23 kb	Genomic libraries	EMBL4

Transformation

Transformation refers to the procedure of introducing DNA into live cells. Following the incorporation of exogenous DNA into a vector, the subsequent procedure involves inserting the resulting construct into a compatible host cell. There are various methods of transformation depending on the cloning objectives and the type of host system involved. Some of the methods of transformation are described. Bacterial cells can readily incorporate exogenous DNA when they have been pre-treated with $CaCl_2$ or a mixture of other salts. Cells that have been treated are called competent cells because they can effectively absorb DNA. Electroporation involves subjecting cells to a short electrical pulse, causing localized and transient damage and disintegration of the cell membrane. This enables DNA molecules to pass through the membrane and diffuse into the cells. The vector DNA, whether it contains foreign DNA or not, can subsequently be taken up by the cells. Transformation by *Agrobacterium* is widely used in plants either by the explant inoculation technique, the protoplast co-cultivation technique, or the seedling inoculation technique. Recent advances have refined transformation approaches specifically for vegetable crops. Agrobacterium-mediated transformation remains the most widely used method for tomato, brinjal, pepper, and cucurbits due to its higher efficiency and lower copy-number insertions compared to biolistics (Gelvin, 2020). However, biolistic methods are still preferred in recalcitrant species like onion and garlic, where Agrobacterium infection efficiency is low (Eady et al., 2000). Emerging techniques such as nanotechnology-mediated gene delivery, CRISPR-based ribonucleoprotein (RNP) delivery, and protoplast electroporation are being tested for precision transformation in vegetables (Demirer et al., 2019). These novel systems hold promise to overcome species-specific barriers and improve stable transformation rates in diverse vegetable crops.

Identification of transformants

Transformants are the cells that have successfully incorporated the foreign DNA. Identifying them involves usage of various screening methods like antibiotic selection, blue-white-screening, reporter genes, colony PCR, positive selection vector, diagnostic restriction digest, and DNA sequencing. Each method provides a different level of confirmation, from initial selection to precise verification of the gene of interest, ensuring that the desired genetic modification has been achieved.

Techniques used in cloning

The choice of technique depends on the specific goals of the cloning project, such as gene function study, protein production, or genetic modification. Several of these are elucidated below.

DNA isolation

The most common approach employed in cloning is the isolation, followed by purification of plasmid DNA from E. coli cells that have been transformed. The miniprep process causes alkaline lysis of the cultured cells and, can be carried out using just 1 ml of an overnight culture. The NaOH/SDS alkaline solution dissociates the cell wall, causing the release of the cellular contents into a solution. Subsequently, the process involves neutralization using potassium acetate, followed by DNA precipitation using 95% ethanol.

Gel electrophoresis

A technique that separates proteins, DNA, RNA, or other molecules by exploiting their differences in size and charge is used extensively. This is achieved by subjecting them to an electric field within a gel matrix. Various molecules exhibit differential migration through the gel, with smaller molecules displaying more mobility compared to bigger ones. Polyacrylamide Gel Electrophoresis (PAGE) is a type of gel electrophoresis specifically used for separating proteins or nucleic acids based on their size and charge using a polyacrylamide gel matrix. It provides high resolution and can distinguish small differences in molecular size, often used for protein analysis under denaturing (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)) or native conditions.

Southern Transfer

Technique used to transfer resolved DNA bands from an agarose gel onto a membrane, often made of nitrocellulose. Following this, hybridization of the probe with the target sequence is known as Southern transfer. (Wong, 2018).

Colony PCR

A technique for examining bacterial colonies to identify the target DNA sequence. It involves picking bacterial colonies directly from a plate and using them as a template in a PCR reaction to amplify the target DNA region, allowing for the identification of clones that contain the desired insert.

Advanced Enzyme-Dependent & Modular Cloning Systems

Golden Gate and Gibson Assembly (fragments with overlapping ends are seamlessly joined through the coordinated action of T5 exonuclease, DNA polymerase, and DNA ligase), 3A BioBrick Assembly (A high-throughput, antibiotic-based assembly of BioBrick parts without gel purification). (Sun et al., 2023).

Recombination-based and ligase-independent techniques

They depend on T4 DNA polymerase catalysis, forming long single-stranded overhangs that allow vector and insert to anneal in the absence of DNA ligase. It streamlines cloning and is helpful for high-throughput applications. It includes SLiCE (Seamless Ligation Cloning Extract) (employs E. coli cell extracts with recombination machinery to covalently

join DNA fragments with short homologous overlaps), TEDA (T5 exonuclease-based assembly uses T5 exonuclease to generate single-stranded overhangs for efficient assembly) (Wang et al., 2021; Zhang et al., 2012; Sun et al., 2023). Additional techniques employed in cloning experiments include DNA sequencing, PCR, site-directed mutagenesis, immunological techniques, and non-radioactive detection methods (Lodges et al., 2007).

Applications of Gene Cloning in Vegetables

Gene cloning is not a direct process but a crucial intermediary in various advanced technologies such as RNA Interference (RNAi), CRISPR-Cas, rDNA technology, gene therapy, synthetic biology, and other molecular diagnostic systems. Gene cloning has numerous applications in vegetable cultivation, enhancing both quality and yield.

Gene cloning for disease resistance

Cloning is essential for resistance breeding because it accurately identifies, isolates, and transfers resistance genes into susceptible crop varieties. Cloning techniques enable the isolation of specific resistance genes from plant varieties that exhibit innate resistance to particular pathogens. They can be achieved through transposon tagging, map-based cloning, etc. Map-based cloning is a method to determine the genetic cause of a mutant trait by searching for genetic markers that are known to be located at specific positions in the genome (Jander et al., 2002). The first step here is to pinpoint the markers close to the target trait to narrow down the region of the genome where the gene is located. Candidate genes are located by narrowing them down using genetic and physical mapping. The candidate genes are then isolated and cloned, and this process is then validated. Cloning can be performed by identifying and mapping genes that confer disease resistance. Multiple replicas of the resistance gene fragment are required in order to transfer the genes to different crop varieties that need protection against similar infections, after identifying the disease resistance gene in the parent variety. Scientists strive to conserve multiple copies of genes for practical use or intentionally disseminate copies of disease-resistant genes to species and genera that are impacted by an identical class of pathogens (Singh et al., 2016). Map-based cloning was conducted to recognize the candidate gene responsible for Fusarium yellows resistance in *Brassica oleracea* (Shimizu et al., 2014). They fine-mapped the *FocBo1* locus using F_2 plants derived by crossing cabbage (resistant) and broccoli (susceptible) lines. An examination of BAC and cosmid sequences associated with the *FocBo1* locus revealed the presence of a gene, known as *Bra012688*, which has been identified as a potential candidate gene in control of providing yellows disease resistance in Chinese cabbage. Transposon tagging is a technique used to identify and isolate genes by utilizing transposable elements that insert themselves into a genome, causing mutations. Through the process of mapping the sites of transposon insertion and establishing a correlation between these spots and observable changes in phenotype, it becomes possible to precisely identify and then study individual genes (Jones and Dangl, 2006). This technique was used for cloning and characterization of the gene responsible for resistance to tomato mosaic virus in *Solanum lycopersicum*. Durable *Tm-22* resistance effectively controls infections caused by tomato mosaic virus in *Solanum lycopersicum* plants (Lanfermeijer et al., 2003). Sharma et al. (2023) employed TA cloning for generating RNAi plants resistant to *Begomovirus* infecting okra using *Agrobacterium*-mediated genetic transformation. TA cloning is a simple and efficient molecular cloning technique that takes advantage of the properties of Taq polymerase, that adjoins single adenosine (A) nucleotide to 3' ends of PCR products. This characteristic allows these PCR products to be directly ligated into cloning vectors with complementary 3' thymidine (T) overhangs (Clark et al., 2019). Molecular cloning is widely used to characterize genes responsible for resistance in various vegetables. The NBS-LLR gene – *SacMi* found in a wild species of brinjal, *Solanum aculeatissimum,* was characterized by its resistance to *Meloidogyne incognita* (Zhou et al., 2018). The entire cDNA sequence of the *SacMi* gene was acquired using the rapid amplification of cDNA ends (RACE) technique and amplified. The amplified cDNA fragments were then cloned into suitable vectors, which were further sequenced. The full-length cDNA clone, named *BcTuR3*, was isolated from Chinese cabbage plants (non-heading) in response to Turnip mosaic virus infection. When being infected with TuMV, the BcTuR3 transcript quickly accumulated in the infected leaves and stems.

A deeper integration of molecular markers such as SSRs, SNPs and CAPS has strengthened map-based cloning strategies by providing fine-scale genetic maps for vegetables. For instance, SNP-based linkage maps have enabled cloning of resistance QTLs against late blight in tomato and pepper (Zhang et al., 2013; Kang et al., 2021). Functional genomics approaches, including RNA-seq and GWAS, further accelerate candidate gene discovery, as seen in cucumber and brassicas for virus and Fusarium resistance (Win et al., 2011; Matsumoto et al., 2022). Omics platforms such as transcriptomics, metabolomics, and proteomics are increasingly combined with molecular markers to narrow candidate regions prior to cloning, ensuring more accurate identification of resistance genes. Marker-assisted cloning is now a standard bridge between QTL mapping and functional validation in vegetable crops (Collard & Mackill, 2008; Varshney et al., 2014). These observations may influence the development of advanced strategies to enhance disease resistance in crops, thereby shifting the evolutionary advantage from the pathogen to the crop (Jones et al., 2014).

Enhancing nutritional quality through gene cloning

RNAi technology is utilized in tomatoes to enhance shelf life. The control of the ripening process is a crucial focus in studying fleshy fruits and vegetables. Tomato plants are a great model for studying development and ripening as it has short lifespan, ease of transformation, and successful multiplication (Das et al., 2023). Polygalacturonase (PG) is an enzyme involved in the degradation of pectin, a polymer made up of galacturonic acids. Pectin is a crucial constituent of the cell wall, which offers essential structural support. In conventional gene functioning, the gene undergoes transcription to produce mRNA, which is subsequently translated to form the enzyme polygalacturonase. An antisense RNA molecule was introduced to attach to the mRNA and inhibit its translation, blocking the creation of the enzyme. This enhances firmness, facilitating efficient long-distance transportation (Sheehy, 1998). The processing of N-glycans influences ripening-associated fruit softness. The analysis of transgenic tomatoes showed that the β-Hex and α-Man RNAi lines had fruits that were approximately 2 and 2.5 times firmer, respectively. Additionally, these tomatoes had an extended shelf life of approximately 30 days (Meli et al., 2010). Schijlen et al. (2007) carried out the production of seedless tomatoes, which was achieved by suppressing the flavonoid biosynthesis pathway by RNAi targeting chalcone synthase (initial gene) in the pathway. Efforts were made to enhance the nutritional content of tomato fruits by inhibiting the activity of a gene involved in photomorphogenesis regulation, known as DET1. This was achieved by utilizing certain fruit-specific promoters. RNAi was employed to suppress the expression of the gene bch, beta-carotene hydroxylase, responsible for the conversion of beta-carotene into zeaxanthin in potatoes to enhance the levels of two carotenoids, such as beta-carotene and lutein (Van Eck et al., 2007). The utilization of CRISPR/Cas9 to silence the SlORRM4 gene resulted in a delay in the ripening of tomato (Yang et al., 2017). Polyphenol oxidases facilitate the oxidation of polyphenols, resulting in the discoloration of the eggplant berry flesh upon being cut. Nutritional gene cloning in vegetables has also been inspired by biofortification successes in cereals and fruits, such as Golden Rice in rice and folate-enriched banana (Paine et al., 2005; Paul et al., 2017). In vegetables, several case studies demonstrate translational success: provitamin A and folate-enriched tomatoes developed through metabolic engineering (Diaz de la Garza et al., 2004; Zhang et al., 2019) and iron- and zinc-enhanced spinach lines using targeted gene overexpression (Garg et al., 2018). These examples highlight how cloning enables not only extended shelf life and texture modification but also targeted nutritional enhancement, providing new avenues for combating micronutrient deficiencies.

Pest and herbicidal resistance

Cloning is used to introduce desired genes into the plant genome to obtain genetically modified organisms. This method effectively protects crops and meets the requirements of sustainable agriculture (Kumar and Srivastava, 2015). The sources of insect resistance genes include microorganisms, plants, and animals. The insect-resistance genes have been identified, replicated and inserted into several commercially significant crop plants (Srivastava et al., 2016). Microbial-derived insect resistance genes encompass vegetative insecticidal protein (*vip* gene(s)) and crystal protein (cry gene(s)) and (Kumar and Srivastava, 2015). *Bacillus thuringiensis* is a prevalent bacterium found in soil, which was initially discovered in Thuringia. *B. thuringiensis* (Bt) generates '*cry*' gene that encodes an insecticidal crystal protein. This protein can paralyze the larvae of some destructive insects. The ingestion of *vip* proteins leads to the enlargement and disruption of the cells lining the midgut of the target insect through the process of osmotic lysis. A major case study illustrating real-world application is Bt brinjal, where the cloned *Cry1Ac* gene was introduced to combat fruit and shoot borer. Bt brinjal has been commercialized in Bangladesh since 2013 and has resulted in over 80% reduction in insecticide use, significantly improving farmer income and reducing pesticide exposure (Shelton et al., 2018). Similar insect-resistance trials in tomato and cabbage using *Cry* and *Vip* genes are ongoing, showing the translational success of cloning-based strategies in vegetables (Poysa et al., 2020; Kumar et al., 2022). Approximately 50 *VIP*s have been identified. Among which, the categories of *vip1* and *vip2* are effective against coleopteran. insects, and *vip3Aa1,* and *vip3Bb1*, which are effective against lepidopteran insects. Plant-derived insect resistance genes encompass the *protease inhibitor* (*PI*) gene and the *lectin gene*. Protease inhibitors function as antimetabolic proteins that disrupt the digestion process of insects. They suppress the function of the digestive enzyme in the gastrointestinal tract. Insects are affected by a decrease in digestible proteins and an increase in digestive enzyme production, resulting in a loss of sulfur amino acids. This leads to weakened insects, slowed growth, and eventual death (Srivastava et al., 2016). Numerous studies have focused on developing tomato resistance against lepidopteran and coleopteran insect pests. Insect resistance genes from microbial sources include *cry IAb* (Delannay et al., 1989), *cry1Ac* (Mandaokar et al., 2000), *cry6A* (Li et al., 2008), and *cry IAa* (Sharma and Srivastava, 2013). Additionally, insect resistance genes from plant sources encompass *Tomato Proteinase Inhibitor-II* (McGarvey et al., 1994), *Tobacco Anionic Peroxidase* (Dowd and Lagrimin, 1997), and *CpTI* (Cowpea Trypsin Inhibitor) (Gatehouse, 1995), among others. The *cryIA (a)* gene was cloned to bring resistance against the pest *Plutella xylostella* in brinjal (Kumar and Srivastava, 2015).

Tolerance to herbicides is the predominant trait in transgenic crops commercially. Transgenesis for herbicide selectivity entails the identification, isolation and modification of a herbicide-tolerant gene from a plant or microbe to enable effective expression in plants. To develop herbicide tolerance in crop plants, various strategies are employed, including the development of tolerance to photosystem II (PSII) inhibitors, resistance to glufosinate, glyphosate, acetolactate synthase (ALS) inhibitors, mitotic disruptor herbicides and plant pigment biosynthesis inhibitors (Mulwa and Mwanza, 2006).

Gene cloning for stress tolerance

The development and crop production are affected by many stresses, such as limited water availability, high temperatures, severe flooding, salinity and freezing temperatures. These stresses typically induce a cascade of alterations in crop plants, leading to damage to cellular machinery (Rai et al., 2011). The *mtID* gene, which codes for a bacterial mannitol-1-phosphate dehydrogenase, was inserted into tomato plants using the promoter, CaMV 35S. This genetic modification resulted in enhanced tolerance to abiotic stress in the transformed tomato plants (Khare et al., 2010). A salt-induced gene, *MdCIPK6L*, was isolated from apple and transferred into tomato plants. The overexpression of *MdCIPK6L* was observed to confer tolerance to drought, salt, and chilling stresses in the transgenic tomatoes (Wang et al., 2012). Cheng et al. (2013) introduced the *choline oxidase* gene (*CodA*) from *Arthrobacter globiformis*, which is responsible for producing glycine betaine, into the potato variety 'Superior' using the SWAP2 promoter to enhance drought tolerance. Fan et al. (2012) genetically modified sweet potato cv. Sushu-2 by introducing a gene from spinach, the *chloroplastic betaine aldehyde dehydrogenase* (*SoBADH*) gene, responsible for the production of glycine betaine. Transgenic sweet potato plants that had the *SoBADH* gene overexpressed demonstrated enhanced resistance to salinity, low temperature, and oxidative stress. This was achieved by safeguarding cell integrity, promoting robust photosynthetic activity, reducing the production of harmful ROS, and activating mechanisms to eliminate ROS. To enhance the ability of bottle gourd to tolerate high levels of salt, Han et al., (2015) introduced the *AVP* gene obtained from Arabidopsis thaliana's H+-pyrophosphatase into a bottle gourd line called 'G5'. The transgenic lines expressing *AVP1* demonstrated enhanced salt tolerance and retained higher relative water content when subjected to salt stress in a glasshouse environment. Integration of next-generation sequencing (NGS) and high-throughput screening platforms has greatly accelerated the discovery of stress-related genes in vegetables, particularly for drought, salinity and temperature tolerance (D'Agostino & Tripodi; Razzaque et al., 2019). Multi-gene cloning and gene stacking approaches are now being applied to pyramid tolerance traits, enabling simultaneous resistance to multiple abiotic stresses in tomato, sweet potato and bottle gourd (Qin et al., 2020; Liu et al., 2021). Comparative analyses also highlight key differences with cereals and fruits: cereals often focus on yield-related QTLs, while fruits face challenges of long generation times and woody growth habits. Vegetables, with shorter cycles, ease of transformation and greater consumer-driven nutritional demands, represent a uniquely suitable system for rapid cloning-based improvement (Varshney et al., 2021).

Cloning Strategies to Address Regeneration Bottlenecks in Recalcitrant Vegetable Crops

The increase in sequenced plant genomes, combined with the information provided by functional genomics and improvements in gene cloning and tissue culture methods, is fuelling fast advances in crop improvement and trait development. Despite the significant advances, some crop species show poor transformable ability by remaining recalcitrant to tissue culture and regeneration protocols (Anjanappa and Gruissem, 2021). *Capsicum annum*. L has a recalcitrant nature in in vitro manipulation, showing poor regeneration efficiencies. Naeem et al. (2025) optimized the regeneration protocol for the variety Zunla-1 with cotyledon and hypocotyl explants subjected to CaREF1 peptide in conjunction with certain growth regulators for increased induction of callus, shoot and root regeneration and elongation. Addition of CaREF1 increased overall regeneration efficiency by almost a factor of two (27.2–55.0%) while enhancing cellular organization, pointing towards its ability for regeneration recalcitrance. Negara et al. (2024) developed an optimal tape-sandwich protoplast isolation technique generating high protoplast yields (~1.3 $\times$ 10^6 cells/mL for 0.1 g leaf tissue). Through PEG-mediated protoplast transfection, close to 49% efficiency for plasmid DNA delivery as well as ~2.9% efficiency for Cas9 protein incorporation were achieved with a significant improvement over the conventional method. Clonal multiplication of effectively transformed lines was made possible in the study by Eady et al. (2000) by directly inducing multiple shoots from transgenic tissues. In addition to ensuring genetic homogeneity and reducing the loss of desirable events during successive regeneration, this raised the likelihood of producing viable transgenic plants even in the face of low initial transformation efficiency. Thus, obtaining a significant number of true-to-type transformants for subsequent breeding and functional studies—one of the main obstacles in onion genetic improvement—was practically addressed by the use of cloning techniques. Kumar et al. (2021) established a tissue culture- and selection-independent Agrobacterium-mediated transformation system for cowpea (*Vigna unguiculata*), a seed legume that is recalcitrant for genetic alteration. Using 1-day-old germinated seedlings infected by wounding, sonication, and vacuum infiltration,

up to 90% transient GUS expression was attained along with production of stable transformants (1.9%), which were stably inherited in a Mendelian manner in subsequent generations. Such a method is noteworthy inasmuch as it circumvents cumbersome and inefficient tissue culture, providing an efficient practical option for genetic improvement in cowpea. Crucially, cloning and clonal multiplication are still imperative for verifying stable inheritance, generating uniform transgenic lines and allowing downstream trait construction and functional genomics, thus vindicating cloning as a pillar for crop transformation approaches.

Regulatory, Ethical, and Intellectual Property Considerations

Beyond scientific advances, the application of gene cloning in vegetable crops is tightly linked with regulatory frameworks, ethical debates and consumer acceptance. Regulatory oversight differs widely across countries; for instance, the United States often applies product-based evaluation, while the European Union follows a strict process-based approach, resulting in longer approval timelines for genetically modified (GM) vegetables (Eriksson et al., 2018). Ethical concerns primarily involve food safety, environmental biosafety and potential impacts on biodiversity. Consumer acceptance also remains a barrier: while Bt brinjal adoption in Bangladesh has shown positive outcomes, resistance persists in other regions due to public perceptions and labeling issues (Shelton et al., 2018). Intellectual property (IP) rights pose another major challenge. Key cloning technologies, including CRISPR/Cas systems and proprietary vectors, are often protected by patents, limiting access for public sector breeders and researchers in developing countries (Koltay et al., 2020). These IP restrictions can slow down the development of open-access, farmer-friendly, improved varieties. Balancing innovation incentives with equitable access is thus crucial for maximizing the global benefits of gene cloning. Therefore, future research and policy dialogues must not only prioritize technical innovations but also integrate regulatory harmonization, ethical safeguards, consumer engagement and IPR reforms. Addressing these aspects will ensure that gene-cloned vegetables are not only scientifically viable but also socially acceptable and globally accessible.

Limitations and Challenges in Gene Cloning of Vegetable Crops

While gene cloning has enabled significant progress in vegetable improvement, several technical, biological, and ecological challenges remain. One major bottleneck is low transformation efficiency in recalcitrant vegetable species, such as onion, garlic and certain legumes, which hinders reproducibility and large-scale application (Altpeter, Springer & Bartley, 2016). Additionally, the genetic background of diverse cultivars strongly influences transgene expression, meaning that a gene conferring resistance or quality traits in one genetic background may not perform consistently in another (Tester & Langridge, 2010). From an environmental perspective, gene flow from transgenic vegetables to wild relatives or non-GM cultivars raises biosafety concerns, especially in centers of crop diversity (Ellstrand, Meirmans & Rieseberg, 2013). There are also risks of unintended effects, such as off-target genetic changes, metabolic trade-offs, or pleiotropic effects that could compromise yield or quality. Furthermore, scaling lab-based successes to field environments often exposes gene–environment interactions that limit the stability of trait expression. Beyond biology, public acceptance, ecological sustainability, and regulatory hurdles continue to be challenges. Taken together, these limitations highlight the need for continued research in refining transformation methods, developing genotype-independent protocols, improving biosafety assessments and engaging in transparent communication with stakeholders.

Conclusion and Future prospects

Advances in gene cloning have significantly improved our understanding of resistance to pests, herbicides, abiotic stresses and fruit ripening pathways, laying the foundation for targeted vegetable improvement. The scope of future research is moving beyond stress resilience toward traits that directly impact farmers, consumers and markets. One major area is post-harvest shelf life, where cloned genes in the ethylene biosynthesis and signaling pathways are being manipulated; delayed-ripening tomato lines generated through antisense suppression of polygalacturonase or CRISPR-based editing are showing promise in reducing post-harvest losses. Another focus is the enhancement of flavor and aroma compounds, with metabolic engineering approaches being applied to fine-tune volatile biosynthesis in tomato and pepper to improve consumer acceptance. Nutritional biofortification is also a key priority, as illustrated by biofortified tomato varieties enriched with provitamin A and folate, as well as iron- and zinc-enhanced spinach and lettuce currently under evaluation. Successful field trials highlight the translational value of gene cloning, i.e., Bt brinjal carrying the *Cry1Ac* gene has already been commercialized in Bangladesh and is under testing in other regions to reduce pesticide dependence against fruit and shoot borer, while cloned resistance genes in pepper are being tested against viral pathogens and cucumber and squash programs are targeting virus tolerance through molecular approaches. By integrating molecular cloning with genome editing and translational research, the next generation of vegetable crops will combine high yield, stress tolerance, extended storage life, superior nutrition and improved sensory qualities, thereby reducing production costs for farmers, improving consumer health and satisfaction and lowering environmental impacts, ultimately paving the way toward a more resilient and sustainable food system.

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सारांश

जीन क्लोनिंग कृषि जैव प्रौद्योगिकी में एक क्रांतिकारी तकनीक है, विशेष रूप से सब्जी खेती में, जो फसलों के गुणों को सुधारने के लिए जैसे की कीट प्रतिरोध, रोग सहनशीलता और पोषण में सुधार, जीन में सटीक आनुवंशिक संशोधन करने की क्षमता प्रदान करती है। इस प्रक्रिया में डीएनए के टुकड़ों को वेक्टर में डालना होता है, जो बैक्टीरियल मेज़बानों के भीतर उत्पन्न होते हैं। इससे जीन की बड़े पैमाने पर प्रतिकृति संभव होती है। पीसीआर से अलग, जीन क्लोनिंग लंबी डीएनए अनुक्रमों की पुनरावृत्ति की अनुमति देती है, जो जीन कार्यों का अध्ययन करने के लिए आवश्यक होती है। पहला कदम लक्षित जीन को अलग करना होता है, जो सामान्यतः cDNA पुस्तकालय से लिया जाता है ताकि पौधों की बड़ी जीनोम और इंट्रॉन्स की चुनौतियों से निपटा जा सके। Agrobacterium tumefaciens का Ti प्लास्मिड पौधों के आनुवंशिक अभियांत्रिकी में व्यापक रूप से उपयोग किया जाता है, हालांकि संशोधित बाइनरी वेक्टर प्रणालियाँ इसकी क्षमता को सुधारती हैं। आवश्यक एंजाइम जैसे रेस्ट्रिक्शन एंडोन्यूक्लियेज़, डीएनए लिगेज़ और पॉलीमरेज़ डीएनए हेरफेर को सक्षम बनाते हैं। Escherichia coli क्लोनिंग के लिए प्राथमिक मेज़बान है, क्योंकि इसे उगाना और तेजी से बढ़ना सरल होता है, जबकि यीस्ट और पौधों की कोशिका संस्कृतियाँ युकैरियोटिक जीन अभिव्यक्ति के लिए वैकल्पिक रूप से सेवा देती हैं। ट्रांसफॉर्मेशन विधियाँ जैसे $CaCl_2$ उपचार, इलेक्ट्रोकोरेशन, Agrobacterium-प्रेरित स्थानांतरण और बायोलिस्टिक डिलीवरी मेज़बान कोशिकाओं में पुनः संयोजित डीएनए को प्रस्तुत करती हैं। सफल ट्रांसफॉर्मेंट्स को एंटीबायोटिक चयन, ब्लू-व्हाइट स्क्रीनिंग, पीसीआर और डीएनए अनुक्रमण के माध्यम से पहचाना जाता है। जीन क्लोनिंग का सब्जी विज्ञान में महत्वपूर्ण अनुप्रयोग है, जो तनाव प्रतिरोधी, पोषण-संवर्धित फसलों के विकास को सक्षम बनाता है, और इस प्रकार सतत कृषि और खाद्य सुरक्षा में योगदान करता है।