A Brief History and Outlook on Plant Engineering

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Advances in plant mutagenesis and transgenesis will enable more sophisticated and precise engineering efforts in plants.

For decades, plant biologists have sought the means to manipulate plant genomes in a targeted and simple manner. Although some of the first experiments to develop these technologies were carried out more than a century ago *(1)*, we are in a new renaissance of scientific advancements and renewed interest in improving tools for plant engineering.

Plant synthetic biology is in its infancy, lagging behind its microbial counterparts primarily due to the technical challenges of working with plants, as well as a disconnect between synthetic biologists and the plant biology community. The plant synthetic biology technologies that exist today are the result of decades of research and scientific advances (Figure 1). This article outlines a brief history of modern agricultural advances to explain how these historical events have laid the foundation for the current renaissance in plant engineering.

The first foray into modifying genomes: Early plant mutagenesis

Modern breeding and agricultural biotechnology can be broadly summed up by two overarching processes: mutagenesis and transgenesis.

Some of the earliest agricultural goals were to devise technologies that would affect the genetics and heredity of plants via external treatments. In 1928, researchers demonstrated for the first time that X-rays could induce mutations in DNA and alter traits in tobacco and maize plants *(2, 3)*. Before that, plant breeding efforts were limited to traits and variations found in nature; the ability to induce mutations enabled biologists to produce variation at their will (Figure 2).

Mutagenesis was sold as a technology capable of creating new varieties and even new species that could be used to address any agricultural trait of interest. For the next half-decade, scientists used X-rays and neutron bombardment to induce mutations and screen for mutants of interest. Although the original hope was to find mutants that had agricultural benefits (*e.g.*, pathogen resistance, drought tolerance, etc.), few of these were found. Interesting new varieties were discovered that could be marketed for novelty rather than practical, agricultural uses. For example, researchers used radiation mutagenesis in grapefruit orchards and discovered a new grapefruit variant that had red flesh instead of the traditional white flesh — they had identified ruby grapefruits.

Researchers later discovered that chemical mutagens could also be used to mutagenize plant genomes. Certain chemical compounds could induce nucleotide-level mutations (*e.g.*, ethyl methanesulfonate) or even chromosomal mutations (*e.g.*, colchicine) that would affect the ploidy (*i.e.,* the number of chromosome sets) of the plant. By increasing the number of chromosomes in plants, many interesting ornamental plants were discovered, such as new

varieties of marigolds, lilies, and snapdragons. Beginning in the 1940s, many of these varieties were commercially sold and marketed by seed companies as novelties that were created using chemicals *(1)*.

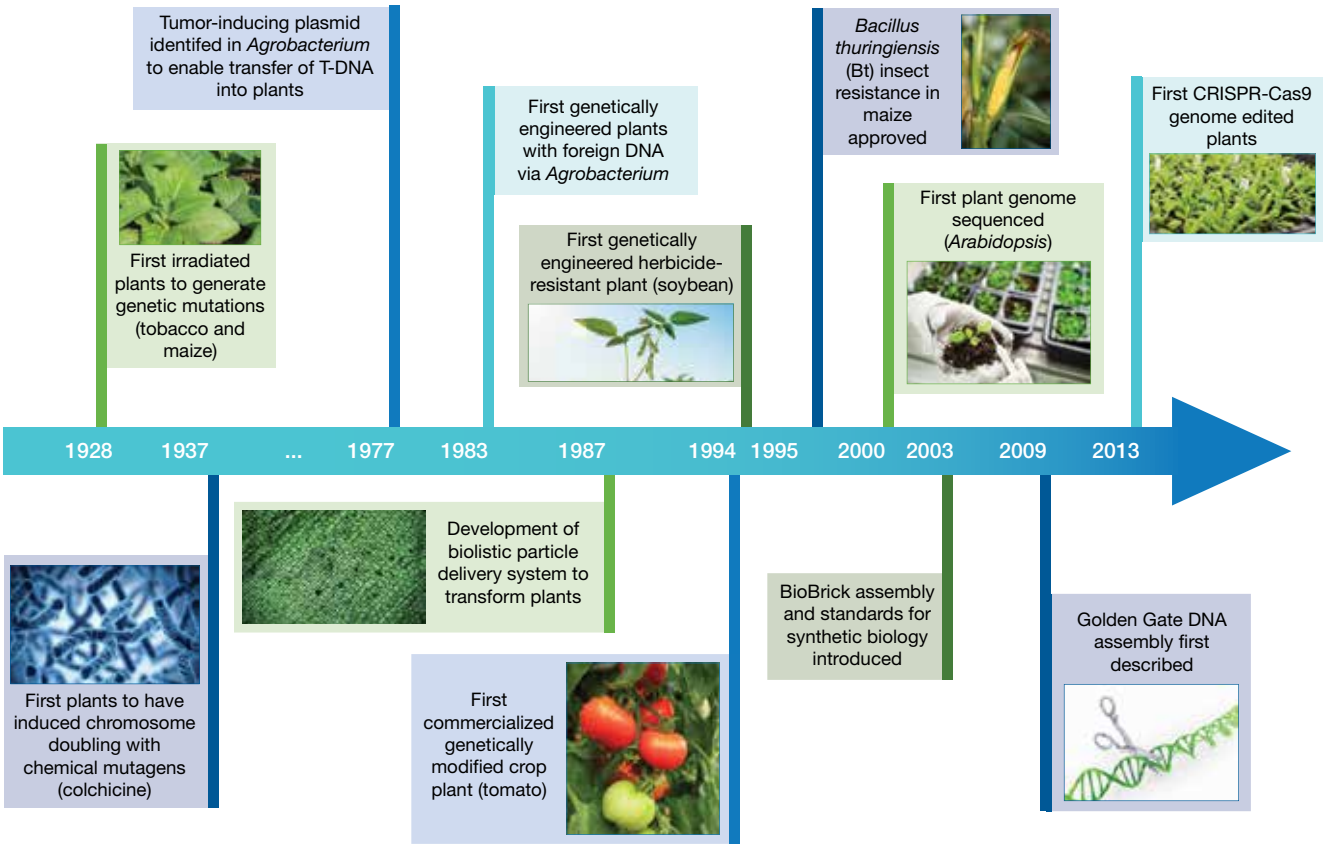
The excitement surrounding these new mutagenesis techniques invigorated the scientific community. Researchers hoped that these approaches could be used to investigate the genetic basis for plant phenotypes. These advances also stoked the enthusiasm of home gardeners, who wanted to learn how to leverage these new technological advances. To that end, home gardeners began purchasing and utilizing mutagens, such as colchicine, to try to create homegrown mutant varieties of their own personal plants. In an era when technological advances were just beginning to enable scientists to understand the genetic basis and underpinnings of life, it is remarkable that nonscientists not only supported that research, but also wanted to participate in manipulating plant genomes.

The hope was that chromosomal and ploidy mutagenesis would be adopted as a revolutionary agricultural breeding practice. However, like X-ray mutagenesis, mutants resulting from chemical mutagens did not yield agricultural improvements for many crop species. Therefore, traditional breeding methods continued to dominate as the main approach for generating commercially relevant varieties. Although these early techniques created few agriculturally relevant varieties of crop plants, these methodologies became invaluable tools in basic plant biology research, as they have generated countless mutant libraries that have been instrumental to our understanding of basic plant genetics.

A new approach: Developing techniques for transgenesis

In the 1980s, an entirely new method of modifying plants, called transgenesis, was developed (Figure 3). Although mutagenesis did not quite deliver on the promise of improving agricultural crops, transgenesis opened the door to modern plant molecular biology.

Researchers employed mutagenesis hoping for mutations that would somehow confer a novel trait. That approach was limited to inducing mutations within the existing plant genome. Breeding efforts enabled scientists to introduce new traits from natural populations of a species into commercially relevant lines. Nonetheless, that still limited plant biologists to the traits that could only arise naturally from these plant populations.



▲ **Figure 1.** Agricultural and technological advances throughout the 20th century influenced the plant synthetic biology tools of the 21st century.

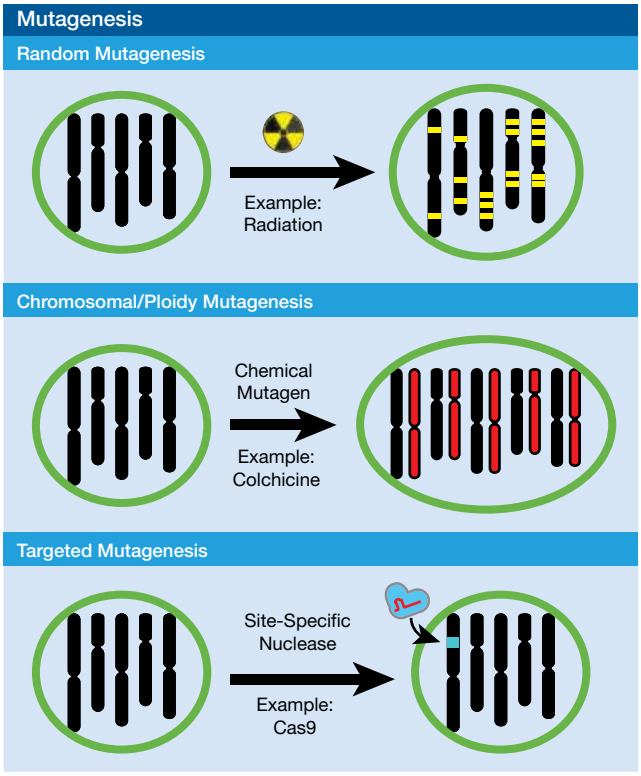
Transgenesis was a technological breakthrough that gave researchers the ability to introduce *any* gene into a plant. No longer constrained to working with existing plant genetic material in a given species, scientists could introduce novel traits of interest to many plant species.

The reports of the first plant transformations in 1983 changed the course of plant biology forever. Three independent groups demonstrated how they could exploit a natural plant pathogen, *Agrobacterium tumefaciens*, to deliver foreign DNA into plant genomes. *Agrobacterium* is responsible for crown gall disease, which forms tumors in many plant species. The pathogen hijacks plant cells to produce specialized amino acids, called opines, which the bacterium can metabolize as a carbon and nitrogen source.

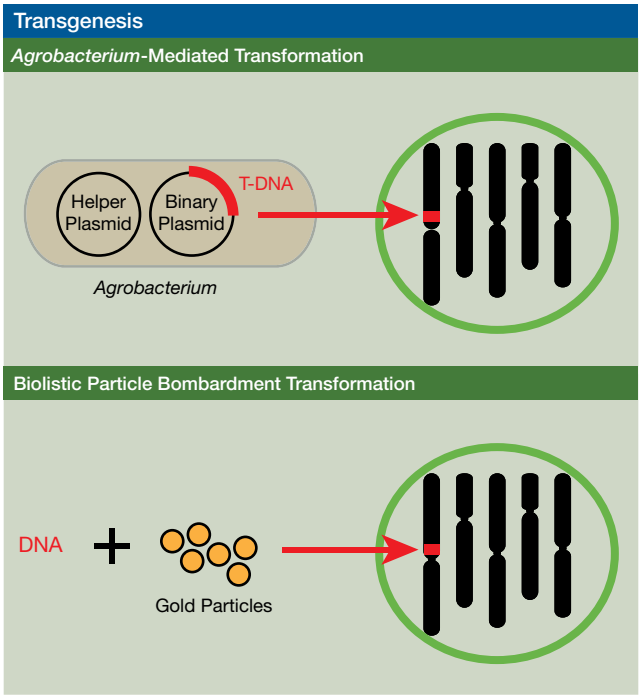
Agrobacterium has the unique ability to commandeer plant metabolism by delivering DNA into the plant genome on a small fragment of DNA called transfer DNA (T-DNA). The foreign T-DNA forces transformed plant cells to produce plant hormones to stimulate cell proliferation and form the tumors that compose the gall formation. The T-DNA also modifies the plant host's metabolism by encoding the genes involved in opine production to feed the *Agrobacterium*.

The key to unlocking transgenesis came when researchers discovered that they could split the genetic components that were necessary for *Agrobacterium* T-DNA delivery onto two separate plasmids (*i.e.,* circular strands of DNA that are separate from the bacterial genome). The bacterial machinery necessary to deliver DNA from *Agrobacterium* to the plant was encoded on one plasmid, called the helper plasmid. A second plasmid, called the binary plasmid, encoded the T-DNA. Genes of interest could be subcloned into the T-DNA region of the binary plasmid, which would then be transferred into the plant via a disarmed nonpathogenic strain of *Agrobacterium*.

The discovery and development of *Agrobacterium* to deliver foreign DNA into plant genomes has been one of the greatest technological achievements in the history of plant science and cannot be understated. Scientists were no longer constrained to randomly mutagenizing genomes to study plants. Today, plant biologists can introduce transgenes (*i.e.,* genes from a source other than the host) into the plant genome to study plants at the genetic and molecular levels. Although the development of *Agrobacterium*-based plant transformations was a landmark achievement, it was limited to plants that could be transformed by *Agro-*



▲ **Figure 2.** There are two overarching methods of modifying plant genomes: mutagenesis and transgenesis. In random mutagenesis, radiation can induce multiple random mutations (yellow) across all chromosomes in the plant nucleus. Chromosomal or ploidy mutations are induced by chemical mutagens and can increase the copies of chromosomes (red). Targeted mutagenesis approaches via site-specific nucleases, like Cas9, can be utilized to produce direct mutagenesis of single loci (blue) in plant genomes.



▲ **Figure 3.** Transgenesis is the incorporation of foreign DNA into the plant genome. Foreign T-DNA inserted via *Agrobacterium* is shown in red. Biolistic particle bombardment can also be used to transform plant genomes by shooting foreign DNA mixed with gold particles into plant cells, which integrate the foreign DNA into the plant genome.

Transgenesis provides a powerful means to engineer plants and study their basic science.

bacterium. As a result, new methods were developed to facilitate the transformation of other plants via mechanical delivery of transgenes.

The most notable innovation was the biolistic particle delivery system, more commonly known as the gene gun. This device launches metal particles coated with exogenous DNA into plant nuclei, and the foreign DNA is randomly integrated into the genome. This approach expanded the toolbox for plant molecular biologists to introduce transgenic genes into plants. This brute-force technique has been widely used and has expanded the number of plant species that can be transformed, pushing the boundaries of plant molecular biology even further.

To this day, these two transgene delivery methods, which were both developed more than three decades ago, are the most widely used techniques to engineer plants. The ability to deliver genes of interest is the foundation of all plant molecular, genetic, and metabolic engineering, and thus the development of these techniques has been the cornerstone of all plant synthetic biology.

Transgenesis has dramatically revolutionized and transformed the agricultural landscape, as 92% of corn and 94% of soybean currently grown in the U.S. are genetically engineered *(4)*. In stark contrast to the era of community engagement in developing new plant varieties via mutagenic approaches in the 1940s, modern transgenic approaches have not captivated or excited nonscientists — perhaps because the inherent technical barriers make this technology less accessible to laypeople. Nonetheless, transgenic approaches provide a powerful means to engineer plants and study their basic science.

Gene stacking in binary plasmids

Early efforts in plant engineering were simple and limited to the number of transgenes that were delivered into plants. The first genetically modified plant approved for human consumption had a single antisense gene introduced that interfered with the expression of the enzyme polygalacturonase. This gene slowed the degradation of plant cell walls during tomato ripening, thereby preventing the softening of tomatoes for easier handling and a longer shelf life.

As complex engineering efforts required more transgenes to be delivered into plant genomes, more sophisticated methodologies needed to be developed to keep pace. One option researchers considered was to sequentially transform plants by delivering transgenes in a piecemeal manner — adding one transgene into a plant one generation at a time. However, given the slow generation time of plants, this was a cumbersome and time-consuming approach. The ability to stack multiple genes into one T-DNA would make this process much faster and facilitate complex engineering efforts.

DNA assembly into binary plasmids became a key technical focal point *(5)*. The first binary plasmids were engineered with multiple cloning sites (MCSs) within the T-DNA region. This allowed biologists to insert a piece of DNA into the region of the MCS so a single gene could be introduced into the plasmid using specific endonucleases. However, this method was not amenable to efforts to introduce more than one gene into the T-DNA.

One of the earliest methods to facilitate gene stacking in binary plasmids was the BioBrick standard *(6)*. The synthetic biology community pursued a method of standardizing DNA parts that was analogous to the standardization of electrical components — parts that would not have to be redesigned and refashioned for each specific design. These parts would be composed of promoters to drive expression of genes, specific coding sequences, and terminators. All parts could be built so that they would fit into the BioBrick cloning system, enabling different research groups to swap and borrow DNA parts to build various designs.

The BioBrick system is a reliable approach to combining standardized parts. From a library of smaller components of DNA parts, longer strands of DNA could be assembled in a systematic way to build a specific DNA sequence. Eventually, plant binary plasmids were modified to be BioBrick-compatible in order to facilitate DNA assembly *(6)*. One caveat to the BioBrick approach is that the slow pairwise assembly of the DNA parts means long, multipart assemblies take longer to assemble. Although BioBrick cloning is currently not as widely used as other more-advanced techniques, many of the perspectives and infrastructure laid down by early BioBrick efforts have fundamentally shaped the synthetic biology community.

MCS and BioBrick cloning rely on Type II restriction enzymes that cut double-stranded DNA and enable new pieces of DNA to be ligated into the cut plasmid. One limitation of restriction-enzyme-based gene stacking methods is the need to ensure that there are no extraneous cut sites in either the plasmid or parts that need to be assembled. This can often lead to cumbersome cloning logistics that require actively avoiding specific restriction enzymes, or point mutating to eliminate specific restriction sites. Several methods have been developed to address some of the technical limitations of creating binary plasmids with MCS and BioBrick cloning strategies (Table 1).

One popular approach that has been embraced by many plant scientists is Gateway cloning. Unlike MCS and Biobrick cloning, Gateway cloning does not use restriction enzymes for cutting and assembling DNA. Instead, it uses

recombination sites to stitch together DNA fragments *(7)*. One drawback of Gateway cloning is that the number of recombination sites available limits the number of genes that can be assembled, and most multifragment assemblies do not extend beyond five fragments.

Gibson assembly also avoids the use of restriction enzymes. With this technique, researchers can assemble multiple overlapping DNA fragments using exonuclease, DNA polymerase, and DNA ligase enzymes *(8)*. This is a very quick and efficient method of DNA assembly, because the isothermal reaction can be performed in a single tube. Typically, the DNA fragments are generated as polymerase chain reaction (PCR) amplicons. However, the PCR process may introduce point mutations, so it is good practice to thoroughly resequence the assembled construct afterwards. Moreover, assembly of more than five DNA fragments by Gibson assembly becomes increasingly difficult.

One of the most popular approaches that has addressed many of the constraints of other techniques is Golden Gate cloning, which uses Type IIS restriction enzymes to simultaneously assemble multiple DNA parts *(9)*. Binary plasmids have been adapted to be compatible with Golden Gate cloning and have drastically increased the DNA assembly capacity for plant engineering efforts, since a large number of fragments can be assembled simultaneously.

Using Golden Gate cloning for gene stacking in binary plasmids is a hierarchical process. First, Type IIS restriction enzymes are used to assemble gene cassettes, which consist of a promoter, coding sequence, and terminator. Type IIS restriction enzymes are then used a second time to stack multiple cassettes together. One limitation of this technique is the necessity to "domesticate" all DNA parts to remove all Type IIS restriction enzymes so they do not interfere with the second step of stacking multiple cassettes together. (To learn more, see Weber, *et al., (9)* for more detail and a thorough review of Golden Gate cloning.)

An improvement on Golden Gate assembly, called jStack, has been developed recently. The jStack technique uses yeast homologous recombination to stitch together multiple gene cassettes into a binary vector, instead of using a second round of restriction enzymes *(10)*. This enables scientists to avoid the domestication step, because only the first round of cloning requires restriction enzymes. Additionally, yeast in homologous recombination has been shown *in vivo* to be a robust method to assemble larger DNA fragments, and whole bacterial genomes have been assembled with this method.

In a practical sense, it does not matter what method is used to assemble DNA into binary plasmids. However, depending on the project, a significant amount of time, capital, and labor can be saved by choosing the appropriate technique.

Advancing plant synthetic biology with targeted nucleases

Although random mutagenesis approaches using radiation or chemical mutagens provided limited advances in agriculturally relevant traits, it did generate plant mutants that were critical to understanding key aspects of plant biology. One key area that has limited plant molecular biology was the lack of tools to generate targeted alterations to the

Table 1. There are many different methods for assembling DNA into binary plasmids.

Method	Description	Pros	Cons
Traditional (Multiple Cloning Site)	Enables subcloning of one coding sequence into a binary vector	—	Can only add one gene into transfer DNA (T-DNA)
BioBrick	Iterative DNA assembly to build constructs using standardized DNA parts	Standardized parts that allow for an iterative assembly of parts	Only two parts can be assembled at a time, so multipart assemblies take longer to make
Gateway	Commercialized system to utilize recombinases to recombine plasmids together	Circumvents restriction enzyme cloning	DNA assembly is limited by the number of recombination sites
Gibson Assembly	Assembly of multiple parts using a one-pot isothermal reaction	Multiple fragments of DNA can be assembled simultaneously via overlaps	Largely PCR-based, so the construct needs to be thoroughly resequenced after it is cloned into the destination vector
Golden Gate	Type IIS restriction enzymes are used to assemble gene cassettes, which are then stacked together	Multiple fragments of DNA can be assembled simultaneously	Requires domestication of parts to eliminate all unique restriction cut sites within DNA parts
jStack	A combination of Golden Gate cloning and yeast homologous recombination for hierarchical cloning into binary vectors	Multiple fragments of DNA can be assembled simultaneously	Requires no homologous sequences in assembled parts

genome. In organisms that efficiently undergo homologous recombination (*e.g.*, *Saccharomyces cerevisiae*), scientists could knock out or swap one gene for another; however, this is nearly impossible to do in plants.

With the recent discovery of site-specific nucleases (*e.g.*, CRISPR-Cas9 technology), targeted mutations can be generated within a genome in a relatively simple manner. This technology has enabled the generation of genetic lesions through double-stranded breaks in targeted regions of a given genome. Not only has this technology revolutionized our ability to generate targeted mutants, but it also may improve the transgenic approaches we use to modify and manipulate plants.

One limitation of *Agrobacterium*- and biolistic-based delivery is the fact that transgenes are randomly inserted into the genome. Inherently, transgenes placed in different regions of the genome will produce different expression levels. This is a key hurdle in consistently reproducing plant traits. Leveraging new site-specific nuclease or Cas9 approaches to insert transgenes within a specific location in the genome via non-homologous end joining or targeted homologous recombination may become routine in the near future. Currently, the targeted insertion of genes into the genome via Cas9 has been demonstrated in plants, but increasing the frequency of successful integration events has been a technical challenge.

Advances in both modern targeted mutagenesis and transgenic efforts are beginning to converge and enable key improvements in plant synthetic biology. Many of the targeted approaches we are just beginning to develop were some of the first goals of plant engineering outlined over a half century ago. Advances in targeted nucleases are quickly refining the tools and approaches for carrying out many sophisticated plant engineering efforts.

The future of plant engineering

For nearly a century, humans have been directly modifying the genomes of plants in hopes of producing and engineering crops with improved traits. Although this began with rudimentary mutagenic approaches that had limited agricultural successes, it led to the development of transgenic approaches to introduce novel traits into crop species. The advancements in sophisticated DNA assembly methodologies to stack multiple genes and traits into a single T-DNA improved and facilitated plant transgenic efforts in engineering plant physiology and metabolism. With the discovery of site-specific nucleases, like Cas9, we have a new set of molecular tools to enable more sophisticated and more precise engineering efforts in plants. However, these efforts have not yet been fully developed to robustly and reliably enable targeted insertion of transgenes into the genome.

In the near future, we will have a full toolset to interrogate, manipulate, and engineer plant genomes, through advances in both mutagenesis and transgenesis. A combination of these two approaches will lay down the foundation for advanced engineering efforts in plants. Agriculture is a key component of civilization. Moving forward, the ability to methodically and precisely modify the genomes of species that are critical to the fabric of society will have a large impact on many aspects of our lives. CEP

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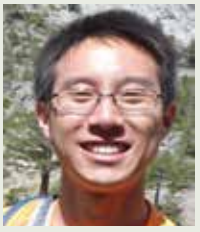

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