

Transformative biotechnology methods: precision in genetically modified crops through trait modification

Abstract

The formidable challenges arising from global climate change, compounded by unfavourable abiotic and biotic factors, are imposing substantial restrictions on agricultural productivity. This poses heightened difficulties for crop scientists who are endeavouring to meet the escalating global demand for food. The primary goal of plant biology research is to fortify food security by elevating crop yield, reinforcing resilience against biotic and abiotic stress, and amplifying nutrient content. Despite the success of traditional breeding methods in generating enhanced crop varieties, including high-yielding hybrid grain crops, persistent challenges remain. Advanced technologies offer promising solutions to address these challenges, employing both direct and indirect approaches. Direct techniques, such as overexpression, RNA interference, and genome editing, coexist with the predominantly indirect approach adopted in breeding. The integration of cutting-edge tools and technologies has hastened the progress of these strategies, propelling the enhancement of crop species. Innovations like next-generation sequencing, high-throughput genotyping, precision editing, and the utilization of space technology for accelerated growth have introduced a new dimension to crop improvement programs. Their aim is to produce superior varieties capable of addressing contemporary challenges. Site-specific nucleases like TALENs and CRISPR/Cas systems, capable of high-efficiency genome engineering across diverse eukaryotic species, have revolutionized biological research and found applications in crop plants. These nucleases facilitate various site-specific genome modifications for agricultural biotechnology purposes. CRISPR/Cas genome-wide screens open avenues for discovering and expanding traits crucial for food security, accelerating trait development in key crops. This discussion delves into the development and utilization of different site-specific nuclease systems for diverse plant genome-engineering applications, emphasizing the opportunities presented by these technologies to precisely enhance traits, thereby boosting crop productivity and fortifying resilience against climate change. Cutting-edge genome-editing technologies, particularly CRISPR/Cas systems, are poised to transform the agricultural landscape and contribute significantly to ensuring future food security. While next-generation genetics and genomics have made remarkable strides in delivering improved varieties, there is untapped potential to extend these advancements to underutilized species. This chapter systematically analyses various genomics approaches employed in major species for trait discovery and improvement, providing a roadmap for implementing similar strategies in other crop species. By enabling targeted improvements in crops, these technologies offer a glimpse into a future where agriculture can adapt to changing environmental conditions and meet the growing global demand for food. Advances in crop trait modifications have been propelled by various technologies such as genetic engineering, gene editing, and synthetic biology. These innovations aim to enhance crop

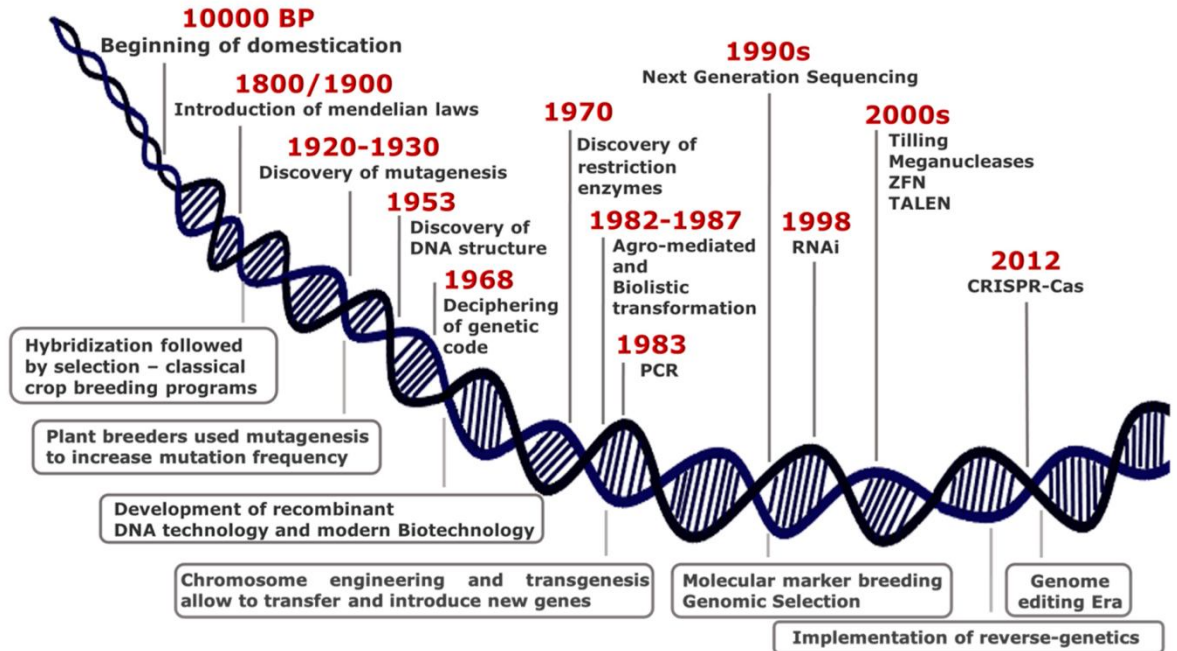
productivity, improve nutritional content, and confer resistance to pests, diseases, and environmental stresses.

Keywords: Molecular markers, Space technology, Next-generation Sequencing, Precision editing, Gene editing

Introduction

We face the critical challenge of producing sufficient food for a growing human population living in a changing and unstable climate. Substantial public research investments have been made to sequence, assemble, and characterize the genomes of major crop plants. This investment in plant science has enabled foundational discoveries of crop genes and their functions. This knowledge is poised to be leveraged for increased agricultural production by using synthetic biology, including tools for precise plant breeding knowledge is poised to be leveraged for increased agricultural production by using synthetic biology, including tools for precise plant breeding [8-11].

It all started with the identification of DNA as the storage polymer for genetic information [2]. The subsequent elucidation of the double-helical structure of DNA is generally regarded as the most important discovery in molecular biology. Subsequently, different types of RNA (mRNA, rRNA, tRNA) were identified as key players in gene expression, and relevant mechanistic details of the transcription and translation processes were unraveled. Eventually, by analyzing the translation of each nucleotide triplet to the corresponding amino acids, the universal genetic code was deciphered [12,13]. This resulted in the Central Dogma of Molecular Biology that is defined as 'the directional flow of detailed, residue-by-residue, sequence information from one polymer molecule to another'. The DNA polymerase, the RNA polymerase (RNAP), and the ribosome were identified as key players in replication, transcription, and translation, respectively.



(Valentina Bigini *et al.*, 2021)

Fig 1. **Historical Progression of Biotechnology**

Genetic information, pioneering genetic studies were performed on bacteria and/or bacterial viruses (bacteriophages). This has resulted in unraveling many basic genetic principles, including gene expression and control thereof (e.g., the lac operon of *Escherichia coli*). But also in revealing a wide range of bacterial defense systems as well as phage attack strategies. Altogether, this fundamental research led to the discovery of enzymes with the potential for genetic engineering, such as specific DNA endonucleases [14-17]. Combining specific type II restriction nucleases with DNA ligase allowed ‘cut-and-paste’ engineering of DNA fragments of a primate virus, simian virus 40 (SV40). Even more spectacular was an experiment in which a combination of enzymes (restriction enzyme, two exonucleases, a poly-A polymerase, a DNA polymerase, and a DNA ligase) allowed the transplantation of a DNA fragment from a bacteriophage into the SV40 genome. Next, an *Escherichia coli* plasmid was used as a vector for inserting a DNA fragment containing a penicillin resistance gene from another bacterium (*Staphylococcus aureus*); upon transformation [18-20]

Around the same time, major technical advances were made, including gel chromatography and DNA sequencing. By combining the new insights in biology/biochemistry with the spectacular technological progress, the stage was set for a new phase to take off: the development of molecular biotechnology with unprecedented applications! A few years later, another milestone in the molecular biology field was the groundbreaking discovery of the polymerase chain

reaction (PCR) by Kary Mullis (1983). The impact of PCR on the development of both fundamental and applied research has been truly overwhelming.

Plant transformation is now a core research tool in plant biology and a practical tool for transgenic plant development. There are many verified methods for stable introduction of novel genes into the nuclear genomes of diverse plant species [21-25]. As a result, gene transfer and regeneration of transgenic plants are no longer the factors limiting the development and application of practical transformation systems for many plant species.

Plant transformation encompasses two distinct consecutive steps:

- (1) DNA introduction into plant cells (sometimes known as transient transformation, in which transgenes have not yet integrated into the genome),
- (2) integration of the introduced DNA into the plant genome (stable transformation). Each step is useful in basic plant research and biotechnology, but the second step is necessary to produce transgenic plants with heritable traits of interest.

For most crops, transgenic plant production requires the ability to regenerate plants from transformed tissues. Although considered part of the transformation process, the regeneration step is often a greater bottleneck than is the stable integration of DNA sequences. Transformation methods Gene delivery systems involve the use of several techniques for transfer of isolated genetic materials into a viable host cell. At present, there are two classes of delivery systems

(a) Non-biological systems (which include chemical and physical methods)

Non-biological based transformation Particle bombardment/Biolistics Particle bombardment was first described as a method for the production of transgenic plants in 1987 (Sanford et al., 1987) as an alternative to protoplast transformation and especially for transformation of more recalcitrant cereals. Unique advantages of this methodology compared to alternative propulsion technologies are discussed elsewhere in terms of range of species and genotypes that have been engineered and the high transformation frequencies for major agronomic crops (McCabe and Christou, 1993).

(b) Biological systems.

Biological gene transfer Agrobacterium mediated transformation The natural ability of the soil bacteria, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenus*, to transform host plants has been exploited in the development of transgenic plants. In 1970s the prospect of using *A. tumefaciens* for the rational gene transfer of exogenous DNA into crops was revolutionary. Despite the development of other nonbiological methods of plant transformation (Shillito et al., 1985; Uchimiya et al., 1986; Sanford, 1988; Arenchibia et al., 1992, 1995), *Agrobacterium* mediated transformation remains popular and is among the most effective. This is especially true among most dicotyledonous plants, where *Agrobacterium* is naturally infectious.

Agrobacterium-mediated transformation consists of bacterial attachment, T-DNA and virulence (vir) effector protein transfer, cytoplasmic trafficking of T-DNA/protein complexes, nuclear entry, removal of proteins from the T-strand, T-DNA integration, and transgene expression. We have a basic understanding of the plant and bacterial virulence proteins that are important for these processes. For example, altered production of the plant proteins has increased host susceptibility to transformation (Gelvin, 2010).

In planta transformation methods

In planta transformation methods provide such an opportunity. Methods that involve delivery of transgenes in the form of naked DNA directly into the intact plants are called as in planta transformation methods. These methods exclude tissue culture steps, rely on simple protocols and required short time in order to obtain entire transformed individuals. In many cases in planta methods have targeted meristems or other tissues with the assumption that at fertilization, the egg cell accepts the donation of an entire genome from the sperm cell that will ultimately give rise to zygotes (Chee and Slighton, 1995)

Arabidopsis thaliana was the first plant that saw successful in planta transformation. Early stages of success in *Arabidopsis* transformation came from the work of Feldmann and Marks (1987). At present, there are very few species that can be routinely transformed in the absence of a tissue culture based regeneration system. *Arabidopsis* can be transformed by several in planta methods including vacuum infiltration (Clough and Bent, 1998), transformation of germinating seeds (Feldmann and Marks, 1987) and floral dipping (Clough and Bent, 1998). Other plants that were successfully subjected by vacuum infiltration include rapeseed, *Brassica campestris* and radish, *Raphanus sativus* (Ian and Hong, 2001; Desfeux et al., 2000). The labor intensive vacuum infiltration process was eliminated in favor of simple dipping of developing floral tissues (Clough and Bent, 1998). Also, the results indicate that the floral spray method of *Agrobacterium* can achieve high rates of in planta transformation comparable to the vacuum infiltration and floral dip methods (Chung et al., 2000).

Plant transformation methods (Birch et al., 1997)

Table 1. Precision in genetically modified crops through trait modification

Plant transformation	
Non-biological based transformation (Direct method)	Biological gene transfer (Indirect method)

A) DNA transfer in protoplasts 1) Chemically stimulated DNA uptake by protoplast 2) Electroporation 3) Lipofection 4) Microinjection 5) Sonication	1) Agrobacterium mediated transformation Primarily two methods a) Co-cultivation with the explants tissue b) In planta transformation 2) Transformation mediated by viral vector
B) DNA transfer in plant tissues 1) Particle bombardment / Biolistics 2) Silicon carbide fiber mediated gene transfer 3) 3) Laser microbeam (UV) induced genetransfer	

As originally performed, plant transformation results in random integration of new sequences into plant genomes. Remarkable advances over the past 15 years now provide more control over integration and permit precise, targeted modifications to DNA sequences in plant cells (genome editing) (Voytas and Gao, 2014; Baltes and Voytas, 2015). Genome editing uses customizable, sequence-specific nucleases (SSNs) that generate a DNA doublestrand break (DSB) at a specific genomic target. These sites allow targeted mutagenesis or specific editing depending on how the cell repairs the break.

The most common cellular mechanism of break repair in angiosperms is nonhomologous end joining (NHEJ). This pathway often results in small changes at the repaired site and can be used to perform targeted mutagenesis to alter gene expression or function (Puchta, 2005; Wang et al., 2014; Li et al., 2012). To achieve targeted mutagenesis, SSNs are either transiently delivered to protoplasts or stably incorporated into the genome as a transgene. In the latter case, during transgenic plant growth, at some frequency the SSN mutates the lineages later incorporated into reproductive cells, enabling mutations to be transmitted to progeny. In subsequent generations, the nuclease transgene can be segregated away, to obtain a nontransgenic plant with mutations in the target locus of interest.

Biolistic gene transfer can be applied to a wider range of genotypes than Agrobacterium-mediated gene transfer (Altpeter et al., 2005) but can be limited by the inability to regenerate plants after bombardment. Regeneration response and transgene performance following biolistic gene transfer depend on particle type, size, quantity and acceleration, DNA amount and structure during particle coating, tissue type, and pretreatment (Klein et al., 1988).

One notable study demonstrating precision in genetically modified (GM) crops through trait modification is the development of Bt cotton. *Bacillus thuringiensis* (Bt) is a naturally occurring bacterium that produces proteins toxic to certain insects. In the case of Bt cotton, scientists incorporated the Bt gene into the plant's genome, endowing it with the ability to produce these insecticidal proteins. This trait modification confers resistance to bollworms and other pests, reducing the need for chemical insecticides. This precision targeting of specific pests not only enhances crop yield but also minimizes the environmental impact associated with broad-

spectrum pesticide use. The adoption of Bt cotton has been successful in various countries, including India and China, where it has significantly contributed to increased cotton production while mitigating the environmental footprint.

The development of herbicide-tolerant crops, such as Roundup Ready soybeans. This genetically modified crop was engineered to withstand the application of the herbicide glyphosate, allowing farmers to effectively control weeds without harming their crops. By introducing a gene that confers tolerance to glyphosate, farmers can use this specific herbicide to target unwanted plants, ensuring precision in weed management. This approach has been widely adopted in several countries, demonstrating the economic and environmental benefits of reduced herbicide usage and increased crop productivity.

Precision in trait modification is also evident in the creation of drought-tolerant crops, as seen in the case of drought-resistant maize. With climate change posing challenges to agricultural productivity, scientists have focused on developing crops capable of withstanding water scarcity. Through genetic modification, specific genes associated with drought resistance are incorporated into maize plants. This precision in trait modification enables crops to thrive in arid conditions, ultimately ensuring food security in regions prone to water scarcity. The success of these genetically modified drought-tolerant crops has been observed in various field trials, highlighting the potential for such innovations to address the impacts of climate change on agriculture.

Ethical and Regulatory considerations :

Genome editing and transformation technologies, such as CRISPR-Cas9, have ushered in remarkable advancements in genetic research and biotechnology, but they also bring forth a host of ethical and regulatory considerations. Ethically, concerns arise regarding the potential misuse of these technologies, particularly in the context of human germline editing. The ability to modify the genetic code of embryos raises profound ethical questions about the unintended consequences and long-term effects on future generations. There is an ongoing debate about the ethical boundaries of genome editing, with calls for responsible and transparent practices to ensure the technology is used for beneficial purposes and does not lead to unintended genetic consequences or ethical violations.

From a regulatory standpoint, governing bodies worldwide face the challenge of keeping pace with the rapid advancements in genome editing. Developing and implementing robust regulations to oversee the ethical use of these technologies is crucial. Striking a balance between fostering scientific innovation and protecting against potential risks and misuse is a delicate task. Regulatory frameworks must address issues such as informed consent, data privacy, and equitable access to emerging genetic therapies. Furthermore, international collaboration is essential to establish consistent standards and guidelines that transcend borders and prevent the emergence of regulatory gaps that could be exploited.

Balancing scientific progress with ethical responsibilities and regulatory oversight is essential to ensure that these powerful tools are harnessed for the betterment of humanity while minimizing potential risks and societal concerns. Ongoing dialogue among scientists, policymakers, and the public is crucial to navigating the complex landscape of genome editing and transformation in a manner that aligns with ethical principles and societal values.

Challenges and controversies :

While genetically modified (GM) crops, specifically those developed through trait modification, offer numerous advantages, they are not without drawbacks. One significant concern is the potential for unintended ecological consequences. Introducing modified traits into crops may inadvertently affect non-target organisms or disrupt local ecosystems. For example, the cultivation of Bt crops, engineered to produce insecticidal proteins, could lead to the development of resistant insect populations over time. This resistance evolution may necessitate increased pesticide use, partially negating the initial environmental benefits of GM crops. Another drawback involves the issue of genetic diversity. The widespread adoption of a few genetically uniform crop varieties may reduce overall genetic diversity within a plant species. This lack of diversity could render crops more susceptible to new diseases or environmental changes, potentially compromising long-term food security.

the social and economic impacts of GM crops pose challenges. The high costs associated with developing and patenting genetically modified seeds may limit access for small-scale farmers in developing countries. This economic barrier raises questions of equity and could contribute to the consolidation of agricultural practices among larger, more financially robust farming operations. There is a risk that modified genes could unintentionally spread to wild or traditional crop varieties, raising questions about the potential impact on biodiversity and the cultural heritage of certain agricultural practices. The lack of consensus on acceptable thresholds for gene flow adds complexity to the regulatory landscape.

Biotechnology methods for genetically modified crops through trait modification

1: Precision Genome Editing Techniques

1.1 CRISPR/Cas9 Technology:

The advent of CRISPR/Cas9 technology has revolutionized crop breeding by enabling precise and efficient genome editing. This section delves into how this powerful tool has been employed to target specific genes responsible for traits such as disease resistance, drought tolerance, and improved nutritional content. Case studies of successful CRISPR-edited crops and their real-world applications illustrate the transformative potential of this technology.

Table 2. Genetically engineered crops either released or having the potential to be released.

Crop species	Gene	Technology	Trait improved	References
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Rice	Phytoenesynthase, phytoene desaturase, lycopene- β -cyclase	Overexpression	Goldenrice-provitaminA-rich rice	Ye et al. (2000)
Rice	Phytoenesynthase, phytoene desaturase, β -carotene ketolase, and β -carotene hydroxylase	Overexpression	aSTARice-astaxanthin-rich biofortified rice	Zhu et al. (2018)
Tomato	Self-pruning, ovate, fasciated, fruit weight 2.2, multiflora and lycopene- β -cyclase	Gene editing	Improved size, number and lycopene content of fruit	Zsögön et al. (2018)
Cotton	Crystalline endotoxin	Overexpression	Insect-resistant cotton	Umbeck (1992)
Tomato	Polygalacturonase	RNAi	Flavr Savr tomato reduction in polygalacturonase activity leading to delayed fruit ripening	Sheehy et al. (1988)
Maize	Waxy	Gene editing	High-amylopectin-content corn	Waltz (2016)

1.2 RNA Interference (RNAi):

RNA interference has emerged as a valuable tool in regulating gene expression at the post-transcriptional level. This section explores how RNAi techniques have been harnessed to enhance crop traits, including pest resistance and improved stress tolerance. The chapter also addresses the challenges and ethical considerations associated with deploying RNAi in agriculture.

2: Accelerating Crop Adaptation to Climate Change

2.1 Drought-Tolerant Varieties:

As climate change continues to pose challenges to global agriculture, the development of drought-tolerant crops has become imperative. This section examines the strategies and genetic modifications that contribute to enhanced water-use efficiency and resilience in crops, showcasing the potential to mitigate the impact of water scarcity on food production.

2.2 Heat and Cold Resistance:

The rising frequency of extreme temperature events necessitates the development of crops resilient to heat and cold stress. Here, we explore how genetic modifications can bolster the adaptability of crops, ensuring stable yields in the face of unpredictable climate patterns. Case studies highlight successful implementations of these advancements in various agricultural settings.

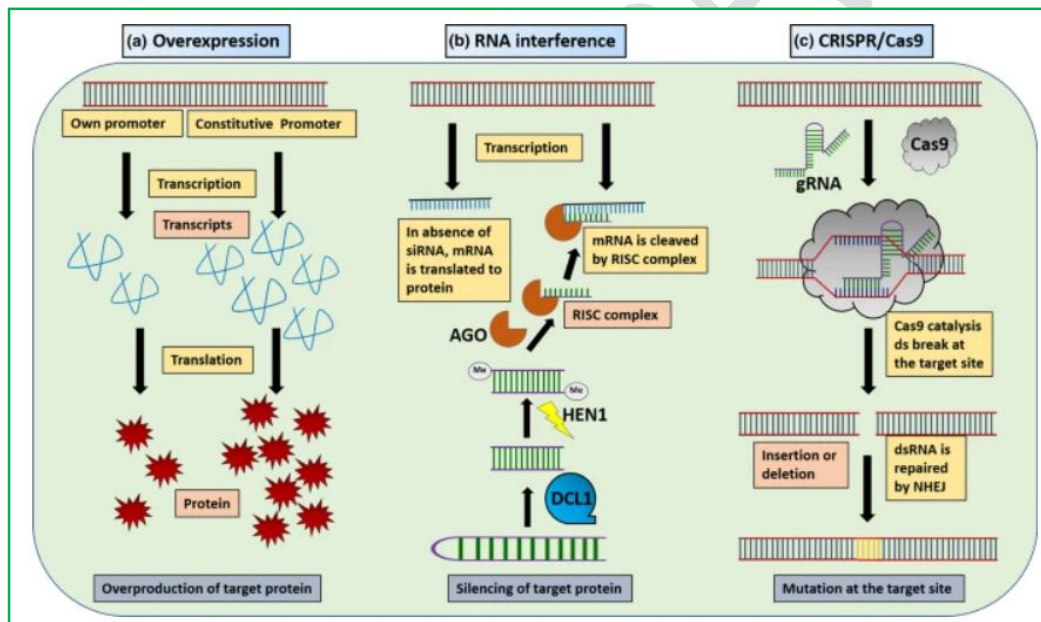
3: Improving Nutritional Content for Enhanced Human Health

3.1 Biofortification:

Crop trait modification extends beyond traditional agronomic traits to address nutritional deficiencies in human diets. This section focuses on biofortification techniques, emphasizing the enhancement of essential micronutrients such as iron, zinc, and vitamins in staple crops. The chapter discusses the potential impact of biofortification on addressing malnutrition and promoting global health.

3.2 Allergen Reduction and Hypoallergenic Crops:

Genetic modifications have also been employed to reduce allergenic components in crops, ensuring food safety for individuals with allergies. This section explores the progress made in developing hypoallergenic varieties of common crops and discusses the implications for public health and consumer acceptance.



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Fig 2. Strategies for crop trait modification through biotechnological approaches. **a** Overexpression leads to greater transcription of target gene which can be translated into protein; **b** RNA interference leads to downregulation of target gene; **c** Gene editing through CRISPR/Cas9 leads to insertions or deletions at target site which gives rise to mutations

Conclusion:

The chapter concludes by summarizing the transformative advances in crop trait modification and their profound implications for global agriculture. As we navigate the complex challenges of the 21st century, these innovations stand as beacons of hope, offering sustainable solutions to feed a growing population and mitigate the impact of climate change on our food supply.

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