Targeted Mutagenesis in Polyploid Species

Timothy J Wiegman

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Abstract

Polyploidy poses a challenge to crop improvement, as it requires prospective hybrids to be painstakingly backcrossed many times in order to incorporate even a single new or changed allele; for some lines, such crosses are actually impossible with the final elite cultivar. This paper presents the four major mutagenesis technologies available to plant genetic engineers and gives a brief overview of their history and mechanisms. Their uses for genomic manipulation of plants are discussed, and the typical constraints for working with highly polyploid cultivars are explored, including insights from a case study from the primary literature. In general, site-specific endonuclease technologies (i.e. CRISPR/Cas9) are recommended for targeted gene editing in plants, delivered either through biolistic bombardment or *Agrobacterium*- (or virus-) mediated infiltration.

1 Background

1.1 Polyploidy

All modern angiosperms have undergone at least one kind of genome duplication event in their evolutionary history, if not multiple cycles of polyploidization (Wendel 2000). However, not all polyploidization events are the same. They are conventionally divided into two kinds: autopolyploidy and allopolyploidy, which refer to the doubling of a species's own genome, and the summation of two genomes via hybridization, respectively (Kihara and Ono 1926). Duplicate chromosomes with the same genes and overall structure (as in diploids and recent cases of autopolyploidy) are called *homologous pairs*, while those that have begun to diverge (but are still recognizably related) are called *homoeologous pairs* (Lyons and Freeling 2008; Spies and Fishel 2015; Glover, Redestig, and Dessimoz 2016). For a more visual explanation of polyploidy and its various forms, see Figure 1, reprinted from the excellent review article by Leitch and Bennett (1997).

Overall, polyploidy is "recognized as a major force in plant evolution, affecting diversification and speciation" (Weiss-Schneeweiss et al. 2013), but it can be a roadblock to traditional crop improvement programs (Laurie and Bennett 1986; Annaliese S. Mason and Batley 2015; Soares et al. 2021), so there exists significant interest in novel methods to drive crop improvement. In this paper, historical and current methods and techniques for manipulating the plant genome are discussed, and particular attention is paid to how polyploidy does (or does not) complicate the operation.

1.2 Mutagenesis Technologies

Many different technologies have been developed for genome manipulation. The following subsections give a brief overview of the four most prominent options. They are presented roughly in order of earliest to most recent development, which coincidentally also arranges them roughly in order of least to most precisely targeted.

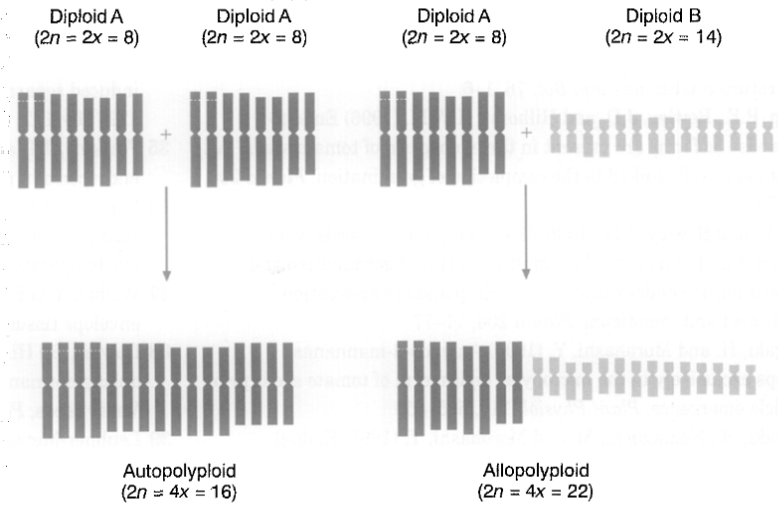
Untargeted Mutagenesis

The simplest methods of mutagenesis are those that cause untargeted, random mutations in the target organism. These generally work by causing small breaks in embryonic DNA, which are repaired (with errors) by endogenous mechanisms. The two most common processes used to cause this are irradiation—historically

Box 1. What is a polyploid?

Polyploids contain more than two genomes:

- A genome is the total DNA in one basic set of chromosomes (x), as found in a mature pollen or egg cell nucleus prior to fertilization (n is the gametic chromosome number)
- A diploid cell nucleus contains two genomes, as found in egg cells following fertilization.
- A polyploid cell nucleus contains more than two genomes. Two basic types of polyploids are recognized: autopolyploids, which contain more than two genetically identical genomes; and allopolyploids, which combine genomes from more than one ancestral species (e.g. an allotetraploid contains four genomes from two different diploid ancestral species).



In nature, auto- and allopolyploids are not always clearly recognizable, and a whole spectrum exists with many polyploids falling somewhere in between these two extremes. The identification of a polyploid of unknown genome composition is often difficult, because many polyploids [e.g. hexaploid wheat (*Triticum aestivum*)] are known to behave cytogenetically as diploids, with strict bivalent pairing and recombination at meiosis. Although the genes responsible for this have been mapped in some cases [e.g. the *Ph1* ('pairing homologous') in wheat] their precise mode of action is still unknown.

Figure 1: Reproduction of Box 1 from Leitch and Bennett (1997), giving a brief overview of polyploidy and visualizing it with a useful embedded figure.

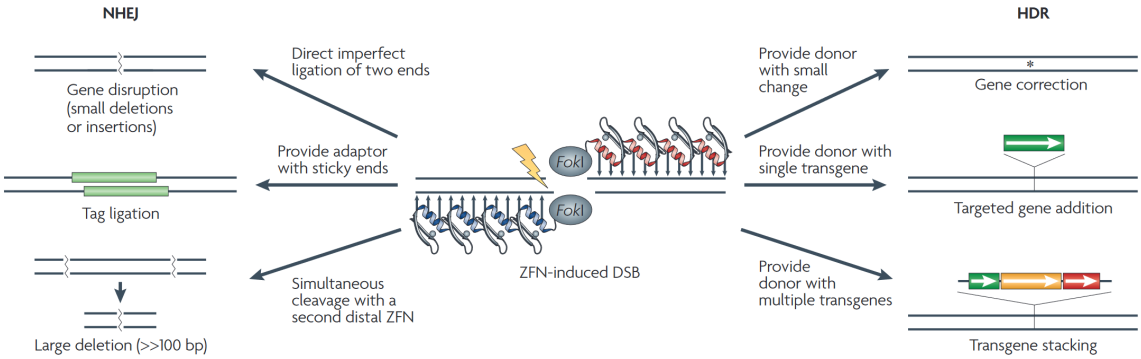


Figure 2: Reproduction of Figure 3 from Urnov et al. (2010), showing the types of genetic changes that can be effected via site-specific DNA nuclease technologies such as ZFNs, TALENs, or CRISPR/Cas.

with X-rays but now mostly with gamma rays—and exposure to chemicals such as ethyl methane sulfonate (EMS) (Micke, Donini, and Maluszynski 1990; Davey and Anthony 2010). However, this method requires preparing huge numbers of mutated offspring so that the desired mutations can be screened from that population, which is a rather labor-intensive task (Freisleben and Lein 1942; Nicolia et al. 2015), even if it is slightly easier in the present era of high-throughput genomics (Collard and Mackill 2008).

Direct Gene Transfer

While the above physical and chemical mutagens are random and cannot be guided, there exist several newer technologies that allow more directed genetic changes. The simplest method is called direct gene transfer (DGT), and it simply functions by inserting foreign DNA fragments into target cells (Gasser and Fraley 1989). Many different variations on this method exist: stimuli like heat shock or electroporation can be applied to plant protoplasts in order to encourage uptake from surrounding solution, chemical mediators like polyethylene glycol (PEG) can be used to make the cell membrane permeable to naked DNA, biolistic "gene guns" or microscopic needles can be used to physically inject the DNA fragments into target cells, or DNA fragments can even be placed into artificial liposomes for endocytosis by the plant cell membrane (Potrykus et al. 1987; Johnson, Carswell, and Shillito 1989; Lacroix and Citovsky 2020).

Agrobacterium-mediated Transformation ("Agroinfiltration")

One of the most influential early discoveries in the field of directed mutagenesis was that of *Agrobacterium tumefaciens*-mediated gene transfer by Chilton et al. (1977). This landmark study showed that a particular pathogen caused tumors by a then-novel mechanism—transferring some of its own genes into its host, which were then integrated into the host plant's genome. The cellular machinery of *Agrobacterium* that produces this effect primarily comes from one large plasmid, which has two sections: the DNA segment to be transferred, called T-DNA, and a set of virulence (or *vir*) genes, which cause the T-DNA to be inserted into the host genome but are not supposed to be integrated themselves.

Once that structure was understood, it was relatively straightforward for a genetic engineer to replace the tumor-causing T-DNA on that plasmid with the gene they want to transform into the target plant instead (Gasser and Fraley 1989). For a notable example, the "SALK lines" of sequence-indexed *Arabidopsis* mutants (Alonso et al. 2003) were created by using a non-coding sequence of T-DNA: when inserted into a gene, that gene is knocked out.

That said, another very important use of this system (Schmitz et al. 2020) and others like it (Yin et al. 2015) is to deliver other mutagens, as it is an effective way to smuggle foreign materials into mature plant cells. However, this method is limited to plants that are susceptible to the original pathogen.

Site-Specific Nuclease Technologies

The newest and most precise method for targeted mutagenesis uses a specialized endonuclease, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or a system of clustered regulatory interspaced short palindromic repeats (CRISPR) and associated (Cas) protein, in order to induce a DNA double-strand break (DSB) at a specific location in the target genome. The organism can then be induced to repair the break in one of two primary ways: either via the sloppy non-homologous end joining (NHEJ) process, which is often used to knock out genes with a small frameshift mutation, or via homology-directed repair (HDR), which can alter or insert genes near the DSB (Urnov et al. 2010). The common uses and outcomes of these technologies are summarized in Figure 2. The core technology underpinning this family of methods "is based on the use of engineered nucleases composed of sequence-specific DNA-binding domains fused to a nonspecific DNA cleavage module" (Gaj, Gersbach, and Barbas 2013), and the fact that the site-specificity is decoupled from the DNA cleavage functionality means that these methods are easy to adapt for nearly any target gene.

Note that CRISPR/Cas methods have largely replaced both ZFN- and TALEN-based methods in recent years, as they are generally cheaper and simpler while still being of comparable or superior precision and reliability

for most applications. However, there are still niche cases where the older technologies are a better choice, so CRISPR/Cas is not an unqualified upgrade (Budhagatapalli et al. 2016; Shamshirgaran et al. 2022).

2 Polyploidy & Genetic Engineering

As discussed in section 1.1, polyploid organisms often hold more than just one or two alleles for a given gene, as they can have multiple homologous or homoeologous pairs of chromosomes. This can pose a challenge for modifying the genome in many cases (A. S. Mason and Snowdon 2016), as is discussed below.

2.1 Backcrossing in Highly Heterozygous Species

Many of the most elite crop varieties are highly heterozygous, painstakingly cultivated over many generations of crosses with carefully selected parent and donor plants in order to combine all the desired alleles into one cultivar. In order to prevent the accumulated traits from segregating out of the offspring, these lines are propagated by vegetative cloning instead of conventional breeding (Udall and Wendel 2006; Schaart, Wiel, and Smulders 2021).

Therefore, bringing a new allele into the elite variety is no simple task: first, the parent plant must be crossed with a new donor plant in order to make a hybrid. Then, the crop improvers must backcross the hybrid progeny with the elite donors or their own parent lines multiple times, which can take many seasons; for polyploid varieties—as most commercial crops are—this workload is multiplied several-fold, as there are that many more alleles to put into one line (Ahmad et al. 2023). This exhaustive process is necessary to ensure that only the new allele is incorporated into the finely-tuned genome of the elite line, preventing any unwanted genes from tagging along in the process. However, diligently following the best practices is still not a guarantee of success: unlucky breeders may find their new line suffering from so-called "yield drag", as their backcrosses fail to eliminate some unwanted genes and have a decreased yield compared to the original elite line (Dhugga 2022).

Because it is so laborious to work new genes into existing crop varieties, there is great interest in using targeted mutagenesis technologies to directly introduce new alleles into elite crops in a much more direct and predictable fashion. Of those discussed in section 1.2, some methods hold more promise than others.

Untargeted Mutagenesis

Fully random mutagens like radiation or EMS are the least effective for this sort of work. Since they are completely untargeted, they are much more likely to disrupt the carefully maintained genotype of the elite cultivar than they are to introduce a beneficial allele with absolutely no other changes. In general, these methods are now primarily used to generate large libraries of mutants for basic research, though some of those mutants may still be used as donors for traditional crosses if they are characterized as having a desirable allele (Nicolia et al. 2015; Tarakanov 2022).

Direct Gene Transfer

DGT can be used to insert new genes into elite cultivars, but there are limitations to the technology that often make it less desirable than newer methods. The greatest downside of DGT is that almost all methods for getting foreign DNA into the target plant cells require them to first be converted to protoplasts by removing or digesting their cell wall. For the plant tissues that are amenable to this treatment, it is not an issue for most *in vitro* research, but many plant species cannot be regenerated into mature plants from protoplast-derived clones, so it is of limited use for developing agriculturally useful crops (Potrykus et al. 1987; Lacroix and Citovsky 2020).

The other main limitation of DGT is that, even for plant tissues suitable for protoplast preparation, it is not very precise. Naked DNA plasmids "routinely undergo rearrangement and concatenation reactions before insertion and can lead to chromosomal rearrangements during insertion" into target cells (Gasser and Fraley 1989). In the modern day, there is rarely a reason to use this basic technique rather than a newer and more precise one.

That said, many of the delivery methods that were originally developed for use with DGT can be repurposed for use with high-precision gene editing technologies. Microinjection and biolistic bombardment, for example, can be used just as well to deliver RNA for transient expression or site-specific endonucleases as they could for naked DNA plasmids, and they do not even require their target cells made into protoplasts (Lacroix and Citovsky 2020).

Agroinfiltration

Traditional agroinfiltration, using *Agrobacterium*-derived virulence genes to insert an engineered T-DNA segment, is still commonly used in crop improvement work on production cultivars. However, the method gives relatively poor control over where the gene is inserted and how many copies are inserted; even the most state-of-the-art technique still inserts multiple copies in half of all transformed specimens (Sahab and Taylor 2022). As a result, it is suboptimal for manipulating elite cultivars without causing ill effects on their carefully-constructed genome.

That said, agroinfiltration may be preferred for other reasons, such as familiarity or ease of regulatory compliance, given its long history of study. Also, agroinfiltration has been adapted to deliver other gene editing technologies—many studies that use site-specific nucleases, for example, utilize some kind of *Agrobacterium* to put their endonuclease system into the target plant (Nadakuduti et al. 2018; Schmitz et al. 2020; Yasumoto et al. 2020).

Site-Specific Nuclease Technologies

As has been implied throughout this section, endonuclease-based technologies like CRISPR/Cas9 are overwhelmingly considered "the king of genome editing tools" (Bannikov and Lavrov 2017) and are expected to be "an important contribution to the future breeding of improved polyploid crops" (Schaart, Wiel, and Smulders 2021). The most powerful aspect of these technologies is that they are extremely precise, such that they can reliably edit specific target genes without disrupting the rest of an elite cultivar's genome (Dhugga 2022). That alone would ensure that these are the modern genetic engineer's first choice for improving a carefully bred crop, but they also bring another powerful ability for working with polyploid plants in particular: all copies of a given gene, across any number of chromosomes, can be targeted in the same multiplex mutagenesis procedure (Ma et al. 2020; Li et al. 2021). This means that genetic engineers can use this technology to edit all homoeologous genes at once, if needed, even in a plant with a highly complex and heterozygous allopolyploid genome.

2.2 Sterility and Hybridization

Another quirk of polyploid species is their propensity for producing sterile hybrids, which may be desirable in spite of (or because of) their sterility. Sex-selective sterility can be a desirable trait to induce in certain cases (Saxena et al. 2010), and fully sterile hybrids may be propagated vegetatively even if they cannot be bred (Schaart, Wiel, and Smulders 2021).

Since such plants cannot be improved by traditional breeding, the only conventional option would be to recreate the variety by crossing whatever parent lines were originally used to develop it. With the power of directed mutagenesis, however, genetic engineers have the ability to directly manipulate individual alleles in even sterile cultivars. This obviates the need for tedious crossing and backcrossing in much the same way as was discussed in section 2.1, so most of the mutagenesis technologies discussed then have the same tradeoffs in this application.

3 Case Study: Multiplex Genome Editing in Hexaploid Wheat

3.1 Background

For a contemporary example of targeted mutagenesis in a complex polyploid crop, consider the study by Li et al. (2021). This study tested how three different variations on CRIPSR/Cas9, all delivered via agroinfiltration,

were able to create mutations at a total of eight different sites across the three homoeologous genomes of Fielder wheat.

Fielder wheat is a hexaploid plant, with three genomes labeled A, B, and D. This study targeted the gene *TaDA1*, which has homoeologs on A and B, and the genes *TaPDS* and *TaNCED1*, both of which have homoeologs on all three genomes. *TaDA1* is a (negative) regulator of kernel size (Liu et al. 2020). *TaPDS* encodes for phytoene desaturase, a carotenoid pathway enzyme that is commonly used for a simple visual screen: knockout or reduced function of this gene results in an "albino" photobleaching phenotype (Howells et al. 2018). *TaNCED1* is a homoeolog of 9-*cis*-epoxycarotenoid dioxygenase (NCED), an enzyme critical to the biosynthesis of abscisic acid (ABA), a plant hormone that regulates responses to environmental stresses like droughts (Zhang et al. 2014).

3.2 Gene Editing Technique

This study (Li et al. 2021) used a combination of several technologies discussed earlier in this paper. Gene edits were performed using CRISPR/Cas9, a site-specific endonuclease that induces DSBs where it encounters a sequence in the host genome that matches a given "single guide RNA" (sgRNA) (Bannikov and Lavrov 2017). The endonuclease system was delivered to target cells using *Agrobacterium tumefaciens*.

The primary intent of the study was to compare the transformation efficiency and robustness of different methods for including multiple sgRNAs in a single transformation vector. Multiplex gene editing, where multiple sgRNAs are provided in the vector, allows targeting multiple genes in a single treatment.

The first multiplexing method, called tandem repeats of separate promoters (TRSP), was the most traditional one. In this scheme, each sgRNA sequence is provided as a separate gene with its own promoter so that it can be transcribed independently of others on the same cassette.

The second multiplexing method was created by Xie, Minkenberg, and Yang (2015). This method adds specific sequences to the flanks of the sgRNA to be recognized by RNase P and RNase Z (or RNase E in bacteria). These RNases are highly conserved across all living organisms, so a genetic engineer can rely on the target cell's own RNases, avoiding the need to include an extra gene on the cassette to encode a post-processing enzyme. Once recognized, these endogenous RNases cut each sgRNA out of the strand, allowing a single transcript to generate multiple separate sgRNAs. This allows fitting more sgRNAs into a cassette of the same length, and has different limitations on precise sequences than the traditional TRSP method. Since these endogenous RNases were originally created in nature to process tRNA, this study calls it the "tRNA" method for shorthand.

The third multiplexing method was created by Gao and Zhao (2014). This method adds an engineered gene called *RGR* to the flanks of each sgRNA that, once transcribed, self-assembles into a ribozyme that cleaves itself on each end, leaving only the sgRNA behind. Just as the tRNA method before, it allows putting more sgRNAs into the same cassette without relying on multiple promoters.

3.3 Results

The ribozyme methods generally performed best, followed by tRNA, and both were always at least as good as the naïve TRSP. Figure 3 shows the editing efficiency of each method for each gene in each genome. While it lacks error bars to give a measure of the uncertainty involved, it shows that generally the ribozyme method performed best, with the tRNA method performing as well or slightly better than the baseline TRSP method. Figure 4 shows that, though none of the methods performed very well to edit *TaNCED1*, the ribozyme method successfully performed its edits significantly more often than the other two methods for most genes, based on sequencing the genomes of transformed plants.

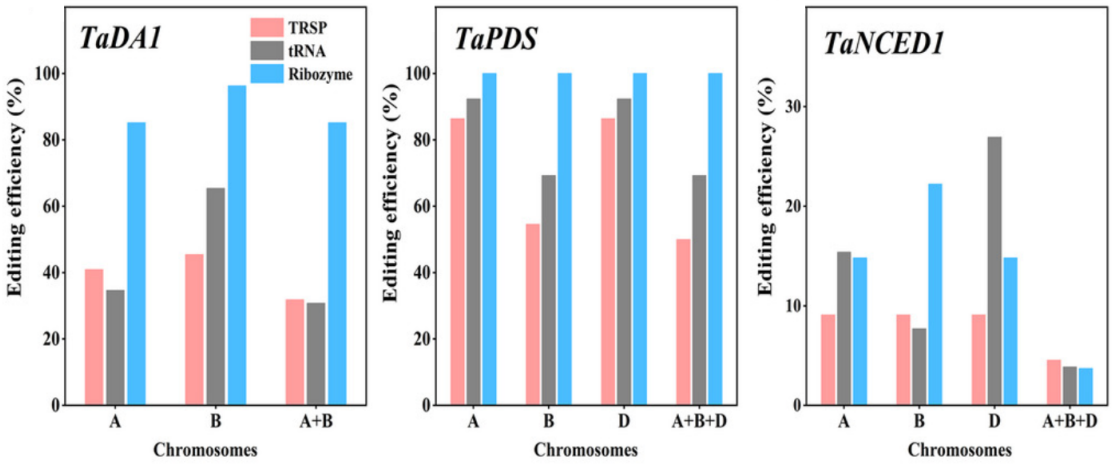


Figure 3: Reproduction of Figure 1c from Li et al. (2021). The original caption was: "Editing efficiencies of homoeologous genes at different subgenomes in three multiplex editing systems. A + B, plants with genes edited at both A and B chromosomes. A + B + D, plants with genes simultaneously edited at A, B, and D chromosomes."

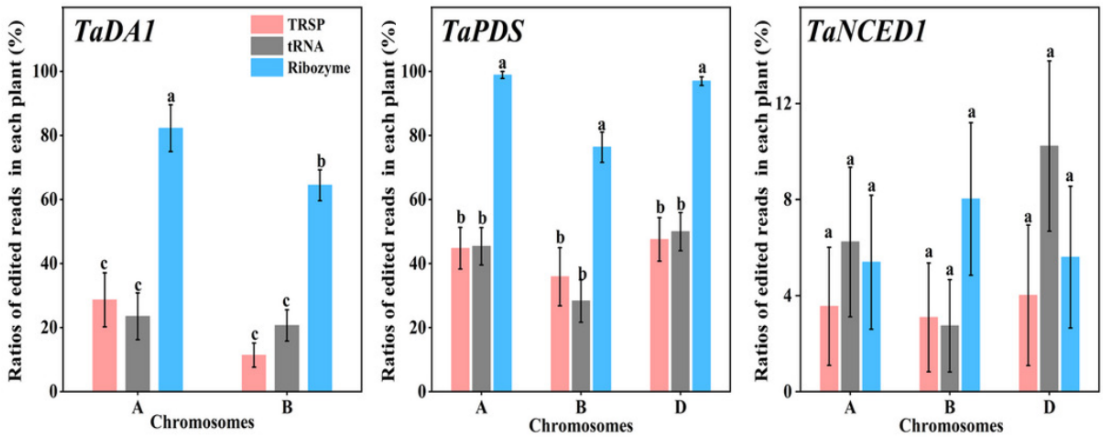


Figure 4: Reproduction of Figure 1g from Li et al. (2021). The original caption was: "Ratios of edited reads for homoeologous genes at different subgenomes in each plant. Data are presented as the mean ± SE. n = 22, 26, 27 for TRSP, tRNA, and ribozyme systems, respectively. Different letters above the columns indicate groups with significant differences (ANOVA, $P < 0.05$)."

4 Conclusion

4.1 Discussion

Of all the mutagenesis techniques presented in this paper, the clear winner for working in highly polyploid plant lines is site-specific nuclease technology. Gene editing with something like CRISPR/Cas9 is highly precise, flexible enough for multiplex operations, and eliminates the need for laborious backcrossing after changing alleles. It is not perfect—figure 4 shows that particularly transformation-resistant genes may be transformed with less than something like 10% efficacy—but it is far better than the alternatives developed throughout the history of plant genetics research. Future work in basic science should seek to identify why certain genes (e.g. *TaNCED1*) are more difficult to transform than others (e.g. *TaPDS*) and develop more effective procedures to overcome that.

4.2 Summary

This paper presented an overview of four mutagenesis techniques: random mutagens (i.e. gamma rays and EMS), direct gene transfer (e.g. electroporation), *Agrobacterium*-mediated infiltration, and site-specific nucleases (i.e. CRISPR/Cas). Their uses for genomic manipulation of plants were discussed, and the typical constraints for working with highly polyploid cultivars were explored. In general, site-specific endonuclease technologies (i.e. CRISPR/Cas9) are recommended for targeted gene editing in plants, delivered either through biolistic bombardment (Lacroix and Citovsky 2020) or *Agrobacterium*- (or virus-) mediated infiltration (Schaart, Wiel, and Smulders 2021). Finally, a case study was presented from the primary literature, showing how CRISPR/Cas9 methods can induce transformation with up to 100% efficiency, even across multiple homoeologous genomes (Li et al. 2021).

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Bing Chat (GPT-4) was used to locate some relevant sources and to translate old and foreign texts (*viz.* Kihara and Ono (1926), Freisleben and Lein (1942)) into modern English for review, but it was **not** used to generate any original text in this paper.

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