

1     **REVIEW**

2     **Edit at Will: Genotype Independent Plant Transformation in the Era of**

3     **Advanced Genomics and Genome Editing**

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15    **Abstract**

16    The combination of advanced genomics, genome editing and plant transformation biology  
17    presents a powerful platform for basic plant research and crop improvement. Together these  
18    advances provide the tools to identify genes as targets for direct editing as single base pair  
19    changes, deletions, insertions and site specific homologous recombination. Recent breakthrough  
20    technologies using morphogenic regulators in plant transformation creates the ability to  
21    introduce reagents specific toward their identified targets and recover stably transformed and/or  
22    edited plants which are genotype independent. These technologies enable the possibility to alter  
23    a trait in any variety, without genetic disruption which would require subsequent extensive  
24    breeding, but rather to deliver the same variety with one trait changed. Regulatory issues  
25    regarding this technology will predicate how broadly these technologies will be implemented. In  
26    addition, education will play a crucial role for positive public acceptance. Taken together these  
27    technologies comprise a platform for advanced breeding which is an imperative for future world  
28    food security.

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58 **Key Words: Transformation biology; morphogenic regulators; genome editing; functional**  
59 **genomics; advanced breeding.**

## 60 **1. Introduction**

61           Recent advances in genomics, computational plant biology and genome editing  
62 capabilities have created a great potential to actuate this progress into a wealth of scientific  
63 information as well as result in new crops and varieties [1, 2]. The ability to conduct genome  
64 editing directly depends on robust genomics platforms, but then also on plant transformation  
65 technologies in order to functionalize genomics studies and trait development [2]. Genomics,  
66 genome editing and plant transformation biology are therefore an interdependent triad of  
67 technologies. Given advances in genomics and genome editing, the need to improve plant  
68 transformation technologies has been apparent as an obvious bottleneck for analysis of functional  
69 genomics [2]. The problems associated with standard plant transformation include that it is: (1)  
70 genotype and explant dependent; (2) tissue culture intensive, resulting in long process times,  
71 involving prolonged time in tissue culture, which may result in somaclonal variation and  
72 deviation from the initial variety or genotype; (3) prone to low efficiencies for genotypes of  
73 interest; (4) expertise and labor intensive; (5) expensive per transformation event, and (6)  
74 requires extensive and expensive regulatory approval for commercialization of outcomes. While  
75 advances in genomics and genome editing technologies have seen exponential growth in the last  
76 few years, plant transformation biology has lagged, but has recently seen significant progress.

77           Initially plant transgenesis was used for biological analysis and then quickly became  
78 applied for development of new crop varieties of agricultural importance. This technology was  
79 familiarly termed as Genetically Modified Organisms (GMO) which drew widespread public  
80 controversy which still persists. The current terminology (which is broader) is genetic  
81 engineering (GE) covering various applications, but these terms are used interchangeably.  
82 Overall, these applications to basic plant science and agricultural applications are necessary and

83 crucial now for investigations of plant biology and for world food security. Given the tools of  
84 advanced genomics and genome editing, the obstacle has remained in the ability to transform and  
85 regenerate plants.

86 Breakthrough advances using morphogenic regulators for improved transformation and  
87 genetic modification have addressed the bottlenecks which have long encumbered plant  
88 transgenic biology [3-5]. Current results with cereal crops demonstrate rapid results, genotype  
89 independence, and utilize a range of explants resulting in reasonably high transformation  
90 frequencies. This enabling technology in monocots is a watershed for the application of genome  
91 editing allowing higher throughput for analysis of genomic targets. These approaches show  
92 significant promise for utility in genome editing which can be *Agrobacterium* free, DNA free,  
93 rapid and for these reasons potentially non-GE [6]. These advances have made significant  
94 progress toward a systems wide approach to plant biology involving genomics, genome editing,  
95 and plant transformation which can be practically achieved. In this review, the key  
96 developments in genomics, genome editing and plant transformation biology are evaluated.

97 Recent advances in genomics and computational biology provide a strong basis for trait  
98 identification, accelerated breeding, the development of new varieties, and proprietary protection  
99 (See Section 1a., below). In fact, the deep layers of genomic technologies will provide the  
100 foundational information that when paired with genome editing technology will offer the  
101 capability to (1) pursue new avenues in investigating basic plant biology, and (2) rapidly develop  
102 new crop varieties with specific traits having market and consumer value, and will likely  
103 underlie nearly all future crop improvement. Sequence information is crucial for target  
104 evaluations; however, there is limited use for sequence information by itself. The consequence  
105 of the data gained through genomics and sequencing and the capabilities afforded by genome

106 editing allows for an exponential increase in the potential introduction of variation. Nonetheless,  
107 this can be functionalized for phenotypic analysis *only through* the applications afforded by  
108 robust plant transformation technologies. Without the ability to introduce the appropriate  
109 molecular constructs through plant transformation, these tools will be somewhat limited in  
110 application. Thus, genomics, computational biology, and genome editing function as  
111 independent disciplines as well as collaborative sub-disciplines of plant transformation science  
112 involving the transfer of DNA, RNA, and proteins to plant cells and tissues and recovery of  
113 regenerated plants.

114         This approach requires a coordinated effort spanning several disciplines and areas of  
115 expertise - namely, sequencing, data analysis, computational biology, genomics, target  
116 identification, genome editing, target validation, plant transformation, phenotype analysis, and  
117 whole genome sequencing for detection of off target effects or inclusion of foreign DNA. A  
118 functional plant genome editing program is dependent upon a deep genomics program utilizing  
119 an efficient DNA, RNA and protein delivery system operating within the context of an efficient  
120 plant transformation program. This, in turn, should lead to high-throughput recovery and  
121 analysis of edited plants. The outcome of using these synergistic technologies for basic science  
122 and plant biotechnology will establish a robust genome editing platform that provides important  
123 new variants and commercially significant varieties which are scientifically important and/or  
124 financially viable in a time frame that is reasonable. The ability to transfer DNA, RNA, proteins  
125 and RNPs (ribonucleoproteins) into plants with the recovery of inheritable traits results in the  
126 fertile collaboration of these three disciplines. The broader application of genomics and genome  
127 editing is therefore dependent on the ability to transfer biological molecules to any plant and any

128 variety of interest. Toward this goal, plant transformation technologies have rapidly advanced in  
129 recent years and this review analyzes the interactive relationships.

## 130 **2. Advanced breeding in plants as a requirement for future food security.**

131 Advanced breeding programs must comprise the interaction with: (1) a robust genomics  
132 platform; (2) the ability to conduct gene editing at will; and, (3) the capability for genotype  
133 independent transformation for any species and any variety. The development of a synergistic  
134 effort with these programs is now essential for world-wide crop improvement. Recent advances  
135 in genomics provide a sufficient and necessary platform for advanced breeding, independent of  
136 the other functions of this article.

### 137 **2.1 Advances in plant genomics platforms and genome editing technologies, mechanisms,** 138 **design, and validation**

139 Genomics assisted breeding will greatly facilitate and accelerate the production of new  
140 hybrid varieties in all crops species. Advanced genomic assisted breeding will also be a major  
141 plank for the identification of novel traits which can be developed through advanced breeding  
142 programs and the applications of genome editing and facilitated by advanced plant  
143 transformation biology.

144 The advent of genomics and associated data analytics has had an enormous impact on the  
145 biological sciences. Agriculture is no exception, where genomics-assisted breeding and trait  
146 identification has already had a tremendous influence on food security. Advanced genomics-  
147 assisted breeding will continue to support efforts to identify novel traits and useful genetic  
148 diversity through molecular-assisted breeding programs and applications of genome editing for  
149 trait improvement.

150           The era of genomics in the plant sciences has seen a wealth of information and resources  
151 become available to breeders and geneticists. Over 50 reference genomes are now available for  
152 cereals, fruit trees, forage grasses, tubers, and other crops and model plant species. Moreover,  
153 this information is highly accessible. General web-based resources such as Ensembl Plants [7],  
154 Gramene [8], and Phytozome [9] in addition to species- and genus-specific websites [10-13],  
155 allow for data ranging from tissue-specific gene expression, sequence conservation, sequence  
156 variation, gene homology, and the 3-dimensional structure of DNA and DNA binding proteins to  
157 be anchored to precise locations in reference genomes. Genomics-assisted breeding utilizes  
158 these resources to improve crops through methods such as marker-assisted selection and genome  
159 editing for simple traits, and genomic selection for complex traits.

160           The genetic architecture of simple and complex traits is an area of genomics facilitated by  
161 quantitative trait loci (QTL) mapping and genome-wide association study (GWAS)-based  
162 approaches. The identification of genetic markers that are proxies or causative genetic variants  
163 provides a rapid means to introduce useful genetic diversity directly into breeding programs.  
164 Genetic variants with strong effects, especially in simple traits matched with tightly linked  
165 markers, enable selection by genotype, not phenotype, in plant breeding programs. Association  
166 studies, utilizing the historical recombination within populations of crops with limited  
167 coancestry, can be used to identify markers closely linked to agronomic traits, such as flowering  
168 time, plant height, yield, and grain traits in tropical rice [14]. Additional resources, such as  
169 mapping populations, are created through specially designed crosses between parental lines with  
170 differing phenotypes. A species-wide rare variant otherwise undetectable in an association study,  
171 if introduced into a mapping population by a parental line, is subjected to greater mapping power  
172 in recombinant offspring. Advanced population designs, such as bi-parental nested association

173 mapping (NAM) [15-17] or multi-parent advanced generation inter-cross (MAGIC) populations  
174 [18], can combine the advantages of association studies with the power of QTL mapping  
175 populations. The ancestral recombination between the parentals, in addition to the recent  
176 recombination within the population, further increases mapping resolution by reducing the size  
177 of statistically independent haplotypes. These trait mapping resources—pedigrees, germplasm,  
178 sequencing, phenotyping data, and mapped QTLs, are publicly available for trait discovery  
179 marker development and, most importantly, as targets for improvement by gene editing  
180 technologies.

181         One area that has had a considerable impact in plant genomics is advances in reduced  
182 representation sequencing, especially low coverage whole genome sequencing and genotyping  
183 by sequencing [19, 20], in conjunction with variant imputation methodologies design specifically  
184 for plant populations [21-23]. These genomics technologies have greatly reduced the cost per  
185 sample of mapping populations. Densely genotyped parental lines allow for progeny haplotypes  
186 to be inferred despite reduced representation. Earlier consortia-level projects describing SNP  
187 panels for crop species in the thousands of markers [24-28] have transformed into species-level  
188 resequencing projects such as the 3,000 Rice Genomes Project [29, 30] that further enable  
189 precise genotype imputation, more detailed analyses of fine-scale population structure, and the  
190 identification of underutilized gene pools. Finally, improvements to sequencing technology  
191 itself, such as the decreasing cost of high-coverage whole genome sequencing, and the increasing  
192 accuracy of long-read sequencing, is democratizing genome assemblies [31]; for example, recent  
193 species-wide resequencing in rice is approaching a paradigm where reference genomes will  
194 become commonly available on the varietal level and a single reference can describe the  
195 structural variation present in multiple sub-populations [29, 32].

196           The early translational effect of these technologies and data availability was marker-  
197 assisted selection (MAS). The recent proliferation of trait dissection studies has improved our  
198 understanding of the genetic architecture of agronomic traits, in addition to the identification of  
199 SNP markers closely linked to causative variants. The increase in genetic gain, culling of  
200 inferior progeny, and the decreased emphasis on phenotyping had substantial food security  
201 outcomes, especially for specific, regional concerns. For example, marker assisted selection  
202 breeding programs introduced submergence resistance into South Asian rice [33], rust resistance  
203 into wheat [34], and self-compatibility into cherry [35], in a much more expedient manner than  
204 traditional backcrossing. In crops with resource-intensive cultivation or those which take years  
205 to achieve reproductive maturity, such as fruit trees, reducing breeding population size is one  
206 main objective of genomics-assisted breeding. Through marker-assisted seedling selection, apple  
207 breeding populations have been reduced by 50-60% [36, 37] and sweet cherry populations by 50-  
208 85% [38, 39].

209           Selection by markers, however, fell short of the goal of a second Green Revolution,  
210 where global yield increases would satisfy both anticipated food security needs and decrease the  
211 material input for agriculture [40]. This is largely because, outside of very simple traits with a  
212 small number of strong-effect alleles, MAS is greatly limited in its predictive capability [41-44].  
213 Many agronomic traits are complex with numerous weak-effect alleles. Moreover, markers  
214 linked to QTLs as identified in mapping populations, may not be easily replicated in separate  
215 breeding populations due to differences in genetic background and population structure.  
216 Association studies, even if conducted within breeding populations, are similarly limited—even  
217 if QTL validation is no longer necessary, large numbers of markers linked to weak-effect alleles  
218 are still unsuitable for MAS [43, 45]. Finally, an inherent limitation of marker assisted

219 backcrossing—the number of generations needed to remove unwanted donor haplotypes—makes  
220 varietal development a time intensive process. Improved translational methods were needed in  
221 order to manifest the benefits of trait dissection and genetic diversity as elucidated by genomic  
222 approaches, both in terms of informed selection processes and biotechnology.

223         Fortunately, as our understanding of plant genomes and biotechnology has improved in  
224 the past decade, two methods have emerged to remedy these challenges 1) genome editing [46,  
225 47], especially allele replacement, carries the benefits of marker-assisted selection in simple  
226 traits without the need for repeated backcrossing, and 2) by performing selection on a genome-  
227 spanning set of markers [42, 44], the heritability of complex traits can be better modeled within a  
228 breeding population itself.

229         Genomic selection (GS) has been successfully implemented in the breeding programs for  
230 a number of crops. Some of the first successful examples of GS were in maize, where stover and  
231 grain yield genetic gain was shown to be 14 to 50% greater than MAS [48]. A comparison of GS  
232 vs. traditional selection for yield in drought conditions demonstrated that, in the third breeding  
233 cycle, yield was 7.3% higher in GS [49]. Genomic selection has been additionally used or tested  
234 in breeding programs for crops as varied as cassava [50], forestry tree species such as eucalyptus  
235 [51], sugar beet[52], rice [53], oats [54], and barley [55]. Although genomics-assisted breeding  
236 could be seen as existing along a continuum of genomics-assisted or “DNA-informed” [56]  
237 approaches, the use of genome-wide marker sets has shown or is predicted to improve  
238 performance for genetic gain, especially for complex traits.

239         Genomics assisted breeding in the future will likely blur the boundaries between genome  
240 editing and informed crossing. A better understanding of breeding population design through  
241 both selection and editing, as described by machine learning approaches and informed by the

242 widespread availability of pan-genome and varietal-level genomics, could bring agriculture  
243 closer to a second Green Revolution. Recent advances in machine learning and artificial  
244 intelligence complement both selection and editing through added predictive power for  
245 biological processes, at all stages of experimental design, from non-linear modeling of the  
246 genetic basis of traits, optimal cross selection, to guide RNA design. In the immediate term, a  
247 parallel approach employing genome editing for simple traits and genomic selection for complex  
248 traits could be effective.

### 249 **2.1.1 Mechanisms and guide RNA design**

250 Biologically-informed predictive models and empirical datasets of guide sequence  
251 activity are used to select guide RNA sequences and design genome editing experiments.  
252 Kanchiswamy et al. provides an excellent review of (non-plant specific) guide RNA design tools  
253 available in 2016 [57]. CRISPR-Plant [58] (with version 2 under review) and CRISPR-P [59, 60]  
254 are examples of two web-based tools designed specifically for plant genome research, with  
255 numerous commonly studied plant reference genomes available. Predictive algorithms for guide  
256 RNA design use sequence similarity in reference genomes to identify features including off  
257 target site potential and upstream/downstream sequence attributes affecting PAM site  
258 recognition, in addition to RNA sequence features such as self-binding potential [57, 61, 62].  
259 Empirical datasets of Cas9 efficiency given permuted guide sequences, mostly in human and  
260 mouse systems [63, 64], offer further capacity to predict on target cutting efficiency given RNA  
261 sequence composition. Moreover, ongoing efforts to better understand the physical and chemical  
262 properties of Cas9 activity *in vivo* [65] will improve guide sequence design in the future. The  
263 conjunction of more informative datasets, in addition to better machine learning approaches [66],  
264 will lead to improved guide RNA design for experiments in plants.

### 265 **2.1.2 Target validation by CRISPR**

266 Despite the proliferation of trait mapping in plants, and the publication of numerous  
267 agronomically-relevant QTLs, even early in the plant genomics era it was observed only a small  
268 subset of mapped genetic variation could be successfully implemented in breeding programs [41,  
269 43]. One issue, as mentioned earlier, is that trait complexity stymies marker assisted breeding—  
270 genomic selection, however, has emerged as a solution to describe the genetic basis of complex  
271 traits [41-44]. Another bottleneck in introducing mapped variation is the difficulty of  
272 independently validating the effects of gene candidates contained within QTL loci. Many QTLs  
273 may be genetic background dependent [67-69]—some yield QTLs identified in indica rice, when  
274 introduced into japonica rice, have been observed to depress yield in select varieties [70]. This is  
275 critical information for the translational effectiveness of plant genomics research. Fortunately,  
276 the targeted mutagenesis of candidate genes via CRISPR/Cas9 offers the potential for rapid and  
277 high-throughput validation of gene candidates. Multiplexed gene editing [71-73] can target  
278 multiple candidate genes simultaneously, allowing for significant candidate reduction before  
279 individual gene targeting in different genetic backgrounds. By rapidly evaluating gene candidates  
280 in several genetic backgrounds, the results from both published and novel trait mapping  
281 experiments can be translated more expediently into breeding programs.

282 Target validation using *in silico* and transient expression assays has accelerated the  
283 processes for genome editing. In addition, the use of Southern-by-Sequencing [74] and whole  
284 genome sequencing have allowed significant biological and commercial advances [75, 76].  
285 Integrated transgenes can be segregated away from edited events and thoroughly sequenced to  
286 create new varieties which should be considered as non-GMO (see below section on Regulatory  
287 Considerations).

288 Technically, genome editing resulting from the natural or induced processes of random  
289 mutagenesis has contributed to crop improvement since the beginning of agriculture [77]. The  
290 early history of genome editing relied upon selection and propagation of the consequences of  
291 spontaneously occurring random mutations [78]. Subsequently, chemical and physical  
292 mutagenesis techniques increased the frequency of mutations and significantly shortened the  
293 time for selection. Techniques for mutagenesis induction have included: (1) chemical  
294 mutagenesis (e.g. ethyl methane-sulfonate, EMS); (2) radiation mutagenesis including the use of  
295 X-rays, gamma rays, fast neutrons, beta irradiation and ultraviolet rays; (3) insertional  
296 mutagenesis including transposon mutagenesis and gene tagging, T-DNA insertional  
297 mutagenesis and tagging, and targeted induced local lesions in genomes (TILLING; [79]). By  
298 any measure the use of induced mutagenesis in breeding programs has been enormously  
299 successful over the years contributing over 3200 officially released new varieties [80]. However,  
300 more precise methods for genome modification were needed.

301 RNA interference (RNAi) is a RNA-dependent gene silencing process, historically also  
302 known as co-suppression or post-translational gene silencing (PTGS), which can be used to  
303 control of gene expression [81]. RNAi provides an approach whereby gene expression or  
304 translation can be inhibited via specific RNA molecules [82]. Gene silencing is initiated by  
305 double-stranded RNA (dsRNA) molecules in the cytoplasm. The cellular mechanisms involved  
306 with RNAi have been well studied and shown to be more stable and efficient than previously  
307 discovered antisense techniques for gene suppression (reviewed by Suarabh et al. [83]). In most  
308 cases RNAi does not result in total ablation of gene expression but will often drastically reduce it  
309 resulting in 'knockdown' mutants [84]. Constructs can therefore be developed to knockdown  
310 expression of a specific gene of interest by generating double stranded RNA with a sequence that

311 is complementary to the gene. One drawback to RNAi technology for use in advanced plant  
312 breeding is that this mechanism imparts an incomplete modification which may revert to wild-  
313 type levels [85] in subsequent generations. However, RNAi has proven to be a robust technique  
314 to influence gene expression in plants, animals, and fungi and has become an important basic  
315 research tool as well as a practical tool in biotechnology, medicine, pharmacy and agriculture  
316 [83].

317 RNAi technology has been used in many basic and applied applications in plants.  
318 Transgenic crops typically use integrated constructs which express dsRNA to specific  
319 knockdown targets. These constructs result in the functional analysis of gene expression and/or  
320 practical product development of new varieties with specialized traits. In one example, plants  
321 producing dsRNA have been designed which affect only insect pests. This technique has proven  
322 to be an effective biological insecticide [86]. More recently RNAi technology has been used to  
323 silence the gene encoding polyphenol oxidase (PPO) in apple. This enzyme is required to convert  
324 chlorogenic acid into the quinone product responsible for the browning phenotype after the fruits  
325 are sliced or damaged. The non-browning apple, called Arctic apple, was approved by the FDA  
326 in 2015. Similarly, the Innate™ potato [87] is also the result of RNAi technology targeting PPO,  
327 resulting in non-browning potatoes. Cotton seeds would be a nutritionally rich source of protein  
328 except for the production of the toxic terpenoid gossypol. RNAi technology has been applied to  
329 reduce the key enzyme involved in gossypol synthesis, delta-cadinene synthase, in the seed  
330 without affecting its synthesis in other part of the plant [88]. Similarly, using RNAi resulted in  
331 the reduction of linamarin in cassava to produce lines with low cyanogenic properties [89].  
332 RNAi technology set the stage for the ability to manipulate gene expression profiles to  
333 interrogate basic gene function and to produce practical outcomes. Nevertheless, the need for

334 precisely targeted stably inherited mutations in plants had presented an elusive goal until  
335 recently.

336 Over the past several years new molecular approaches have been developed to  
337 specifically target and modify DNA sequences in plants (reviewed in Weeks et al [90].; Songstad  
338 et al. [77]). A history of important milestones in genome editing of plants is shown in Table 1.  
339 These genome editing functions all rely on the specific sequence recognition of targeted sites on  
340 the genome. Site-directed nucleases (SDNs) also known as sequence specific nucleases (SSNs),  
341 have been developed which recognize specific target sequences resulting in a double stranded  
342 break (DSB). These SDN directed approaches result in DSBs which are then repaired by  
343 endogenous non-homologous end joining (NHEJ) or homology-directed recombination (HDR).  
344 NHEJ typically produces small deletions or insertions resulting in heritable loss of gene function.  
345 Genome editing technologies using SDNs have been rapidly developing including  
346 meganucleases [91-93], Zinc Finger Nucleases (ZNF) [94-101], Transcription Activation-Like  
347 Effector Nucleases (TALENs; [102-111]) and most recently Clustered Regularly Interspaced  
348 Short Palindromic Repeats (CRISPRs; [6, 46, 47, 112-124]). CRISPR-Cas9, derived from  
349 *Streptococcus pyogenes*, is currently the most widely used SDN system [46, 113, 114]. It is now  
350 widely recognized that genome editing presents a historic opportunity in modern plant breeding  
351 providing a clear methodology to improve phenotypic traits including biotic and abiotic stress  
352 tolerance, yield, growth, water use efficiency, herbicide tolerance and a plethora of consumer  
353 traits across diverse crops [115-118, 125-137].

354 It is clear that CRISPR systems are the major genome editing application in many plant  
355 species [46, 113, 114]. CRISPR utilizes RNA rather than proteins (as in meganucleases, ZNF, or  
356 TALENs) to specifically target DNA sequences, have allowed a greater ease of use in

357 comparison to alternative systems, and have been widely applied in many plant systems [112,  
358 138]. Genomic sequences which contain a protospacer adjacent motif (PAM) sequence NGG  
359 can be targeted by the CRISPR-Cas9 ribonucleoprotein (RNP) complex. CRISPR-Cas9  
360 recognizes RNA-DNA base pairing to specifically recognize target DNA sequences and Cas9  
361 activity results in precise blunt end DSBs at target sites. When repaired by NHEJ, these sites  
362 result in heritable null expression of the target gene that is irreversible (in comparison with RNAi  
363 which results in incomplete modification). The demonstrated utility of the CRISPR-Cas9  
364 complex for genome editing led to the identification of variants in other bacteria (i.e., *Prevotella*,  
365 *Francisella*; [137], and; *Lachnospiraceae*; [139, 140]) which have been investigated for specific  
366 applications. The molecular mechanisms for CRISPR-Cas9 and recent variants (Figure 1) have  
367 been well studied [77, 141].

368 Variants of Cas9 (i.e., CRISPR-Cpf1) were first used to edit genomes in humans, [46,  
369 113, 114] mice [142, 143] and flies [144]. CRISPR-Cpf1 from *Lachnospiraceae* bacterium  
370 (LbCpf1) is one of the most potent mutagens currently available [139, 140]. CRISPR-Cpf1 relies  
371 on base-pairing between the DNA target and the CRISPR RNA. The PAM region in Cpf1 is  
372 'TTTN' facilitating targeting to AT rich regions. Cpf1 creates 5' staggered ends allowing larger  
373 deletions than Cas9 and improving mutant identification. Additional variants allowing  
374 epigenetic modification, gene activation, and base editing [145] are currently being explored for  
375 their specific applications in plant genome editing. Next generation genome sequencing has  
376 allowed rapid, inexpensive and robust genomics platforms and identification of specific genome  
377 targets of interest. Genome editing technologies provide the basis through which those sequences  
378 may be altered.

379 DNA editing outcomes have been described in three tiers, or classes, designated SND1,  
380 SND2, and SND3 [1]. The events included in the SDN-1 class comprise targeted modifications  
381 resulting from NHEJ repair of broken chromosomes, including small deletions or insertions (or  
382 collectively, indels). Deletions are most often the frequent outcome with insertions being rarer  
383 [1, 105]. SDN-2 events occur when a targeted chromosomal break is repaired by HDR using  
384 sequence information from a DNA template resulting in single nucleotide substitutions or small  
385 indels [146]. The template sequence can be supplied as part of the genome editing process,  
386 where the template contains homology to the target DNA flanking the break site. The template  
387 sequence can also be provided by a sister chromatid or a homologous chromosome. In either case  
388 the template sequence is typically intraspecific. In contrast, SND3 events involve the site-  
389 specific insertion of large DNA sequences which may be cisgenic, transgenic or intragenic [1].  
390 The types of outcomes have distinct utility and application, and will likely be regulated  
391 differently. It is also important to note that the products of genome editing will likely be  
392 regulated as non-GMO by the USDA, FDA, the EU and many developing countries, which is  
393 covered later in this review.

394 However, these technologies are of limited use unless the reagents can be delivered and  
395 fertile plants recovered in desired plant varieties via transgenesis.

### 396 **3. Advances in plant transformation biology**

397 In order to understand the present technologies, needs, and future advances in plant  
398 transformation biology related to genomics and genome editing, it is important to put this into  
399 the context of historical milestone achievements. Plant transformation systems are complex;  
400 involving an understanding of plant developmental biology, molecular biology, plant physiology,

401 plant tissue culture, media modifications, explant biology, DNA delivery methods, selection of  
402 transformed cells, plant regeneration and genetics.

### 403 **3.1 Perspective on plant transformation**

404 A fundamental but often underappreciated technology for understanding the plant  
405 genome and utilizing plants to their greatest potential involves the capability to create, test and  
406 cultivate transgenics. This technology can introduce valuable agronomic genetic variation into  
407 crops, functionally link genes to biological functions, modify metabolic pathways, and create  
408 robust plant-abiotic and biotic stress resistance. Some of the most innovative and important  
409 scientific discoveries and agricultural enablements would not have been possible without  
410 transgenic technologies. Transgenics have created crops resistant to environmentally sustainable  
411 herbicides [147], plants resistant to viral and microbial pathogens [148-150] and stress-resistant  
412 crops [151]. Vitamin A and iron biofortification in maize and rice was made possible by  
413 transgenic technologies. The rice *sub1* gene was [152] discovered by genetic mapping and  
414 genomics and was an enabling technology that was made possible by transgenic technology.  
415 Indeed, a great number of trait genes that have been examined and demonstrated to be effective  
416 in plants have been made possible by transgenic biology.

417 At the basic scientific research level, transgenic technology in plants is essential to the  
418 actualization of functional genomics and the applicability of genome editing. The application of  
419 Koch's Principles renders robust transgenic capabilities an imperative functional genomics  
420 technology. The ability to knock-out or knock-down gene expression, conduct promoter  
421 analyses, observe over-expression characteristics, interrogate the effect of single base pair  
422 changes, make specific adjustments in protein structure and function, and the introduction of  
423 novel genes that convey traits pertinent to pest, stress and drought tolerance and a host of other

424 important agronomic characteristics, all depend on the ability to create and test transgenics.  
425 While the floral dip method of transformation has been very effective for a select few number of  
426 plants, such as *Arabidopsis thaliana* and *Camelina sativa*, this technique has not proven to be  
427 transferrable to the major crop species. The majority of plant transgenic biology is  
428 interdependent on cell and tissue culture.

### 429 **3.1.1 Morphogenic plasticity in tissue culture related to plant transformation**

430 Plants exhibit a wide array of morphogenic responses in tissue culture (Figure 2). While  
431 not the direct subject of this review it is worth noting in this context the relationship between  
432 tissue culture response, plant transformation, and regeneration. There are also generalized  
433 differences in plant transformation biology between the monocots and dicots. Plant tissue  
434 culture exploits the *in vitro* plasticity of existing plant growth and developmental pathways and  
435 there are many books and reviews devoted to this topic (e.g., George et al. [153] ). New cell  
436 types are not created in culture but rather existing cell types respond to environmental cues, such  
437 as media components, to undergo developmental gene expression patterns already present in the  
438 plant (Sussex, 1996, personal communication). These responses are influenced by the species or  
439 genotype, explant source, media components including plant hormone types and concentrations,  
440 carbon source, macro- and micro-nutrients and a host of other variables. Cell division and callus  
441 formation, embryogenesis and organogenesis can be manipulated in many plants through the  
442 control of these parameters. Using tissue culture regimes, fertile plants can be regenerated from a  
443 wide range of isolated plant cells or tissues from most plants. Figure 2 shows the various  
444 developmental pathways which have been demonstrated in tissue culture of various plants and  
445 from various explant sources. Appropriate tissue culture pathways and protocols can be used for

446 plant transformation, but this is usually genotype dependent, tissue culture and selectable marker  
447 responsive and explant dependent.

448 Plant transformation is considered the major bottleneck for the actualization of functional  
449 genomics and analysis of genome editing outcomes [2]. Reproducibility is essential and  
450 reliability is relative, however, transformation efficiency for some plants is considered by some  
451 still to be quite low [154]. The limitation of genotype dependence and/or low transformation  
452 efficiencies in some species has been due to several mutually inclusive criteria: the capability to  
453 produce developmentally significant events; proliferation of transformed cells amidst the  
454 senescing cells of the untransformed culture during selection, and retaining the potential for  
455 subsequent regeneration to fertile plants (totipotency). While much work has been done on media  
456 composition related to morphogenic plasticity, many plants have remained recalcitrant to  
457 transformation selection and subsequent regeneration. There are significant general  
458 developmental differences between many monocot versus dicot transformation and regeneration  
459 systems.

460 Efficient plant regeneration systems do not necessarily lead to efficient transformation  
461 and vice versa. In many monocot transformation systems there is a predominant reliance on  
462 direct or indirect somatic embryogenesis as the basis for transformation. In contrast many dicot  
463 species exhibit regenerable callus formation as a result of direct or indirect organogenesis from  
464 various explant sources which can be readily regenerated to plants. Some of these systems can  
465 also be used for robust and reliable plant transformation (e.g., tobacco, petunia, potato), while  
466 others remain remarkably recalcitrant. The process of selection also plays an important role.  
467 Transformed cells need to be selected from non-transformed cells and still be able to reiterate the  
468 culture and retain the ability to regenerate to fertile plants. A single cell must be able to survive

469 amidst non-transformed and dying cells and still retain the capabilities to reiterate a regenerable  
470 culture. Many results from both somatic embryogenic and organogenic pathways have shown  
471 that these events are initiated from a single cell transformants as deduced from Southern blot and  
472 sequencing analyses (See, for example [3]. Transformation systems are fundamentally different  
473 in dicots and monocots, between genera and species and are vrietally independent. on the basis  
474 of their tissue culture response.

475 Any plant transformation protocol which is based on a tissue culture intermediates will be  
476 subject to somaclonal variation. Somaclonal variation results from a variety of sources,  
477 including, single base pair substitutions, insertions and deletions, chromosomal rearrangements,  
478 and stochastic epigenetic changes. While these occur commonly in nature, in the context of plant  
479 transformation these variations have a specific significance, as the starting material from a  
480 specific variety may not be identical to the outcome due to these aberrations. This will mean that  
481 transformed plants will require significant backcrossing to recover a specific genotype with only  
482 the modification included. The preferred option would be to eliminate or drastically reduce tissue  
483 culture during transformation protocols.

484 Challenges to improved transformation include an increased and integrated understanding  
485 of the culture systems and the genetic basis of transformation parameters. Factors which  
486 influence transformation efficiencies such as genotype specific biology, media composition,  
487 temperature, recipient cell biology, necrosis inhibition and other media addenda [155] need to be  
488 tested and extended to other species and cultivars. For translational research, the required  
489 number of independent transformation events may reach large numbers (over 50) to recover  
490 simple insertions that express stable transgenes. Robust systems are needed that are genotype  
491 independent for both monocot and dicot systems with minimal tissue culture..

### 492 **3.1.2 A brief history of plant transformation**

493 Plant transformation was first achieved in the early 1980s and has been extensively  
494 reviewed elsewhere (for example, see [156] and [157]). The ability to transform plants is widely  
495 understood as a major scientific breakthrough that has fundamentally changed world agriculture  
496 and plant biology. Some of the key events and milestones in plant transformation are shown in  
497 Table 2. The first report of *Agrobacterium* DNA transfer to plant cells was in 1977 with the  
498 identification of *Agrobacterium* Ti plasmid DNA in plants exhibiting crown gall tumorigenesis  
499 [158]. Soon thereafter, *Agrobacterium rhizogenes* T-DNA was found to be localized to the  
500 nucleus [159]. However the use of *Agrobacterium* as a vector for plant transformation could not  
501 be realized until the genes involved with pathogenesis were removed to create 'disarmed' Ti  
502 plasmids into which DNA constructs could be inserted [160-162]. Expression of bacterial genes  
503 in plant cells was demonstrated in 1983 [161]. A binary vector concept for *Agrobacterium*-  
504 mediated DNA delivery was developed [163] and enabled a 'simple and general method' for  
505 genetically engineered plants with foreign DNA in 1984 [164, 165], followed by transgenic  
506 carrot plants were developed [166]. Antibiotic resistance genes, such as *nptII* and *hpt*, allowed  
507 selection of transformants from the non-transformed background. Herbicide resistance markers,  
508 such as the *bar* and EPSPS genes, allowed for applications in plants where antibiotic resistance  
509 was not applicable. Various promoters were then investigated for driving transgene expression  
510 including constitutively expressed promoters such as the 35S cauliflower mosaic virus  
511 (CaMV35S) promoter [167, 168].

512 Initial plant transformation methods relied on *Agrobacterium*-mediated gene transfer and  
513 was restricted to dicot plants (see review by [169]). Both direct and indirect organogenic tissue  
514 culture systems were amenable to DNA transfer, selection and subsequent plant regeneration in

515 some species. However, stable gene transfer to monocots remained elusive for nearly a decade  
516 despite extensive efforts, causing some to doubt as to its possibility [170]. Overcoming the  
517 obstacle of DNA delivery in monocots was first accomplished using rice protoplasts to produce  
518 the first transgenic monocots [171-174]; however, maize protoplast transformation and antibiotic  
519 selection resulted only in infertile plants [175, 176]. The GUS reporter gene system allowed  
520 visualization of delivered DNA allowing optimization to occur more rapidly [177].

521 Microprojectile bombardment ('the gene gun') was invented by John Sanford and Ted Klein in  
522 1987 [178] to overcome the barrier to *Agrobacterium* infection in monocots. The first  
523 transformed monocots were produced in 1988 via a protoplast based system and direct DNA  
524 delivery [174] but this approach was not shown to be effective for other cereals. Particle  
525 bombardment obviated the need for cumbersome protoplast systems, resulting in the first fertile  
526 transgenic maize using indirect somatic embryogenic cultures and improved herbicide resistance  
527 selection protocols [179, 180], specifically embryogenic suspension cultures and the *bar* gene as  
528 a selectable marker [180]. The development of fertile wheat transformation quickly followed  
529 using the same approach [181], and this technology transferred rapidly to successful  
530 transformation of sugarcane [182], rye [183] and tritordeum [184] and eventually other  
531 monocots. While enabling early monocot transformation, biolistic technology had several  
532 intrinsic disadvantages for cereal transformation, such as high frequencies of i) multicopy inserts  
533 resulting in transgene silencing, ii) integration of the vector backbone, iii) loss of transgene  
534 cassette integrity, and iv) the complications for gene expression studies. There are also basic  
535 biology and regulatory issues caused by these consequences. The pendulum swung back in the  
536 other direction when improvements to *Agrobacterium* vectors resulted in successful  
537 transformation and regeneration of monocots [185].

538 The discovery of tissue culture free transformation via the 'floral dip method' in  
539 *Arabidopsis* [186] has unfortunately not been widely applied to other plants. Currently,  
540 *Agrobacterium* remains the method of choice for DNA delivery in most monocot and dicot  
541 systems in combination with sophisticated tissue culture technologies.

542 These transformation technologies led to the development of many commercialized crops  
543 based on single gene transfer events, including: Bt maize developed for insect resistance [187];  
544 the first commercialized GE crop in U.S. (the FlavrSavr tomato); herbicide resistant soybean  
545 [188], and eventually, widespread commercialization of GE maize, soybean, papaya, sugarbeet,  
546 cotton, canola, and alfalfa. While many plants have been transformed with valuable trait genes,  
547 the high cost of de-regulation and negative public perception has prevented many of these from  
548 being agriculturally realized. Advances in genome sequencing have led to a fundamental  
549 understanding of plant genetics [189, 190]. The ability to use this information for advanced  
550 breeding is a significant achievement to world agriculture. Now the ability to harness this  
551 information for genome editing is another exponential advancement [106] [118]. The remaining  
552 piece is the advance of transformation technologies.

553 Fundamentally, standard plant transformation systems are complex but depend on three  
554 key processes: 1) DNA transfer and integration into a host recipient cell; 2) ability to select  
555 transformed cells from non transformed cells; and 3) ability to regenerate adult fertile plants  
556 from single totipotent transgenic cells. Basic biological differences between monocots and  
557 dicots play a major role in successful transformation protocols. The current systems for  
558 transformation of monocots and dicots provide insight into the future needs and developments.

### 559 **3.1.3 Monocot transformation now and the future**

560 Significant improvements in reliable monocot transformation technologies have relied on  
561 *Agrobacterium*-based protocols [191, 192]. *Agrobacterium*-mediated transformation of cereals,  
562 such as maize, rice, wheat, barley, sorghum, millet, oats, Triticale, and rye (references cited in  
563 Table 2), is the current method of choice for cereal transformation and has largely superseded  
564 direct DNA transformation methods for generating transgenics [185, 191, 192]. *Agrobacterium*-  
565 mediated transformation is also widely applied in many genome editing strategies [193]. The  
566 reasons for the widespread utility of *Agrobacterium*-based methods are that it provides a number  
567 of significant improvements over direct DNA transformation. These are: i) reliability and the  
568 ability to generate large numbers of independent events; ii) low copy integration of transgenes  
569 with defined borders; iii) transfer of relatively large intact DNA constructs; iv) consistent and  
570 stable integration of transgenes and their transmission to progeny; and, v) consistent transgene  
571 expression have all contributed to the widespread use of *Agrobacterium* DNA transfer.

572 It is often assumed that significant improvements to monocot transformation were the  
573 result of developments in enhanced *Agrobacterium* strains [194, 195] for DNA delivery. What is  
574 not as well appreciated, however, includes that a number of complementary factors are essential  
575 for reliable and efficient cereal transformation including: i) advances in monocot tissue culture of  
576 'early' embryogenic cells (such as [196]) as transformation competent recipients [154]; ii)  
577 identification of suitable explant sources [191] that produce these types of cell cultures (e.g.,  
578 immature embryos and embryogenic callus); iii) careful selection of genotypes amenable to  
579 production of such embryogenic cultures [191]; iv) use of appropriate selectable markers for  
580 monocots; v) complex media alterations [191]; and vi) a number of species-specific refinements  
581 including genotype dependent cell culture responses [155].

582 Improved tissue culture protocols primarily from immature embryo explants and calli  
583 derived from scutellar tissue coupled with the development of super-binary T-DNA vectors  
584 [194, 195] enabled highly efficient *Agrobacterium*-mediated rice transformation without  
585 restricted genotype dependence for indica, japonica and javanica cultivars (reviewed by [197]).  
586 However, in most other cereal crops (e.g., maize, wheat, barley and sorghum) *Agrobacterium*  
587 transformation remained restricted primarily to genotypes selected for their ability to produce  
588 friable Type II [196, 198-201] embryogenic callus such as: the maize inbred line A188 [202], Hi  
589 II [198]; the Bobwhite line in wheat [203]; and Schooner in barley [204]. Some success has been  
590 achieved with extension of transformation capabilities to elite cereal germplasm [205-210]  
591 through media modifications to enhance embryogenic culture production, but genotype  
592 independence has been elusive until recently (See section 6.4 below). While more  
593 developmentally organized and compact tissue culture systems referred to as Type I (in  
594 comparison to the 'friable embryogenic culture called Type II), in maize [201] and barley [211],  
595 are often highly regenerable, such systems have been only marginally successful for  
596 transformation. Most maize varieties are capable of producing Type I cultures, which are not  
597 amenable to transformation, selection and subsequent plant regeneration (Kausch, 1995,  
598 unpublished). A paucity of recipient cells that remain totipotent and independent of surviving  
599 selection to form regenerable transgenic colonies are characteristics understood to be under  
600 cultivar specific genetic control and a major limiting factor to cereal transformation technology  
601 development [154]. Currently, transgene introgression is used for modification of elite  
602 germplasm.

603 Efficient selection of stably transformed cells from the overwhelming number of non  
604 transformed cells is another essential component for successful transformation and regeneration

605 of transgenic plants. The early dicot transformation systems relied on resistance to  
606 aminoglycosides [156], such as kanamycin, neomycin and G418, which proved ineffective in  
607 most cereal crops [154]. Selection of maize, wheat, and barley transformants has been most  
608 effectively achieved using herbicide resistance markers including the *bar* gene [180, 181] for  
609 bialaphos resistance; the *als* gene [179] for chlorsulfuron resistance; the mutant *epsps* gene [212]  
610 for glyphosate resistance and for metabolic selection, the *pmi* gene [213, 214] using mannose.

#### 611 **3.1.4 The centrality of early somatic embryogenic cultures in monocot transformation**

612 The centrality of early somatic embryogenic (SE) cultures in monocot transformation has  
613 been seen as essential for improved transformation [4, 215, 216]. This is understood as a  
614 generality and there are many exceptions. Somatic embryos are differentiated outcomes derived  
615 from vegetative cells (single or a small group) which undergo development to become  
616 independent mature plants. Unlike zygotic embryos, which are formed from the outcome of  
617 fertilized gametes, somatic embryos are genetic clones from the explant parent [217]. Direct  
618 somatic embryogenesis refers to the formation of embryos from a single mature cell whereas  
619 indirect somatic embryogenesis can result via a callus intermediate. Given the attributes of single  
620 cell origin and the capability for regeneration to fertile plants renders somatic embryogenesis as  
621 ideal for production of transgenic or genome edited plants. 'Early' somatic embryogenic cultures  
622 resemble the early stages of zygotic embryogenesis and are retained in that developmental state  
623 by genetic and tissue culture response. In many monocots, for example, these stages in culture  
624 resemble zygotic embryos at the 5-8 days after pollination (DAP), hence the descriptor of 'early'.  
625 The stages of somatic embryogenesis are very similar to the stages of zygotic embryogenesis in  
626 monocots and dicots [218, 219].

627 Broad scale molecular and cellular changes are required for somatic embryogenesis. In  
628 some plants this requires an initial de-differentiation step where the differentiated cells in the  
629 explant must undergo a re-programming in order to become stem-cell like. In general, less  
630 developed tissues are more amenable to de-differentiation and somatic embryo formation. In  
631 many monocots, the scutellar epidermal cells of immature zygotic embryo explants are often a  
632 preferred source of these cell types. In order to acquire totipotency, cells must be competent to  
633 respond to signals which will cause a gene expression pattern profile recapitulating that of  
634 zygotic embryogenesis. Lastly, initiated cells undergo wide-ranging gene expression patterns  
635 involving cell cycle, signal transduction pathways, meristematic axis development and  
636 commitment to embryogenesis [220]. Genomic [216] and molecular genetic studies [3] show  
637 that expression of embryo and meristem specific transcription factors and/or loss of chromatin  
638 modifying proteins are involved with the induction of somatic embryogenesis (see review by  
639 Horstman et al. [221], and references therein).

640 A typical procedure to initiate somatic embryogenic callus was first developed by Green  
641 and Phillips [201] and relies on immature (10-12 DAP) zygotic embryo explants. Studies with a  
642 focus on plant somatic embryo development in culture have shown that the ability to produce  
643 friable embryogenic cultures (Type II) are genotype dependent [222, 223], and largely controlled  
644 by auxin/cytokinin ratios and other media components to achieve transcriptional reprogramming  
645 [221]. Friable somatic embryogenic cultures often provide the basis for successful transformation  
646 and plant regeneration protocols, meeting all of the criteria previously described.

647 Although transformation (DNA transfer and integration) may be achieved at reasonably  
648 high frequencies in many cell types, the ability to transform, and generate totipotent target cells  
649 which can be recovered and regenerated to plants may be a significant limiting factor.

650 Furthermore, *Agrobacterium*-mediated transformation, most often, especially in the monocots,  
651 must involve only recipient target cells at the surface of infected tissues. For many monocot  
652 systems, transformation procedures rely on scutellar epidermal cells of immature zygotic  
653 embryos. Particle bombardment also involves mainly surface cells [215]. Another limitation of  
654 genome editing and transformation is the relatively few numbers of effective selectable markers  
655 available for use with most plant species. The ability to test multigenic constructs and gene  
656 stacking capabilities in standard transgenic applications currently rely on multiple selection  
657 strategies. For multiple genome editing events, this will be less of a factor. Hence, the relatively  
658 low transformation efficiency, as measured by number of independent events per treated explant,  
659 is not understood as the major obstacle, but rather the ability to reliably recover the independent  
660 number of transgenics to biologically replicate and address a given experimental questions is the  
661 major obstacle.

662 Somatic embryogenesis is most often genotype limited [216, 224] despite long efforts to  
663 improve the conditions for tissue culture. The somatic embryogenic (Type II) tissue culture  
664 response is now understood as a quantitative trait locus (QTL) controlling gene networks  
665 involved with the developmental responses for embryo formation *in vitro* [216]. Reverse  
666 genetics has been used to identify several genes involved with somatic embryogenesis in various  
667 plant species, including maize, wheat, pine, soybean, cotton, *Arabidopsis*, and coffee [220, 225-  
668 227]. QTL mapping has been used across several plant species for identification of genetic  
669 factors using populations segregating for embryogenic tissue culture response [199, 228-231].  
670 Lowe et al. [229] used marker assisted breeding to move the high tissue culture response  
671 phenotype to the recalcitrant maize inbred FBL1 further substantiating the genetic underlying  
672 mechanisms. Using a QTL fine mapping approach, Salvo et al. [216] identified and validated

673 novel genes involved with somatic embryogenesis. From work done on mutants in the model  
674 system *Arabidopsis*, a network of transcription factors are known to be involved with somatic  
675 embryogenesis [221].

### 676 **3.1.5 Dicot transformation now and the future**

677 Dicot plants are composed of a huge group of plant species including well-known  
678 legumes, vegetables, fiber producing plants (e.g., cotton), as well as horticulture crops. As a  
679 result, regeneration and transformation systems are much more diverse than with the monocots.  
680 Historically, dicot plants have been considered to be more amenable to tissue culture,  
681 transformation and plant regeneration than monocots. Both *Agrobacterium*-mediated and  
682 particle bombardment methods for transformation have played important roles in advancing  
683 plant biology studies and production of commercial crop varieties [232]. Dicot transformation  
684 was first successfully achieved through introduced DNA constructs into the root cells of the dicot  
685 *Daucus carota* (carrot) via *Agrobacterium rhizogenes*-mediated transformation [233]. The very  
686 first successful transformation recovering fertile plants was achieved in *Nicotiana tobacum*,  
687 another dicot species, using *Agrobacterium tumefaciens*-mediated transformation of leaf disks  
688 [165]. This 'simple and efficient transformation' system quickly made this plant species a model  
689 system and was then subsequently adopted by worldwide researchers. Soon afterwards, stable  
690 transgenic tomato and *Arabidopsis* plants were developed via *Agrobacterium*-mediated  
691 transformation of leaf-disks and roots, respectively [234, 235]. The tissue culture regime in  
692 these studies was based on organogenesis with a short immediate callus phase, followed by  
693 regeneration, provided a fast and simple transformation process. By using selectable marker  
694 genes and the use of chemical selection only transgenic events were recovered against the  
695 background.

696 Two decades of extensive efforts in development of simple and efficient transformation  
697 protocols led to the establishment of *in planta* transformation systems in a limited number of  
698 plants. The most significant accomplishments resulted from methods for *in planta* transformation  
699 of *Arabidopsis*, by 'floral dip' which opened the way to significant research. Tissue culture free  
700 floral dip in *Arabidopsis* revolutionized model dicot plant functional genomics and substantially  
701 accelerated research in basic plant molecular biology [236, 237]. However, despite these  
702 efforts, this approach has only been successful in a very few dicot or monocot plants, as  
703 demonstrated by the lack of references in the field, and therefore these procedures have not been  
704 widely adopted. Other attempts to circumvent the *in vitro* tissue culture phase for plant  
705 transformation have not borne the test of scientific scrutiny or reproducibility, including  
706 nanotechnology approaches [238], pollen tube-mediated transformation [239], pollen  
707 bombardment [240], pollen electroporation or sonication [241] , and pollen magnetofection  
708 [238]. If any of these procedures were robust, they would already be widely used. Clearly more  
709 research into these methods is necessary.

710 The first ground breaking research in dicot transformation for agriculture  
711 biotechnological applications was accomplished by Monsanto research group led by Horsch  
712 [242]. Hinchee and her co-workers developed a successful *Agrobacterium*-mediated  
713 transformation system in soybean (*Glycine max*), a highly recalcitrant dicot crop species, that is  
714 still difficult to transform to date. This success relied on utilization of totipotent stem cells  
715 located in the cotyledonary-node area of germinating seeds and plant regeneration was through  
716 direct shoot organogenesis where no intermediate callus phase was involved. This regeneration  
717 regime offers certain advantages over indirect organogenesis in that the transformation process is  
718 fast and simple, enables high fertility and retains genome stability of transgenic plants.

719 Subsequently, similar systems were developed or modified to transform a number of other  
720 legume species [243]. Meanwhile, other stem cell containing explant tissues from the same or  
721 different plant groups have been also explored to determine alternative yet simple regeneration  
722 processes. For example, regeneration and transformation systems have been developed using  
723 primary leaf node, hypocotyl, shoot tip, embryogenic axis, or immature cotyledonary-node with  
724 varying successful rates [244]. Therefore the dependency on organogenesis in dicot tissue  
725 culture systems has provided the basis for transformation protocols.

726 As more transformation efforts were made for various dicot species, plant genotype,  
727 explant tissue, *Agrobacterium* strain and infection conditions, selectable markers and agents,  
728 gene delivery systems, etc. were all found to influence transformation outcomes [245-248]. For  
729 example, *Agrobacterium* strains EHA101, EHA105, and GV3101 were found to be effective to  
730 transform soybean, citrus, and tomato, respectively [249-251]. Thus, researchers made intense  
731 efforts in optimization of *Agrobacterium* infection conditions for dicot transformation. Of many  
732 studies, employment of various media components, such as antioxidants, in particular L-cysteine  
733 and dithiothreitol, during the infection stage made new major breakthroughs in dicot  
734 transformation, leading to multiple fold increases in transformation efficiencies in several dicot  
735 plant species [252-257].

736 In addition to the *Agrobacterium*-mediated transformation, other gene delivery systems  
737 have been also employed early and successfully to transform various dicot plant species. For  
738 example, a number of legume species have been also transformed by microinjection, silicon  
739 fibers, electroporation, and micro-projectile bombardment [245, 246, 248]. Nonetheless,  
740 transformation of dicot species has been primarily most efficiently accomplished via  
741 *Agrobacterium tumefaciens*-mediated methods.

742 Plant regeneration through somatic embryogenesis regime has been also deployed for  
743 those dicot plant species where organogenesis was found to be difficult. For example, plant  
744 regeneration via somatic embryogenesis from cotton and coffee as well as many woody plants  
745 has been a routinely practice [258-260]. Somatic embryogenesis has been also used in soybean  
746 [261, 262]. As a result, somatic embryogenesis system lays a foundation for the transformation  
747 or micropropagation of these plant species. However these approaches remain heavily genotype  
748 dependent.

749 The majority of transformation efforts and success in the dicots have been made by use of  
750 organogenic systems, appropriate explant types, more virulent *Agrobacterium* strains, media  
751 modifications during the infection stage, suitable vectors, function promoter driven gene  
752 expression, and appropriate selectable markers. Moreover, *Agrobacterium*-mediated  
753 transformation via a shoot organogenesis regime has been a dominant system in the dicot  
754 systems, enabling integration of low copy transgene and stable transgene expression.

### 755 **3.1.6 The centrality of organogenesis in dicot transformation**

756 Organogenesis in plants results in the differentiation of various organs, including roots,  
757 shoots, stems leaves and flowers without the requirement of preexisting initials. In tissue culture  
758 organogenesis, usually involves the dedifferentiation of mature cells in an explant followed by  
759 cell differentiation and the formation of organ primordia [263]. Direct organogenesis refers to the  
760 formation of primordia without an undifferentiated phase whereas indirect organogenesis can  
761 result via a callus intermediate. Organ development is an iterative process in plant development  
762 initiated by meristems. All above-ground organs in plants are derived eventually from a shoot  
763 apical meristem (SAM) which differentiates lateral organs in phylotactic patterns. Cell layers in  
764 the SAM are defined as L1, L2, L3 and a central zone of cells which replenish the meristem

765 [263]. From the SAM, cells transition from the morphogenetic zone to become differentiated  
766 organs according to the Hofmeister principle where new organ form maximally distant from  
767 previous organs, and auxin plays a major role [264]. Organogenesis in tissue culture requires  
768 fundamental changes in gene expression regulatory networks, developmental biology and cell  
769 physiology. Somatic direct organogenesis is most often derived from a single cell, as has been  
770 demonstrated by Southern blot analysis on many dicot transgenic plants. It appears that a single  
771 cell may also be the origin of multicelled groups which will form a SAM in tissue culture via  
772 indirect somatic embryogenesis as also indicated by Southern blot analysis. Organogenenic  
773 pathways toward success plant transformation, selection and regeneration has been clearly  
774 identified using GUS and GFP reporter genes. The process or organogenesis is critical for  
775 efficient transformation and regeneration in many dicot species.

776         The transition from differentiated cells (direct organogenesis) or undifferentiated callus  
777 (indirect organogenesis) involves changes in gene expression, auxin mediated development, and  
778 miRNAs (reviewed by [265]). A number of factors involved in organogenesis have been  
779 identified, including transcription factors such as KNOTTED-LIKE HOMEBOX (KNOX)  
780 genes, the MAB4 gene family, including MEL1 and MEL2, are involved with expression of PIN  
781 efflux proteins directing polar auxin transport [265]. However, auxin transport is not the sole  
782 controlling factor in organogenesis as cytokinin also plays a critical role. For example the  
783 *Arabidopsis* mutant for the cytokinin ARABIDOPSIS HISTIDINE PHOSPHOTRANSFERASE  
784 PROTEIN 6 (AHP6) affects phyllotactic patterning [266]. Also in *Arabidopsis* CLAVATA3  
785 (CLV3) negatively regulates *WUS* which is required for cell division and presumably for the  
786 maintenance of the central zone in SAM [267]. Increased *WUS* expression in *clv* mutants results  
787 in an enlarged and fasciated SAM. Manipulation of tissue culture response in some systems

788 results in robust SAM formation and has become the basis for many dicot transformation  
789 protocols.

#### 790 **4. Looking towards the future in transformation, genomics, and genome editing**

##### 791 **4.1 Monocots**

792 'Transformability' is now understood as a complex of interdependent and inclusive  
793 systems characteristics. The systems comprise a number of biological processes such as: i)  
794 accessibility to DNA introduction; ii) and stable chromosomal integration of the transgene; iii)  
795 genotype-specific cell culturability; iv) selectability of totipotent transformed cells; and, v) fertile  
796 plant regeneration from stably transformed cells. These mutually exclusive criteria have  
797 rendered many transformation methods that are complicated, only partially communicated in any  
798 single reference, evolving incrementally over time and are species and genotype specific.  
799 Relative low efficiency (as measured by number of independent events per treated explant) was  
800 not understood as the major obstacle, but rather the ability to reliably recover the required  
801 transgenics to address a given experimental criteria was the primary goal. While reliable,  
802 transformation efficiency for some crops may be considered to be low transformation is nearly  
803 always the result of single cell events giving rise to homogeneous transformed plants. This is  
804 determined from Southern blot analyses, where extremely high efficiencies would result in  
805 complex heterogeneous events. Transformation and regeneration has been limited to those  
806 genotypes which provide embryogenic or organogenic cultures that are capable of reiteration  
807 from a single transformed cell with and callus proliferation amidst the senescing cells of the  
808 untransformed culture during selection and capable of subsequent whole plant regeneration to  
809 fertile plants. As a result successful monocot transformation methods are often, *complex, multi-*  
810 *step protocols*, whereby small improvements are accrued over time, minute method compliance

811 is mission-critical, and hands-on experience matters significantly. Genotype independence for  
812 monocot transformation is a major goal.

813           In order to meet the growing need for transgenics for plant genome editing and analysis,  
814 the transformation platform of the future must not only integrate the required characteristics of  
815 current systems and facilitate high-throughput generation of events [191], but utilize a set of new  
816 tools currently available for genome modification and analyses. Recent advances in plant  
817 transgenic biology are rapidly changing the landscape towards the long range goals of being able  
818 to transform and hence edit any plant with any DNA target sequence. Currently, there are  
819 various pathways to produce genome edited events using plant transgenics. The selection of  
820 strategy is predicated on various issues already discussed including efficiency, intellectual  
821 property, regulatory issues and crop species applications.

822           Significant advances in monocot transformation have been realized by utilizing genes  
823 involved in somatic embryogenesis [3-5]. Manipulation of gene expression through differential  
824 expression of morphogenic regulators, such as BBM and WUS2, involved with somatic  
825 embryogenesis has provided a new platform for transformation.

## 826 **4.2 Dicots**

827           Due to the economic importance of diverse dicot plant species, it is imperative to take  
828 advantages of genomic resources and genome editing technology to advance dicot plant biology  
829 study and variety development. Evidently, efficient transformation systems are a prerequisite for  
830 accomplishing this goal. Despite significant progress has been made in dicot transformation (see  
831 previous section), there are still several factors that constrain efficient dicot transformation in  
832 some recalcitrant species. First, genotype dependency is common rather than exception for the  
833 transformation of most of dicot species. For example, success in cotton transformation has

834 achieved in only one cotton genotype, i.e., the Coker line or its derivatives. Second, high rates of  
835 chimerism and non-germline events are common in those dicot transformation systems whose  
836 regeneration regime is based on direct shoot organogenesis without intermediate callus phase.  
837 The mechanisms of these constraints are still unknown. Third, although the present predominant  
838 view considers plant regeneration rather than gene transfer process as the bottleneck, numerous  
839 dicot plant species do display good regeneration ability through shoot organogenesis but suffer  
840 from poor *Agrobacterium* infection. Use of BBM/WUS has enabled efficient somatic  
841 embryogenesis in monocots [3-5]. Use of plant morphogenic regulators offers at least two key  
842 advantages: 1) it enables formation of totipotent cells to become regenerable from non-  
843 regenerable somatic cells; 2) it may induce indirect shoot organogenesis with a short  
844 intermediate callus phase. In dicots, shoot organogenesis without an intermediate callus stage  
845 rarely produces chimeric or non-germline events, even though the mechanism of this observation  
846 remains elusive (Zhang, unpublished data). Embryogenic and organogenic pathways are not  
847 mutually exclusive. There are monocots which exhibit organogenic pathways which can be  
848 exploited for transformation. Similarly, in dicots there are embryogenic pathways. Efficient  
849 somatic embryogenesis has been achieved in recalcitrant sweet pepper (*Capsicum* L.) using  
850 BBM/WUS [268]. However, as of today, there have not been any reports on enabling dicot plant  
851 shoot organogenesis by co-expression of WUS and a gene underlying organogenesis. Thus, it  
852 will be interesting to investigate if this approach would allow making breakthroughs in  
853 transformation and plant regeneration of dicot species.

#### 854 **5. Pathways to produce viable plant genome edited outcomes**

855 Genomics advances clearly have improved DNA sequence target identification.

856 Advances in genome editing have enabled modification of those targets. Plant transformation

857 biology has extended this information to testable events. The ability to transfer genes in plants  
858 for genetic improvement has revolutionized many aspects of basic plant science but also for  
859 practical agricultural applications. These technologies have enabled the development of new  
860 commercial crop varieties that have been planted on over 2 billion ha (4.9 billion acres) over the  
861 past 20 years [269]. Taken together, genomics, genome editing and plant transformation  
862 together will forever change agriculture. There has not been a more significant improvement to  
863 agriculture since the plow. Current work will provide the basis for precise genome editing in  
864 multiple crops which is now essential.

865         The application of stably integrated events by standard plant transformation technology  
866 to produce desired outcomes has now been widely applied across many crops, but has been  
867 hampered by several bottlenecks [2] including technological, intellectual property and regulatory  
868 issues. Some of the considerations for the choice of plant transformation technologies in  
869 commercial settings involve Freedom to Operate (FTO) and subsequent regulatory concerns (see  
870 later section). These restrictions govern some decisions regarding plant transformation strategies.  
871 Subsequent to the recovery of the targeted events, backcrossing is usually used to introduce the  
872 genome edited event into the varieties of commercial interest. Many events using this approach  
873 have already been commercialized [270]. Yet this approach has several downsides, including: (a)  
874 long development time, due to the backcrossing process; (b) genotype limitations, due to tissue  
875 culture abilities, or explant limitation such as the requirement for immature embryos or other  
876 tissues; and, (c) costly if they need to be deregulated as GMO. Direct genotype independent  
877 transformation, especially for genome edited outcomes then has become a significant goal.

## 878 **5.1 Protoplast based systems**

879 Transformation of protoplasts as recipient cells has been utilized for decades [271].  
880 Macromolecules can be delivered to protoplasts via various techniques including electroporation,  
881 PEG, liposomes, and heat shock [272]. The protoplast systems were developed early in plant  
882 transformation history, but then fell out of favor for the use of easier technologies. While some  
883 systems, particularly many dicots, can be transformed with high efficiencies using protoplasts,  
884 others are prohibitively recalcitrant. The first transgenic rice plants were created using  
885 protoplasts [174]. For plant which are amenable to this approach, this system provides a high  
886 degree of efficiency for genome editing results and has resulted in many significant commercial  
887 products. In addition, protoplasts provide a robust system to test transient gene expression [273].  
888 Protoplasts derived from embryogenic suspension cultures became an early focus of cereal crop  
889 transformation research [274], but it became quickly apparent that these systems are extremely  
890 labor intensive, did not provide recovery of fertile transgenic plants [175, 176]. There is an  
891 inverse correlation between the production of viable protoplasts from maize embryogenic  
892 suspension cultures and their ability to be recovered as fertile plants (Kausch, unpublished).  
893 However, protoplast systems have seen a recent renaissance in current applications of genome  
894 editing for the introduction of DNA, RNA, and ribonuclear proteins (RNPs). These  
895 macromolecules can be directly delivered to protoplasts at high efficiencies, and can be  
896 regenerated to plants with or without selectable markers (Figure 3A). These plants can be  
897 subsequently screened using various molecular techniques for recovery of stably edited fertile  
898 plants in some species. For some plants amenable to protoplast technology, this represents a very  
899 attractive and viable approach which negates some difficulties with other methods, including  
900 regulatory and FTO restrictions. The negative aspects are that this approach remains very labor  
901 intensive and restricted to only some plants or genotypes.

902 **5.2 Particle bombardment**

903 Particle bombardment (biolistics) can also be used to deliver various macromolecular  
904 reagents used for gene editing and obviates the use of protoplasts, but suffers from various other  
905 drawbacks. While the macromolecules of interest for genome editing can be delivered by  
906 particle bombardment without the use of a selectable marker ([123, 124], see 3B) this approach  
907 has relatively lower efficiencies of delivery, species and explant dependence. This approach is  
908 attractive for a variety of reasons (see below) but requires a robust tissue culture and  
909 regeneration response which will not be genotype independent. Particle bombardment has been  
910 used to introduce genome editing functions with and without selectable marker genes, where  
911 stably integrated events can be subsequently segregated away in subsequent generations.  
912 Particle bombardment has an apparent regulatory advantage over *Agrobacterium*-mediated  
913 transformation since it does not involve the use of a plant pathogen. This difference may have a  
914 regulatory advantage. In addition, it may be possible to use particle bombardment to produce  
915 genome edited events which are DNA free, using only RNAs and RNPs, providing perhaps  
916 another regulatory advantage as a mutagenesis approach to advanced breeding [123, 124].  
917 Particle bombardment also eliminates specific *Agrobacterium* plant specific interactions.  
918 Segregation of introduced transgenes can be achieved and verified with whole genome  
919 sequencing and other methods. This approach may be considered as advanced mutagenesis and  
920 regulated as non-GE in some cases and perhaps only by some countries.

921 **5.3 *Agrobacterium*-mediated transformation**

922 The use of *Agrobacterium* vectors to deliver constructs that include genome editing  
923 functions using well known transformable genotypes, recovering stably integrated events and  
924 subsequently segregating the transgene away from the edited target has been demonstrated [77].

925 However, *Agrobacterium* is widely considered as a plant pest and therefore edited plants may be  
926 regulated as a GE organism. The additional bottlenecks with this approach include the use of  
927 *Agrobacterium* for construct delivery, genotype dependence, explant dependence, tissue culture  
928 response, regulation hurdles and time in development. The next step(s) subsequent to recovery of  
929 targeted events require backcrossing the targeted edited event into the varieties of commercial  
930 interest. Many events using this approach are already in the process of commercialization (SIVB  
931 meeting 2018). This is largely the current state of the art, but has several downsides, including:  
932 (a) long development time, due to the backcrossing process and linkage drag; (b) genotype  
933 limitations, whether due to tissue culture abilities, or explant limitations, such as the requirement  
934 for immature embryos or other tissues; and, (c) costly if they need to be deregulated as GE  
935 organism. The technology using gene editing including ZNFs [94-101], TALENS [102-111] and  
936 CRISPR ; [6, 46, 47, 112-124], have already been widely applied. Therefore, both particle  
937 bombardment and *Agrobacterium*-mediated delivery can be applied for genome editing purposes  
938 (Figure 3C) and with or without selection and without stable DNA integration (Figure 3D).

#### 939 **5.4 Morphogenic regulator-mediated transformation**

940 The development of advanced transformation technologies has been explored through the  
941 manipulation of key early somatic embryogenesis genes to promote transformation and plant  
942 regeneration. Promoting early stages of somatic embryogenesis (SE) in recalcitrant cereal crop  
943 species and varieties has been a key for making a breakthrough in developing advanced cereal  
944 transformation technologies. Presently, however, our knowledge about the mechanisms  
945 underlying this somatic embryogenesis (SE) is limited. On the other hand, SE in general mirrors  
946 zygotic embryogenesis (ZE). Studies on ZE are more advanced and most of key genes  
947 underlying early ZE have been identified. Thus, the knowledge of early ZE can be translated to

948 SE. Since no new cell types are actually created in culture (Sussex 1995, personal  
949 communication) we assume that a common set of genes govern both SE and ZE in their early  
950 developmental stages except that gene functions for a transition from somatic cell to embryonic  
951 competent cells are required in SE. Indeed, emerging evidences show that early SE includes  
952 three steps: dedifferentiation, expression of totipotency, commitment, and development and each  
953 step requires SE-related genes (Figure 4). Efficient transgenic cell selection requires early  
954 embryogenic totipotent cell types. Therefore, it is early SE that plays a critical role in  
955 determining the success in SE and plant transformation, especially in most monocots. Recent  
956 experiments show that a novel strategy to promote early somatic embryogenesis through  
957 differential expression of key developmentally important candidate genes, alone or coordinately  
958 can promote genotype independent transformation. Additional candidate genes involved in each  
959 of these processes which can be manipulated are shown in Figure 4.

960 Cereal crops, specifically maize sorghum and rice, have varieties which have been  
961 traditionally recalcitrant to transformation, including the reference genomes. Many sorghum  
962 varieties, exhibit low rates of SE, transformability and plant regeneration. Sorghum SE is quite  
963 representative of many other cereal plant species especially recalcitrant species which show a  
964 typical “Type I” embryogenic callus and relatively short developmental time frame. Type I callus  
965 is not efficient and often incompetent for plant transformation, yet exhibits misleadingly highly  
966 regenerability. The sorghum genome has been sequenced, lacks genome duplications (as  
967 compared with maize), and shares a high sequence homology and synteny with maize and, to  
968 large degree, with rice (gene/sequence resources are available). Therefore, results from our  
969 sorghum SE studies is applicable to other cereals including recalcitrant sorghum genotypes,  
970 maize inbred lines, millet, *Setaria* and extending to other species.

971           It remains possible that other early ZE genes such as *PIN1* or 7 (*PINFORMED 1, or 7*) as  
972 well as *WRKY 2* (*WRKY DNA-BINDING PROTEIN 2*) and *WOX2, 8, and 9* (*WUSCHEL*  
973 *RELATED HOMEODOMAIN 2, 8, and 9*) may play a critical role in SE as it related to efficient event  
974 recovery for some plants. These genes are essential in cell polarity and asymmetric division as  
975 well as apical-basal axis formation which may cause somatic cells to convert to embryonic cells.  
976 These experiments should be tested by direct effects on not only SE but also transformation and  
977 subsequent plant regeneration efficiency. A comprehensive plan to differentially express SE  
978 genes responsible for totipotency and commitment, individually or pair-wisely could be  
979 implemented. For pair-wise differential expression, each pair of *WUS* and *BBM*, *WUS* and *LEC1*,  
980 *SERK1* and *BBM*, or *STM* and *BBM* would need to be differentially co-expressed to be  
981 adequately tested. Moreover, additional co-expression may include each pair of *PIN7* and *BBM*,  
982 *WOX2* and *BBM*, *PIN7* and *WUS*, or *WOX2* and *WUS*. The co-expression of some of these pair  
983 genes may lead to a conversion from somatic cells to embryogenic cells and even to somatic  
984 embryo development. Further analysis of these genes may lead to enhanced SE in plants,  
985 enabling genome editing and would inform our understanding of plant development and  
986 differentiation.

987           It is likely that the morphogenic regulators that are involved with organogenesis will also  
988 have a significant impact on the ability to affect plant transformation and regeneration. This  
989 approach may be most significantly impactful with recalcitrant dicot species. Although, the  
990 approaches through somatic embryogenesis and organogenesis are not mutually exclusive or  
991 divided between the monocots and dicots. There is a need to understand organogenesis as a  
992 viable route to transformation pathways.

993 Various genes have been overexpressed in transgenic plants to test their ability to induce  
994 a somatic embryogenic response in tissue culture (reviewed by [221, 222]). Some of these  
995 genes are morphogenic regulators encoding transcription factors including: Agamous-Like-15,  
996 AGL15 [275]; Baby boom, BBM [276]; Leafy Cotyledon1 [277]; *Lec1* [278]; Somatic  
997 Embryogenesis Receptor-Like Kinases, SERK [279]; and Wuschel, WUS [280]. Differential  
998 overexpression of AGL15, BBM, SERK and WUS has been demonstrated to improve  
999 embryogenic response and reduce genotype dependence [278, 281-283]. Recently published  
1000 research from Lowe et al. [3, 4] and Mookkan et al. [5] confirmed the effective use of genetic  
1001 mechanisms to control somatic embryogenic callus formation, and the re-differentiation of  
1002 organs and somatic embryos from different tissues through discriminatory expression of  
1003 morphogenic regulators, WUS2, and *BBM*. Initial attempts utilized an Oleosin promoter driving  
1004 expression of WUS2 were not successful resulting in chimeric events [4]. The differential  
1005 expression of BBM and WUS results in direct somatic embryogenesis. However, resulting  
1006 transgenic cultures either could not be regenerated or resulted in aberrant phenotypes [4]. Further  
1007 experimentation utilized the strong maize *Ubiquitin* promoter (*ubi*; [284]) driving BBM  
1008 expression and the weak nopaline synthase (*nos*) promoter from *Agrobacterium* [285] driving  
1009 WUS2. In order to recover regenerated plants the expression of BBM, WUS2 must be removed.  
1010 To accomplish excision, the desiccation inducible *rab17* promoter from maize [286, 287] was  
1011 used to express the CRE recombinase gene [288-290]. One of the constructs (Figure 5A)  
1012 described by Lowe et al. [4] was designed with loxP sites flanking the BBM, WUS, CRE  
1013 cassettes such that upon desiccation stress, *rab17* would activate CRE expression resulting in  
1014 removal of the cassettes and allow plant regeneration. These constructs can be delivered via  
1015 particle bombardment or *Agrobacterium* (as in Figure 4C) and result in stable integration of T-

1016 DNA; transgenic events can be recovered with the use of a selectable marker, as in this case,  
1017 (Figure 5A) phosphinothricin (maize Ubi driving moPAT). The fluorescent visual marker YFP  
1018 was used to visualize events following excision of the *BBM*, *WUS*, *CRE* cassette. In some  
1019 cases, "helper plasmids" as DNA constructs harboring the *BBM* and *WUS2*, driven by differential  
1020 expression, results in the genotype independent development of somatic embryos in maize [3].  
1021 This system [3, 4] demonstrated genotype independence for large numbers of maize inbred lines,  
1022 sorghum and sugarcane which had been shown previously to be recalcitrant. Soon thereafter, the  
1023 results described above were confirmed and extended through differential co-expression of *BBM*  
1024 and *WUS2* coupled with the desiccation inducible *CRE/lox* excision system to enable recovery of  
1025 stable transgenics without a chemical selectable marker in recalcitrant maize inbred B73 and  
1026 sorghum P898012 (Figure 5B; [5]). Genome editing reagents can also be supplied using this  
1027 approach (Figure 3C,D; [123]). This technology is a paradigm shift in this field. In addition, this  
1028 new technology has opened the possibility for genotype independent transformation. The use of  
1029 morphogenic regulators to overcome genotype dependence is a breakthrough technology in  
1030 cereal transformation, and can be coupled with genome editing capabilities to bring about a  
1031 change in the study of basic plant biology as well as crop plant breeding [291]. This approach  
1032 will also enable new plants to become chassis for synthetic biology and the development of new  
1033 biochemical pathways in plants.

1034 Various improved strategies have been already developed. In an effort to circumvent  
1035 pleiotropic effects of *BBM* and *WUS* and the need for excision, Lowe et al. [3] demonstrated the  
1036 use of a system using specific promoters to drive expression of the morphogenic regulator genes  
1037 (Figure 5C). A survey of an extensive maize transcriptome atlas of over sixty-five thousand  
1038 gene models from a gene dataset sequenced via Illumina RNAseq identified a viable candidate,

1039 Zm-PLTP. The promoter from a maize phospholipid transferase protein gene (*Zm-PLTP<sub>pro</sub>*) was  
1040 selected based on its expression pattern in callus, embryos, and leaves but down regulation in  
1041 other tissues. A construct was developed using the *Zm-PLTP<sub>pro</sub>* to drive expression of *BBM*, an  
1042 auxin inducible promoter from maize (*Zm-Axig1pro*) to drive expression of *WUS* and an *Sb-*  
1043 *ALS<sub>pro</sub>* driving expression of a double-mutant gene *HRA* [292] as a selectable marker (see Figure  
1044 5C). This construct was introduced into an *Agrobacterium* strain carrying an improved  
1045 accessory plasmid, pPHP71539 [293], which is a derivative of the super binary vector pSB1  
1046 [195]; pPHP71539 carries additional copies of *vir* genes as well as corrections to several  
1047 mutations found in pSB1. When this strain/vector combination was used to transform immature  
1048 zygotic embryos of maize, large numbers of somatic embryos formed directly from scutellar  
1049 epidermal cells which could be directly germinated into fertile plants without an intermediate  
1050 callus phase [3, 293].

1051 Expression of morphogenic regulator constructs paired with gene editing functions  
1052 targeting four genomic regions (liguleless1, *LIG*; acetolactate synthase, *ALS2*; and two male  
1053 fertility genes, *MS26* and *MS45*) was reported by Svitashv et al [123], who used biolistics to  
1054 deliver Cas9-gRNA ribonucleoproteins (RNPs) into immature maize embryos. DNA vectors  
1055 carrying maize *BBM*, *WUS*, and a MoPAT-DSRED visual/selectable marker transgene cassette  
1056 were co-bombarded with the RNPs into the embryos. In this case integrated DNA can be  
1057 segregated away from edited targets. Delivery of either non-integratable DNA or RNA with  
1058 RNPs would accomplish the same goal, with the exception that there would be no original  
1059 integrative event (Figure 3D). Svitashv et al. [123] also deployed this very method and  
1060 successfully obtained edited plants using Cas9-gRNA RNPs alone, targeting three genomic  
1061 regions (*LIG*, *MS26* and *MS45*). Since no DNA has been integrated in these cases, current

1062 considerations render this approach under regulatory review to be determined as an advanced  
1063 mutagenesis approach and hence potentially non-GE, especially when *Agrobacterium* is not used  
1064 as a delivery system. In this scenario the introduction of molecules facilitating gene editing  
1065 without integration would result in a rapid recovery of events without the need for backcrossing,  
1066 saving time and resources. Ideally this would allow 'editing at will' in well known established  
1067 varieties or inbreds and recovered without any further breeding or backcrossing.

## 1068 **6. Regulatory considerations**

1069         The direction in future research in this area will be strongly determined around regulation  
1070 of genome edited outcomes. If genome edited events are treated as GE organisms, the technology  
1071 will remain in the control and use of the private sector and limited to only those crops which  
1072 merit the cost of deregulation, which could amount to tens of millions of dollars [294].  
1073 University research will also be stymied by standards for controlled used and outdoor growth and  
1074 testing. This effect is illustrated by the fact that since 1992, when USDA-APHIS issued its first  
1075 deregulation petition, only two out of 162 (1%) genetically engineered crop products have been  
1076 deregulated by the public sector: virus resistant papaya from Cornell University, and virus  
1077 resistant plum from USDA-ARS [295]. In contrast, when applicants have successfully  
1078 demonstrated that they are not regulated articles according the criteria established by USDA-  
1079 APHIS, 21 public sector products out of 68 (31%) have come from the public sector [296]. The  
1080 “Am I Regulated?” pathway requires significantly lower costs than the deregulation pathway.

1081         Worldwide, there is a lack of agreement regarding whether or not the products of gene  
1082 editing will be regulated as GE organisms. Countries that have rendered opinions regarding  
1083 which techniques should be treated as GE include the United Kingdom, Germany, Sweden,  
1084 Switzerland, and South Africa [297]. In these countries, there is incomplete agreement regarding

1085 the GE status of certain organisms. For example, in the United Kingdom, offspring of edited  
1086 products of oligo-directed mutagenesis and zinc-finger nucleases are considered non-GE;  
1087 Germany agrees with the exception of offspring of edited products obtained through the use of  
1088 zinc finger nuclease-3, while South Africa considers oligo-directed mutagenesis and use of site  
1089 directed nucleases, with the exception of intragenesis achieved with these nucleases, as non-GE  
1090 [297]. Some uniformity among European countries will be established by the Court of Justice of  
1091 the European Union following its decision in July 2018, in a court case involving this issue. In  
1092 advance of this decision, the Advocate General of the court rendered an opinion, stating that  
1093 gene edited organisms are

1094

1095 “...exempt from the obligations of the GMO Directive *provided* that they do not involve  
1096 the use of recombinant nucleic acid molecules or GMOs other than those produced by  
1097 mutagenesis or cell fusion of plant cells of organisms, which can exchange genetic material  
1098 through traditional breeding methods.” [298]

1099

1100 This opinion signaled to many that edited organisms would not be classified as GE.  
1101 However, the Court finally ruled that organisms obtained via mutagenesis are genetically  
1102 engineered, but that only those that were obtained via conventional mutagenesis were exempt  
1103 from the EU Directive on GMOs, thus subjecting gene edited organisms in the European Union  
1104 to the same extensive review system that applies to transgenic organisms (Court of Justice of the  
1105 European Union, 2018). The consequences through past history are apparent.

1106 In order to update its regulations to account for new developments in breeding  
1107 techniques, including gene editing, USDA-APHIS proposed new rules in 2017, which were

1108 subsequently withdrawn. A tiered approach was submitted by the Society for In Vitro Biology  
1109 (Kausch et al. SIVB, 2017) during the public commentary period for consideration in response to  
1110 USDA proposed rule, consistent with an issue paper by Bogdanove, et al. [1]. Tier one would  
1111 consist of events comprising single nucleotide changes, which arguably could happen in nature  
1112 or be the result of chemical mutagenesis techniques and therefore should be regulated as non-  
1113 GE. The second tier would describe events including gene deletion such as those caused by  
1114 double stranded breaks and non-homologous end joining resulting in heritable gene silencing.  
1115 Also in Tier 2 would be events involving in insertions resulting in gene silencing. Again, this  
1116 approach should be considered non-GE since it does not involve foreign DNA and is considered  
1117 advanced mutagenesis. The third Tier would include site directed insertion of cis elements,  
1118 including the insertion of regulatory elements, such as promoters and introns, into site directed  
1119 loci, or the insertion of cis coding regions and the multiple insertion of cis genes or elements.  
1120 These also should be considered as non-GE without the introduction of any foreign DNA. The  
1121 fourth tier would involve events including the introduction of foreign DNA facilitated by these  
1122 new technologies. These likely should be regulated according to current guidelines used for GE  
1123 organisms.

1124         The goal of non-GE, DNA-free, genotype independent varietal development would  
1125 obviate many of the obstacles preventing a more active contribution of the public sector to the  
1126 development of new crop varieties. Also paramount is the development of an education and  
1127 outreach component which includes the public consumer interests. The outcome would be a vast  
1128 benefit to society and world agriculture. It would be of considerable interest to develop a  
1129 situation whereby university investigators and small start-up companies could actuate their  
1130 results through these combined technologies.

1131 **7. Education and the public interests**

1132           Ultimately, the successful implementation of these technologies will be dependent on  
1133 public perception and resultant regulatory policies. Regardless of the advances in agricultural  
1134 biotechnology, if not implemented, the benefits will not be realized. In a world of rapidly  
1135 expanding population, diminishing land and water resources, climate change, energy demands,  
1136 and geopolitical strife, all of the tools for advanced agriculture must be brought to bear. The  
1137 applications of the technologies described in this review are among the most provocative and  
1138 socially relevant topics today. Perhaps the greatest danger of this technology is that it would not  
1139 be broadly put into practice. Norman Borlaug, founder of the Green Revolution, stated that  
1140 *"Biotechnology is not a threat, starvation is."*

1141           What divides our thinking about genetic modification (GMO) in crop plants? A lack of  
1142 education about basic biology lies at the heart of misconceptions and fears which has had a large  
1143 affect on public perception of biotechnology in general [299-301]. There is a fundamental and  
1144 growing educational gap about biology, genetics and agriculture creating a fear of the unknown.

1145           "Currently there is a wide disparity between the knowledge of the general public about  
1146 DNA and biotechnology and the actual science and its applications. This gap is growing wider  
1147 every day and has created a current educational crisis about DNA, how life works and  
1148 biotechnology. A working knowledge of DNA, genetics and biotechnology has become as  
1149 fundamental to a basic education as an understanding of the solar system. The largest  
1150 challenges in biotechnology right now are not technological, but those of public perception, as  
1151 biotechnology education have not kept pace with the rapid growth of its science." (Kausch,  
1152 unpublished).

1153           In addition, there is, despite the facts, a lack of public trust about GMO plants, even  
1154 though the same technologies are commonly used in pharmacy and medicine with little concern.  
1155 The technological growth in biotechnology is now in an exponential phase, with major new  
1156 discoveries happening daily. "*Science gathers knowledge faster than society gathers wisdom*"  
1157 (Isaac Asimov, ref) and in no other field is this more obvious than biotechnology. Trust in the  
1158 technology then, is the basis of the issue which can only be established and maintained through  
1159 fundamental education (Bobo SIVB 2018). Only through trust will these technologies be  
1160 embraced encouraged and, even celebrated. Education will encourage trust, reliable and safe  
1161 regulatory policies and governance, and the sound stewardship of the technologies to the benefit  
1162 of the world wide public.

1163           The application of genetic modification (GMO) in crop plants by standard technologies  
1164 has largely been restricted to a low number of traits in high volume row crops controlled by the  
1165 largest agricultural companies. Despite the very large number of studies and evaluations over  
1166 three decades every claim of negative health or environmental consequence from genetically  
1167 modified crops has not stood up to scientific validation [302]. Meanwhile the anti-GMO  
1168 mentality has persisted through fear mongering with a substantial negative impact to world  
1169 agriculture and food security. To be clear, the anti-GMO movement has not been benign but has  
1170 resulted in serious agricultural setbacks and world food production issues. Simply put, the world  
1171 cannot afford another equivalent setback regarding the current technologies.

1172           We, the public, need to make informed decisions. Never before have we been challenged  
1173 with so many crises and with so many tools to address them. We should not do just because we  
1174 can, but we should do what is most responsible. This is necessary to maintain a high degree of

1175 public trust and integrity. The roles of education, governance and stewardship are hugely  
1176 important to these goals. What we teach our students now will inform our future public policy.

## 1177 **8. Conclusion**

1178         This article provides the basis for an intellectual and agricultural movement. The crucial  
1179 goal of the collective research on genomics, genome editing and plant transformation is to be  
1180 able to edit at will resulting in an altered genotype in a single generation. This ability will  
1181 fundamentally change plant science and world agriculture. The ability to transform any species  
1182 or variety will enable new chassis for synthetic biology. It should be possible to take a single  
1183 variety and change a single trait (disease resistance, for example) to make an appropriate edit and  
1184 return the same variety with a single gene edit without extensive backcrossing. This technology  
1185 will enable the addition of new biochemical pathways in plants, perhaps enabling the  
1186 development of a plant source that is one hundred percent nutritionally effective for humans.  
1187 This new era of plant transformation will provide a pan-application systems approach to plant  
1188 biology and crop development. Collectively the capabilities described in this review will provide  
1189 relatively rapid approaches to produce insertions, deletions, single nucleotide substitutions, and  
1190 site specific integration that are species, genotype, and varietal independent. These advances  
1191 will also provide explant and genotype independence which is nearly tissue culture free with  
1192 limited or no somaclonal variation at relatively high frequencies. DNA-free approaches may be  
1193 (and perhaps should be) regulated as non-GE as an advanced mutagenesis or advanced breeding  
1194 technique which will make these applications available to university investigators and small  
1195 companies, broadening the applicability and benefit to the society and world agriculture. The  
1196 future of genotype independent plant transformation in the era of genomics and gene editing will

1197 continue to accelerate and become more defined. Synergistically these developments will prove  
1198 to be the best improvement in agriculture since the plow.

1199           However this success will rely on public perception and acceptance. It is well known that  
1200 world population will increase from its current 7.2 billion to over 9 billion over the next thirty  
1201 years. It is also known that the available arable land and water resources will not meet the need  
1202 for increased food production without dramatic increases in yields. Increase of arable land and  
1203 water will come with the destruction of already decreasing wild habitats, such as rainforest and  
1204 other fragile habitats. During the next 30 years world food production will need to be increased  
1205 by 70% to meet these needs [193]. Given world food security issues, implementation of the  
1206 technology discussed in this review is imperative. The world simply cannot afford another  
1207 information, public perception and regulatory debacle as that which was associated with GE  
1208 organisms. Therefore, it is incumbent on all participants to enhance education efforts in this area,  
1209 not only with the general public, but also starting at an early age. What is happening in this field  
1210 now will change world agriculture forever and these accomplishments should be celebrated as  
1211 the 'moon landing' for global food security.

1212

### 1213 **Acknowledgements**

1214

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1216 SC0018277.

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1218 **Table 1. History of Genome Editing in Plants**

<b>Year</b>	<b>Milestones in Plant Genome Editing</b>	<b>Ref</b>
2003	ZFNs for targeted modification in eukaryotic genes	[94]
2005	First gene mutations induced via ZFNs in <i>Arabidopsis</i>	[96]
2005	Repair of a mutant transgene via HR using ZFNs in transgenic tobacco	[99]
2008	Improved ZFN design methods developed	[97]
2009	Elucidation of the TALE DNA-binding code	[103]
2009	Targeted insertion of transgenes and mutation of maize <i>IPK1</i> by ZFNs	[98]
2009	Targeted transgene integration in tobacco using ZFNs	[95]
2009	Efficient ZFN editing of tobacco acetolactate synthase genes	[100]
2010	First successful use of TALENs for gene editing tested <i>in vivo</i> in yeast	[104]
2010	Mutations induced in maize <i>liguleless1</i> gene via meganuclease	[91]
2011	<i>adh1</i> editing in <i>Arabidopsis</i> using TALENs (TALE + nuclease)	[106]
2011	Targeted mutagenesis of a transgene and the paralogous genes DCL4a and DCL4b using ZFNs in soybean	[101]
2012	CRISPR/Cas9 used to cleave dsDNA in bacteria	[113]
2012	TALENs editing of a rice bacterial blight susceptibility gene conferring resistance to blight	[107]
2012	Meganuclease editing of <i>Arabidopsis</i>	[92]
2013	Specific targeting of genes in mammalian cells using CRISPR/Cas9	[46, 114]
2013	Targeted addition of two transgenes in cotton using meganuclease	[93]
2013	Cas9/sgRNA editing of <i>Arabidopsis</i> and tobacco	[115]
2013	Mutation of rice and wheat loci using Cas9/sgRNA editing	[117]
2013	Cas9/sgRNA editing of <i>Arabidopsis</i> , tobacco, sorghum, and rice	[118]
2014	Improved soybean oil quality via TALENs editing of the fatty acid desaturase 2 gene family	[108]
2014	Mutation of four genes in maize using TALENs and ZmIPK using CRISPR/Cas9 in maize	[110]
2014	Simultaneous TALENs editing of three homoeoalleles in hexaploid bread wheat conferring resistance to powdery mildew	[111]
2014	Cas9/sgRNA mutation in tomato	[119]
2015	Reduced acrylamide potatoes via TALENs	[109]
2015	Cas9/sgRNA mutation of potato <i>StIAA2</i> locus	[120]
2015	CRISPR/Cas9 knockout of a transgene (GFP) and modification of nine endogenous loci in soybean	[121]
2015	DNA-free editing using preassembled CRISPR-Cas9 ribonucleoproteins in <i>Arabidopsis thaliana</i> , tobacco, lettuce and rice protoplasts	[122]
2016	DNA and selectable marker-free genome editing using Cas9/gRNA RNPs in maize delivered by particle bombardment	[123]
2016	Genome editing in wheat via transient expression of CRISPR/Cas9 DNA or RNA	[124]

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1222 **Table 2. History of Plant Transformation Biology**

Year	Some Milestones in Plant Transformation	Ref
1977	First identification of <i>Agrobacterium</i> T <sub>i</sub> plasmid DNA in plants exhibiting crown gall tumorigenesis	[158]
1980	T-DNA found to be nuclear localized	[159]
1983	Disarmed Ti Plasmids	[160-162]
1983	Binary vector concept for plant transformation	[163]
1984	A general method for genetically engineered dicots with foreign DNA	[164, 165]
1985	Transgenic carrot plants	[166]
1985	CaMV35S promoter	[167, 168]
1987	Microprojectile bombardment for DNA delivery	[178]
1987	GUS reporter gene	[177]
1987	BAR selectable marker	[303]
1988	Protoplast transformation of rice	[174]
1990	First fertile transformation of maize via microprojectile bombardment	[180]
1990	Bt cotton developed	[304]
1992	Maize ubiquitin constitutive promoter	[284]
1992	First fertile transformation of wheat via microprojectile bombardment	[181]
1993	First fertile transformation of rice by <i>Agrobacterium</i>	[185]
1993	Bt maize developed for insect resistance	[187]
1994	First commercialized GE crop in U.S. (tomato)	[305]
1995	Herbicide resistant soybean developed	[188]
1996	GM tomato paste released in the UK	[306]
1996	GFP reporter gene expressed in plants	[307, 308]
1998	Widespread commercialization of GM maize, soybean, sugarbeet, cotton, and canola	[309]
1998	<i>Arabidopsis</i> floral dip tissue culture-free transformation	[186]
2000	<i>Arabidopsis</i> genome sequenced	[310]
2002	Rice genome sequenced	[30]
2005	ZFN genome editing in plants	[96, 99]
2009	Maize and sorghum genomes sequenced	[189, 190]
2011	TALEN genome editing in plants	[106]
2013	CRISPR/Cas9 genome editing in plants	[118]
2016	Induction of somatic embryogenesis by morphogenic regulators during transformation	[4]
2016	Selectable marker and DNA free genome editing	[123, 124]
2018	Genotype independent transformation in maize and sorghum	[3, 5, 293]
Future	Advanced crop breeding using genomics, genome editing and advanced transgenics; Genomics based transgenics; GMO- free edit at will capabilities without genotype restrictions	

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1225 **Figure Legends**

1226

1227 **Figure 1.** Diagrammatic representation of Cas9 (top) and Cpf1 (bottom). The molecular  
1228 mechanisms for CRISPR-Cas9 and many other variants have now been well studied.

1229

1230 **Figure 2.** Morphogenic plasticity in plants in tissue culture is dependent on genotype, explant  
1231 source, media requirements and other parameters. Few plants display every aspect of the  
1232 diversity of this plasticity, but this diagram represents the range of responses in plants.

1233

1234 **Figure 3.** Pathways to produce viable plant genome edited outcomes. (A) Shows that protoplasts  
1235 can be used as recipient cells for DNA, RNA and RNPs via electroporation, but is highly species  
1236 and genotype dependent. (B) Depicts the use of particle bombardment to deliver DNA, RNA,  
1237 and/or RNPs in wheat followed by indirect embryogenesis or organogenesis without selection to  
1238 derive genome edited events recovered by molecular screening. (C) Shows the delivery of DNA,  
1239 RNA or encoded RNPs via either *Agrobacterium* or particle bombardment using morphogenic  
1240 regulator mediation with or without selection to recover stable integration events which are then  
1241 screened for genome edited events. (D) Demonstrates the use of transient expression of  
1242 morphogenic regulators to recover stable edited plants without stable integration of any DNA  
1243 sequences and hence a mutagenesis technique and non-GMO.

1244

1245 **Figure 4.** Key genes governing early stages of somatic embryogenesis (modified from [297]).

1246 *PRC1* and *PRC2*, *POLYCOMB REPRESSIVE COMPLEX 1* and *2*; *LBD29*, *LATERAL ORGAN*  
1247 *BOUNDARIES DOMAIN 29*; (*KYP*)/*SUVH4*, *KRYPTONITE*; *SERK1*, *SOMATIC*

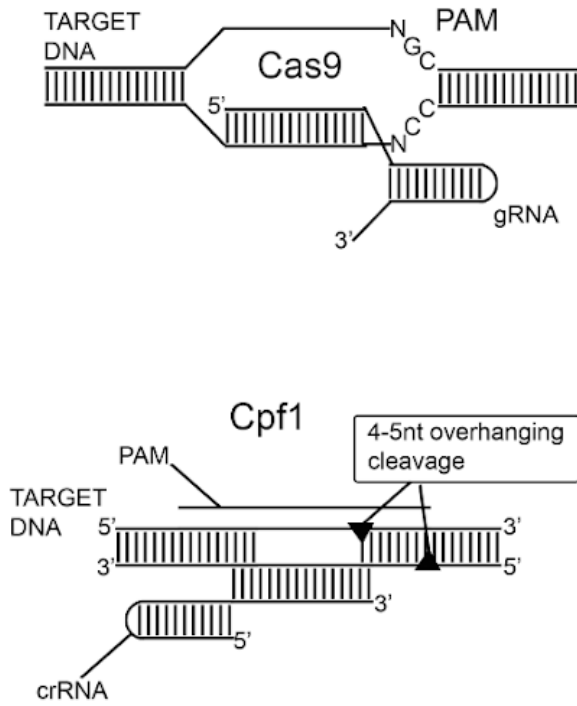
1248 *EMBRYOGENESIS RECEPTOR KINASE 1; WUS, WUSCHEL; LEC1, LEAFY COTYLEDON 1 ;*  
1249 *STM, SHOOT MERISTEMLESS; BBM, BABY BOOM.*

1250

1251 **Figure 5.** Description of constructs used for morphogenic regulator mediated transformation in  
1252 monocots. (A) Shows a diagrammatic representation of the pPHP35648 vector the T-DNA  
1253 vector using a *Nos<sub>SAT</sub>* promoter driving the *Wus<sub>2M</sub>: pinII* and the *Ubi<sub>M</sub> pro* driving *Bbm<sub>M</sub>:pinII*  
1254 for induction of somatic embryogenesis. Desiccation induction of CRE is driven by the maize  
1255 *rab17<sub>M</sub>* promoter. this cassette is bracketed by lox P sites where by on desiccation site is removed  
1256 allowing plant regeneration This vector also includes the selectable marker for bialaphos  
1257 resistance with a *Ubi<sub>M</sub> pro* driving *moPAT*. The excision events can be visualized with the *Ubi*  
1258 *pro* driving expression of YFP [4]. (B) shows a diagrammatic representation of the PHP78891  
1259 vector [5]. Similar to (A), the PHP78891 vector contains four expression cassettes: (i) *RAB17<sub>M</sub>:*  
1260 *CRE*; (ii) *NOS<sub>At</sub>: WUS2*; (iii); *UBI<sub>M</sub>: Bbm<sub>M</sub>* and (iv) *UBI<sub>M</sub>: GFP*. The *CRE/WUS2/BBM* cassette  
1261 is bracketed by lox P sites, but does not have a selectable maker. One lox P site is downstream  
1262 of the right *Agrobacterium* border and the other adjacent to the *UBI<sub>M</sub>: GFP* cassette within the  
1263 left *Agrobacterium* border. (C) shows a construct using the *Zm-PLTP<sub>pro</sub>* to drive expression of  
1264 *Bbm* [3], which presumably initiates cell division, and subsequently down regulated, an auxin  
1265 inducible promoter from maize (*Zm-Axig1pro*) to drive expression of expression of *WUS2*  
1266 allowing plant regeneration when subcultured to auxin free media, and an *Sb-ALS<sub>pro</sub>* driving  
1267 expression of a double-mutant gene *HRA* [292] as a selectable marker.

1268

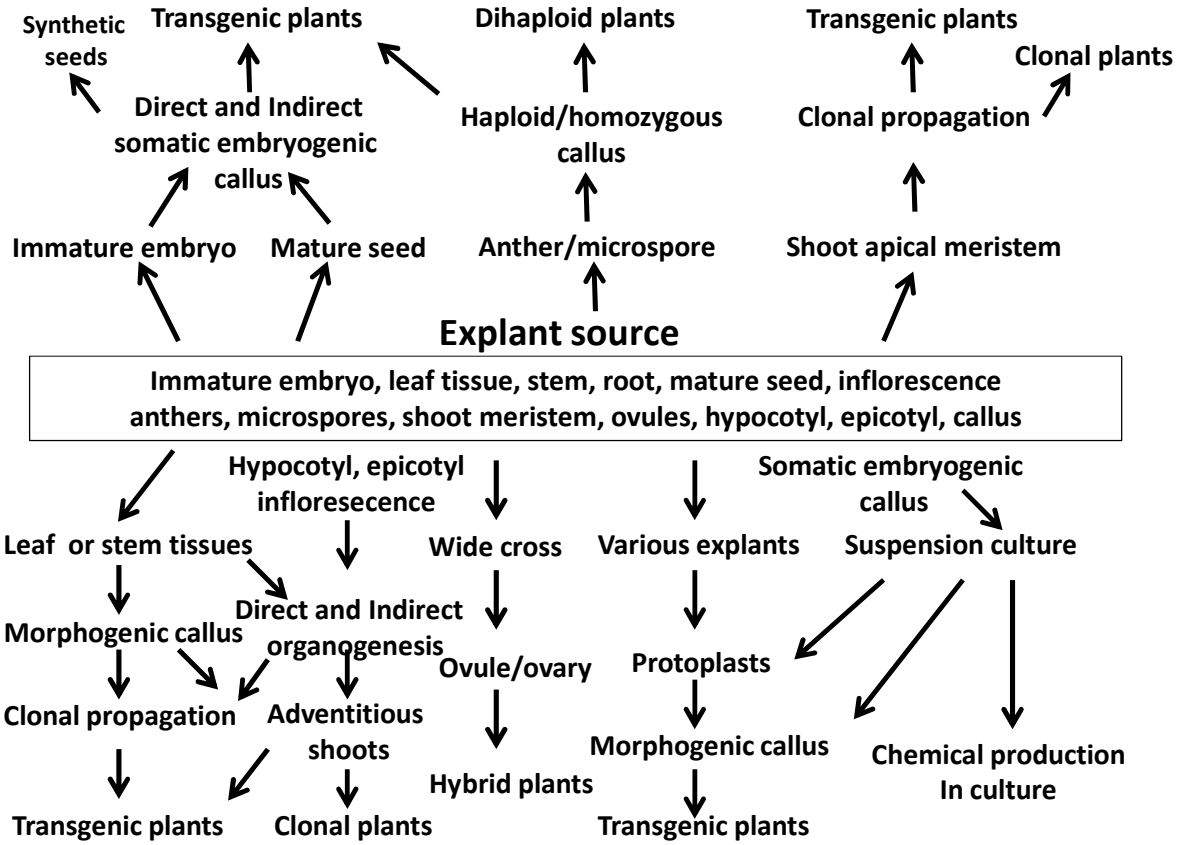
1269 **Figure 1**



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1286 **Figure 2**

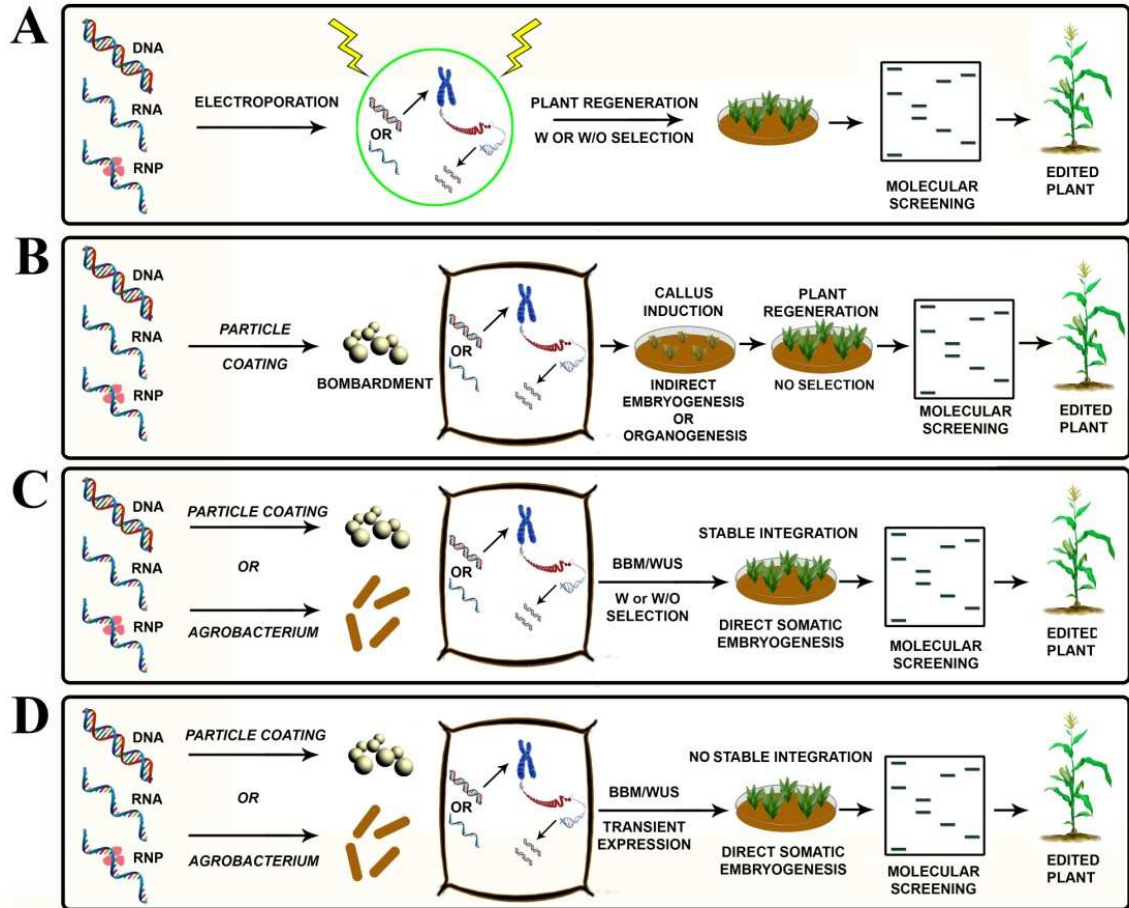
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1289 **Figure 3.**

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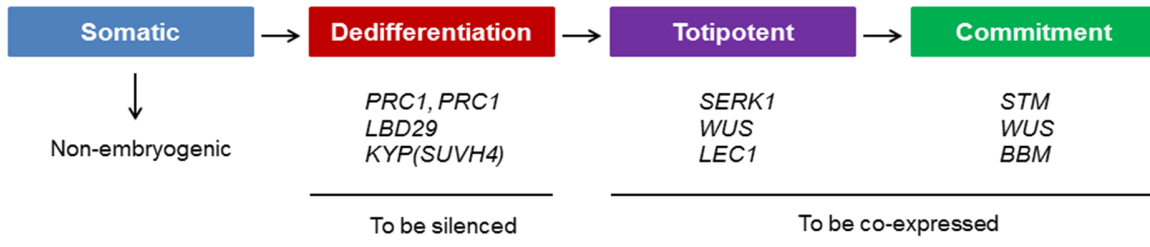


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1293 **Figure 4**

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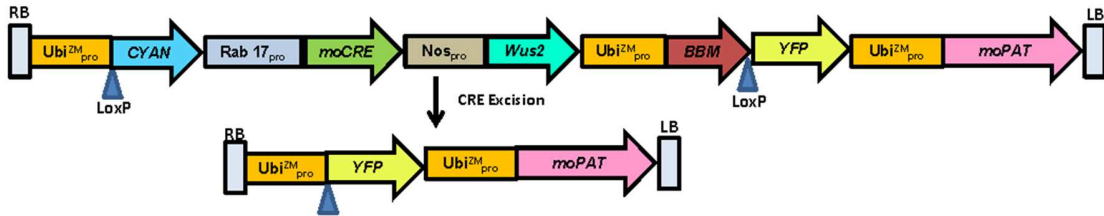
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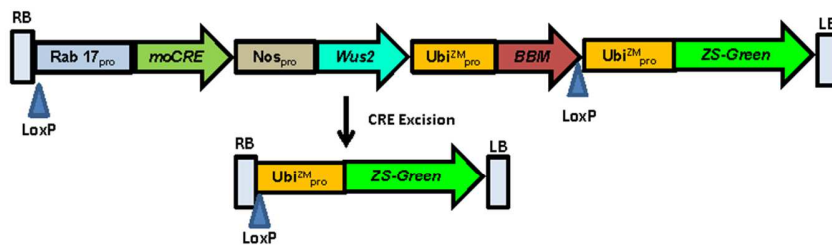
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1298 **Figure 5**

A



B



C



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