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**21st Century Directions in Biology:  
New Transgenic Crops**

# Engineering with Precision: Tools for the New Generation of Transgenic Crops

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*In the past 25 years, a major revolution in agricultural practice and crop production has occurred. Genetically engineered crops with improved agronomic traits have made the transition from laboratory benches and greenhouses to fields all over the world, where they are being grown commercially. Genetic engineering technologies have evolved as a science and continue to provide the tools for making the crops of tomorrow. Armed with genomic information and nanotechnology, plant molecular biologists are redesigning molecular tool kits to engineer plants with more precision. This article describes the current major transformation methods, discusses their strengths and limitations, and focuses on a number of research areas that are likely to be used for producing the new generation of transgenic crops.*

*Keywords:* Agrobacterium tumefaciens, biolistic gun, biotech crops, genetic engineering, transgenic plants

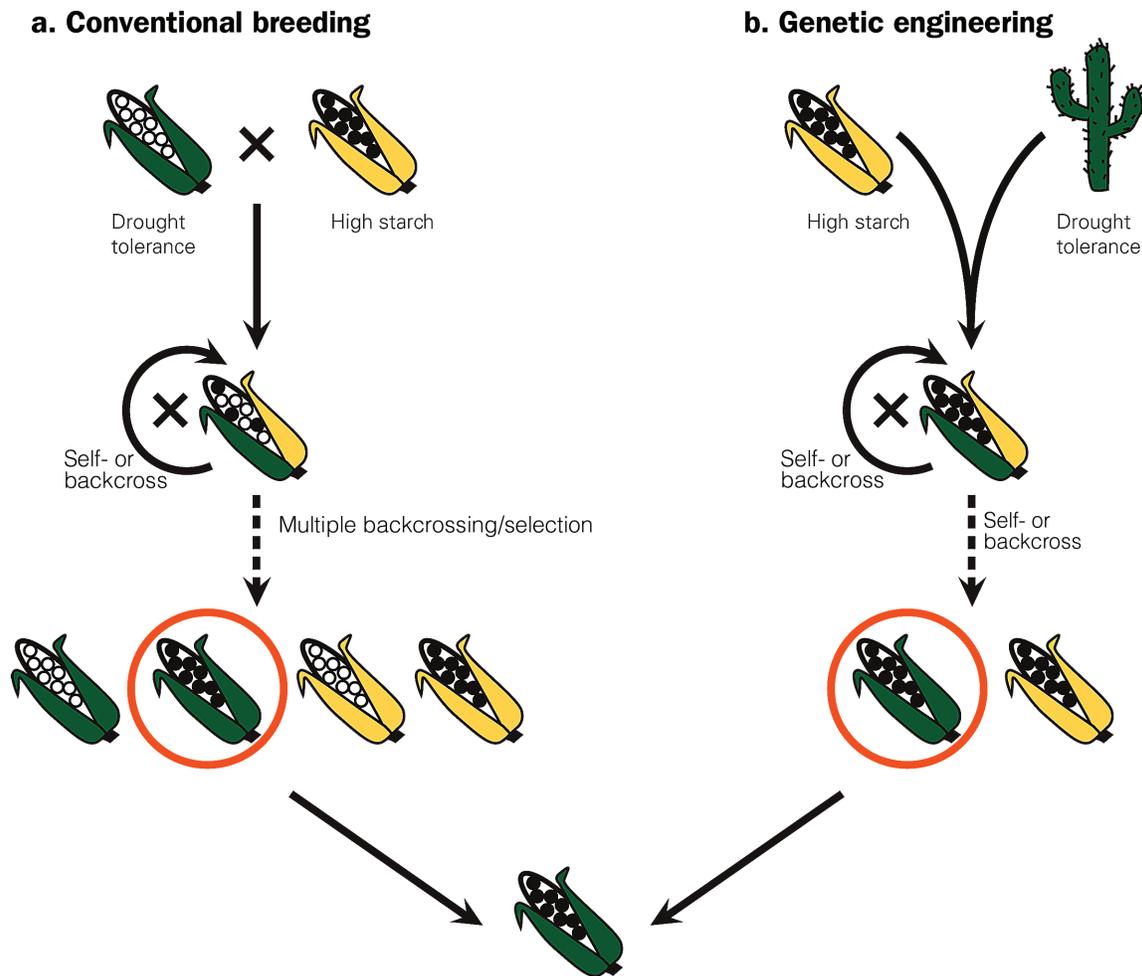
**H**uman efforts in plant breeding have been continuous for the past 10,000 years (Schlegel 2007). Our ancestors have been altering the genetic makeup of crops by selecting for desirable features such as faster growth, larger seeds, or sweeter fruits. As a result of this lengthy selection process, domesticated plant species can be drastically different from their wild relatives. Although such efforts have contributed significantly to the food supply and stability of human society, most modern crops were created with little knowledge of the genetics and fundamentals of modern plant breeding. The 20th century has seen major advances in crop development, following the discovery of Mendel's laws of inheritance. In the 1960s, the breeding of maize, rice, and wheat varieties with better yield potential, disease and stress resistance, and adaptability to different environments resulted in an unprecedented increase in food production that came to be known as the Green Revolution (Hedden 2003). Taking valuable traits and carefully incorporating them into future generations has enabled breeders to produce better crops that possess the desired combination of traits (figure 1a). Many breeding techniques (such as hybrid breeding, mutation breeding, chromosome substitutions and translocations, somaclonal variation using *in vitro* culture) have been developed to enhance the discovery, selection, and integration of important agronomic traits (Jauhar 2006, Schlegel 2007). Although these methods have been useful in generating

improved species, they are limited in correlating traits with specific gene functions—that is, the introduction of a desired trait to a recipient line often leads to the introduction of numerous undesired traits. These breeding methods often require lengthy selection and elimination processes to produce the desired traits and get rid of undesired ones. In addition, it is not possible to introduce genes from a distant species.

Genetic engineering technology, the most recent method for advanced breeding, introduces genes with known functions into plant genomes for crop improvement through recombinant DNA (deoxyribonucleic acid) technology. It not only provides the means for the introduction and expression of genes from distant species (figure 1b) but also allows controlled gene expression (such as developmental or tissue-specific expression). We are not yet able to predict and control the molecular interactions and outcomes of introduced transgenes and endogenous genes. However, we can develop a crop expressing an enhanced trait associated with a specific gene more effectively than we could before.

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**Figure 1. Plant improvement through conventional breeding and genetic engineering.** Conventional breeding (a) identifies traits of interest in parent lines and incorporates them into a new variety through crosses (or hybridization). The resulting product can be further crossed to itself or backcrossed to another line of interest, thus further integrating the trait in the genetic background of choice. This approach, however, often results in a complex mix of genetic sequences integrated with the gene responsible for the desired trait. The process is lengthy and requires large amounts of seed and effort over several generations to achieve the final product. Crosses can be accomplished only between related species. Plant improvement through genetic engineering (b) allows the integration of genes conferring a trait of interest regardless of the donor species. For crop species, the procedure is tissue culture–dependent and often requires the backcrossing of the transgenic line to a line of desired agronomic performance before it can be released to the field. Though a complex task, genetic engineering allows crossing of the species barrier for trait utilization. Traits enhanced through genetic engineering are the same type as those selected for through conventional breeding, though genetic engineering has the added advantage of having no limit on the germplasm that can be used as donor species.

### The gene revolution: A quick look back

Genetically engineered (GE) or transgenic crops are generated by altering the crop's genetic makeup (i.e., introducing, deleting, or silencing a gene or group of genes of interest) using recombinant DNA technology. Whereas transgenic plants are generated by insertion of heterologous DNA sequences from a different species, cisgenic plants are generated by re-introducing or silencing a gene of the same species, usually under regulation of the native elements (Schouten et al. 2006). Today, most GE plants—if not all—can be referred to

as transgenic plants because various heterologous DNA sequences are present, carried over through the engineering process. In this article, the term “transgenic” will be used to refer to both transgenic and cisgenic crops.

The generation of transgenic plants was first reported in 1983 Miami Winter Symposia (Advances in Gene Technology: Molecular Genetics of Plants and Animals) by three independent groups from the University of Ghent in Belgium, Washington University, and Monsanto Company. These groups subsequently published their results on transgenic

tobacco resistant to kanamycin and methotrexate (Herrera-Estrella et al. 1983), on kanamycin-resistant *Nicotiana glauca* (a close relative to ordinary tobacco) (Bevan et al. 1983), and on kanamycin-resistant petunia plants (Fraleigh et al. 1983), respectively.

In 1987 came a report of the successful genetic engineering of tobacco plants expressing a chimeric *Bacillus thuringiensis* toxin encoded by the *bt2* gene, and showing resistance to lepidopteran insect attack (Vaecck et al. 1987). This was the first report of an agronomically relevant trait incorporated into the plants and evaluated. Even though tobacco is not a major food crop species, the transgenic tobacco plants resistant to insect damage marked the beginning of a new era in genetic engineering.

In 1994, after three years of evaluations by the Food and Drug Administration (FDA), the Californian company Calgene was allowed to commercialize the first transgenic crop for food consumption, the Flavr Savr Tomato. The Flavor Savr tomato was designed to delay ripening on the vine, allowing more flavor to build in the fruit before harvesting and extending the fruit's shelf life ([www.cfsan.fda.gov/~lrd/biotech.html](http://www.cfsan.fda.gov/~lrd/biotech.html)). Because of various technical difficulties associated with transgenic tomato production, as well as consumer concerns about the presence of an antibiotic selectable marker gene in the plants, Flavr Savr tomatoes were removed from the shelves after only a couple of years on the market. However, by 1995, applications had been made to the FDA for review and approval of various new GE crops, including virus-resistant squash; potatoes resistant to the Colorado potato beetle; and herbicide-tolerant soybean, corn, canola, and cotton ([www.cfsan.fda.gov/~lrd/biocon.html](http://www.cfsan.fda.gov/~lrd/biocon.html)).

The boom in the era of plant genetic engineering can be appreciated by comparing the numbers of species and permits for regulated-article field release in the last 20 years. In 1987, tomato and tobacco were the only organisms with field release permits, with a total of five permits. In 2007, the US Department of Agriculture (USDA) and its Animal and Plant Health Inspection Service (APHIS) unit authorized a total of 671 field release permits ([www.nbiap.vt.edu/cfdocs/fieldtests1.cfm](http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm)) for 54 organisms and several phenotype categories (figure 2). According to reports published by the International Service for the Acquisition of Agri-biotech Applications, 102 million hectares of GE crops were planted worldwide in 2006, a 60-fold increase since 1996 (Brookes and Barfoot 2006). This figure makes biotechnology the fastest adopted crop technology in recent history. In 2005 alone, the economic benefit of GE crop adoption to farm income was about \$5 billion (Brookes and Barfoot 2006). The biotech generation of crops has resulted in a 15.3% net reduction in environmental impact on GE crop farming land since 1996, owing in great measure to significant reductions in the application of herbicides and insecticides worldwide. It is estimated that more than 10 million farmers have adopted GE crop technology and have experienced the benefits and profits that biotechnology can bring (Brookes and Barfoot 2006).

For basic research purposes, transgenic plants are generated to understand gene function and regulation mechanisms. Overexpression or down regulation of key enzymes or regulatory factors often perturb known or unknown pathways that can lead to significantly altered phenotypes. For crop improvement purposes, transferred genes usually confer an output trait that renders the transgenic plant superior to its nontransgenic counterpart. Desirable output traits include pest and herbicide resistance; resistance to environmental stress conditions; enhanced yield; improved nutritional value; improved shelf life; altered oil, sugar or starch contents; production of plant-based pharmaceuticals or industrial products; and potential for bioremediation.

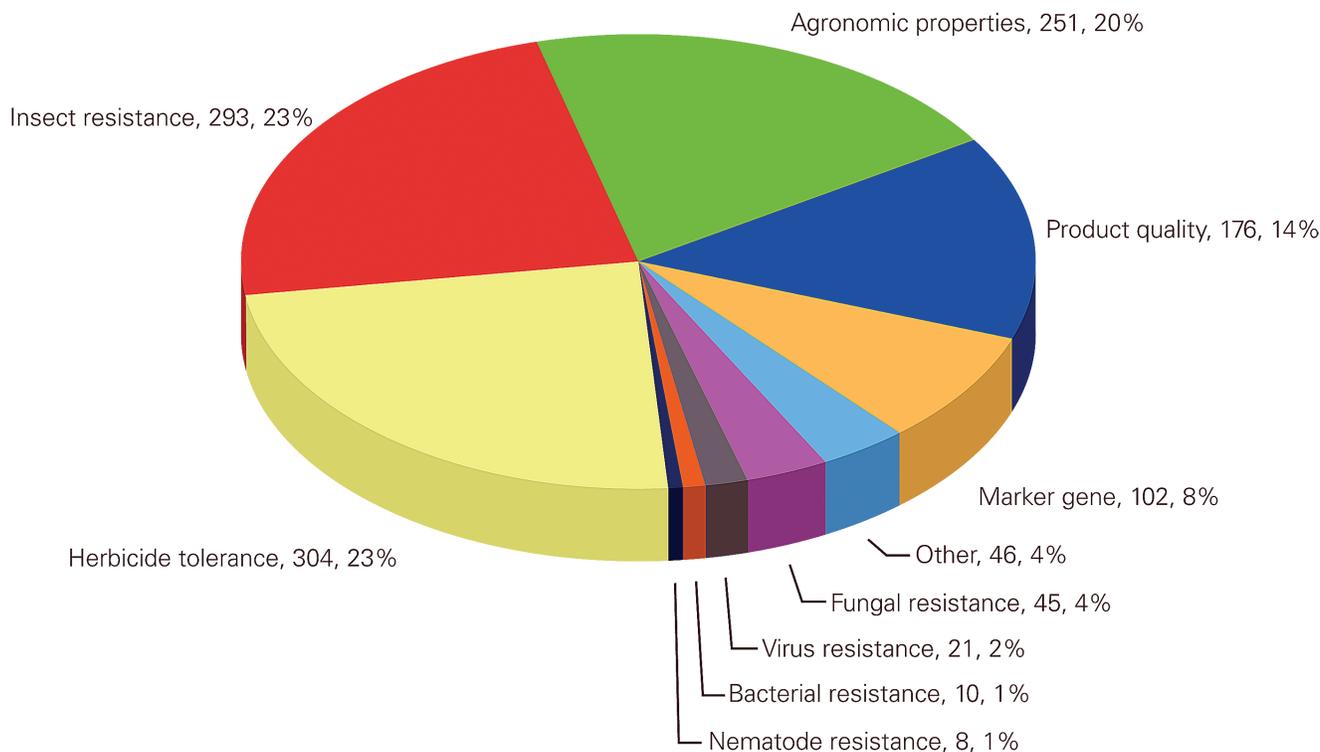
The term “genetically modified organism” is frequently used to describe transgenic crops. In fact, all crops generated and cultivated through conventional breeding have been genetically modified as well. Therefore, “genetically engineered” or “biotech” should be used to more precisely describe transgenic crops created through biotechnology.

### Smart bug and top gun: Delivering genes into plants

The revolution in genetic engineering of plant species can be attributed to the remarkable research progress on studying a plant pathogen, *Agrobacterium tumefaciens*. In nature, this soil bacterium infects a wide range of host plants. It transfers a gene-encoding segment DNA (T-DNA) from its own plasmid (the circular extrachromosomal molecule in *Agrobacterium*) into the plant genome to promote production of opines, nutrients that only *Agrobacterium* is able to use for survival and reproduction. Along with the opine genes, the bacterium also transfers phytohormone genes to promote cell proliferation, resulting in the formation of tumors commonly known as crown galls.

The molecular dissection of the *Agrobacterium* tumor-inducing plasmid (Ti plasmid) allowed researchers to design a “disarmed” vector system, in which the virulence functions of *Agrobacterium* Ti plasmid were removed and replaced with a gene of interest, while retaining its DNA transfer abilities. By simply infecting plant cells or tissues with the engineered strain, the researchers were able to generate healthy transgenic plants carrying the gene of interest (figure 3a; Gelvin 2003).

Although the *Agrobacterium*-mediated method generated numerous transgenic plants, it appeared to be ineffective for many crop species, especially during the early years of genetic engineering. To overcome this problem, many physical delivery methods have been tested. Use of plant protoplasts for methods such as electroporation, injection, or delivery of DNA using polyethylene-glycol proved successful, but efficiencies were very low, costs were high, and applications were limited to certain plant species. Other methods such as tissue electroporation and silicon carbide whisker-mediated transformation have yielded transgenic plants but have not found wide applications (Potrykus and Spangenberg 1995). The biolistic gun, a device that literally shoots tungsten or gold particles coated with the DNA of interest, came to be the delivery



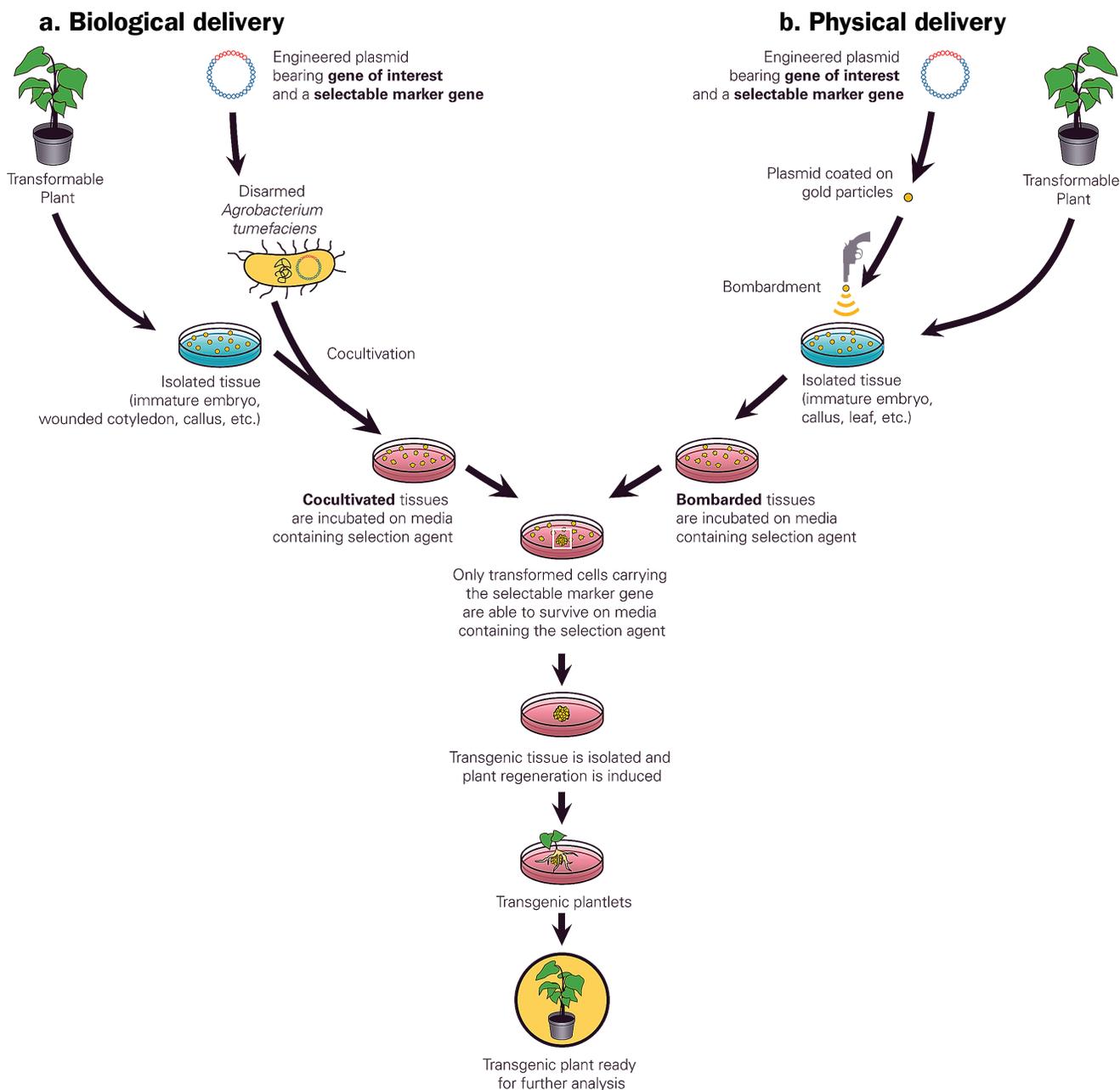
**Figure 2.** Permits approved by the Animal and Plant Health Inspection Service for field testing in 2007. A total of 671 release permits were issued for field-testing transgenic crops in the United States. The label for each category indicates the phenotype, the number of permits issued for that category, and the percentage it represents of the total number of traits tested. One permit can contain multiple phenotypes or phenotype categories. It is noteworthy that approximately 54% of traits pertain to crop protection through biotic stress resistance or tolerance. The phenotype category for agronomic properties includes traits such as resistance and tolerance to abiotic stress, altered amino acid composition, coloration, fertility, kernel development, lignin content, nitrogen metabolism, oil profile, floral development, and germination. The phenotype category “product quality” includes accelerated ripening, altered secondary metabolites, amino acid and oil levels, altered coloration, altered heat-stress response, altered kernel development, altered lignin biosynthesis, altered oxidative-stress response, altered cadmium metabolism, altered caffeine levels, improved digestibility, altered fiber properties, production of industrial enzymes, enhanced iron absorption, reduction of nicotine levels, reduction of phytate levels, and altered vitamin C content, among others. The phenotype category “others” includes antibody and antibiotic products, altered carbohydrate metabolism, anthocyanin levels, susceptibility to *Agrobacterium*, accumulation of fluorescent proteins, pharmaceutical protein production, protein tagging, and value-added protein production, among others. Details of phenotypic categories can be found at [www.isb.vt.edu/cfdocs/isblists2.cfm?opt=28#AP](http://www.isb.vt.edu/cfdocs/isblists2.cfm?opt=28#AP).

method of choice for species recalcitrant to *Agrobacterium*-mediated transformation (figure 3b).

The success of the transformation does not depend only on the gene delivery method. The combination of selection markers and gene regulatory elements also determines the success of both the generation of the transgenic events and the expression of the transgene of interest. The choice of appropriate target cell or tissue and the judicious treatment of the target tissue before and after transformation are important for the survival and selection of the transgenic events.

The effect of the number of transgene copies on the transgene’s expression level is still an amply debated issue. Transgenic plants carrying more copies of a transgene in their genomes do not always give a high level of transgene expres-

sion. In fact, integration of high numbers of transgene copies often leads to transgene silencing, a phenomenon in which the expression of the transgene or endogenous gene with sequence homology is partially or completely reduced in transgenic plants (Shou et al. 2004, Travella et al. 2005). Transgene transcript dosage (amount of RNA [ribonucleic acid] transcripts) is responsible for some of the silencing and variability observed in transgene expression among transgenic lines carrying the same construct (Schubert et al. 2004). Compared with the biolistic gun method of DNA delivery, *Agrobacterium*-mediated transformation results in more transgenic events with a lower or single-copy number of transgene integrations, and thus is currently the preferred system for most plant scientists.



**Figure 3. Two major approaches for the plant genetic transformation. (a) Agrobacterium-mediated transformation.** This biological delivery system utilizes the natural DNA transfer ability of a soil bacterium, *Agrobacterium tumefaciens*. For plant transformation, researchers use a laboratory strain of *Agrobacterium* (i.e., disarmed strain) that has been engineered to remove its pathogenicity but retain its DNA transfer ability. The target plant tissue (e.g., leaf disc, immature embryos, callus material) is “infected” with the disarmed *Agrobacterium* carrying a plasmid that bears the gene of interest and an appropriate selectable marker gene (e.g., herbicide or antibiotic resistance). **(b) Biolistic gun-mediated transformation.** This is one of the most effective physical gene delivery systems for plants. Tungsten, gold, or silica nanoparticles are coated with a plasmid carrying the gene of interest and an appropriate selectable marker gene, and are bombarded into the tissue to be transformed. After delivery of the DNA via the *Agrobacterium*-mediated (a) or biolistic gun-mediated (b) method, the tissue is incubated on media containing nutrients and plant growth hormones for a short time before being taken to a similar medium that includes the phytotoxic selection agent (herbicide or antibiotics). Because of the presence of a selectable marker gene, the transformed cells can survive the media containing phytotoxins, whereas nontransformed cells will die. Herbicide- or antibiotic-resistant tissue can be further cultured on media for regeneration to become a whole plant. These plantlets can be grown in the soil to maturity. Molecular and biological analysis of these plants can be conducted from this point forward.

### Successes, limitations, and future outlook of current genetic engineering technology

The past 25 years have seen tremendous technological advances in plant biotechnology and rapid adoption of biotech crops in agricultural practice. At the same time, limitations associated with early transgenic technology have raised concerns about the long-term impacts that GE crops may have on health and the environment. From a scientific standpoint, the successful application of genetic engineering to more crops for more traits has been hindered in more than one way. The addition of two or more genes or desired traits into a single species of interest is known as gene stacking. Gene stacking in plant biotechnology is important because it makes the design of transgenic plants highly versatile, allowing, for example, the engineering of resistance to multiple pathogens, multitoxin resistance, and metabolic pathway engineering (Halpin 2005). Many of the new technologies for developing transgenic plants are designed with their ability for gene stacking in mind.

### Location, location, location: Overcoming transgene position effects and insertion-site complexity.

Although genetic engineering has the advantage of allowing defined segments of DNA and genes with known functions to be introduced into a genome of a distant plant species, it cannot yet control the location and numbers of the introduced gene fragments. One inherent problem with current transformation technologies is that transgenes are inserted randomly into the plant genome, and consequently each transgene is inserted into a different genomic environment that can significantly affect its expression (position effect). Furthermore, transgene expression can vary depending on insertion-site complexity. Analysis of transgene insertion sites has shown that the transgene often integrates as multiple copies, especially when biolistics is the gene delivery method (Makarevitch et al. 2003). Transgene copies may be incomplete or arranged in direct or inverted repeats, often resulting in silencing the transgene.

The position effects and insertion-site complexity that arise from the location of the transgene in the genome have been an issue of concern for plant biotechnologists since the early days of the modern biotechnology era. The current approach to solving the problem is to screen hundreds to thousands of independent transformation events for the few with simple integration structures that express the transgene at the desired level. One of the early strategies to reduce complexity problems was to bombard linear DNA molecules, yielding DNA insertions of less complexity but still random integration into the genome (Breitler et al. 2002). Shielding the transgene from the genomic environment by flanking it with matrix attachment regions (MARs) was another strategy tested. Although the technique seemed promising, the reported findings from this approach were highly variable and showed dependence on the transgene construct and the MAR used (Mankin et al. 2003).

Site-specific recombination is a strategy explored actively in plant systems to circumvent the position effect. Many vari-

ations of site-specific integration, deletion, or inversion of transgenes have been used in plants, including at least three different recombination systems: the Cre-*lox* from bacteriophage P1, the FLP/*FRT* from yeast, and INT from phiC31 *Streptomyces* phage (Ow 2005). The results of recombination depend on the initial location of the recombination site, as well as on the inherent capability of the recombination system. Flanking a transgene with directly oriented *lox* or *FRT* sites can lead to deletion of the flanked transgene, such as selectable marker genes no longer needed after transformation (Ow 2007). Flanking a transgene with inversely oriented sites can resolve multiple transgene integration patterns, as shown with the Cre-*lox* system in wheat, rice, and maize (Ow 2005). Recombination among the different integrated copies leads to the simplest recombination unit, generally a single copy that can only invert, not delete. In rice, Cre-directed site-specific integration places a precise single-copy DNA fragment into the target site in nearly half of the selected events. These precise, single-copy-insertion plants express the transgene within a range that is predictable and reproducible, indicating that once a suitable target site is found, the plant line can be used for predictable insertion and expression of genes (Ow 2005). Recently, high-lysine corn LY308, developed by Monsanto Company using this technology, obtained USDA regulatory approval to have nonregulated status for commercial release in the poultry industry ([www.aphis.usda.gov/brs/aphisdocs/04\\_22901p\\_com.pdf](http://www.aphis.usda.gov/brs/aphisdocs/04_22901p_com.pdf)).

One limitation with the current recombinase-based system is that it first requires development of well-characterized lines carrying target sites that will permit stable and predictable transgene expression. These founder lines can then be used for the introduction of a gene of interest. Because the generation of founder lines uses conventional transformation methods (biolistic or *Agrobacterium*), one needs to characterize a large transformation population to identify suitable founder lines. Nevertheless, once the founder lines are established, they can be used to precisely insert any compatible transgene construction. Inducible systems for recombinase gene Cre expression and self-excision (Ow 2007) have recently been used to accelerate the process. It is expected that further technological advances will help to create a new generation of transgenic plants with the desired insertion locations and more predictable gene-expression levels. Moreover, this technology will allow the removal of unwanted transgene sequences and improve the ability for gene stacking in transgenic plants (Ow 2005).

The use of zinc-finger nucleases (ZFNs) as molecular scissors for gene targeting was recently adapted to animal and plant systems (Durai et al. 2005, Porteus and Carroll 2005). Zinc fingers (ZFs) are highly specific, DNA-binding motifs found widely across all eukaryotic genomes. ZFNs are engineered proteins carrying highly specific ZF domains and a nonspecific nuclease domain, such as the one from endonuclease Fok I, that deliver a double-strand break at a targeted site in the genome. The generation of a double-strand break in the genome, in turn, induces homologous recombination

to mediate site-specific recombination for genome repair. Unlike other forms of genome repair, homologous recombination is accurate and uses a homologous sequence (from an undamaged sister chromatid or the delivered gene of interest) as a source for repairing the DNA break. The specificity of the ZFNs comes from the ZF domain, as each of these domains recognizes specific triplets in the genome. By engineering three or more of these ZFs in tandem, one can achieve the specificity required and potentially target every gene in the genome of most crop plants (Durai et al. 2005, Porteus and Carroll 2005).

The application of ZFNs in plant genetic engineering has been successfully demonstrated in *Arabidopsis* (Lloyd et al. 2005), tobacco (Wright et al. 2005), and maize (Arnold et al. 2007). In tobacco protoplasts, the use of ZFNs greatly enhanced the frequency of localized recombination when measuring homologous recombination through restoration of a defective GUS::NPTII reporter gene. Gene targeting in the proper location was as high as 20% (Wright et al. 2005). In maize, multiple ZFNs were designed to target an herbicide-resistance marker (PAT) to the ZmIPK1 gene, an endogenous maize gene encoding for inositol 1,3,4,5,6-pentakisphosphate 2-kinase, the enzyme responsible for the terminal step in phytic acid biosynthesis. PCR (polymerase chain reaction)-based genotyping and sequencing confirmed integration of the PAT cassette into the ZmIPK1 target (Arnold et al. 2007), though no integration fidelity and random transgene insertions in the genome were analyzed. These two studies are important confirmation that the use of ZFNs can overcome the hurdles of using homologous recombination for plant genome modification.

Researchers moved recently from working with small gene sequences (approximately 200 base pairs) to test the possibility of using ZFNs for "gene addition" or site-directed integration of long segments of DNA. Moehle and colleagues (2007) showed that ZFNs can direct the integration of an 8-kilobase (kb) sequence carrying three different transcriptional units into an endogenous locus of the human genome. This study demonstrates the versatility of ZFN technology for simultaneous gene targeting and gene stacking (Moehle et al. 2007). Although ZFN technology for gene targeting has been proven to work in several eukaryotic systems and presents the potential for numerous applications in genome remodeling, it is not yet widely used. One major limitation is the off-target DNA cleavage of ZFNs, which leads to cellular toxicity. Several groups have worked on redesigning the structure of the dimerization complex of the two ZFs in the ZFN to reduce the off-target cleavage frequency and cytotoxicity (Miller et al. 2007, Szczypek et al. 2007). Until recently, the design of highly specific ZFNs was difficult and required a considerable amount of time and input. Resources are now available for engineering ZFNs to be used in several systems (Wright et al. 2006).

Another strategy for overcoming the position effect and size restriction of transgenes is the development of plant artificial chromosomes for delivery of large DNA sequences, includ-

ing large genes, multigene complexes, or even complete metabolic pathways (Preuss and Copenhaver 2006). In mammals and yeast (Houben and Schubert 2007), and more recently in plants (Yu et al. 2006, 2007, Carlson et al. 2007), artificial chromosomes have been produced by *de novo* construction using cloned components of chromosomes (Carlson et al. 2007) or through telomere-mediated truncation of endogenous chromosomes (Yu et al. 2006, 2007).

Telomeres are composed of tandem motifs of highly conserved sequences found at the ends of chromosomes, where they play a protection role. By introducing telomeric sequences through *Agrobacterium*-mediated transformation or particle bombardment, it is possible to generate chromosomes with new telomeres, after generating a truncation at the end of the recipient chromosome. Yu and colleagues (2006, 2007) used a 2.6-kb telomeric sequence from *Arabidopsis* to transform immature zygotic embryos of maize. The telomeric sequence was flanked by sites for future manipulations in site-specific recombination such as the Cre/*lox* or FRT/*FLP* systems and by marker genes that could be visualized to detect the sites of integration and chromosomal truncation. Truncation of the maize chromosomes was demonstrated using fluorescent *in situ* hybridization karyotyping, showing expression of the marker genes at the chromosome ends (Yu et al. 2006). The utility of the site-specific recombination systems in the engineered chromosomes was also demonstrated (Yu et al. 2007). A transgenic plant line carrying the Cre recombinase controlled by a constitutive 35S promoter (35S-*lox66*-Cre) was crossed with another transgenic plant line carrying a red fluorescent protein marker gene with no promoter (*lox71*-DsRed). The cross yielded progeny plants that showed the expression of the DsRed gene, indicating the integration of the 35S promoter at the 5' end of the DsRed gene via precise recombination at the *lox* sites. This study exemplified the potential for using engineered chromosomes in crop species for site-directed integration and gene stacking. An added advantage of this system is that the engineered chromosome can be introduced or removed from a particular genotype through conventional breeding techniques.

More recently, the generation of *in vitro* assembled chromosomes was demonstrated in maize as well. Carlson and colleagues (2007) designed a minichromosome vector that contained two marker genes and 7–190 kb of maize genomic sequences such as satellites, retroelements, and repeats found in centromeric regions. The vectors were delivered into maize embryogenic tissue, and the plants were recovered and analyzed for functionality of the maize minichromosomes, inheritance, and stability of the marker gene through four generations. The research group was able to demonstrate that a maize minichromosome can be an autonomous chromosome stably inherited through at least four generations, and can be maintained through meiosis and mitosis. Expectations are high for this new achievement, as it is a valuable tool for achieving gene stacking in corn, presumably reducing the amount of time required for the lengthy breeding programs that follow a single-trait transformation. Applications can also

go beyond traditional agriculture: this technology allows the integration of metabolic pathways that can be used in the production of pharmaceuticals and industrial products in plants (Carlson et al. 2007).

**Not all plants are created equal: Toward genotype independent transformation.** One of the major impediments in plant genetic engineering is the fact that not all plant species are equally transformable. Even for a species that can be transformed by either *Agrobacterium* or biolistic gun, not all of the genotypes or varieties within that species are necessarily able to be transformed. For example, Nipponbare, a japonica rice variety, can be transformed readily by the *Agrobacterium*-mediated method, but most indica rice varieties are recalcitrant to transformation regardless of the gene delivery method. The inability to transform any genotype at any time was one of the major drawbacks during the early development of GE crops. The transgene carrying the trait of interest has to be introgressed from a transformable laboratory genotype—often inferior in agronomic performance—into an elite genotype through hybridization and a lengthy breeding and selection program, followed by extensive evaluation to eliminate deleterious genomic background effects.

Great effort has been devoted to exploring and establishing tissue and cell cultures with transformation competency—the ability to integrate exogenous genes into the genome and the ability to regenerate to a fertile plant. With the exception of *Arabidopsis*, for which a nontissue culture-based approach to transformation is available, most plant transformation involves tissue culture processes via organogenesis or embryogenesis (Potrykus and Spangenberg 1995, Wang 2006). Although research to fine-tune culture media and incubation conditions will continue to be important in improving transformation incrementally, more attention is now turning to understanding the molecular basis of plant regeneration and the interaction between plant and *Agrobacterium*.

Nishimura and colleagues (2005) identified and isolated rice genes that are involved in regeneration. They found that a main quantitative trait loci gene encoding ferredoxin-nitrite reductase (*NiR*) determines regeneration ability in rice. The rice variety Koshihikari has poor tissue culture regeneration ability and a lower expression of the *NiR* gene compared with the highly regenerable variety Kasalath. When the Kasalath *NiR* gene was introduced into Koshihikari callus culture using the *Agrobacterium*-mediated method, the regenerability of Koshihikari was vastly improved (Nishimura et al. 2005).

It was also found that stimulation of the cell cycle by disruption of the plant retinoblastoma pathway can lead to enhanced maize transformation frequency (Gordon-Kamm et al. 2002). When a replication-associated protein (RepA) from wheat dwarf virus was introduced into a tobacco suspension BY-2 cell culture by bombardment, significantly more cells were found to have undergone cell division. In maize, the transgenic maize plants carrying the RepA gene were observed to have greater embryogenic callus growth and could be retransformed with higher frequency.

We expect to gain further insights into better selection of target tissues and developmental stages for genetic transformation as more genomic information becomes available. Many groups have attempted nontissue culture-based methods for various plant species, inspired by the great success of the *Arabidopsis* floral dipping method. However, few reproducible protocols have been developed and reported to date. Although it is important to identify genes that may stimulate transformation or tissue culture responsiveness, transformation would be difficult if one first had to establish transgenic plants expressing the transformation-enhancing gene and introduce the gene of interest by the second transformation. Regardless of the method of choice, the most desired transformation technology should require the least input—labor, supplies, and growth space—and offer the highest efficiency and broadest spectrum.

**In mother we trust: Plastid transformation.** All deregulated commercial transgenic crops are, to date, nuclear transformants, with transgenes inserted into the nuclear genome of plants. In addition to transgene instability caused by position effects and complex transgene integration, nuclear transformation can also be of concern for undesired transgene dissemination through cross-pollination or pollen drift. This is especially important when pollen from transgenic plants is intended for nonfood production (e.g., pharmaceuticals or industrial enzymes). One of the proposed biological confinement measures is to express transgenes in the plastids, which are inherited through maternal tissues in most species (Bock 2007). Because plastid genomes are of prokaryotic origin, they can express several genes from single operons, a desirable characteristic for gene stacking. The plastid also has prokaryotic-type gene expression machinery, which allows versatility in codon usage for recombinant protein production. A single cell in higher plants can contain thousands of copies of a single plastid gene, which for transgenes can result in high levels of protein expression—up to 45% of total soluble protein (Maliga 2004). Epigenetic interference with the stability of transgene expression is reduced in transplastomic plants (those whose plastid genome has been engineered), leading to reproducible and stable transgene expression.

Plastid transformation represents a major technological challenge to plant biotechnologists. Although chloroplast transformation has been achieved in several plant species such as tobacco, tomato, and soybeans (Bock 2007), only tobacco chloroplasts can be routinely transformed. Plant mitochondria, on the other hand, have never been transformed.

The difficulty of engineering the plastid genome lies in the large number (1000 to 10,000) of genome copies per cell distributed among numerous (10 to 100) plastids. Incorporation of the transgenes into the plastid genome through either homologous recombination or site-specific recombination is only the first step in obtaining a genetically stable plant, as it takes several cell generations and selection to dilute wild-type plastid genome copies. The use of spectinomycin and streptomycin resistance as a selective marker for

plastid transformation has limited to some extent its application in cereal crops. Current cereal transformation protocols mostly use somatic embryogenesis processes that require incubation in the dark, a condition under which spectinomycin becomes less effective. To date, no cereal chloroplast transformation has been reported (Maliga 2004, Bock 2007).

Even though plastid transformation is an attractive alternative for transgene containment, the technology still has several limitations that must be overcome before it can be widely used. It remains to be seen whether expression of a wide array of proteins that require certain posttranslational modifications, multimerization, or secretion is successful in the plastidial environment. Researchers should also focus efforts on deciphering the mechanisms of transgene expression in nonphotosynthetic plastids for their potential use in other tissues. Finally, control of transgene expression continues to be a major element in the success and safety of this technology.

**Think small: Plant genetic transformation goes nano.** Using nanoparticles for transmembrane delivery of DNA and drugs into animal cells is becoming a popular trend worldwide (Yih and Al-Fandi 2006). However, the fundamental differences between cell-wall-free animal cells and cell-wall-bearing plant cells present a major challenge for using these nanoparticles in plant research. In the recently published first report on using nanoparticles for gene transformation in plants (Torney et al. 2007), it was demonstrated that mesoporous silica nanoparticles (MSNs), small surface-functionalized silica particles, can be used to deliver both DNA and chemicals into either isolated plant mesophyll cells or intact plant leaves. The honeycomb-like nanoparticles can encapsulate chemicals in the pores, and their surface can be coated with DNA molecules. The most distinct advantage of this nanoparticle system over the current plant transformation methods is its ability to deliver more than one biological species to cells. Torney and colleagues (2007) loaded and capped a chemical (gene inducer or effector) inside the MSN, then coated the particles with DNA fragments carrying an inducible marker gene. Not only did they successfully deliver both chemicals and DNA into plant cells using the biolistic gun, they also were able to release the encapsulated chemical in a controlled manner to trigger the expression of the co-delivered transgene in the same cell.

The application of nanotechnology in plant transformation presents new possibilities for plant basic research and biotechnology. Because the MSN system has the ability to codeliver more than one type of molecule, it provides opportunities to combine genes, hormones, enzymes, regulatory elements, RNA molecules, and chemicals in the study of gene function and cell development. For example, one may enhance the integration or recombination frequencies in plants by codelivering molecular components required for integration or recombination together with the targeted DNA fragments. Because the chemicals in the particle mesopores are encapsulated with covalently bound caps, they can, when necessary, be released by the introduction of uncapping triggers (chem-

icals that can cleave the covalent bonds attaching the caps to the MSN). This can be very useful if toxic compounds need to be delivered and released in a specific cellular compartment. Environmental changes (such as temperature or pH) could also be used as uncapping triggers to study the gene function and regulation at different plant developmental stages or environmental condition. Finally, it is possible to customize the MSN system by modifying the pore size and surface function of the nanoparticles. For example, functionalization with targeting sequences on the particle surfaces could allow the MSN and its contents to be delivered to specific cellular compartments.

### From lab to field: Transgenics in action

Genetically engineered plants have played a pivotal role in the study of gene function and metabolic pathways. Historically, gene function in plants has been understood through the study of genetics as practiced by Gregor Mendel, or by using populations obtained through chemical and radiation mutagenesis. The T-DNA random insertion ability of *Agrobacterium* has been exploited to generate populations of transgenic plants carrying tagged T-DNA insertions for disruption of gene function and further biological study. Unlike mutation populations generated by chemical or radiation treatment, T-DNA tagged transgenic lines can readily be characterized at the molecular level. Using the known sequence of T-DNA, the site of insertion can be identified and the gene of interest can be cloned. The T-DNA tagged lines of multiple species are now available, including *Arabidopsis* (Galbiati et al. 2004, Ostergaard and Yanofsky 2004), rice (An et al. 2005, Hsing 2007), and barley (Ayliffe et al. 2007), among others. For studies on essential genes for which no viable mutants can be recovered, transgenic technology offers a tool for knockdown or knockin gene expression. Gene functions of these essential genes can be studied by overexpressing them (knockin) or by down regulating or silencing them (knockdown), and observing the resulting phenotypes.

Perhaps the holy grail of breeders and biotechnologists is to be able to develop crops with significant improvements in yield. Transgenic approaches have been used for improving yield in the field by increasing biomass and biomass stability through crop protection, and more recently, by working on yield-enhancing genes. Crops have been engineered for traits such as sink strength, plant architecture, plant development, root growth, source strength, and assimilate partitioning (Van Camp 2005).

In crop protection, genetic engineering has provided the world with successfully commercialized insect-resistant and herbicide-tolerant crops, benefiting corn, soybean, and cotton growers around the globe ([www.isaaa.org/resources/publications/briefs/35/highlights/default.html](http://www.isaaa.org/resources/publications/briefs/35/highlights/default.html)). As APHIS data on field release permit applications for 2007 show (figure 2), efforts are ongoing to generate transgenic crops resistant or tolerant to biotic stresses such as viruses, fungi, bacteria, and insects (Castle et al. 2006). Abiotic stresses such as cold,

drought, and salinity are also of major importance in agriculture, and numerous transgenic approaches have been taken to engineer tolerance in crops (Vinocur and Altman 2005).

Biotechnology has also made it easier to improve the nutritional quality of crops. Even though conventional breeding has been used successfully for this purpose since the beginning of agriculture, genetic engineering provides the added advantage of being able to supply traits or genes that are not present in available germplasm of some crops. Two landmark studies in this area are the engineering of the  $\beta$ -carotene biosynthetic pathway in rice for enhanced provitamin A content (Ye et al. 2000) and the engineering of tomatoes for increased folate production (Diaz de la Garza et al. 2007). Interest is keen in using plant systems for “molecular pharming”—that is, using plants as biofactories for the production of recombinant proteins for pharmaceutical or industrial applications. This is largely due to the plant’s ability to produce a large quantity of “foreign protein” free of mammalian pathogen contamination. Over the last 10 years, molecular pharming has included production of proteins for pharmaceutical applications; enzymes, monoclonal antibodies, and antigens for vaccines or vaccine components; and other industrial products (Boehm 2007). Maize-derived avidin,  $\beta$ -glucuronidase, and trypsin have already gone through the regulatory process and are available commercially (Dunwell 1999, Horn et al. 2004, Boehm 2007). Recent interest in bioethanol has triggered interest in the production of cellulase enzymes in the plant system, which has been achieved with some success in maize (Biswas et al. 2006, Sticklen 2006). The study by Biswas and colleagues (2006) exemplifies the feasibility of producing heterologous cellulases in plants for biomass conversion to sugars and possibly biofuels.

Genetic engineering has been used in other areas for industrial and agronomic applications. For example, lignin content has been altered for use in paper pulping (Baucher et al. 2003) and for improved feedstock digestibility (Reddy et al. 2005). Polyhydroxyalkanoates, macromolecule polyesters considered ideal for replacing petroleum-derived plastics, have been produced in several plant species such as *Arabidopsis*, tobacco, maize, *Brassica napus*, alfalfa, flax, sugar beets, potato, and cotton (Suriyamongkol et al. 2007). Over the past decade, transgenic approaches have also focused on designing plants for phytoremediation (the use of plants for removing contaminants from the environment; Salt et al. 1998, Kramer 2005). Much interest has been shown in phytoremediation of heavy metals from soil and water, contamination caused by mining, industry, agriculture and military practices. Transgenic plants capable of removing, containing, or sequestering cadmium, lead, mercury, arsenic, and selenium have been produced (Eapen and D’Souza 2005, Kramer 2005). New or improved varieties of floricultural crops have also been obtained by engineering traits such as color, shape, fragrance, vase life (in cut-flower species), rooting potential, and general plant morphology (Dunwell 1999, Casanova et al. 2005).

## Concluding remarks

It is not an exaggeration to say that plant genetic engineering technology has changed the face of plant science, both in field practice in agriculture and in laboratory approaches in plant biology research. Despite concerns and skepticism, the first generation of commercialized transgenic crops has become the most rapidly adopted technology in modern agriculture. With rapidly expanding genomic information and improved transformation technologies, it is hoped that advances in biological research will bring a new generation of improved crop species to meet world demands for food, feed, fiber, and fuel.

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