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Review article

Edit at will: Genotype independent plant transformation in the era of advanced genomics and genome editing

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ABSTRACT

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

1. Introduction

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

Initially plant transgenesis was used for biological analysis and then quickly became applied for development of new crop varieties of agricultural importance. This technology was familiarly termed as Genetically Modified Organisms (GMO) which drew widespread public controversy which still persists. The current terminology (which is broader) is genetic engineering (GE) covering various applications, but these terms are used interchangeably. Overall, these applications to basic plant science and agricultural applications are necessary and crucial now for investigations of plant biology and for world food security. Given the tools of advanced genomics and genome editing, the obstacle has remained in the ability to transform and regenerate plants.

Breakthrough advances using morphogenic regulators for improved transformation and genetic modification have addressed the bottlenecks which have long encumbered plant transgenic biology [3–5].

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Current results with cereal crops demonstrate rapid results, genotype independence, and utilize a range of explants resulting in reasonably high transformation frequencies. This enabling technology in monocots is a watershed for the application of genome editing allowing higher throughput for analysis of genomic targets. These approaches show significant promise for utility in genome editing which can be *Agrobacterium* free, DNA free, rapid and for these reasons potentially non-GE [6]. These advances have made significant progress toward a systems wide approach to plant biology involving genomics, genome editing, and plant transformation which can be practically achieved. In this review, the key developments in genomics, genome editing and plant transformation biology are evaluated.

Recent advances in genomics and computational biology provide a strong basis for trait identification, accelerated breeding, the development of new varieties, and proprietary protection (See Section 1a., below). In fact, the deep layers of genomic technologies will provide the foundational information that when paired with genome editing technology will offer the capability to (1) pursue new avenues in investigating basic plant biology, and (2) rapidly develop new crop varieties with specific traits having market and consumer value, and will likely underlie nearly all future crop improvement. Sequence information is crucial for target evaluations; however, there is limited use for sequence information by itself. The consequence of the data gained through genomics and sequencing and the capabilities afforded by genome editing allows for an exponential increase in the potential introduction of variation. Nonetheless, this can be functionalized for phenotypic analysis *only through* the applications afforded by robust plant transformation technologies. Without the ability to introduce the appropriate molecular constructs through plant transformation, these tools will be somewhat limited in application. Thus, genomics, computational biology, and genome editing function as independent disciplines as well as collaborative sub-disciplines of plant transformation science involving the transfer of DNA, RNA, and proteins to plant cells and tissues and recovery of regenerated plants.

This approach requires a coordinated effort spanning several disciplines and areas of expertise - namely, sequencing, data analysis, computational biology, genomics, target identification, genome editing, target validation, plant transformation, phenotype analysis, and whole genome sequencing for detection of off target affects or inclusion of foreign DNA. A functional plant genome editing program is dependent upon a deep genomics program utilizing an efficient DNA, RNA and protein delivery system operating within the context of an efficient plant transformation program. This, in turn, should lead to high-throughput recovery and analysis of edited plants. The outcome of using these synergistic technologies for basic science and plant biotechnology will establish a robust genome editing platform that provides important new variants and commercially significant varieties which are scientifically important and/or financially viable in a time frame that is reasonable. The ability to transfer DNA, RNA, proteins and RNPs (ribonucleoproteins) into plants with the recovery of inheritable traits results in the fertile collaboration of these three disciplines. The broader application of genomics and genome editing is therefore dependent on the ability to transfer biological molecules to any plant and any variety of interest. Toward this goal, plant transformation technologies have rapidly advanced in recent years and this review analyzes the interactive relationships.

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

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

2.1. Advances in plant genomics platforms and genome editing technologies, mechanisms, design, and validation

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

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

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

The genetic architecture of simple and complex traits is an area of genomics facilitated by quantitative trait loci (QTL) mapping and genome-wide association study (GWAS)-based approaches. The identification of genetic markers that are proxies or causative genetic variants provides a rapid means to introduce useful genetic diversity directly into breeding programs. Genetic variants with strong effects, especially in simple traits matched with tightly linked markers, enable selection by genotype, not phenotype, in plant breeding programs. Association studies, utilizing the historical recombination within populations of crops with limited coancestry, can be used to identify markers closely linked to agronomic traits, such as flowering time, plant height, yield, and grain traits in tropical rice [14]. Additional resources, such as mapping populations, are created through specially designed crosses between parental lines with differing phenotypes. A species-wide rare variant otherwise undetectable in an association study, if introduced into a mapping population by a parental line, is subjected to greater mapping power in recombinant offspring. Advanced population designs, such as bi-parental nested association mapping (NAM) [15–17] or multi-parent advanced generation inter-cross (MAGIC) populations [18], can combine the advantages of association studies with the power of QTL mapping populations. The ancestral recombination between the parentals, in addition to the recent recombination within the population, further increases mapping resolution by reducing the size of statistically independent haplotypes. These trait mapping resources—pedigrees, germplasm, sequencing, phenotyping data, and mapped QTLs, are publicly available for trait discovery marker development and, most importantly, as targets for improvement by gene editing technologies.

One area that has had a considerable impact in plant genomics is advances in reduced representation sequencing, especially low coverage whole genome sequencing and genotyping by sequencing [19,20], in conjunction with variant imputation methodologies design specifically for plant populations [21–23]. These genomics technologies

have greatly reduced the cost per sample of mapping populations. Densely genotyped parental lines allow for progeny haplotypes to be inferred despite reduced representation. Earlier consortia-level projects describing SNP panels for crop species in the thousands of markers [24–28] have transformed into species-level resequencing projects such as the 3000 Rice Genomes Project [29,30] that further enable precise genotype imputation, more detailed analyses of fine-scale population structure, and the identification of underutilized gene pools. Finally, improvements to sequencing technology itself, such as the decreasing cost of high-coverage whole genome sequencing, and the increasing accuracy of long-read sequencing, is democratizing genome assemblies [31]; for example, recent species-wide resequencing in rice is approaching a paradigm where reference genomes will become commonly available on the varietal level and a single reference can describe the structural variation present in multiple sub-populations [29,32].

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

Selection by markers, however, fell short of the goal of a second Green Revolution, where global yield increases would satisfy both anticipated food security needs and decrease the material input for agriculture [40]. This is largely because, outside of very simple traits with a small number of strong-effect alleles, MAS is greatly limited in its predictive capability [41–44]. Many agronomic traits are complex with numerous weak-effect alleles. Moreover, markers linked to QTLs as identified in mapping populations, may not be easily replicated in separate breeding populations due to differences in genetic background and population structure. Association studies, even if conducted within breeding populations, are similarly limited—even if QTL validation is no longer necessary, large numbers of markers linked to weak-effect alleles are still unsuitable for MAS [43,45]. Finally, an inherent limitation of marker assisted backcrossing—the number of generations needed to remove unwanted donor haplotypes—makes varietal development a time intensive process. Improved translational methods were needed in order to manifest the benefits of trait dissection and genetic diversity as elucidated by genomic approaches, both in terms of informed selection processes and biotechnology.

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

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

Genomics assisted breeding in the future will likely blur the boundaries between genome editing and informed crossing. A better understanding of breeding population design through both selection and editing, as described by machine learning approaches and informed by the widespread availability of pan-genome and varietal-level genomics, could bring agriculture closer to a second Green Revolution. Recent advances in machine learning and artificial intelligence complement both selection and editing through added predictive power for biological processes, at all stages of experimental design, from non-linear modeling of the genetic basis of traits, optimal cross selection, to guide RNA design. In the immediate term, a parallel approach employing genome editing for simple traits and genomic selection for complex traits could be effective.

2.1.1. Mechanisms and guide RNA design

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

2.1.2. Target validation by CRISPR

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

Table 1
History of Genome Editing in Plants.

Year	Milestones in Plant Genome Editing	Ref
2003	ZFNs for targeted modification in eukaryotic genes	[94]
2005	First gene mutations induced via ZFNs in *Arabidopsis*	[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 *IPK1* 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 *in vivo* in yeast	[104]
2010	Mutations induced in maize *liguleless1* gene via meganuclease	[91]
2011	*adh1* editing in *Arabidopsis* 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 *Arabidopsis*	[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 *Arabidopsis* and tobacco	[115]
2013	Mutation of rice and wheat loci using Cas9/sgRNA editing	[117]
2013	Cas9/sgRNA editing of *Arabidopsis*, 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 *StIAA2* 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 *Arabidopsis thaliana*, 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]

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

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

RNA interference (RNAi) is a RNA-dependent gene silencing process, historically also known as co-suppression or post-translational gene silencing (PTGS), which can be used to control of gene expression [81]. RNAi provides an approach whereby gene expression or translation can be inhibited via specific RNA molecules [82]. Gene silencing is initiated by double-stranded RNA (dsRNA) molecules in the cytoplasm. The cellular mechanisms involved with RNAi have been well studied and shown to be more stable and efficient than previously discovered antisense techniques for gene suppression (reviewed by Suarabh et al. [83]). In most cases RNAi does not result in total ablation of gene expression but will often drastically reduce it resulting in 'knockdown' mutants [84]. Constructs can therefore be developed to knockdown expression of a specific gene of interest by generating double stranded RNA with a sequence that is complementary to the gene. One drawback to RNAi technology for use in advanced plant breeding is that this mechanism imparts an incomplete modification which may revert to wild-type levels [85] in subsequent generations. However, RNAi has proven to be a robust technique to influence gene expression in plants, animals, and fungi and has become an important basic research tool as well as a practical tool in biotechnology, medicine, pharmacy and agriculture [83].

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

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

It is clear that CRISPR systems are the major genome editing application in many plant species [46,113,114]. CRISPR utilizes RNA rather than proteins (as in meganucleases, ZNF, or TALENs) to specifically target DNA sequences, have allowed a greater ease of use in comparison to alternative systems, and have been widely applied in many plant systems [112,138]. Genomic sequences which contain a protospacer adjacent motif (PAM) sequence NGG can be targeted by the CRISPR-Cas9 ribonucleoprotein (RNP) complex. CRISPR-Cas9 recognizes RNA-DNA base pairing to specifically recognize target DNA sequences and Cas9 activity results in precise blunt end DSBs at target sites. When repaired by NHEJ, these sites result in heritable null expression of the target gene that is irreversible (in comparison with RNAi which results in incomplete modification). The demonstrated utility of the CRISPR-Cas9 complex for genome editing led to the identification of variants in other bacteria (i.e., *Prevotella*, *Francisella*; [137], and; *Lachnospiraceae*; [139,140]) which have been investigated for specific applications. The molecular mechanisms for CRISPR-Cas9 and recent variants (Fig. 1) have been well studied [77,141].

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

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

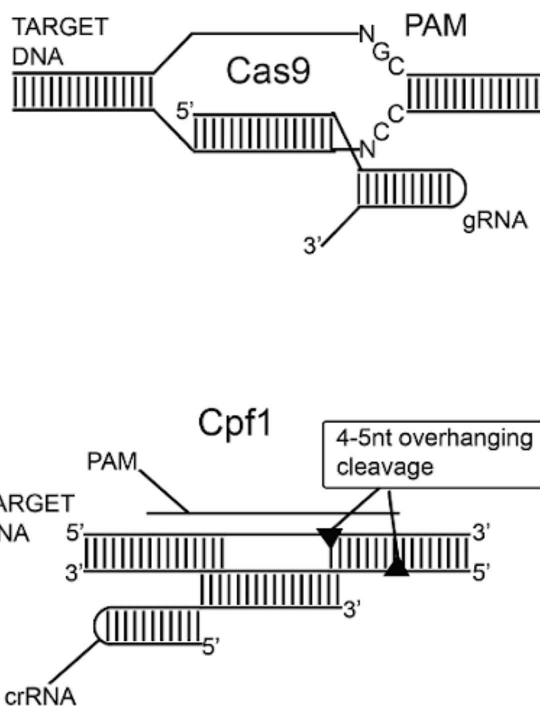

Fig. 1. Diagrammatic representation of Cas9 (top) and Cpf1 (bottom). The molecular mechanisms for CRISPR-Cas9 and many other variants have now been well studied.

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

3. Advances in plant transformation biology

In order to understand the present technologies, needs, and future advances in plant transformation biology related to genomics and genome editing, it is important to put this into the context of historical milestone achievements. Plant transformation systems are complex; involving an understanding of plant developmental biology, molecular biology, plant physiology, plant tissue culture, media modifications, explant biology, DNA delivery methods, selection of transformed cells, plant regeneration and genetics.

3.1. Perspective on plant transformation

A fundamental but often underappreciated technology for understanding the plant genome and utilizing plants to their greatest potential involves the capability to create, test and cultivate transgenics. This technology can introduce valuable agronomic genetic variation into crops, functionally link genes to biological functions, modify metabolic pathways, and create robust plant-abiotic and biotic stress resistance. Some of the most innovative and important scientific discoveries and agricultural enablements would not have been possible without transgenic technologies. Transgenics have created crops

resistant to environmentally sustainable herbicides [147], plants resistant to viral and microbial pathogens [148–150] and stress-resistant crops [151]. Vitamin A and iron biofortification in maize and rice was made possible by transgenic technologies. The rice sub1 gene was [152] discovered by genetic mapping and genomics and was an enabling technology that was made possible by transgenic technology. Indeed, a great number of trait genes that have been examined and demonstrated to be effective in plants have been made possible by transgenic biology.

At the basic scientific research level, transgenic technology in plants is essential to the actualization of functional genomics and the applicability of genome editing. The application of Koch's Principles renders robust transgenic capabilities an imperative functional genomics technology. The ability to knock-out or knock-down gene expression, conduct promoter analyses, observe over-expression characteristics, interrogate the effect of single base pair changes, make specific adjustments in protein structure and function, and the introduction of novel genes that convey traits pertinent to pest, stress and drought tolerance and a host of other important agronomic characteristics, all depend on the ability to create and test transgenics. While the floral dip method of transformation has been very effective for a select few number of plants, such as *Arabidopsis thaliana* and *Camelina sativa*, this technique has not proven to be transferrable to the major crop species. The majority of plant transgenic biology is interdependent on cell and tissue culture.

3.1.1. Morphogenic plasticity in tissue culture related to plant transformation

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

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

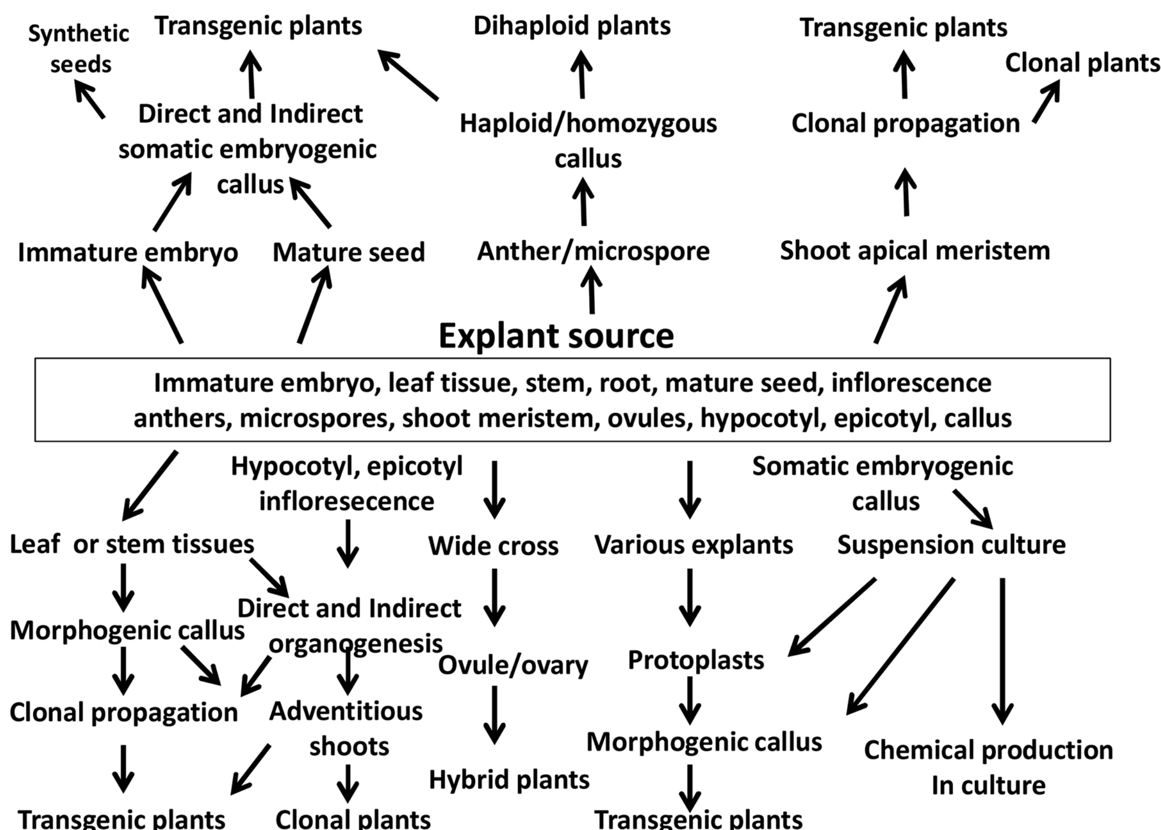

Fig. 2. Morphogenic plasticity in plants in tissue culture is dependent on genotype, explant source, media requirements and other parameters. Few plants display every aspect of the diversity of this plasticity, but this diagram represents the range of responses in plants.

There are significant general developmental differences between many monocot versus dicot transformation and regeneration systems.

Efficient plant regeneration systems do not necessarily lead to efficient transformation and vice versa. In many monocot transformation systems there is a predominant reliance on direct or indirect somatic embryogenesis as the basis for transformation. In contrast many dicot species exhibit regenerable callus formation as a result of direct or indirect organogenesis from various explant sources which can be readily regenerated to plants. Some of these systems can also be used for robust and reliable plant transformation (e.g., tobacco, petunia, potato), while others remain remarkably recalcitrant. The process of selection also plays an important role. Transformed cells need to be selected from non-transformed cells and still be able to reiterate the cultre and reatin the ability to regenerate to fertile plants. A single cell must be able to survive amidst non-transformed and dying cells and still retain the capabilities to reiterate a regenerable culture. Many results from both somatic embryogenic and organogenic pathways have shown that these events are initiated from a single cell transformants as deduced from Southern blot and sequencing analyses (See, for example [3]. Transformation systems are fundamentally different in dicots and monocots, between genera and species and are varietally independent. on the basis of their tissue culture response.

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

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

3.1.2. A brief history of plant transformation

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

Initial plant transformation methods relied on *Agrobacterium*-mediated gene transfer and was restricted to dicot plants (see review by [169]). Both direct and indirect organogenic tissue culture systems were amenable to DNA transfer, selection and subsequent plant regeneration in some species. However, stable gene transfer to monocots remained elusive for nearly a decade despite extensive efforts, causing some to doubt as to its possibility [170]. Overcoming the obstacle of DNA delivery in monocots was first accomplished using rice protoplasts to produce the first transgenic monocots [171–174]; however, maize protoplast transformation and antibiotic selection resulted only in infertile plants [175,176]. The GUS reporter gene system allowed visualization of delivered DNA allowing optimization to occur more rapidly [177]. Microprojectile bombardment ('the gene gun') was invented by John Sanford and Ted Klein in 1987 [178] to overcome the barrier to *Agrobacterium* infection in monocots. The first transformed monocots were produced in 1988 via a protoplast based system and direct DNA delivery [174] but this approach was not shown to be effective for other cereals. Particle bombardment obviated the need for cumbersome

Table 2
History of Plant Transformation Biology.

Year	Some Milestones in Plant Transformation	Ref
1977	First identification of *Agrobacterium* T_i plasmid DNA in plants exhibiting crown gall tumorigenesis	[158]
1980	T-DNA found to be nuclear localized	[159]
1983	Disarmed Ti Plasmids	[160,161,162]
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 *Agrobacterium*	[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	*Arabidopsis* floral dip tissue culture-free transformation	[186]
2000	Arabidopsis 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	

protoplast systems, resulting in the first fertile transgenic maize using indirect somatic embryogenic cultures and improved herbicide resistance selection protocols [179,180], specifically embryogenic suspension cultures and the *bar* gene as a selectable maker [180]. The development of fertile wheat transformation quickly followed using the same approach [181], and this technology transferred rapidly to successful transformation of sugarcane [182], rye [183] and tritordeum [184] and eventually other monocots. While enabling early monocot transformation, biolistic technology had several intrinsic disadvantages for cereal transformation, such as high frequencies of i) multicopy inserts resulting in transgene silencing, ii) integration of the vector backbone, iii) loss of transgene cassette integrity, and iv) the complications for gene expression studies. There are also basic biology and regulatory issues caused by these consequences. The pendulum swung back in the other direction when improvements to *Agrobacterium* vectors resulted in successful transformation and regeneration of monocots [185].

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

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

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

3.1.3. Monocot transformation now and the future

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

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

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

Efficient selection of stably transformed cells from the overwhelming number of non transformed cells is another essential component for successful transformation and regeneration of transgenic plants. The early dicot transformation systems relied on resistance to aminoglycosides [156], such as kanamycin, neomycin and G418, which proved ineffective in most cereal crops [154]. Selection of maize, wheat, and barley transformants has been most effectively achieved using herbicide resistance markers including the *bar* gene [180,181] for bialaphos resistance; the *als* gene [179] for chlorsulfuron resistance; the mutant *epsps* gene [212] for glyphosate resistance and for metabolic selection, the *pmi* gene [213,214] using mannose.

3.1.4. The centrality of early somatic embryogenic cultures in monocot transformation

The centrality of early somatic embryogenic (SE) cultures in monocot transformation has been seen as essential for improved transformation [4,215,216]. This is understood as a generality and there are many exceptions. Somatic embryos are differentiated outcomes derived from vegetative cells (single or a small group) which undergo development to become independent mature plants. Unlike zygotic embryos, which are formed from the outcome of fertilized gametes, somatic embryos are genetic clones from the explant parent [217]. Direct somatic embryogenesis refers to the formation of embryos from a single mature cell whereas indirect somatic embryogenesis can result via a callus intermediate. Given the attributes of single cell origin and the capability for regeneration to fertile plants renders somatic embryogenesis as ideal for production of transgenic or genome edited

plants. 'Early' somatic embryogenic cultures resemble the early stages of zygotic embryogenesis and are retained in that developmental state by genetic and tissue culture response. In many monocots, for example, these stages in culture resemble zygotic embryos at the 5–8 days after pollination (DAP), hence the descriptor of 'early'. The stages of somatic embryogenesis are very similar to the stages of zygotic embryogenesis in monocots and dicots [218,219].

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

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

Although transformation (DNA transfer and integration) may be achieved at reasonably high frequencies in many cell types, the ability to transform, and generate totipotent target cells which can be recovered and regenerated to plants may be a significant limiting factor. Furthermore, *Agrobacterium*-mediated transformation, most often, especially in the monocots, must involve only recipient target cells at the surface of infected tissues. For many monocot systems, transformation procedures rely on scutellar epidermal cells of immature zygotic embryos. Particle bombardment also involves mainly surface cells [215]. Another limitation of genome editing and transformation is the relatively few numbers of effective selectable markers available for use with most plant species. The ability to test multigenic constructs and gene stacking capabilities in standard transgenic applications currently rely on multiple selection strategies. For multiple genome editing events, this will be less of a factor. Hence, the relatively low transformation efficiency, as measured by number of independent events per treated explant, is not understood as the major obstacle, but rather the ability to reliably recover the independent number of transgenics to biologically replicate and address a given experimental questions is the major obstacle.

Somatic embryogenesis is most often genotype limited [216,224] despite long efforts to improve the conditions for tissue culture. The somatic embryogenic (Type II) tissue culture response is now understood as a quantitative trait locus (QTL) controlling gene networks involved with the developmental responses for embryo formation *in vitro* [216]. Reverse genetics has been used to identify several genes involved with somatic embryogenesis in various plant species, including maize, wheat, pine, soybean, cotton, *Arabidopsis*, and coffee [220,225–227]. QTL mapping has been used across several plant species for identification of genetic factors using populations segregating for embryogenic tissue culture response [199,228–231]. Lowe et al. [229] used marker assisted breeding to move the high tissue culture response phenotype to the recalcitrant maize inbred FBLL further substantiating the genetic underlying mechanisms. Using a QTL fine mapping approach, Salvo et al. [216] identified and validated novel genes involved with somatic embryogenesis. From work done on mutants in the model system *Arabidopsis*, a network of transcription factors are known to be involved with somatic embryogenesis [221].

3.1.5. Dicot transformation now and the future

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

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

The first ground breaking research in dicot transformation for agriculture biotechnological applications was accomplished by Monsanto research group led by Horsch [242]. Hinchee and her coworkers developed a successful *Agrobacterium*-mediated transformation system in soybean (*Glycine max*), a highly recalcitrant dicot crop species, that is still difficult to transform to date. This success relied on utilization of totipotent stem cells located in the cotyledonary-node area of germinating seeds and plant regeneration was through direct shoot organogenesis where no intermediate callus phase was involved. This regeneration regime offers certain advantages over indirect organogenesis in that the transformation process is fast and simple, enables high fertility and retains genome stability of transgenic plants. Subsequently, similar systems were developed or modified to transform a number of other legume species [243]. Meanwhile, other stem cell containing explant tissues from the same or different plant groups have

been also explored to determine alternative yet simple regeneration processes. For example, regeneration and transformation systems have been developed using primary leaf node, hypocotyl, shoot tip, embryogenic axis, or immature cotyledonary-node with varying successful rates [244]. Therefore the dependency on organogenesis in dicot tissue culture systems has provided the basis for transformation protocols.

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

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

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

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

3.1.6. The centrality of organogenesis in dicot transformation

Organogenesis in plants results in the differentiation of various organs, including roots, shoots, stems leaves and flowers without the requirement of preexisting initials. In tissue culture organogenesis, usually involves the dedifferentiation of mature cells in an explant followed by cell differentiation and the formation of organ primordia [263]. Direct organogenesis refers to the formation of primordia without an undifferentiated phase whereas indirect organogenesis can result via a callus intermediate. Organ development is an iterative process in plant development initiated by meristems. All above-ground organs in plants are derived eventually from a shoot apical meristem (SAM) which differentiates lateral organs in phylotactic patterns. Cell layers in the SAM are defined as L1, L2, L3 and a central zone of cells which replenish the meristem [263]. From the SAM, cells transition from the morphogenetic zone to become differentiated organs according to the Hofmeister principle where new organ form maximally distant from previous organs, and auxin plays a major role [264]. Organogenesis in tissue culture requires fundamental changes in gene expression regulatory networks, developmental biology and cell physiology. Somatic direct organogenesis is most often derived from a single cell, as has been demonstrated by Southern blot analysis on many dicot transgenic plants. It appears that a single cell may also be the origin of multicelled groups which will form a SAM in tissue culture via indirect somatic embryogenesis as also indicated by Southern blot analysis. Organogenic pathways toward success plant transformation, selection and regeneration has been clearly identified using GUS and GFP reporter genes. The process or organogenesis is critical for efficient transformation and regeneration in many dicot species.

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

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

4.1. Monocots

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

In order to meet the growing need for transgenics for plant genome editing and analysis, the transformation platform of the future must not only integrate the required characteristics of current systems and facilitate high-throughput generation of events [191], but utilize a set of new tools currently available for genome modification and analyses. Recent advances in plant transgenic biology are rapidly changing the landscape towards the long range goals of being able to transform and

hence edit any plant with any DNA target sequence. Currently, there are various pathways to produce genome edited events using plant transgenics. The selection of strategy is predicated on various issues already discussed including efficiency, intellectual property, regulatory issues and crop species applications.

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

4.2. Dicots

Due to the economic importance of diverse dicot plant species, it is imperative to take advantages of genomic resources and genome editing technology to advance dicot plant biology study and variety development. Evidently, efficient transformation systems are a prerequisite for accomplishing this goal. Despite significant progress has been made in dicot transformation (see previous section), there are still several factors that constrain efficient dicot transformation in some recalcitrant species. First, genotype dependency is common rather than exception for the transformation of most of dicot species. For example, success in cotton transformation has achieved in only one cotton genotype, i.e., the Coker line or its derivatives. Second, high rates of chimerism and non-germline events are common in those dicot transformation systems whose regeneration regime is based on direct shoot organogenesis without intermediate callus phase. The mechanisms of these constraints are still unknown. Third, although the present predominant view considers plant regeneration rather than gene transfer process as the bottleneck, numerous dicot plant species do display good regeneration ability through shoot organogenesis but suffer from poor *Agrobacterium* infection. Use of BBM/WUS has enabled efficient somatic embryogenesis in monocots [3–5]. Use of plant morphogenic regulators offers at least two key advantages: 1) it enables formation of totipotent cells to become regenerable from non-regenerable somatic cells; 2) it may induce indirect shoot organogenesis with a short intermediate callus phase. In dicots, shoot organogenesis without an intermediate callus stage rarely produces chimeric or non-germline events, even though the mechanism of this observation remains elusive (Zhang, unpublished data). Embryogenic and organogenic pathways are not mutually exclusive. There are monocots which exhibit organogenic pathways which can be exploited for transformation. Similarly, in dicots there are embryogenic pathways. Efficient somatic embryogenesis has been achieved in recalcitrant sweet pepper (Capsicum L.) using BBM/WUS [268]. However, as of today, there have not been any reports on enabling dicot plant shoot organogenesis by co-expression of WUS and a gene underlying organogenesis. Thus, it will be interesting to investigate if this approach would allow making breakthroughs in transformation and plant regeneration of dicot species.

5. Pathways to produce viable plant genome edited outcomes

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

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

5.1. Protoplast based systems

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

5.2. Particle bombardment

Particle bombardment (biolistics) can also be used to deliver various macromolecular reagents used for gene editing and obviates the use of protoplasts, but suffers from various other drawbacks. While the macromolecules of interest for genome editing can be delivered by particle bombardment without the use of a selectable marker ([123,124], see 3B) this approach has relatively lower efficiencies of delivery, species and explant dependence. This approach is attractive for a variety of reasons (see below) but requires a robust tissue culture and regeneration response which will not be genotype independent. Particle bombardment has been used to introduce genome editing functions with and without selectable marker genes, where stably integrated events can be subsequently segregated away in subsequent generations.

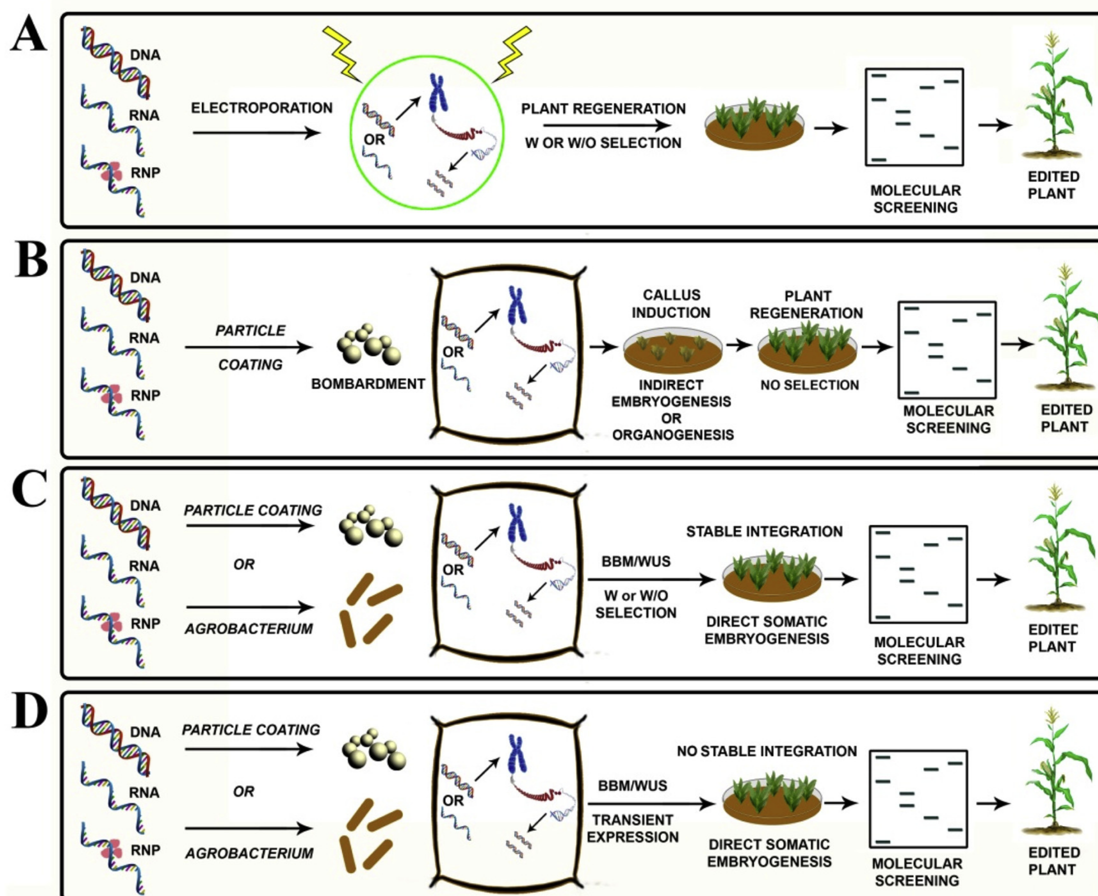

Fig. 3. Pathways to produce viable plant genome edited outcomes. (A) Shows that protoplasts can be used as recipient cells for DNA, RNA and RNPs via electroporation, but is highly species and genotype dependent. (B) Depicts the use of particle bombardment to deliver DNA, RNA, and/or RNPs in wheat followed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

Particle bombardment has an apparent regulatory advantage over *Agrobacterium*-mediated transformation since it does not involve the use of a plant pathogen. This difference may have a regulatory advantage. In addition, it may be possible to use particle bombardment to produce genome edited events which are DNA free, using only RNAs and RNPs, providing perhaps another regulatory advantage as a mutagenesis approach to advanced breeding [123,124]. Particle bombardment also eliminates specific *Agrobacterium* plant specific interactions. Segregation of introduced transgenes can be achieved and verified with whole genome sequencing and other methods. This approach may be considered as advanced mutagenesis and regulated as non-GE in some cases and perhaps only by some countries.

5.3. *Agrobacterium-mediated transformation*

The use of *Agrobacterium* vectors to deliver constructs that include genome editing functions using well known transformable genotypes, recovering stably integrated events and subsequently segregating the transgene away from the edited target has been demonstrated [77]. However, *Agrobacterium* is widely considered as a plant pest and therefore edited plants may be regulated as a GE organism. The additional bottlenecks with this approach include the use of *Agrobacterium* for construct delivery, genotype dependence, explant dependence, tissue culture response, regulation hurdles and time in development. The next step(s) subsequent to recovery of targeted events require backcrossing the targeted edited event into the varieties of commercial interest. Many events using this approach are already in the process of commercialization (SIVB meeting 2018). This is largely the current state of the art, but has several downsides, including: (a) long development time, due to the backcrossing process and linkage drag; (b) genotype limitations, whether due to tissue culture abilities, or explant limitations, such as the requirement for immature embryos or other tissues; and, (c) costly if they need to be deregulated as GE organism. The technology using gene editing including ZNFs) [94–101], TALENS [102–111] and CRISPR ; [6,46,47,112–124], have already been widely applied. Therefore, both particle bombardment and *Agrobacterium*-mediated delivery can be applied for genome editing purposes (Fig. 3C) and with or without selection and without stable DNA integration (Fig. 3D).

5.4. *Morphogenic regulator-mediated transformation*

The development of advanced transformation technologies has been explored through the manipulation of key early somatic embryogenesis genes to promote transformation and plant regeneration. Promoting early stages of somatic embryogenesis (SE) in recalcitrant cereal crop species and varieties has been a key for making a breakthrough in developing advanced cereal transformation technologies. Presently, however, our knowledge about the mechanisms underlying this somatic embryogenesis (SE) is limited. On the other hand, SE in general mirrors

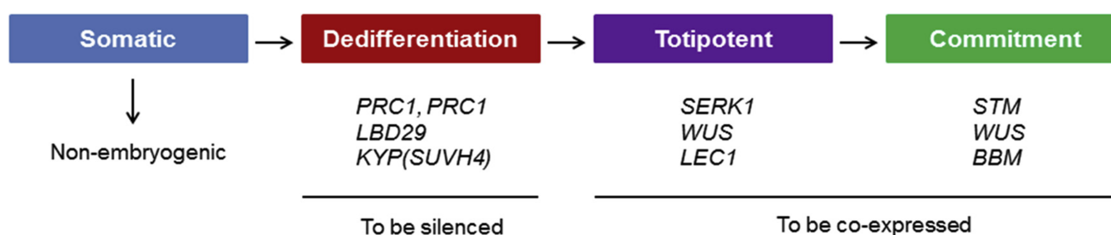

Fig. 4. Key genes governing early stages of somatic embryogenesis (modified from [297]). *PRC1 and PRC2, POLYCOMB REPRESSIVE COMPLEX 1* and *2*; *LBD29, LATERAL ORGAN BOUNDARIES DOMAIN 29; (KYP)/SUVH4, KRYPTONITE; SERK1, SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1; WUS, WUSCHEL; LEC1, LEAFY COTYLEDON 1 ; STM, SHOOT MERISTEMLESS; BBM, BABY BOOM*.

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

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

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

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

Various genes have been overexpressed in transgenic plants to test their ability to induce a somatic embryogenic response in tissue culture (reviewed by [221,222]). Some of these genes are morphogenic regulators encoding transcription factors including: Agamous-Like-15, AGL15 [275]; Baby boom, BBM [276]; Leafy Cotyledon1 [277]; *Lec1* [278]; Somatic Embryogenesis Receptor-Like Kinases, SERK [279]; and Wuschel, WUS [280]. Differential overexpression of AGL15, BBM, SERK and WUS has been demonstrated to improve embryogenic response and reduce genotype dependence [278,281–283]. Recently published research from Lowe et al. [3,4] and Mookkan et al. [5] confirmed the effective use of genetic mechanisms to control somatic embryogenic callus formation, and the re-differentiation of organs and somatic embryos from different tissues through discriminatory expression of morphogenic regulators, WUS2, and *BBM*. Initial attempts utilized an Oleosin promoter driving expression of WUS2 were not successful resulting in chimeric events [4]. The differential expression of BBM and WUS results in direct somatic embryogenesis. However, resulting transgenic cultures either could not be regenerated or resulted in aberrant phenotypes [4]. Further experimentation utilized the strong maize *Ubiquitin* promoter (*ubi*; [284]) driving BBM expression and the weak nopaline synthase (*nos*) promoter from *Agrobacterium* [285] driving WUS2. In order to recover regenerated plants the expression of BBM, WUS2 must be removed. To accomplish excision, the desiccation inducible *rab*17 promoter from maize [286,287] was used to express the CRE recombinase gene [288–290]. One of the constructs (Fig. 5A) described by Lowe et al. [4] was designed with loxP sites flanking the BBM, WUS, CRE cassettes such that upon desiccation stress, *rab*17 would activate CRE expression resulting in removal of the cassettes and allow plant regeneration. These constructs can be delivered via particle bombardment or *Agrobacterium* (as in Fig. 4C) and result in stable integration of T-DNA; transgenic events can be recovered with the use of a selectable marker, as in this case, (Fig. 5A) phosphinothricin (maize Ubi driving moPAT). The fluorescent visual marker YFP was used to visualize events following excision of the BBM, WUS, CRE cassette. In some cases, "helper plasmids" as DNA constructs harboring the *BBM* and *WUS2,* driven by differential expression, results in the genotype independent development of somatic embryos in maize [3]. This system [3,4] demonstrated genotype independence for large numbers of maize inbred lines, sorghum and sugarcane which had been shown previously to be recalcitrant. Soon thereafter, the results described above were confirmed and extended through differential co-expression of *BBM* and *WUS2* coupled with the desiccation inducible *CRE*/lox excision system to enable recovery of stable transgenics without a chemical selectable marker in recalcitrant maize inbred B73 and sorghum P898012

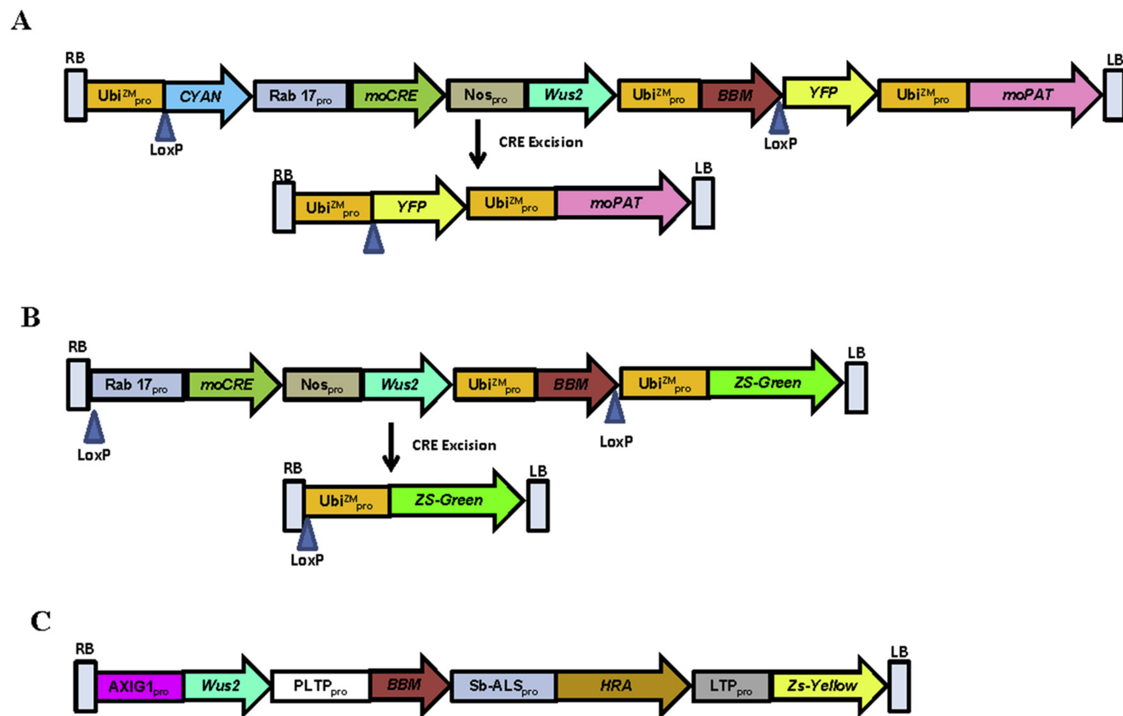

Fig. 5. Description of constructs used for morphogenic regulator mediated transformation in monocots. (A) Shows a diagrammatic representation of the pPHP35648 vector the T-DNA vector using a Nos_{AT} promoter driving the $Wus2_{M}$: pinII and the Ubi_{M} pro driving Bbm_{M}:pinII for induction of somatic embryogenesis. Desiccation induction of CRE is driven by the maize $rab17_{M}$ promoter. this cassette is bracketed by lox P sites where by on desiccation site is removed allowing plant regeneration This vector also includes the selectable marker for bialaphos resistance with a Ubi_{M} pro driving moPAT. The excision events can be visualized with the Ubi pro driving expression of YFP [4]. (B) shows a diagrammatic representation of the PHP78891 vector [5]. Similar to (A), the PHP78891 vector contains four expression cassettes: (i) $RAB17_{M}$: *CRE*; (ii) NOS $_{At}$: *WUS2*; (iii); UBI_{M}: Bbm_{M} and (iv) UBI_{M}: GFP. The *CRE/WUS2/BBM* cassette is bracketed by lox P sites, but does not have a selectable maker. One lox P site is downstream of the right *Agrobacterium* border and the other adjacent to the UBI_{M}: GFP cassette within the left *Agrobacterium* border. (C) shows a construct using the *Zm-PLTP$_{pro}$* to drive expression of *Bbm* [3], which presumably initiates cell division, and subsequently down regulated, an auxin inducible promoter from maize (*Zm-Axig1pro*) to drive expression of expression of *WUS2* allowing plant regeneration when subcultured to auxin free media, and an *Sb-ALS$_{pro}$* driving expression of a double-mutant gene *HRA* [292] as a selectable marker.

(Fig. 5B; [5]). Genome editing reagents can also be supplied using this approach (Fig. 3C,D; [123]). This technology is a paradigm shift in this field. In addition, this new technology has opened the possibility for genotype independent transformation. The use of morphogenic regulators to overcome genotype dependence is a breakthrough technology in cereal transformation, and can be coupled with genome editing capabilities to bring about a change in the study of basic plant biology as well as crop plant breeding [291]. This approach will also enable new plants to become chassis for synthetic biology and the development of new biochemical pathways in plants.

Various improved strategies have been already developed. In an effort to circumvent pleiotropic effects of *BBM* and *WUS* and the need for excision, Lowe et al. [3] demonstrated the use of a system using specific promoters to drive expression of the morphogenic regulator genes (Fig. 5C). A survey of an extensive maize transcriptome atlas of over sixty-five thousand gene models from a gene dataset sequenced via Illumina RNAseq identified a viable candidate, Zm-PLTP. The promoter from a maize phospholipid transferase protein gene (*Zm-PLTP$_{pro}$*) was selected based on its expression pattern in callus, embryos, and leaves but down regulation in other tissues. A construct was developed using the *Zm-PLTP$_{pro}$* to drive expression of *BBM*, an auxin inducible promoter from maize (*Zm-Axig1pro*) to drive expression of *WUS* and an *Sb-ALS$_{pro}$* driving expression of a double-mutant gene *HRA* [292] as a selectable marker (see Fig. 5C). This construct was introduced into an *Agrobacterium* strain carrying an improved accessory plasmid, pPHP71539 [293], which is a derivative of the super binary vector pSB1 [195]; pPHP71539 carries additional copies of *vir* genes as well as corrections to several mutations found in pSB1. When this strain/vector combination was used to transform immature zygotic embryos of maize, large numbers of somatic embryos formed directly from scutellar epidermal cells which could be directly germinated into fertile plants without an intermediate callus phase [3,293].

Expression of morphogenic regulator constructs paired with gene editing functions targeting four genomic regions (liguleless1, *LIG*; acetolactate synthase, *ALS2*; and two male fertility genes, *MS26* and *MS45*) was reported by Svitashev et al [123], who used biolistics to deliver Cas9-gRNA ribonucleoproteins (RNPs) into immature maize embryos. DNA vectors carrying maize BBM, WUS, and a MoPAT-DSRED visual/selectable marker transgene cassette were co-bombarded with the RNPs into the embryos. In this case integrated DNA can be segregated away from edited targets. Delivery of either non-integratable DNA or RNA with RNPs would accomplish the same goal, with the exception that there would be no original integrative event (Fig. 3D). Svitashev et al. [123] also deployed this very method and successfully obtained edited plants using Cas9-gRNA RNPs alone, targeting three genomic regions (*LIG*, *MS26* and *MS45*). Since no DNA has been integrated in these cases, current considerations render this approach under regulatory review to be determined as an advanced mutagenesis approach and hence potentially non-GE, especially when *Agrobacterium* is not used as a delivery system. In this scenario the introduction of molecules facilitating gene editing without integration would result in a rapid recovery of events without the need for backcrossing, saving time and resources. Ideally this would allow 'editing at will' in well known established varieties or inbreds and recovered without any further breeding or backcrossing.

6. Regulatory considerations

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

Worldwide, there is a lack of agreement regarding whether or not the products of gene editing will be regulated as GE organisms. Countries that have rendered opinions regarding which techniques should be treated as GE include the United Kingdom, Germany, Sweden, Switzerland, and South Africa [297]. In these countries, there is incomplete agreement regarding the GE status of certain organisms. For example, in the United Kingdom, offspring of edited products of oligo-directed mutagenesis and zinc-finger nucleases are considered non-GE; Germany agrees with the exception of offspring of edited products obtained through the use of zinc finger nuclease-3, while South Africa considers oligo-directed mutagenesis and use of site directed nucleases, with the exception of intragenesis achieved with these nucleases, as non-GE [297]. Some uniformity among European countries will be established by the Court of Justice of the European Union following its decision in July 2018, in a court case involving this issue. In advance of this decision, the Advocate General of the court rendered an opinion, stating that gene edited organisms are

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

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

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

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

7. Education and the public interests

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

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

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

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

The application of genetic modification (GMO) in crop plants by standard technologies has largely been restricted to a low number of traits in high volume row crops controlled by the largest agricultural companies. Despite the very large number of studies and evaluations over three decades every claim of negative health or environmental consequence from genetically modified crops has not stood up to

scientific validation [302]. Meanwhile the anti-GMO mentality has persisted through fear mongering with a substantial negative impact to world agriculture and food security. To be clear, the anti-GMO movement has not been benign but has resulted in serious agricultural setbacks and world food production issues. Simply put, the world cannot afford another equivalent setback regarding the current technologies.

We, the public, need to make informed decisions. Never before have we been challenged with so many crises and with so many tools to address them. We should not do just because we can, but we should do what is most responsible. This is necessary to maintain a high degree of public trust and integrity. The roles of education, governance and stewardship are hugely important to these goals. What we teach our students now will inform our future public policy.

8. Conclusion

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

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

Acknowledgements

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