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REVIEW

Maize transformation: history, progress, and perspectives

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Abstract Maize functional genomics research and genetic improvement strategies have been greatly accelerated and refined through the development and utilization of genetic transformation systems. Maize transformation is a composite technology based on decades' efforts in optimizing multiple factors involving microbiology and physical/biochemical DNA delivery, as well as cellular and molecular biology. This review provides a historical reflection on the development of maize transformation technology including the early failures and successful milestones. It also provides a current perspective on the understanding of tissue culture responses and their impact on plant regeneration, the pros and cons of different DNA delivery methods, the identification of a palette of selectable/screenable markers, and most recently the development of growth-stimulating or morphogenic genes to improve efficiencies and extend the range of transformable genotypes. Steady research progress in these interdependent components has been punctuated by benchmark reports celebrating the progress in maize transformation, which invariably relied on a large volume of supporting research that contributed to each step and to the current state of the art. The recent explosive use of CRISPR/Cas9-mediated genome editing has heightened the demand for higher transformation efficiencies, especially for important inbreds, to support increasingly sophisticated and complicated genomic modifications, in a manner that is widely accessible. These trends place an urgent demand on taking maize transformation to the next level, presaging a new generation of improvements on the horizon. Once realized, we anticipate a near-future where readily accessible, genotype-independent maize transformation, together with advanced genomics, genome editing, and accelerated breeding, will contribute to world agriculture and global food security.

Keywords Advanced breeding · Functional genomics · Genetic modification · Morphogenic regulators · Plant transformation · *Zea mays*

Abbreviations

ABA	Abscisic acid
BBM	Baby Boom transcription factor
BMS	Black Mexican Sweet
CaMV	Cauliflower mosaic virus

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Cas9	CRISPR-associated protein 9
CIMMYT	Centro Internacional de Mejoramiento de Maíz y Trigo (in Spanish) International Maize and Wheat Improvement Centre
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
eSECs	"Early" somatic embryogenic cells
DAP	Days after pollination
EMS	Ethyl methanesulfonate
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
EU	European Union
FTO	Freedom to operate
GM	Genetically modified
GMO	Genetically modified organism
GWAS	Genome-wide association study
HPT	Hygromycin phosphotransferase
IE	Immature embryo
MRT	Morphogenic regulator-mediated transformation
NPT	Neomycin phosphotransferase
PEG	Polyethylene glycol
PGS	Plant Genetic Systems
RNPs	Ribonucleoproteins
SAM	Shoot apical meristem
SiC	Silicon carbide
TALENS	Transcription activator-like effector nucleases
WUS2	Wuschel 2 transcription factor
ZFN	Zinc finger nucleases

Introduction

The significance of maize (*Zea mays* L.) to the study of plant genetics as well as to global agriculture, world economy, and food security is widely known. In just 2019 alone, 60.9 Mha of transgenic maize varieties were planted worldwide (ISAAA 2019). New varieties of maize generated using genetic modification technologies produce up to 10% higher yields than similar conventional varieties (Stokstad 2019). Transformation technology in maize has been central to recent agronomic progress for maize, and will continue to be essential for future development of important new maize varieties as well as basic scientific investigations on functional genomics, phenotypic trait analysis, and plant science (Kausch et al. 2019; Que et al. 2014; Yadava et al. 2017).

Our understanding of the maize genome continues to progress rapidly, as maize researchers increasingly focus the power of deep genome learning on the breadth of maize diversity and the rich historical genetic knowledge of this crop. Deep sequencing is increasing our understanding of maize diversity (Wang et al. 2019), transposon biology (Springer et al. 2018), and maize miRNA expression (Liu et al. 2019). In parallel with the explosion in maize genomics, CRISPR/Cas-9-based genome editing systems are facilitating precise and efficient maize genome modification that were unimaginable even 10 years ago. Finally, the substantial advances in maize transformation achieved to date will provide the egalitarian access required to realize the potential of this new era of crop improvement. Thus, the synergistic application of plant transformation, advanced genomics, and genome editing provides a potent interdependent triad for functional genomics research and advanced molecular breeding (CAST 2018; Altpeter et al. 2016; Kausch et al. 2019).

More than five decades of research on maize transformation technology have provided the capability to reliably create, test, cultivate, and breed maize transgenics. However, underlying the genomics/transformation/editing triad described above is the often underappreciated requirement to create, test, and cultivate genome-edited, but otherwise non-transgenic, plants. This represents a departure from the previous paradigm where plant transformation was defined by introducing *transgenes* to create regenerable fertile T0 plants. This transgenic model is giving way to today's technology where precise genome modifications are produced (including single-base changes, multi-base edits, deletions, or cisgenic/intragenic alterations) with no incorporation of foreign DNA in the genome. This new generation of genome editing still requires ourplant transformation methods to facilitate this process, and in fact, as the demand for higher numbers and greater intricacy of genome edits increases, this will place a concomitantly greater demand on our underlying transformation methods used to produce such large numbers of genomic changes with surgical precision.

Here we describe how we have arrived at the current state of maize transformation and provide our perspectives for next steps to achieve the necessary transformation methods for "deep genome" level research that will be required to meet future challenges.

A brief history of maize transformation

When the first dicot transformation experiments were reported simultaneously in 1983 (Bevan et al. 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983), it was widely assumed that the technology would be easily extended to all plants including monocots, as indicated in the title of the seminal article “A Simple and General Method for Transferring Genes into Plants” (Horsch et al. 1984). This optimism faded as labs around the world worked to transfer the technology to maize and other cereal crops. Techniques for gene transfer resulting in stable integration events in maize and other cereals that could be selected and regenerated to fertile plants remained elusive for nearly a decade. Arguments ensued over what constituted integrative transformation, what represents a recipient cell that was competent for integrative transformation and regeneration, and even speculation that transformation of monocots in general may not be possible (Potrykus 1989, 1990).

The history of plant transformation has been widely reviewed previously (Vain 2007; Vasil 2008; Ramkumar et al. 2020) and provides an inspiring view of the overall accomplishments which supported maize transformation. A timeline of the supporting events in plant transformation and the key research milestones related to maize transformation is shown in Fig. 1. It is clear that the success of maize transformation is the result of dedicated and multidisciplinary research effort cumulated over many decades. It is also clear that the accomplishments and lessons learnt from transformation of maize impacted and influenced the successful transformation of the other cereal crops.

The first attempt to transform maize with exogenous DNA was reported more than 50 years ago (Coe and Sarkar 1966) through injection of genomic DNA from a purple, red-anther maize variety into apical meristems of 242 wild-type maize seedlings. The outcome was disappointing, as none of the characteristics such as purple sheath, husk, or anthers of the donor plants was seen in the treated plants. Nevertheless, they reported the experiment and concluded that “Competence, nucleases, penetration, susceptibility of loci, numbers treated, and degradation are indicated as problems that may need to be considered and solved in order to achieve transformation in higher plants.” Nearly half a century later, the maize community is still addressing many of these same issues.

A number of challenges would need to be overcome before plant transformation was finally achieved in monocots. Based on the early work in dicots, it became apparent that three processes would need to be merged to form a unified maize transformation method. These included (i) an efficient tissue culture and regeneration system, (ii) a method for introducing foreign DNA into the cell, and (iii) the ability to select clonal transgenic tissue capable of regenerating a T0 plant.

Early success of regeneration from tissue culture in maize inbred A188 (Green and Phillips 1975) stimulated a flurry of research activity. Over the next three decades, both compact embryogenic/organogenic (Type I) and friable embryogenic (Type II) callus culture (Armstrong and Green 1985) systems would become well established within the research community, although such culture responses and the subsequent ability to regenerate fertile plants would remain constrained to only a few model inbreds or derivative hybrids (see Jones 2009 for review). Tomes and Smith (1985) and Hodges et al. (1986) recognized that tissue culture response and plant regeneration capacity were genetically determined by nuclear genes and thus influenced by genotypic variation. Likewise, Willman et al. (1989) and Bohorova et al. (1995) concluded that genotypic variation in tissue culture response for somatic embryogenesis of maize was controlled or influenced by multiple loci. Mapping studies began to identify chromosomal regions that potentially contribute to somatic embryogenesis in culture.

Between the early 1990s (Armstrong et al. 1992) and the mid-2000s (Krakowsky et al. 2006), quantitative trait loci (QTL) were identified that correlated with improved culture/regeneration. It was also demonstrated that QTL-specific markers could be used to aid in introgressing the regenerable culture response into an otherwise unresponsive maize inbred (Lowe et al. 2006). However, despite intense research focus on this topic, regenerable culture response remained extremely limited to a small subset of maize genotypes. During this period, it was tacitly recognized that the rapidly growing, friable, somatic embryogenic culture response would be most amenable to both transformation and selection, and this will be

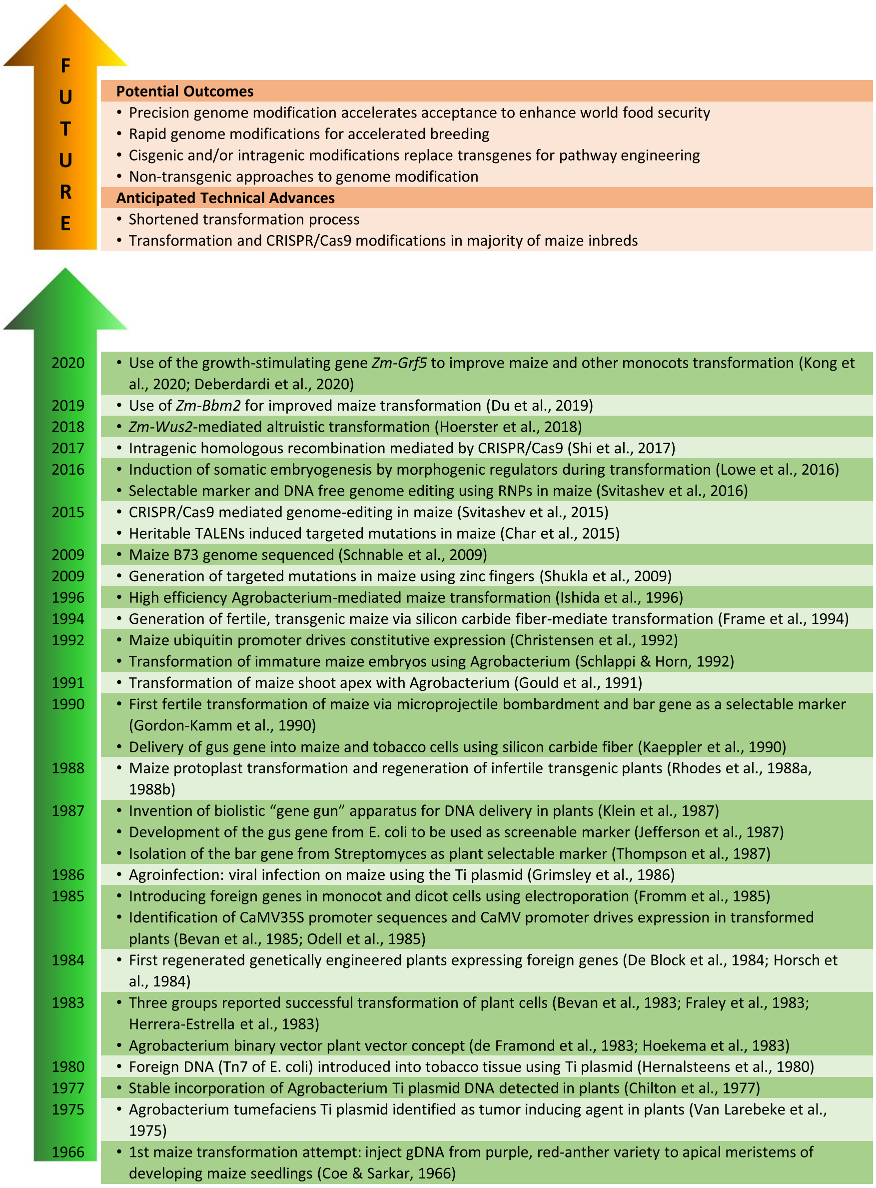

Fig. 1 Timeline of the history of maize transformation

Potential Outcomes

- Precision genome modification accelerates acceptance to enhance world food security
- Rapid genome modifications for accelerated breeding
- Cisgenic and/or intragenic modifications replace transgenes for pathway engineering
- Non-transgenic approaches to genome modification

Anticipated Technical Advances

- Shortened transformation process
- Transformation and CRISPR/Cas9 modifications in majority of maize inbreds

2020	• Use of the growth-stimulating gene *Zm-Grf5* to improve maize and other monocots transformation (Kong et al., 2020; Deberdardi et al., 2020)
2019	• Use of *Zm-Bbm2* for improved maize transformation (Du et al., 2019)
2018	• *Zm-Wus2*-mediated altruistic transformation (Hoerster et al., 2018)
2017	• Intragenic homologous recombination mediated by CRISPR/Cas9 (Shi et al., 2017)
2016	• Induction of somatic embryogenesis by morphogenic regulators during transformation (Lowe et al., 2016) • Selectable marker and DNA free genome editing using RNPs in maize (Svitashev et al., 2016)
2015	• CRISPR/Cas9 mediated genome-editing in maize (Svitashev et al., 2015) • Heritable TALENs induced targeted mutations in maize (Char et al., 2015)
2009	• Maize B73 genome sequenced (Schnable et al., 2009)
2009	• Generation of targeted mutations in maize using zinc fingers (Shukla et al., 2009)
1996	• High efficiency Agrobacterium-mediated maize transformation (Ishida et al., 1996)
1994	• Generation of fertile, transgenic maize via silicon carbide fiber-mediate transformation (Frame et al., 1994)
1992	• Maize ubiquitin promoter drives constitutive expression (Christensen et al., 1992) • Transformation of immature maize embryos using Agrobacterium (Schlappi & Horn, 1992)
1991	• Transformation of maize shoot apex with Agrobacterium (Gould et al., 1991)
1990	• First fertile transformation of maize via microprojectile bombardment and bar gene as a selectable marker (Gordon-Kamm et al., 1990) • Delivery of gus gene into maize and tobacco cells using silicon carbide fiber (Kaeppler et al., 1990)
1988	• Maize protoplast transformation and regeneration of infertile transgenic plants (Rhodes et al., 1988a, 1988b)
1987	• Invention of biolistic "gene gun" apparatus for DNA delivery in plants (Klein et al., 1987) • Development of the gus gene from E. coli to be used as screenable marker (Jefferson et al., 1987) • Isolation of the bar gene from Streptomyces as plant selectable marker (Thompson et al., 1987)
1986	• Agroinfection: viral infection on maize using the Ti plasmid (Grimsley et al., 1986)
1985	• Introducing foreign genes in monocot and dicot cells using electroporation (Fromm et al., 1985) • Identification of CaMV35S promoter sequences and CaMV promoter drives expression in transformed plants (Bevan et al., 1985; Odell et al., 1985)
1984	• First regenerated genetically engineered plants expressing foreign genes (De Block et al., 1984; Horsch et al., 1984)
1983	• Three groups reported successful transformation of plant cells (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983) • Agrobacterium binary vector plant vector concept (de Framond et al., 1983; Hoekema et al., 1983)
1980	• Foreign DNA (Tn7 of E. coli) introduced into tobacco tissue using Ti plasmid (Hernalsteens et al., 1980)
1977	• Stable incorporation of Agrobacterium Ti plasmid DNA detected in plants (Chilton et al., 1977)
1975	• Agrobacterium tumefaciens Ti plasmid identified as tumor inducing agent in plants (Van Larebeke et al., 1975)
1966	• 1st maize transformation attempt: inject gDNA from purple, red-anther variety to apical meristems of developing maize seedlings (Coe & Sarkar, 1966)

more thoroughly described in the "Somatic embryogenic cultures: a prerequisite for maize transformation" section below.

In addition to having the proper tissue culture and regeneration systems as the underlying platform for transformation, an often underappreciated requirement is the appropriate selectable marker gene (and options for selection protocols) needed for the development of successful maize transformation systems (Jones 2009; Que et al. 2014). The ability to provide resistance through expression of an introduced selectable marker gene, allowing survival of that cell amidst an overwhelming population of non-transformed cells, was a prerequisite for the demonstration of successful maize transformation. The establishment of specific maize germplasm with high Type II callus response (Armstrong et al. 1991) was well suited to this co-development of selectable markers.

Early work on transformation in various plant species occurred concurrently (see Fig. 1), which supported and accelerated progress toward maize transformation. Aspects of plant transformation that were pioneered in dicots included DNA delivery mediated by *Agrobacterium tumefaciens*. The first stable incorporation of *Agrobacterium* Ti plasmid DNA in plants was reported by Mary-Dell Chilton's lab (Chilton et al. 1977), taking advantage of the in vitro "hairy root" growth pattern in transgenic tobacco cultures. While an analogous hairy root phenotype was lacking in maize, the development of friable Type II cultures in maize provided a similar tool for early transformation studies. Of special significance across all plant transformation efforts, including maize (Fig. 1), the identification of the CaMV35S promoter and its wide usage in transformed plants (Bevan et al. 1985; Odell et al. 1985) was critical in early successes such as the development of the first herbicide-resistant plant through expression of a mutant 5-enolpyruvylshikimate- 3-phosphate (EPSP) synthase gene to produce a glyphosate-tolerant Petunia line (Shah et al. 1986).

Stable transformation of maize (Fromm et al. 1986) was first achieved using electroporation of protoplasts made from maize "Black Mexican Sweet" (BMS) suspension cultures. While useful for basic experiments testing selective agents or molecular components such as promoters, BMS cell suspensions were of limited value because this cell line had lost the ability to regenerate. Focus thus shifted to embryogenic cultures and how to select and/or visibly screen for transgenic events. Selectable markers first demonstrated in dicots, such as the antibiotic-resistant neomycin phosphotransferase II (*npt*II) gene and the *bar* gene that confers resistance to the herbicide bialaphos (De Block et al. 1989), became logical candidates for monocot transformation. Likewise, the development of the β-glucuronidase (*gus*) gene reporter gene system by Jefferson et al. (1987) enabled visualization of both transient and stable expression of delivered DNA, albeit leading to death of expressing cells following GUS assay treatment. This allowed optimization of delivery, selection for a co-integrated selectable marker, and regeneration of plants with stably integrated transgenes. As a result, the GUS assay was widely used.

The obstacle of DNA delivery in monocots to produce the first regenerated and fertile transgenic monocots was first overcome using rice protoplasts (Zhang and Wu 1988; Toriyama et al. 1988; Shimamoto et al. 1989); however, maize protoplast transformation and antibiotic selection resulted only in infertile plants (Rhodes et al. 1988a, b). To overcome the perceived obstacle of *Agrobacterium* infection in monocots (Potrykus 1990) and the prohibitively laborious nature of protoplast culture, especially in maize, John Sanford and Ted Klein developed the "gene gun" for the direct delivery of DNA into plant cells by acceleration of plasmid DNA-coated micron-sized particles of tungsten into plant cells using a modified 0.22 caliber pistol barrel (Klein et al. 1988a, b, 1987; Sanford 2000). This was truly one of the most creative and innovative solutions to DNA delivery into plant cells ever conceived that has stood the test of time, remaining one of the standard methods of plant transformation today. Microprojectile bombardment obviated the need for cumbersome protoplast systems, resulting in production of the first fertile transgenic maize using somatic embryogenic suspension cultures (SC82 and SC719) (Fromm et al. 1990; Gordon-Kamm et al. 1990; 1991). The ability to select transformed cells from the overwhelming population of non-transformed cells is an indispensable requirement for successful recovery of transformed plant cells, and the *bar* gene is well suited to this purpose especially given its slow kill properties in maize in combination with the rapid, undifferentiated growth of maize Type II callus. Soon after the maize success, the generation of fertile, transgenic wheat was reported using similar protocols (Vasil et al. 1992; Shewry et al. 1995), followed by sugarcane (Bower and Birch 1992), rye (Castillo et al. 1994; Spangenberg et al. 1995), tritordeum (Barcelo et al. 1994), turfgrasses (Zhong et al. 1993), and eventually many other cereals and monocots (Barcelo and Lazzeri 1995).

Biolistic technology, while facilitating early monocot transformation, had several inherent drawbacks for cereal transformation, such as high frequencies of (i) integration of the vector backbone (which can be overcome by delivery of the transgene expression cassette only), (ii) loss of transgene cassette integrity, (iii) multicopy inserts which can result in rearrangements, instability, and transgene silencing, (iv) complications for gene expression analyses, and (v) the resultant impediments in breeding commercial varieties.

Improvements to *Agrobacterium* vectors resulted in enhanced strains finally enabling successful and efficient transformation and regeneration of the maize inbred A188 and its derivatives (Ishida et al. 1996). In the subsequent 25 years, the *Agrobacterium*-mediated method has gone through continued improvements (for example, see Frame et al. 2006; Anand et al. 2018). In combination with sophisticated tissue culture technologies, *Agrobacterium*-mediated transformation has become the method of choice for cereal crops such as rice, maize, barley, oat, wheat, sorghum, millet, triticale, and rye (reviewed by Hiei et al. 2014; Kausch et al. 2019), superseding methods such as direct DNA transformation (Shrawat and Lorz 2006; Barampuram and Zhang 2011) or electroporation of protoplasts (Fromm et al. 1987) for generating transgenics. Still, the transformation of maize is not a simple story and remains a very active field replete with innovation and development (see Fig. 1).

Early failures and the importance of fortitude

It was clear early on that the ability to transfer genetic material into maize would be a significant breakthrough, but it was not obvious how this would be accomplished. Two key obstacles were recognized by Coe and Sarkar (1966) in their unsuccessful attempts to introduce exogenous DNA into maize: (1) the cell wall is an effective barrier to the introduction of macromolecules such as DNA, and (2) it may not be possible to recover the cells which receive foreign DNA from the many which do not (i.e., without selection). In later work by many labs, further obstacles were identified when it became apparent that not all maize varieties were amenable to cell and tissue culture, or regeneration to fertile plants. The intervening years saw many optimistic but unsubstantiated claims of maize transformation (Kivilaan and Blaydes 1974; Korohoda and StrzaŁka 1979).

In hindsight, many of the early unsuccessful attempts at cereal transformation are understandable. While the first transformed dicot species were produced using *Agrobacterium*, it became clear that those techniques could not readily be applied to most monocots. It was generally believed that *Agrobacterium* lacked the molecular machinery to transfer DNA to monocots (Potrykus 1989, 1990), despite elegant but conflicting experiments which indicated otherwise (Grimsley et al. 1987). The lack of competent recipient cells which were selectable and regenerable and the senescent wound response inherent to most monocots were thought to contribute to the inability to recover transformed cells (Potrykus 1989, 1990).

Reports using physical transfection of plasmid DNA into cereal protoplasts started on an encouraging note, with a flurry of reports demonstrating transient expression after DNA delivery (Potrykus et al. 1985; Lörz et al. 1985; Fromm et al. 1986). However, regenerating robust, fertile transgenic plants from protoplasts remained problematic (Rhodes et al. 1988a, b; Shimamoto et al., 1989; Zhang and Wu 1988). Using established maize embryogenic suspension cultures to prepare protoplasts, transformation via direct DNA uptake facilitated by polyethylene glycol (PEG) was used to produce a regenerated T0 plant which unfortunately was not fertile (Rhodes et al. 1988a, b).

For maize, one of the first demonstrations of successful protoplast transformation (and transgene inheritance) would require approaching the problem from the perspective of a geneticist. This approach began with multiple generations of selective breeding to produce germplasm that was either highly regenerable from protoplasts or that produced stable friable suspension cultures, and then crossing these two lines and applying selection for both traits. The result was a single maize genotype capable of regenerating fertile plants from isolated protoplasts (Morocz et al. 1990). This genotype (designated as HE/89) was then used to generate an embryogenic suspension culture from which protoplasts could be produced at high titers, transfected with plasmid DNA using PEG, cultured using the selective antibiotic hygromycin, regenerated to produce fertile T0 plants, and finally demonstrated to transmit the transgenic traits to progeny (Golovkin et al. 1993; Omirulleh et al. 1993). The above attempts and innovations demonstrated that persistence can be an important ingredient in basic research, and likely contributed to the first report of successful protoplast transformation and transgene inheritance in a Novartis inbred a few years later (Wang et al. 2000a, b).

These accomplishments, however, did not resolve the underlying issues that would preclude maize protoplasts becoming generally useful for transformation. The protoplast systems, tantalizingly rich in potential, were notoriously difficult in practice, especially in high-throughput experiments. Such systems had cell culture issues related to “protoplastability” of suspension cultures and longevity in culture resulting in off-type somaclonal variation. Protoplast methods were often unreliable with many parameters not under experimental control. Finally, the level of expertise and commitment required for this technology put this method beyond the reach of most maize research groups.

During this period, various alternative techniques for DNA transfer to plants were explored, which at first looked promising but gave rise to unexpected problems and low reproducibility. These included direct microinjection and electrophoretic transfection to intact tissues, mature pollen transformation, *Agro*-infection of mature pollen, *Agro*listics tissue incubation, use of non-integrating DNA and RNA viral vectors, pollen tube transformation, magnetic mediated transformation, and "floral dip" transformation in plants other than *Arabidopsis* or *Camelina*. Some of these approaches will be discussed later in this review. The fact that some of these approaches are still being actively investigated highlights the need and importance of novel and improved transformation technologies, even today.

Small steps leading to routine maize transformation

"Transformability" is a composite of numerous processes, and their individual improvement has depended on how they can be evaluated and optimized relative to each other. These components include (i) DNA introduction; (ii) depending on the goal, stable integration of the transgene when necessary or target editing; (iii) genotype-specific culture response; (iv) the ability to select totipotent transformed/edited cells; and (v) fertile plant regeneration from stably transformed or edited cells. The early successes in maize transformation foresaw, understood, and incorporated these criteria.

The late 1980s was a period of intense research activity in maize transformation, with competing labs using a variety of approaches for DNA delivery, selection of transformants, and subsequent plant regeneration. Useful and reproducible maize transformation, defined as the production of fertile transgenic plants which produce viable T1 transgenic seed, was first reported 30 years ago (Fromm et al. 1990; Gordon-Kamm et al. 1990) using the biolistic gun delivery system. While these seminal publications provided the means to realize applications and outcomes from maize transformation, the naïve thought that maize transformation had been solved quickly evaporated with the realization that much more improvement was necessary. The next important step occurred with the first published report of fertile, stable transgenic maize using an *Agrobacterium* "super-binary" vector (Ishida et al. 1996). Subsequently, a standard binary *Agrobacterium* vector system was developed (Frame et al. 2002) which has been more generally adopted. Maize transformation was becoming a reality for an ever-increasing number of labs.

It is often presupposed that the development of enhanced *Agrobacterium* strains alone (Hiei et al. 1994; Ishida et al. 1996; Komari et al. 1996) was the sole factor resulting in significant improvements to monocot transformation. However, substantial improvements to maize transformation were also derived from a number of complementary developments. These included advances in underlying tissue culture techniques such as (i) development and culture of the so-called early somatic embryogenic cultures (such as Armstrong 1999; Armstrong and Green 1985), (ii) advancing our understanding of complex media formulations (Bohorova et al. 1999), and (iii) identification of optimal explant sources such as immature embryos, which produce embryogenic cultures (Barampuram and Zhang 2011). Methods for DNA delivery were also rapidly evolving. In addition to *Agrobacterium*-mediated transformation (Ishida et al. 1996), diverse methods also include PEG-mediated protoplast transformation (Wang et al. 2000a) and microprojectile bombardment protocols (Svitashev et al. 2016; Zhang et al. 2016).

Strategies to improve embryogenic response continued to be explored, with many efforts focusing on careful selection and breeding of genotypes amenable to production of an embryogenic culture response (Armstrong 1999; Morocz et al. 1990). Alternatively, departing from conventional tissue culture manipulation to control embryogenic growth, growth-stimulating genes (Gordon-Kamm et al. 2002) or maize morphogenic regulators such *Lec1* (Lowe et al. 2002) and the combination of *Wus2*/*Bbm* genes were used to increase transformation frequencies and extended the range of usable target explants (Lowe et al. 2016, 2018). Use of such genes also reduced the dependence on having an underlying genotype-dependent embryogenic culture response (see the "Morphogenic genes increase transformation and genome editing efficiency" section in this review). Finally, the use of appropriate selectable markers for monocots (Jones 2009) was critically important, along with a variety of species-specific refinements such as optimizing promoters driving constitutive, developmental, inducible, cell- and tissue-specific expression (Datla et al. 1997), and genotype-dependent cell culture responses (Cheng et al. 2004), which all contributed to this overall success.

Somatic embryo formation which underlies the embryogenic culture response has been crucial for improving maize transformation (Kausch et al. 1995, 2019; Lowe et al. 2018; Salvo et al. 2018). Development of the morphogenic regulators WUS2 and BBM to stimulate embryogenesis (Lowe et al. 2016; Mookkan et al. 2017; Lowe et al. 2018; Zhang et al. 2019; Hoerster et al. 2020) continues to improve efficiency, extends genotype range, and improves the potential for high-throughput applications. With routine and reliable protocols now in place, and continuing improvements, the application of transgenic technology in maize production and modern agriculture is now imperative to all serious breeding programs, both academically and commercially. Maize transformation is an essential technology for advanced trait and phenotypic analysis and potential agronomic development. Hopefully, continued improvements through the use of morphogenic regulator genes such as BBM and WUS2 will contribute toward the ultimate goal of true genotype-independent transformation of recalcitrant maize inbreds and other cereal varieties.

Developing tissue culture and selection systems for transformation

Historic work in maize tissue culture established a strong platform for starting to explore transformation. Tissue culture systems and plant regeneration were recognized early on as an important prerequisite to transformation. Tissue culture options center around the morphology (developmental state) of the tissue, and this can have a major impact on the efficacy of selectable marker systems.

The role of tissue culture morphology in maize transformation

Over the past 45 years, many maize genotypes have been tested in tissue culture, with inbreds and hybrids producing a variety of interesting in vitro growth responses. In general, there are three tissue culture responses that have played an important role in transformation, including the “immortalized” cell suspension derived from a maize variety referred to as “Black Mexican Sweet” (BMS), and two types of regenerable cultures that differ in callus morphology, the regenerable cultures having the most impact and utility for maize transformation methods.

BMS cell suspensions: providing early hope that corn could be transformed

Successful maize transformation technologies are currently based on intricate cell and tissue culture systems. Those systems have been painstakingly and meticulously researched over decades. Early on, cell cultures derived from a maize variety known as “Black Mexican Sweet” (BMS) were first developed by William Sheridan (Sheridan 1975; Sheridan and Neuffer 1980; Sheridan 1982) as a system to study and evaluate cell and tissue culture parameters in maize. Previous investigations had already described variation in callus growth of different maize genotypes to a standard culture medium (Green and Phillips 1975) and evaluated media for the induction and maintenance of maize callus (Green and Phillips 1975; Sheridan 1975). BMS cultures became instrumental as an important model in many early studies on maize cell and tissue culture (Sheridan 1982; Kamo and Hodges 1986; Kamo et al. 1987) and in understanding the early stages of the transformation process, such as plasmid DNA delivery, and transient expression (Fromm et al. 1986; Klein et al. 1989). Even though BMS cultures are non-regenerable, they served as a vital proxy for regenerable cultures. Batch culture growth of BMS suspension cultures consists of homogeneous suspensions of single cells or small clusters of cells (Fig. 2a–c), which are rapidly growing, highly reproducible, and easily maintained on a well-defined medium (Kirihara 1994) through routine subculture. Subculture is accomplished on a regular schedule by removing an aliquot of suspension in conditioned medium and transferring to fresh media. These characteristics were conducive for their use in early maize transformation and important selection experiments, including “kill curves” with various selective agents using protocols analogous to those used for microbial systems.

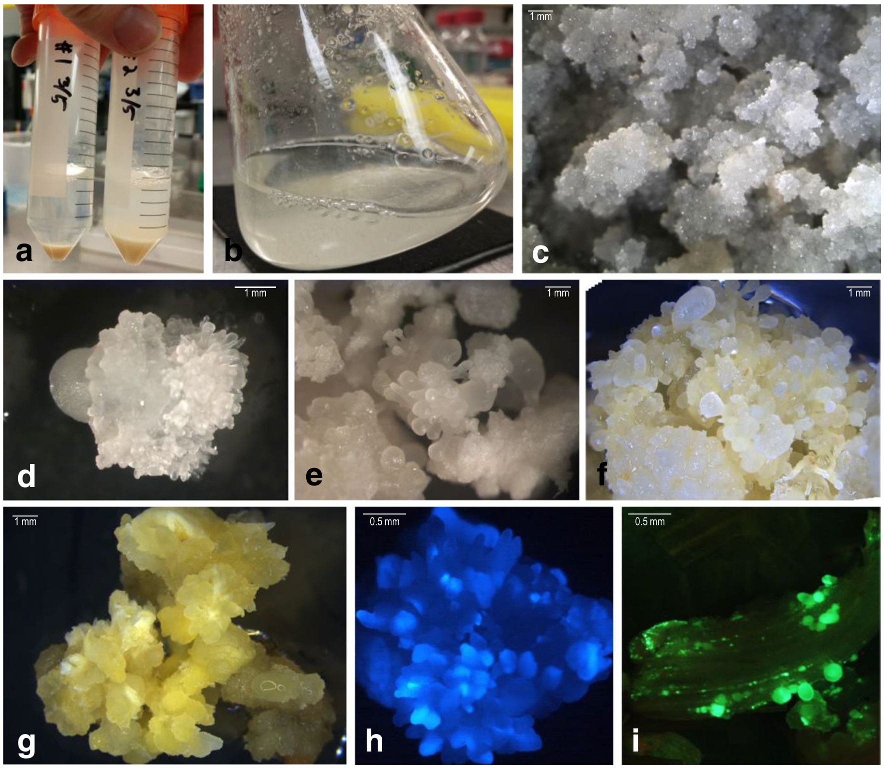

Fig. 2 Tissue culture types used in maize transformation. **a**, **b** Suspension cultures of Black Mexican Sweet (BMS). **c** BMS callus culture. **d**–**f** Type II friable embryogenic callus of Hi II (A × B). **g** Type I embryogenic callus of LH244. **h**, **i** Direct somatic embryo development 2 weeks after *Agrobacterium*-mediated delivery of *Wus2* and *Bbm* morphogenic genes along with either a constitutive CYAN fluorescent gene into immature embryos of Corteva inbred PHB38 (**h**), or a constitutive ZS-GREEN1 into leaf tissue from inbred HC69 (**i**). Credits: **a**–**g** by Frank McFarland (University of Wisconsin); **h** and **i** by Larisa Ryan, Corteva Agriscience

BMS cultures played an important role during the early stages for the determination of various transformation parameters through electroporation of protoplasts (Fromm et al. 1986) and for initial microprojectile bombardment experiments (Klein et al. 1989). BMS suspension cultures were also used in plant cell physiology investigations (Fetter et al. 2004; Cavez et al. 2009; Stadler et al. 2011). It was observed that production of conditioning factors in media derived from BMS cultures (Somers et al. 1987) or using BMS as “nurse cultures” was beneficial for recovery and growth of protoplasts derived from other embryogenic calluses (Kamo and Hodges 1986; Kamo et al. 1987). The use of BMS cultures sets the stage for early transformation experiments on maize using regenerable embryogenic cultures.

Somatic embryogenic cultures: a prerequisite for maize transformation

The first report of plant regeneration from tissue culture of maize established an important benchmark (Green and Phillips 1975). The growth form (or morphotype) of the callus in this early report would later be called Type I. Subsequent work on regenerable maize tissue culture resulted in the description of two general growth forms characterized by their developmental biology in culture. By far the most common are referred to as Type I and Type II (Fig. 3a and b, respectively). These designations for Type I and Type II callus cultures were first established by Armstrong and Green (1985). Developmentally organized and compact tissue was typically referred to as Type I callus and consisted of a mixture of “late” embryogenic, organogenic, and meristematic domes (often fused together), which characteristically present a continuous epidermal layer over their surfaces (Fig. 2g). Type II cultures, in comparison, are “early” embryogenic cultures comprising rapidly growing, friable cell clusters (Fig. 2d). It features recognizable stages of embryogenesis (Fig. 2e, f), which usually do not produce a discernible epidermal layer over the entire callus. Most maize varieties are capable of producing Type I cultures (Fig. 3a), which are typically more difficult for transformation, selection, and subsequent plant regeneration. Despite the added difficulty, there have been reports of transformation and selection of Type I callus to produce transgenic plants (Wan et al. 1995; Frame et al. 2006; Siderov et al., 2006). However, the characteristics of Type II callus (Fig. 3b), rapid cell division and proliferation of somatic embryos, have generally been recognized as important attributes that contribute to successful maize transformation (Kausch et al. 1995; Lowe et al. 2018; Salvo et al. 2018). This culture response would prove to be a foundational requirement for recovery of stably transformed fertile maize, and for continued development of high-efficiency methods that have come since.

The A188 × B73 Hi II hybrid became the genotype of choice because of its tissue culture response to produce "early" somatic embryogenic cultures (Frame et al. 2002; Zhao et al. 2002). Somatic embryos are genetic clones from the explant distinct from zygotic embryos, which are formed from the outcome of fertilized gametes. The term "early" somatic embryogenic cultures derive from the observation that these somatic cultures resemble the early stages of zygotic embryogenesis (see Fig. 4 and Randolph 1936; Poethig and Sussex 1985) and are arrested in that developmental state by genetic and tissue culture response. For example, in many monocots, these stages of somatic embryogenesis in culture strongly resemble the developmental stages of zygotic embryos at 5–8 days after pollination (DAP), hence the descriptor of "early." The ability to produce friable Type II embryogenic cultures is strongly genotype dependent (Loyola-Vargas and Ochoa-Alejo 2016; Tripathi 2017) and promoted by auxin/cytokinin ratios and other media components (Horstman et al. 2017).

The majority of successful maize transformation protocols, especially in early research, relied on Type II cultures. The reasons for this become apparent through an understanding of the developmental biology of the two broad callus morphotypes. Figure 4 shows a series of biological illustrations depicting a comparison between the developmental sequences of zygotic and somatic embryogenesis in maize. The zygotic embryo developmental sequence was first described by Randolph (1936) and Kiesselbach (1949) and is illustrated in Fig. 4. The developmental sequences of somatic embryogenesis from the transformation of immature embryos (Fig. 5a) were informed and modeled based on micrographs provided in Fig. 5c–g of Lowe et al. (2018). Stages of somatic embryogenesis after transformation using *Agrobacterium* to deliver WUS2/BBM into immature embryos harvested 11 DAP are shown in Fig. 5a: (i) initiation of proembryos with the first cell division in epithelial cells; (ii) early globular proembryos begin to form; (iii) continued cell division to produce late globular proembryos; (iv and v) possible apical meristem formation, with the somatic embryo protoderm remaining confluent with the epithelial layer of the originally transformed immature embryo explant.

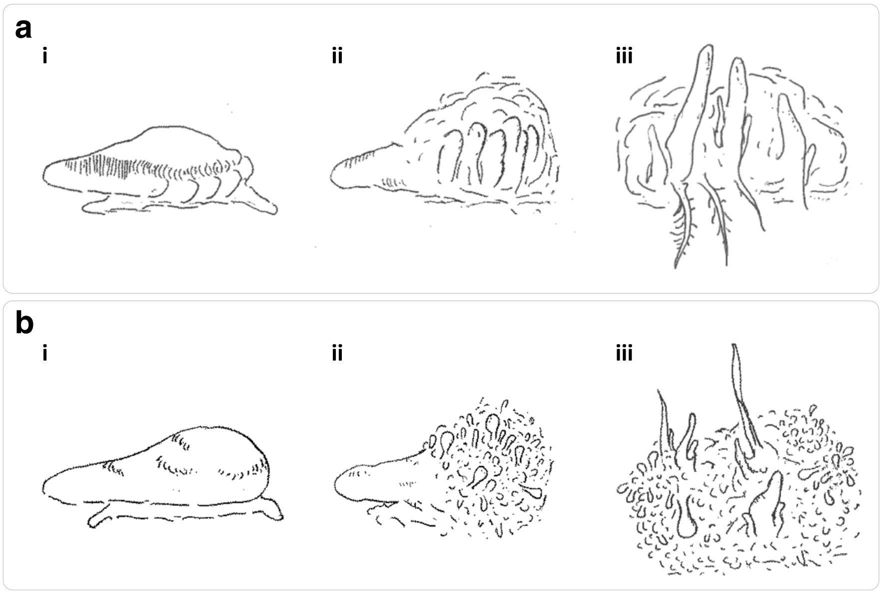

Fig. 3 Diagrammatic representation of the two major different regenerable callus morphotypes in maize. **a** Type I compact and organized callus resulting in plants derived from late stage direct somatic embryogenesis. **b** Type II friable callus resulting in plants germinated from early stage indirect somatic embryogenesis. Illustrations by APK

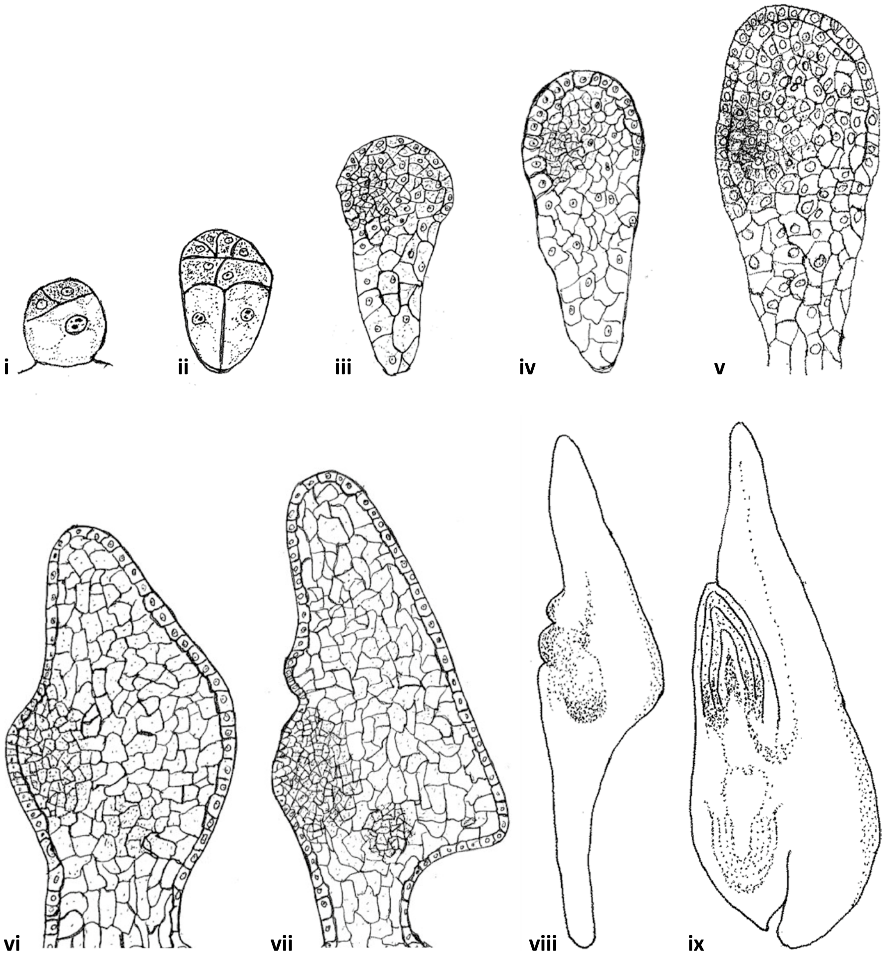

Fig. 4 Zygotic embryogenesis in maize. **i**–**v** Proembryo stages. **i** The three-celled proembryo after the first anticlinal division of the terminal cell. **ii** Proembryo 4-day post-fertilization showing earliest developmental stages of the embryo proper (early globular proembryo), differentiated from the lower cells which will become the suspensor. **iii** Proembryo 6-day post-fertilization shows random and rapid cell division patterns in the embryo proper, lacking a defined epidermal layer or meristemic organization. **iv** Seven-day-old embryo shows delimitation of the protoderm and increased cytoplasmic density in the region that will become the shoot apical meristem (SAM). **v** Late globular stage proembryo. **vi** Transition-stage embryo; embryo is asymmetrical. **vii** Coleoptilar-stage embryo, 11-day-old embryo with further differentiation of the visible SAM, with increased cytoplasmic density in the region that will become the root apical meristem. **viii** L2-stage embryo, 13-day-old embryo with distinct shoot apical meristem with developed coleoptilar ring, a differentiating root apical meristem, a well-defined suspensor, and the development of active mitoses in the abaxial basal area of the scutellum. **ix** L5-stage embryo, with developed leaf primordia and root apical meristem differentiation with files of cells that will form the root epidermis, cortical parenchyma, and stele cells. Illustrations by APK, modified and inspired by Randolph (1936) and Kiesselbach (1949).

The incipient shoot apical meristem development in such developing somatic embryos appears comparable to that observed in the zygotic embryo. Lowe et al. (2016) also provide the first report of morphogenic regulator-mediated transformation (MRT) of seedling-derived leaf tissue and direct formation of embryogenic callus (Fig. 5b). Based on their GUS staining data, it appears that *Agrobacterium*-mediated T-DNA delivery occurs most readily in single mesophyll cells (Figs. 2i and 5b), suggesting that *Agrobacterium* delivery occurs through stomates and intracellular spaces. Lowe et al. (2016) also emphasize that two patterns of stimulated cell division were observed, one type in which the GUS-expressing cells

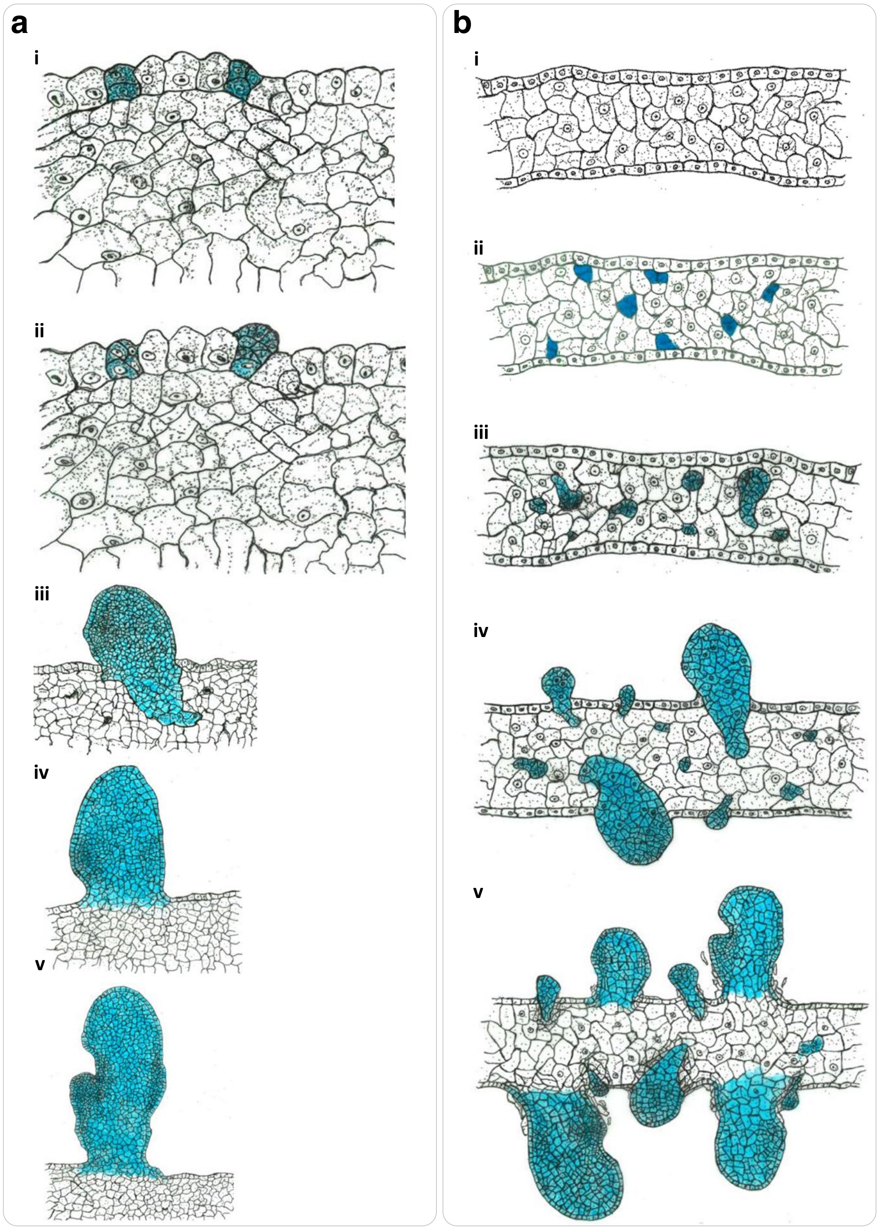

Fig. 5 Developmental sequence of somatic embryogenesis mediated by morphogenic regulators *Bbm* and *Wus2* genes. **a** In 14-DAP immature embryos: (**i**) scutellar epidermal becomes cytoplasmically dense, and expresses reporter genes, such as GUS (in blue); (**ii**) early proembryo stages become apparent; (**iii**) late proembryo stage somatic embryo; (**iv**) early transition-stage embryo; (**v**) early L1-stage somatic embryo. **b** In seedling leaf: (**i**) early proembryo–stage somatic embryos differentiating from mesophyll parenchyma express reporter gene constructs, such as GUS (in blue); (**ii**) late proembryo–stage proembryos in infected young leaf explants; (**iii** and **iv**) early to middle transition-stage somatic embryos (**v**) late stage somatic embryos . Illustrations by APK

divide (consistent with cell-autonomous expression of BBM), and another pattern where non-dividing blue cells (GUS+) appear to stimulate cell division in neighboring cells, which is consistent with WUS2 protein moving from cell-to-cell. We have depicted these early stages of MRT-mediated maize leaf transformation in Fig. 5b. While the earliest stages of cell division in leaf cells are shown, the later stages of somatic embryogenic are illustrations based on known maize embryo developmental stages.

The above comparison provides a simple suggestion. Type II callus has been successful for transformation and selection because of the remarkable similarity between rapidly growing somatic embryos and the early stages of zygotic embryo formation. Similar to cells within the early zygotic embryo, the comparable cells in somatic embryos are totipotent and capable of sustained cell divisions independent of surrounding cells or tissues that are also under stress and senescence during selection. This was observed that the transgenic clonal plants regenerated from the same resistant callus line after selection shared the same Southern blot patterns, suggesting a single cell origin and a homogeneous, non-chimeric insertion event (Gordon-Kamm et al. 1990). This distinctive attribute of Type II callus of being selected amidst surrounding cell death while retaining regenerability is critical to successful transformation. This situation also exists in other cereal species and many other grasses (Jones 2009).

By comparison, Type I morphotypes exist as complex differentiated tissues where single cells are dependent on the surrounding tissue for sustained growth. Even if such a cell has stably integrated the transformation vector DNA, it may not develop further during selection due to its dependency on the surrounding tissue. Stated another way, a transgenic differentiated epidermal cell, within this interdependent mass of cells, would likely remain dependent on the surrounding organized callus tissue for growth, and lacks independent totipotency. This underscores the importance of early embryogenic cultures for historic success in maize transformation and provides the underlying principles for the use of morphogenic regulators (see Kausch et al. 2019, and "Morphogenic genes increase transformation and genome editing efficiency" of this review).

The developmental biology of early embryogenic cultures was an underappreciated yet essential cornerstone to successful cereal transformation. Embryogenic cultures or immature embryo explants would become identified as central in early transformation experiments as most responsive and suitable for the transformation of maize and other cereals (Gordon-Kamm et al. 1990; Ishida et al. 1996; Frame et al. 2002; Huang and Wei 2005; Hiei et al. 2006; Vega et al. 2008). Somatic embryos are differentiated outcomes derived from somatic cells in culture as single or small groups of cells (Emons and Kieft 1995), which undergo development to become independent mature plants. In maize, the developmental stages of zygotic embryogenesis (Fig. 4i–vii) are reiterated in the depiction of somatic embryo formation via MRT from immature embryo explants (Fig. 5a) and young leaf explants (Fig. 5b). A developmental progression is depicted that is likely during somatic embryogenesis mediated by morphogenic regulator overexpression in immature embryos (Fig. 5a), and during development of somatic embryogenic growth mediated by morphogenic regulator overexpression in leaves (Fig. 5b). Figure 5a is based on Lowe et al. 2018 in which cells of immature embryos were transformed and rapidly produced IE-derived somatic embryos within 4–7 days after Agrobacterium infection. It was also informed by the parallel progression observed between zygotic embryonic stages described by Randolph 1936 and Kiesselbach 1949. Figure 5b depicts that this progression might also occur in leaf explants. Lowe et al. 2016 demonstrated that i) mesophyll (or bundle sheath) cells within the leaf segments were observed to be transformed and clearly begin dividing, and ii) the leaf segments produced somatic embryogenic callus from which fertile T0 plants were regenerated. Whether the transformed leaf cells immediately form somatic embryos as depicted in Fig. 5b or divide in a more undifferentiated fashion before forming somatic cells within the leaf segments produced embryogenic callus has not been definitively reported. It is also likely that a similar developmental sequence occurs during microspore-derived somatic embryogenesis (Massonneau et al. 2005). Together, these examples of totipotency illustrate the astonishing developmental plasticity in maize as demonstrated through tissue culture. The late plant developmental biologist Ian Sussex noted that these examples also substantiate that new cell types or developmental programs are not created in culture, but rather that existing cell types go through similar developmental gene expression patterns and progressions already present in the plant (Sussex 1996, personal communication, APK).

Choosing the right selectable or visible marker genes

Early dicot transformation selection systems relied on marker genes for resistance to aminoglycosides (Gasser and Fraley 1989) such as neomycin, kanamycin, and G418, which were ineffective in most cereal crops (Jones 2009). Selection of maize, wheat, sorghum, rice, barley, oat, and pearl millet transformants has been most effectively achieved using herbicide resistance markers including the *bar* gene from *Streptomyces* (De Block et al. 1984; White et al. 1990; D'Halluin et al. 1992; Thompson et al. 1995) for bialaphos resistance (Gordon-Kamm et al. 1990; Vasil et al. 1992; Somers et al. 1992; Rathore et al. 1993; Gallo-Meagher and Irvine 1996; Knapp et al. 2000; Zang et al. 2009). Other useful selectable marker genes were investigated including the hygromycin phosphotransferase gene (*hpt*) for antibiotic hygromycin resistance (van den Elzen et al. 1985; Walters et al., 1992; Hagio et al. 1995; Ortiz et al. 1996); the acetolactate synthase (*Als*) gene for herbicide chlorsulfuron resistance (Fromm et al. 1990; Li et al. 1992); the *highly resistant Als* gene, or *Hra*, for imazapyr selection (Hoerster et al. 2020); the mutant *epsps* gene (encoding 5-enolpyruvylshikimate-3-phosphate synthase) for glyphosate resistance (Howe et al. 2002; Shah et al. 1986); and the phosphomannose isomerase (*pmi*) gene using mannose for metabolic selection (Joersbo and Okkels 1996; Joersbo et al. 1998; Negrotto et al. 2000; Privalle 2002; Wenck and Hansen 2005). Other functional selection systems for plants can utilize alternative antibiotics, including blasticidin, bleomycin, chloramphenicol, gentamicin, and streptomycin; herbicides, including bromoxynil, 2,4-diclorophenoxyacetic acid, phosphinothricin, glufosinate, and glyphosate; or novel metabolic markers, such as threonine dehydratase and phosphomannose isomerase (for reviews, see: Gheysen et al. 1998; Newell 2000; Lee and Gelvin 2008; Anami et al. 2013; Que et al. 2014; Breyer et al. 2014; Hwang et al. 2017). Selectable markers are an indispensable part of successful integrative transformation.

While the above list of potentially useful selectable markers in maize is extensive, a smaller subset has risen to the top, becoming the workhorses for ongoing maize (or cereal) transformation. These include the *bar* gene (or *pat*), *nptII*, *pmi*, and *Hra*. Of these, the *bar* gene (or *pat*) conferring resistance to bialaphos (Gordon-Kamm et al. 1990) and the *pmi* gene which permits growth on mannose-containing media (Negrotto et al. 2000; Wang et al. 2000a, b) have been and continue to be used consistently in maize by many groups since first being reported. The *nptII* gene was the most popular selectable marker gene for most dicot plants and one of the first markers tested by early researchers in maize transformation (for example, Rhodes et al. 1988a, b), but for many years other selectable markers displaced this antibiotic marker for general use in maize. The *bar* gene continues to be a staple tool for *Agrobacterium*-mediated maize transformation (Frame et al. 2002). The *pmi* gene demonstrates its utility in efficient recovery of targeted integration events using FLP-mediated site-specific integration (Anand et al. 2019).

Interestingly, the *nptII* gene was not always considered a suitable selectable marker gene for monocots in early research due to their natural high level of resistance to the antibiotic kanamycin (Dekeyser et al. 1989; Breyer et al. 2014). However, with its robust utility and dependability for selection with G418, the *nptII* gene is again finding use in maize transformation. Recently, it is reported that the *nptII*/G418 selection system can be successfully used in MRT-stimulated rapid somatic embryo formation (Hoerster et al. 2020), *Agrobacterium*-mediated targeted integration using Cas9 for homology-dependent recombination (Gao et al. 2020), and enrichment for recovery of Cas9-mediated intragenomic targeting via homology-dependent recombination (Barone et al. 2020). For a similar reason, the *Hra* gene is also useful for rapid selection during MRT-induced somatic embryo formation in maize (Hoerster et al. 2020).

In addition to selectable markers, effective visual markers have been critical tools for developing plant transformation. From the first demonstration of maize transformation (Gordon-Kamm et al. 1990), using GUS transient assays to confirm and understand DNA delivery, as an invaluable tool for following transgenic sectors in plants (Christou and McCabe 1992; Lowe et al. 1995; Kilby et al. 2000; Dominguez et al. 2004), and to confirm inheritance of transgene expression (for example, Fromm et al. 1990; Gordon-Kamm et al. 1990; Zhong et al. 1996; Petolino et al. 2000), GUS has continued to be used across plant transformation. However, the major difficulty with this marker was the requirement to sacrifice the tissue to use this non-viable staining/assay protocol.

The discovery of green fluorescent protein (GFP) (Prasher et al. 1992; Chalfie et al. 1994) led to its emergence as a powerful new tool as a viable visible reporter gene in plants (Sheen et al. 1995; Reichel et al. 1996; Haseloff et al. 1997; Tian et al. 1997), and it has been an invaluable tool for accelerating progress in monocot transformation (Kaeppler et al. 2001). The ability to visualize GFP expression (or subsequently developed fluorescent markers) has led to great strides in optimization of DNA delivery, as adjusting selection protocols for efficient recovery of transgenic events, and for myriad basic research applications. A spectrum of available fluorescent protein genes rapidly became available (Shaner et al. 2005), which have become critical tools in basic cell biology and transformation studies in maize.

Standard maize transformation approaches

The two mainstream approaches used to generate transgenic maize are mediated by *Agrobacterium tumefaciens* or microprojectile bombardment. These approaches are considered "standard" because they are the most widely used, have reliable well-tested protocols, and can be routinely used to create a "pipeline" of transgenic plant production. Once established, a transformation pipeline can deliver expected transgenics that will be integrated into further studies, phenotypic analyses, or breeding programs.

A successful transformation system is described using the term "transformation frequency" or "transformation efficiency." These two expressions are often used interchangeably. "Transformation frequency" is defined as the percentage of independent transgenic events per 100 explants treated (either by *Agrobacterium* infection or by biolistic bombardment). The term "independent transgenic event" can vary between different labs; usually it refers to a cell line or T0 plant(s) derived from a cell containing a unique, independent transgene insertion. It can also refer to the transgenic T1 event (the herbicide- or antibiotic-resistant plants that produce viable seeds expressing the transgenes). It is important to define explicitly the transformation frequency when describing a transformation system or pipeline. Transformation frequency can be further described in more specific terms such as "quality event," which typically represents a transgenic event with a single copy transgene with no undesired DNA segments such as vector backbones.

"Transformation efficiency" describes how long the transformation procedure takes and how many "people hours" (or full time equivalents (FTEs)) it takes to generate the number of transgenic events for each construct. The number of events required for each construct depends on the nature of the study or program. Therefore, in most scientific publications, the use of the term "frequency" instead of "efficiency" is a more accurate descriptor of a transformation system.

Agrobacterium-mediated transformation

The majority of labs conducting routine maize transformation rely on *Agrobacterium*-mediated delivery. Detailed protocols for standard *Agrobacterium*-mediated transformation and recovery of events have been described (Ishida et al. 1996, 2007; Negrotto et al. 2000; Frame et al. 2002; Huang and Wei 2005; Vega et al. 2008). Various *Agrobacterium* strains harboring modified vectors have been widely used to deliver constructs and generate a useful pipeline for production of transgenics in maize and other cereals (Gelvin 2003). In addition to many examples of using standard *Agrobacterium*-mediated maize transformation for transgene integration, it has been used for various genome editing studies, including zinc finger nucleases (ZFNs: (Bibikova et al. 2003; Cai et al. 2009; Shukla et al. 2009; Townsend et al. 2009), transcription activator-like effector nucleases (TALENS; Cermak et al. 2011; Char et al. 2015; Christian et al. 2013; Li et al. 2012, 2016; Liang et al. 2014), and Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR–associated protein 9 (CRISPR/Cas9; Char et al. 2017; Jiang et al. 2013; Brooks et al. 2014; Jacobs et al. 2015; Li et al. 2016; Shan et al. 2013).

Agrobacterium strains and vector systems

The use of various *Agrobacterium* strains with custom-made modifications has increased over the past years and varies widely (Zhang et al. 2020). T-DNA binary vectors have evolved to be more highly developed as molecular vector construction has become more sophisticated. Binary vectors have become increasingly more specialized (reviewed by Hwang et al. 2017; Zhang et al. 2020) for different applications (Hellens et al. 2000; Chung et al. 2005; Hiei et al. 2006; Lee and Gelvin 2008; Mehrotra and Goyal 2012; Anami et al. 2013), and in industry, decisions may be made according to FTO, licensing, and regulatory constraints. Despite the diversity of *Agrobacterium* strains with various modifications, there are some commonalities in the T-DNA-based binary vectors currently being used. Requisite and consistent characteristics and elements include (1) functional selectable marker genes compatible with *E. coli*, *A. tumefaciens*, and plants; (2) the left and right border sequences defining the T-DNA region; (3) set up for modular construction (Čermák et al. 2017) with a wide assortment restriction site in the T-DNA region allowing versatile customization and insertion of one or more genetic elements of interest; (4) plant promoters, enhancers, UTRs, transit peptide sequences, genes of interest, selectable markers, and reports which do not affect bacterial growth; and (5) origin(s) of replication allowing cloning vectors to replicate in bacteria.

As determined by the resident Ti plasmids and the *Agrobacterium* chromosomal background, the most commonly used "disarmed" *Agrobacterium* strains were derived from two wild-type isolates: C58 (Lin and Kado 1977) and Ach5 (Kovács and Pueppke 1994). Strains with nopaline-type chromosomal background of C58 and modified Ti plasmids of pTiBo542 or pTiC58 (Hood et al. 1986; Koncz and Schell 1986; Lazo et al. 1991) including AGL1, EHA101, EHA105, and GV3101(pMP90) have been widely applied across a range of plant species. The *Agrobacterium* strains from the Ach5 octopine-type chromosomal background are also commonly used, and are from LBA4404 with the octopine-type Ti plasmid pAL4404 (Ooms et al. 1982) derived from the pTi-Ach5 plasmid. Combinations of modified Ti plasmids and genetic backgrounds have pronounced effects on strain host range, T-DNA transfer, and transformation efficiencies of various plant species and genotypes (Lee and Gelvin 2008; Hwang et al. 2010).

Agrobacterium-mediated transformation was first achieved in maize using super-binary vectors such as pSB1 (Ishida et al. 1996). Super-binary vectors, such as pTOK233, were originally thought to be particularly more efficient and, in fact, enabling and required for transformation of the cereals. In addition to the native *vir* genes in the disarmed Ti acceptor vector, these vectors carry additional *vir* genes in the plasmid containing the T-DNA region, conferring a "super-virulent" phenotype. The super-binary pSB1 is the *Agrobacterium* strain LBA4404 disarmed Ti acceptor vector and an intermediate vector, pSB11, that carries the T-DNA region. While the original "super-binary" vector system is effective, the steps involving gene cloning into this large size plasmid and subsequent co-integration required for introduction of the T-DNA vector can be cumbersome. To simplify this process, Anand et al. (2018) reported an improved ternary vector system for *Agrobacterium*-mediated maize transformation. With a compatible origin of replication, the ternary vector (or accessory pVIR plasmid) carrying extra copies of *vir* genes can co-exist with the T-DNA binary vector in the same *Agrobacterium* strain. A comparison between SB1 alone (Anand et al. 2018; Table 3) shows a 6–7 fold increase in transformation frequency. This simpler and smaller ternary vector system has provided enhancement in eleite maize transformation over protocols using non-ternary systems (Anand et al. 2018). The work of the Chen group (Zhang et al. 2019, 2020) made ternary vectors accessible for other academic labs.

Transformable maize genotypes

The first successful and reproducible protocol for *Agrobacterium*-mediated maize transformation was reported in 1996 (Ishida et al. 1996). This was a major accomplishment, overturning the long-held belief that monocots such as maize were somehow recalcitrant to *Agrobacterium* transformation. In this report, immature embryos isolated from the maize inbred A188, an *Agrobacterium* "super-binary" vector system, and the *bar* selectable marker gene were used to produce transgenic events. Using this protocol, frequencies of transformation were between 5 and 30% (reported as independent transgenic events derived per 100 embryos infected). Over 70% of the T0 plants were fertile and morphologically normal. The presence of the transgene, expression, stable integration, and Mendelian inheritance were confirmed by molecular and genetic analysis. They observed low frequencies of transgene cassette rearrangements with between one to three copies of the transgene integrated in most events. Genotypic variation in transformation frequency was observed with successful transformation of F_1 hybrids between five other inbreds and A188.

The first published report of fertile, stable transgenic maize by using a standard binary *Agrobacterium* vector system was by Frame et al. (2002). In this work, immature embryos from a maize genotype with the ability to readily produce Type II callus type (Hi II) were used. For gene delivery, they used an *Agrobacterium* strain EHA101 carrying a standard binary vector system with *bar* and *gus* selectable marker genes. They reported that inclusion of L-cysteine in the co-cultivation medium increased transient *gus* expression and resulted in increased recovery of stable transformants. The transformation frequency, reported as the percentage of bialaphos-resistant events per 100 embryos, was 5.5%. The *bar* and *gus* transgene integration, expression, and inheritance were confirmed by Southern blot and phenotypic analyses in the T0, T1, and T2 generations. The step-by-step protocol of maize transformation using the standard binary vector method was also reported by Lee and Zhang (2016), and can be found in the website for The Plant Biotechnology Laboratory, University of Rhode Island (https://web.uri.edu/ctwg/).

These protocols using Hi II maize represent the most widely used approaches with minor variations and individualized techniques unique to some labs. Maize Hi II F_1 immature embryos used as donor recipients for transformation (or grown into embryo donor plants) are derived from the cross of two inbred lines, Hi IIA and Hi IIB, each of which is maintained as independent lines derived from selection, screening, and several generations of selfing of two separate F_2 embryo progenies of the cross of A188 × B73. Thus, the Hi II F_1 embryos targeted in transformation (or grown as embryo donor plants) are derived from the cross of Hi IIA × Hi IIB, designated as Hi II (A × B). The A and B do not stand for A188 and B73 and thus the use the "Hi II (A × B)" designation in this review refers to germplasm developed by Armstrong et al. (1991). Using maize genotype Hi II (A × B) immature embryos as donor recipient explants and constitutively expressed *bar* as a selectable marker is widely considered the model maize transformation system. For consistent and constant supply of immature embryos, F_1 seeds are used for the production of vigorous donor plants grown in optimal greenhouse conditions.

Ishida et al. (2007) documented an efficient and very detailed step-by-step protocol, including excellent figures of each part of the process in Nature Protocols. Their protocol used the *Agrobacterium* strain LBA4404 with a super-binary vector carrying the *bar* gene for selection and *gus* as a reporter. The Ishida et al. protocol can be followed directly for reliable results with the genotypes A188, A634, H99, and W117 used in their paper. They stress that a decisive factor for success is the co-cultivation of healthy unbroken immature zygotic embryos at the correct developmental stage (1.2–1.5 mm in length) from vigorous donor plants grown in optimal greenhouse conditions. In the inbred A188, 50% of the isolated immature embryos would produce independent events (reported as the number of bialaphos-resistant events per 100 embryos infected). This same protocol in inbred lines H99 and W117 resulted in recovery of approximately 15% of the inoculated immature embryos generating independent resistant events. Plants regenerated from these events with 50% of the transformants contained one to two copies of the insert. The protocol required about 90 days from infection with *Agrobacterium* to transplanting transformants into soil.

Many maize inbred lines, including the genotypes described above, produce embryogenic Type I callus from their immature embryos, when using Murashige and Skoog (MS) salts–based media (Frame et al. 2006). Out of 11 inbreds tested in the work of Frame et al. (2006), three lines (B104, B114, and Ky21) could be transformed. Among these lines, B104 gained popularity among academic researchers because it is a public inbred derived from BS13(S)C5, an Iowa Stiff Stalk Synthetic (BSSS) population (Hallauer et al. 2000). It also shares some percentages (~60%) of genetic similarity with the maize community reference inbred B73 (Liu et al. 2003; Schnable et al. 2009).

The ability to transform inbred lines is highly important to maize researchers. Although the hybrid Hi II can be transformed readily by many groups, it is not a desired genotype for transgene analysis, especially if the gene of interest involved quantitative trait loci (QTL). The segregation of traits in progeny of transgenic Hi II events can make gene functional analysis difficult. To address this issue, the "Morphogenic genes increase transformation and genome editing efficiency" section will discuss the innovation of using morphogenic genes for enhancing maize transformation and expanding the spectrum of transformable inbred lines.

Microprojectile bombardment–mediated transformation

Microprojectile bombardment, also known as biolistics, microprojectile bombardment, or "gene gun" delivery, is the most significant DNA delivery alternative to *Agrobacterium* for plant transformation (Sanford 2000; Biolistic DNA Delivery in Plants 2020b). One of the milestones in maize transformation was the successful generation of transgenic events using the biolistic method (Fromm et al. 1990; Gordon-Kamm et al. 1990). Developed to overcome the cell wall barrier to direct DNA delivery, microprojectile bombardment has been used in countless studies involving transient expression analysis and recovery of plants with stably integrated transgenes, and used to generate the progenitors of many commercial lines.

The advantages of using microprojectile bombardment over *Agrobacterium* or other delivery methods include that it provides (i) an excellent, rapid, and efficient system for transient expression studies using effective reporter genes in intact tissues; (ii) the ability to deliver reagents to a wide array of target plant cells and tissues; (iii) delivery to a sufficient number of recipient cells to recover stable transformants (Gordon-Kamm et al. 1990, 1991); (iv) concurrent delivery of a number of independent genetic elements and macromolecules; and (v) a valuable system for organelle transformation.

Microprojectile bombardment may have an apparent regulatory advantage over *Agrobacterium*-mediated transformation since it does not involve the use of a plant pathogen (CAST 2018). It has been shown that bombardment can be used to generate genome-edited events using DNA, or RNAs and ribonucleoproteins (RNPs) (Svitashev et al. 2016; Zhang et al. 2016). Microprojectile bombardment also eliminates the issue of *Agrobacterium*/plant-specific interactions. This approach is attractive but requires highly efficient tissue culture and plant regeneration responses, which are in many cases strongly species, genotype, and explant dependent.

The disadvantages of microprojectile bombardment are that it often results in multiple copy transgene insertions; there is the possible resultant formation of chimeric or mosaic plants; fragmentation or loss of transgene molecular integrity is common; and there is a size limitation to deliverable DNA constructs (Finer et al. 2000; Newell 2000; Lorence and Verpoorte 2004; Altpeter et al. 2005; Herrera-Estrella et al. 2005). Complex DNA integrations can result in heritable instabilities and/or epigenetic silencing of the transgene. Owing to these disadvantages and the shortcomings of the chemical and physical limitations of bombardment, the most frequently applied system for generation of transgenic plants is still *Agrobacterium*; however, bombardment will likely always have utility in plant transformation research.

Maize transformation using diverse delivery methods or recipient explants

While *Agrobacterium* and microprojectile bombardment are the predominant methods for maize transformation, alternative methods remain attractive for various reasons. These methods include the use of alternative delivery methods or alternative explant donor sources, cells, and tissues. While the current standard maize transformation protocols provide a firm foundation for reliably creating desired outcomes, they have been limited by tissue culture expertise, facility-intensive resources (infrastructure such as greenhouses), genotype restrictions, and explant dependence. In addition, prolonged time in tissue culture, or even using callus cultures as the explant for transformation, often results in somaclonal variation and genetic deviation from the initial genotype (this is a major drawback which must be addressed). Even for genotypes that can be transformed, low efficiencies remain a problem, along with expense in time and labor. Investigations into alternative transformation methods have been aimed at overcoming some or all of the above limitations.

Delivery of DNA to competent recipient cells has long been recognized as a significant barrier to the transformation of maize (Coe and Sarkar 1966). Several delivery methods involve intact tissues as initial starting material with most restricted primarily to surface cells. For example, *Agrobacterium* attachment is limited to exposed surfaces, such as the totipotent epithelial layer on the abaxial side of the scutellum of immature zygotic embryos (Duncan et al. 1985; Ishida et al. 1996), mature seed–derived embryos, cut surfaces of nodal explants, and leaves and leaf bases, as well as embryogenic callus derived from various explants. The restriction of DNA delivery to surface cells is not limited to *Agrobacterium*. Microprojectile bombardment delivery is limited to the outermost (perhaps three) cell layers of immature embryos and embryogenic callus tissues (Kausch et al. 1995). Particle penetration is intended to create a balance between tissue damage and successful DNA delivery (Kausch et al. 1995; Kemper et al. 1996). This is logical since the force to enter the cell is directly proportional to the fixed mass of the particle (using heavy metals such as Tn or Au particles) times the acceleration that is limited by acceleration method and degree of incurred cell damage.

Various explant sources have been used as initial starting materials for maize transformation (Que et al. 2014; Yadava et al. 2017). Robust tissue culture protocols for regeneration of maize plants from cells which are transformation competent have been reported using various explant donors including immature embryos (Duncan et al. 1985; Bohorova N. E. et al. 1995; Ishida et al. 1996; Frame et al.

2002; Aguado-Santacruz et al. 2007), mature zygotic embryos from seed Lowe et al. 2016), seedling-derived callus (Sidorov et al. 2006), leaves and leaf bases (Lowe et al. 2016), tassel and ear meristem (Pareddy and Petolino 1990; Songstad et al. 1992), and shoot meristems (Sairam et al. 2003). Factors which may influence choice of one explant donor source versus another include availability and ease of maintenance (e.g., mature seeds, young leaves), tissue culture response, and genotype of choice.

Delivery methods

Silicon carbide fibers (whiskers)

Demonstration of successful transformation of plant cells via microprojectile bombardment spurred further investigations into alternative direct DNA delivery methods based on physical penetration of plant cell walls. Although the biolistic approach to transformation overcame some of the limitations of earlier protoplast and *Agrobacterium*-based transformation systems, the process of preparing microparticles and target tissues was laborious, and the cost of the bombardment apparatus and associated materials was high. Research efforts aimed at the development of less expensive, simple, rapid direct DNA delivery methods led to exploring the use of silicon carbide (SiC) fibers as a means of DNA delivery into plant cells. Those investigations followed reports from Cockburn and Meier (unpublished) of transmission of functional plasmid DNA into insect embryos following treatment with SiC fibers using a simple protocol that involved vortexing a suspension of fibers, DNA, and embryos in a microfuge tube.

Optimization of the protocol for treatment of plant cells led to the first report of SiC fiber–based delivery of functional transgene DNA into plant cells in maize and tobacco (Kaeppler et al. 1990). In the protocol, SiC fibers with an average diameter of 0.6 µm, and average length of 10–80 µm, were suspended in a liquid plant tissue culture medium in a microfuge tube, along with plasmid DNA carrying selectable/screenable marker genes, and an aliquot of cells from either BMS, tobacco, or regenerable embryogenic maize suspension cultures. The tube was then vortexed for a set amount of time, and the treated cell cultures placed on tissue culture medium without selection in order to observe transient expression of the *gus* gene. GUS expression was observed in hundreds of cells in the BMS and tobacco cell treatments, with many of these transformed cells developing into regenerable cell cultures. Refinement of the initial plant protocol then led to stable transformation of maize cells via SiC fiber–mediated DNA delivery (Kaeppler et al. 1992); however, the transgenic cell lines were not regenerable. Generation of fertile, transgenic maize plants via SiC fiber–mediated transformation was finally achieved in 1994 (Frame et al. 1994), and standardized protocols for SiC fiber–mediated transformation of maize cells were established (Kaeppler and Somers 1994; Thompson et al. 1995; Wang et al. 1995). Extension of the protocol to other plant species resulted in successful SiC fiber–mediated transformation of a diverse group of species, including microalgae, rice, cotton, and peanut (Dunahay et al. 1997; Matsushita et al. 1999; Asad et al. 2008; Akram et al. 2016), and in successful editing of maize (Shukla et al. 2009).

Despite that success, however, SiC fiber–based plant transformation systems have not been widely adopted. Although the method was shown to be simple, inexpensive, and rapid, it was hindered by several limitations. One of the main limitations was in the types and responses of target tissues that could be used in DNA delivery treatments. Fragile or sensitive target tissues (such as immature embryos) could not be targeted due to damage caused during the vortexing step of the protocol. The most efficient recipient cells were suspension culture cells or friable callus cultures. However, the ability of donor explant tissues to generate friable callus cultures and/or fast growing suspension cultures consisting of small cell aggregates was very genotype dependent. Additionally, the long culture times sometimes required to develop friable or finely suspended cultures often resulted in loss of plant regeneration ability or fertility of regenerated plants. Finally, unlike microprojectile bombardment or *Agrobacterium*-mediated DNA delivery, the fibers could not be used to penetrate more than one cell layer, or access inner tissue targets.

Time release and bioactivated beads

Various methods of preparation of DNA for delivery have been developed including immobilization of isolated DNA into or onto dissolvable matrices, including polymeric substrates such as polyethylene-glycol

(PEG), poly(lactic-co-glycolic acid (PLGA), polyethylenimine (PEI) (Cunningham et al. 2018; Demirer et al. 2019), and calcium alginate (Draget et al. 1989; Sone et al. 2002). These types of matrices offer protection of DNA from degradation and shear, time release properties, versatility for combination of various biomolecules, and the ability to increase electrostatic affinity for plant cells (Murakawa et al. 2008). Alginate beads are easily produced by including cells, protoplasts, and/or biomolecules (i.e., DNA) into calcium chloride and drop-mixing this solution into emulsified sodium alginate to form Ca^+-alginate beads.

Ca^+-alginate has several attributes for plant cell culture as a gelling agent (in comparison to conventional agar/agarose-based systems) and as a delivery medium for biomolecules to plants. Improved efficiency of transformation mediated by the Ca^+-alginate bioactive bead system was made by introducing DNA-Lipofectin complexes as the entrapped biomolecular cargo (Liu et al. 2004; Murakawa et al. 2008). Surface immobilization of chromosomal DNA (Mizukami et al. 2003) was reported with stabilization of DNA fragments up to 280 kb in size. Transformation of rice was achieved using bioactive bead–mediated transformation with large DNA fragments (i.e., a 100-kb BAC construct) containing *Aegilops tauschii* genes (Wada et al. 2009, 2012). Currently, the use of alginate and bioactive beads is an underexploited technology and may serve broader applications.

Recipient explants

Transformation using mature seed

Mature seeds have been used as initial donor explants, as an alternative to immature embryos, providing target tissue for successful transformation. Mature seeds present a reliable source which is easily stored that is not greenhouse dependent and less labor intensive and expensive, obviates the maintenance of donor plants (e.g., as required as a source of immature embryos), and can be used on demand, reducing time in culture and resultant somaclonal variation. During the 1990s, a number of publications described the production of seedling-derived meristem cultures from shoot apical meristems (Zhong et al. 1992a, b, 1996; Zhang et al. 1998, 2002; Li et al. 2002). While the media reported in these works could indeed induce meristematic callus culture, the success of using the tissue for genetic transformation was not readily reproducible. In addition, the induction of the multiple shoot clumps was still highly genotype dependent. Sidorov et al. (2006) reported success in producing embryogenic Type I callus from seedling of several maize genotypes including commercial inbred lines, when using MS-based media supplemented with auxins such as picloram and 2–4-dichlorophenoxyacetic acid (2,4-D). *Agrobacterium*-mediated transformation was performed on five genotypes using either paromomycin (for *nptII* gene construct) or glyphosate (for *epsps* gene construct). They were able to achieve transformation with frequencies ranging between 2 and 11%.

Mature seeds of maize have also served as initial primary tissues infected by *Agrobacterium* (Lowe et al. 2016) in the MRT experiments (reviewed below in the "Morphogenic genes increase transformation and genome editing efficiency" section). Young leaves of germinated seedlings from mature seeds are a highly attractive explant source for MRT. In fact, this approach may likely be broadly applicable across other cereal species and recalcitrant genotypes, resulting in a "universal" transformation approach. These advances are rapidly changing approaches to the transformation of maize and other cereals.

Leaf transformation

Leaf tissue is the ideal source material for transgenic experiments and ideally using such a readily accessible tissue for transformation will become the method of choice for all maize transformation. Young leaves have mitotically active intercalary meristems at their bases providing an excellent source of recipient cells. Leaf explants for transformation would obviate the need to maintain reproductive plants in the greenhouse and potentially provide a more consistent source of donor material accessible to a broader range of researchers. The development of efficient and routine leaf-based transformation systems may provide the opportunity to make a fundamental advance in cereal transformation, potentially removing the "bottleneck" (Altpeter et al. 2016) of transformation to advance functional genomics and genome editing.

The choice of leaves as an explant source for maize is not merely intuitive, but has been substantiated by previous reports (Conger et al. 1987; Ray and Ghosh

1990; Gless et al. 1998a, b; Pasternak et al. 1999). However, reliable and scalable leaf-based regeneration and transformation systems for maize had not been realized, until recently. The breakthrough advent of using differential expression of morphogenic regulator genes to initiate embryogenesis from mesophyll cells represents a revolutionary paradigm shift for cereal biotechnology.

This result elevated the prospect of direct leaf transformation in maize using the MRT system. *Agrobacterium* delivery of T-DNA-carrying morphogenic regulator-based constructs (Nos_{pro}::*Wus2* + Ubi_{pro}::*Bbm*) into leaf cells from 15- to 16-day-old seedlings of inbred line PHH5G resulted in a non-uniform distribution of individual dividing leaf cells, many of which developed into various stages of somatic embryogenesis (Lowe et al. 2016; and see Figs. 2i and 5b). Multiple embryogenic GFP positive events were frequently observed from the same original explant (Fig. 2i) as well as various stages of somatic embryogenesis within a single inoculated leaf explant independent of time course post-infection.

Protoplasts

As mentioned in the "Early failures and the importance of fortitude" section, the very first successful maize transformation was, in fact, PEG-mediated DNA delivery in protoplasts prepared out of embryogenic suspension culture (Rhodes et al. 1988a, b). However, establishment of protoplast-to-plant transformation/regeneration systems remains challenging (Yadava et al. 2017). It is important to put these difficulties into a meaningful context. Over time in culture maize cells undergo fundamental changes that have developmental consequences. Developmental and morphogenetic potential has been observed to occur progressively with prolonged maize culture (J. Petolino; C. Armstrong; R. Shillito; T. Hodges; K. Lowe; 1990; unpublished, personal communication). The progressive deterioration of regenerated plant (and culture) phenotypes with increasing time in vitro would be increased frequency of *tassel seed* mutants; loss of male fertility; loss of tassel development; loss of ear formation; stunting; tissue culture–derived plantlets not forming properly or senesce; selected callus being not embryogenic; and culture being capable of division and maintenance but non-regenerable and BMS-like. In addition, when friable embryonic cultures are used to initiate suspension cultures, at the outset the culture yields only low numbers of protoplasts (under typical enzymatic digestion and osmotic conditions) but with increasing time in culture they become more "protoplastable" (higher protoplast yields). The yield of viable protoplasts per milliliter packed cell volume (PCV) of suspension culture cells increases as the number of cell culture passages (or sub-cultures) increases. Ironically, the timeframe to achieve protoplastability coincides with the timeframe typically resulting in loss of regeneration capacity.

On the other hand, protoplasts of maize mesophyll tissue have been invaluable to study gene functions as a transient expression system for maize (Sheen 2001). A maize endosperm protoplast system has also been reported (Hu et al. 2020). Recent years have seen a resurgence in the use of protoplast systems. Protoplasts were used to investigate the transcriptional profiles of 12,525 single cells from developing maize ears (Xu et al. 2021) A renaissance has occurred in protoplast technologies for the introduction of DNA, RNA, and ribonuclear proteins (RNPs) in response to the recent advances in genome editing technologies in plants (Woo et al. 2015; Songstad et al. 2017; Kausch et al. 2019). Reagents for genome editing can be effectively delivered and, because of the sheer number of cells involved, results in a highly efficient method for target validation and stable transformation in amenable systems. Protoplast-facilitated genome editing systems are especially powerful for the plant of choice, once a robust protocol has been established. It is therefore also likely that protoplast systems will be re-discovered for other applications as well, such as single cell genomic studies.

Microspores and mature pollen

Microspores, microspore-derived cultures, and pollen have long been recognized as potential targets for transformation (Ohta 1986; Harwood et al. 1996; Yang et al. 2017). In the male gametophytic pathway of higher plants, microspores develop in planta into pollen grains. The high level of developmental competence and plasticity in plants is beautifully revealed in the ability of the developing male gametophyte to shift its fate determination from pollen maturation to somatic embryogenesis in vitro (Wang et al. 2000b; Soriano et al. 2013). Androgenesis is the development of haploid plants derived from immature pollen, including microspores, isolated mature pollen, and anther culture applications. In this review, transformation approaches using immature pollen (i.e., microspores) will be considered separately from those utilizing mature pollen.

The potential of the microspores of higher plants to develop directly into haploid or doubled haploid (DH) plants through androgenesis has been shown genetically (Blakeslee 1922; Clausen and Mann 1924; Gaines and Aase 1926; Coe 1959; Geiger 2009) and can be induced in tissue culture (Guha and Maheshwari 1964; Soriano et al. 2013). Homozygous lines can be created within a year compared with otherwise laborious, costly, and long inbreeding programs which require several years (Geiger 2009). By contrast, maternal haploids are created by using a haploid inducer (HI) line, such as Stock 6 (Coe 1959) or Stock 6–derived inducer lines, such as CAU5 (Dong et al. 2014). In maize, these embryos progress through developmental stages similar to those in zygotic embryogenesis (Soriano et al. 2013). For paternal DH methods, the androgenic developmental patterns of expression in tissue culture are in response to environmental cues, such as media components, hormones, or stress. It appears that the critical parameter required during tissue culture for embryogenic induction is the introduction to the protocol of an abiotic stress component (Ochatt et al. 2009), including (i) physical stresses such as temperature shock (heat or cold shock), nutrient deprivation, osmotic, oxidative, and drought stresses, exposure to gamma irradiation, reduced atmospheric pressure, and (ii) chemical treatments such as ABA, azetidine, colchicine, EMS, glutathione (gamma glutamyl cysteinyl-glycine), or heavy metals (reviewed by Shariatpanahi et al. 2006; Islam and Tuteja 2012).

In maize, the major obstacle to wider use of paternal DH in transgenic and genome editing functions is genotype dependence associated with the efficiency of androgenesis, the induction of embryogenesis, and the regeneration of fertile plants. A genotype-independent transgenic DH system combined with genome editing functions would be very useful for genome mapping, trait gene identification, and functional genomics, including basic analysis of biochemical and physiological traits and pathways. Perhaps the intersection of morphogenic regulator-mediated transformation and microspore tissue culture technologies could resolve the issue of genotypic recalcitrance in microspore embryogenesis and DH production.

Transformation and somatic embryogenesis through microspore technology have been combined with the CRISPR/Cas9 system to achieve efficient genome editing in wheat (Bhowmik et al. 2018). Recently, *MATRILINEAL* (*ZmMATL*) was shown to be responsible for haploid induction in maize and to encode a pollen-specific phospholipase (Gilles et al. 2017; Kelliher et al. 2017; Liu et al. 2017). Knockout mutation of the *ZmMATL* homolog (*OsMATL*) in rice demonstrated involvement in haploid induction and embryo formation (Yao et al. 2018). Bhowmik et al. (2018) reported on several helpful factors affecting delivery of genome editing reagents into microspores. In maize, haploid formation may be the result of spermatid chromosomal fragmentation (Li et al. 2017) and selective elimination of uniparental chromosomes (Zhao et al. 2013).

Syngenta scientists (Kelliher et al. 2019) demonstrated that genome editing is possible in maize during haploid induction; they called this method “HI-edit”. Using a haploid inducing maize line expressing the CRISPR/Cas9 reagents targeting genes important in yield, they could show that indeed about 5–8% of the recovered haploid seeds showed edits in the targeted genes. They could also demonstrate that HI-edit can target genes in wheat during maize pollen-induced haploid generation in wheat. Using pollen-specific regulatory sequences in their constructs reduced the amount of off-target edits and resulted in higher numbers of edited haploid offspring.

The use of CRISPR/Cas9 editing has also been evaluated by Wang et al. (2019) during the process of HI-induced haploid formation. They used the HI CAU5 line with an integrated CRISPR/Cas9 cassette to pollinate a non-HI maize line and report generation of genome-edited haploids targeting *ZmLG1* and *UB2* in a B73 background. A total of 245 haploid maize plants were recovered from 339 haploid candidate seeds, 10 of which contained mutations at the targeted Lg1 locus, demonstrating a 4.1% editing efficiency (Wang et al. 2019). They called this system “Haploid-Inducer

Mediated Genome Editing (IMGE).” Through the rapid generation of pure DHs, lines can be edited for any desired trait improvement in any elite background and will greatly accelerate advanced maize breeding.

Mature pollen has been an attractive target to deliver exogenous DNA during the natural process of plant fertilization for a long time (Ohta 1986), for fairly obvious reasons (Eapen 2011). Pollen tube transformation, or PTT, has been described to use DNA solutions and *Agrobacterium tumefaciens* applied to stigmas or silk, prior or after pollination in rice (Luo and Wu 1989), and using magnetic particles to introduce DNA in cotton, pepper, and pumpkin (Zhao et al. 2017). Many of the reported techniques for pollen transformation and pollen tube-mediated transformation could not be reproduced by other laboratories, and some of the methods have been disproven, even retracted, or contradictory evidence has been obtained (Vejlupkova et al. 2020).

Morphogenic genes increase transformation and genome editing efficiency

Morphogenic genes such as *Wuschel2* (*Wus2*) and *Baby boom* (*Bbm*) are powerful tools for maize transformation which can impart numerous benefits. These include (i) increasing transformation frequencies via random integration (using either microprojectile bombardment or *Agrobacterium* delivery), (ii) aiding in the recovery of plants containing Cas9-mediated genome modifications, or (iii) permitting transformation of new target explants such as mature seed or seedling-derived leaf tissue. However, sustained expression of *Wus2* and *Bbm* during plant regeneration results in negative pleiotropic phenotypes, necessitating that, once their benefit has been realized, these genes must be down-regulated or removed via excision. These aspects are discussed in more detail below.

Using growth-stimulating genes as transformation tools has percolated through the plant biotechnology literature for many years, starting with early reports of using the cytokinin biosynthesis gene *Ipt* to aid in recovery of transgenic tobacco and poplar plants after excision of *Ipt* (Ebinuma et al. 2001) or cell cycle stimulation using *Ubi*:*RepA* to improve maize transformation (Gordon-Kamm et al. 2002). During this same period, genes involved in morphogenic regulation were being characterized, producing a steady stream of reports demonstrating ectopic formation of embryo- and/or meristem-like structures in *Arabidopsis* or other model dicot species, with early examples including genes such as *Lec1* (Lotan et al. 1998), *Lec2* (Stone et al. 2001), *Esr1* (Banno et al. 2001), *Wus* (Zuo et al. 2002), and *Bbm* (Boutilier et al. 2002). While informative and exciting, the majority of these observations relied on constitutive expression of the morphogenic gene, typically preventing the regeneration of normal fertile plants. To harness such genes as transformation tools, controlling or limiting expression became the next critical step.

Controlled expression of morphogenic genes has predominantly been accomplished by utilizing either inducible expression or transgene excision. Inducible expression has been effectively used in such diverse species as *Capsicum annuum* (Heidmann et al. 2011), *Arabidopsis thaliana* (Lutz et al. 2015), *Theobroma cacao* (Shires et al. 2017), and *Nicotiana tabacum* (Kyo et al. 2018). Similar to earlier reports where excision of *Ipt* from transgenic tobacco shoots (Ebinuma et al. 1997) or *Bbm* from transgenic callus in *Populus tomentosa* (Deng et al. 2009) was required to regenerate normal plants, use of constitutive *Nos*::*Wus2* plus *Ubi*::*Bbm* substantially improves maize transformation but requires excision before regeneration of healthy, fertile T0 plants (Lowe et al. 2016; Mookkan et al. 2017). Another way to solve this problem has been to switch from long-term (10–12 weeks) constitutive expression using *Nos*::*Wus2* + *Ubi*::*Bbm* to short-term expression (i.e., 1 week) using *Axig1*::*Wus2* + *Pltp*::*Bbm*, to rapidly stimulate somatic embryo formation (Fig. 2h) and permit regeneration of healthy, fertile T0 plants—potentially without excision (Lowe et al. 2018). Figure 2 shows the fluorescent somatic embryo formation from the scutellar surface of a maize non-stiff-stalk inbred immature embryo (Fig. 2h) or seedling-derived leaf tissue (Fig. 2i) after *Agrobacterium*-mediated transformation with a T-DNA containing constitutive *Wus2* and *Bbm* expression cassettes. Blue fluorescent embryos form 9 days after agro-infection (Fig. 2h) and green fluorescent somatic embryos form on leaf tissue 15 days after agro-infection (Fig. 2i).

Even though using these two promoters for *Wus2* and *Bbm* is tolerated, allowing T0 growth and reproduction, *Cre*-mediated excision of *Wus2*/*Bbm* is still preferred, in order to obviate any possibility of pleiotropic phenotypes in either the T0 plants or progeny, and simply to remove all unnecessary transgenes (Lowe et al. 2018). Another alternative relies on transient expression of the morphogenic genes to improve transformation, conferred by co-transformation with a mixture of two *Agrobacterium* strains. For example, when one *Agrobacterium* strain delivers a T-DNA containing a strong *Wus2* expression cassette, and a second strain delivers a trait-containing T-DNA, transient expression of *Wus2* is sufficient to stimulate somatic embryogenesis in cells containing only the integrated trait T-DNA, resulting in regeneration of transgenic events without integration of the *Wus2* T-DNA (Hoerster et al. 2020).

New morphogenic and/or growth-stimulating genes continue to be developed as transformation tools. For example, Du et al. (2019) have characterized a maize *Bbm* paralog (*Bbm2*) and demonstrated that 35SPro::*Bbm2* is also effective at increasing transformation frequency in two maize inbreds. Recently, use of growth-stimulating genes has been highlighted by two recent publications (Debernardi et al. 2020; Kong et al. 2020) using *growth-regulating factor* (*Grf*) genes to improve regeneration and hence overall transformation rates in a variety of crops. Accordingly, Kong et al. (2020) demonstrate overexpression of *Grf5* or paralogs increasing regeneration capacity (and thus overall transformation frequency) from 1.5-fold to approximately sixfold (relative to control transformation frequencies) across numerous crops, including sugar beet, canola, soybean, sunflower, and maize. Furthermore, Debernardi et al. (2020) show that a fusion between *Grf4* and *Grf-interacting factor1* (*Gif1*) provides an additional boost in transformation in both wheat and citrus (beyond that of using either *Grf4* or *Gif1* alone). Both groups report that fertile plants are produced even though the *Grf* genes were expressed with constitutive promoters, with Debernardi et al. (2020) providing an explanation by elucidating that post-transcriptional down-regulation of *Grf* by naturally occurring *miRNA396* in the resultant T0 plants appears to provide a built-in mechanism for mitigating pleiotropic problems. Just as *Wus2* and *Bbm* have provided a synergistic improvement in maize transformation, perhaps the *Grf*/*Gif* system can be combined with other morphogenic genes to further improve transformation and genome modification in maize and other crops.

The utility of *Wus2*/*Bbm* goes beyond simply improving recovery of transgenic events. The use of the morphogenic genes *Wus2* and *Bbm* in maize has facilitated recovery of targeted mutagenesis events generated using custom meganucleases (Gao et al. 2010; Svitashev et al. 2015), in addition to transgenic events containing de novo assembled mini-chromosomes (Ananiev et al. 2009). These two morphogenic regulators have also played an important role in rapid implementation of CRISPR/Cas9 genome editing applications. For example, transient expression of *Wus2*, *Bbm*, and CRISPR/Cas9 components through microprojectile bombardment has been used to facilitate recovery of maize T0 plants after (i) targeted mutagenesis of various endogenous genes (*Lig1*, *Ms26*, *Ms45*, *Als1*, and *Als2*), (ii) template-mediated editing of the *Als2* gene to confer chlorsulfuron resistance, and (iii) homology-dependent recombination (HDR) for targeted integration (Svitashev et al. 2015). Microprojectile bombardment–mediated delivery of *Wus2* and *Bbm* along with ribonucleoprotein (RNP) complexes has been used for template-mediated editing of the endogenous *Als2* gene to confer chlorsulfuron resistance in maize (Svitashev et al. 2016). Microprojectile bombardment of separate plasmids for *Wus2*, *Bbm*, Cas9, and gRNA expression cassettes has been successfully used to generate deletions (either 4-kb or 6-kb fragments depending on the pairs of gRNAs used) of the endogenous maize *waxy* gene, rapidly creating agronomically superior WAXY phenotypes directly in 12 elite Pioneer inbreds (Gao et al. 2020). Finally, particle delivery of *Wus2*, *Bbm*, Cas9, gRNA, and a donor template sequence for HDR has been used in two types of examples: (i) for a cisgenic promoter swap where the endogenous maize *Gos2* promoter was precisely integrated to replace the *Argos8* promoter, conferring improved drought resistance (Shi et al. 2017), and (ii) for routine integration of "Site-Specific Integration Landing Pads" at predetermined genomic locations (Gao et al. 2020) for subsequent FLP-mediated recombination (Betts et al. 2019). In this manner, *Wus2* and *Bbm* have been deployed to facilitate a wide range of CRISPR/Cas9-mediated changes in difficult maize inbreds (Chilcoat et al. 2017; Zhang et al. 2019).

The above examples of Cas9-mediated genome modification in maize highlight the advantage of using *Wus2*/*Bbm* in recalcitrant inbreds to aid in the implementation of CRISPR/Cas9 genome editing applications. How *Wus2*/*Bbm* may be aiding in Cas9-mediated editing remains speculative. In addition to simply increasing the number of T0 plants for analysis, *Wus2*/*Bbm* may impart another benefit for genome editing. While WUS2 and BBM proteins do not directly interact with cell cycle components, enhanced somatic embryogenesis results in cell cycle stimulation, which in turn may provide an HDR-conducive cellular environment. Similarly, it has been suggested that Rep/RepA expression in plant cells harboring viral replicons may be favorable to DSB-initiated HDR-based genome editing (Baltes et al. 2014). While examples have continued to arise in which *Wus2* and *Bbm* have been used for Cas9-mediated genome editing, the recent report by Debernardi et al. (2020) suggests that other plant growth-stimulating genes may have a similar utility—as highlighted in the recent report of using *Ubi*::*Grf4*:*Gif1* to aid in Cas9-mediated genome targeted mutagenesis in wheat.

For most maize research labs, maintaining a steady supply of immature embryos for transformation can be prohibitive in terms of greenhouse space and resources. The use of *Wus2*/*Bbm* is helping to provide new alternatives. In a major departure from the literature where immature embryos had been the norm since 1996 (Songstad et al. 1996), the use of *Nos*::*Wus2* plus *Ubi*::*Bbm* results in successful transformation of both embryo sections prepared from mature seed and seedling-derived leaf segments (Lowe et al. 2016). After *Agrobacterium*-mediated transformation of either explant, the combination of constitutive *Wus2* and *Bbm* expression stimulates somatic embryo formation, as shown in Figs. 2i and 5b for leaf tissue. Embryogenic callus from both explants is regenerated to produce healthy fertile T0 plants (Lowe et al. 2016). Hopefully, transformation methods using such starting explants will continue to be improved, potentially making transformation of this crop more accessible to labs where growing mature plants to supply immature embryos is neither onerous nor feasible, or both.

Concluding remarks

The intersection of maize transformation, genomics, and genome editing

The ability to make stable transgenics and edit the genome is both necessary and central to functional genomics, trait gene identification, endogenous gene modification and enhancement, and all future advanced breeding programs. The power of CRISPR/Cas9 has quickly become the major focus for both basic research and trait development in maize, including such modifications as single-base pair mutagenesis, small-scale edits, and the ultimate challenge of homology-dependent recombination (HDR) resulting in targeted sequence-specific integration. Looking to the future, genome editing will increasingly extend beyond single-base pair changes, and with such increasingly complex, sophisticated, and large-scale engineering will come a concomitant need for more efficient transformation methods to support editing technology.

The ability to edit at will is entirely dependent on transgenic capabilities and the need will drive innovations and improvements. Looking at progress in HDR puts this in perspective. Plant HDR has been an active field for many years, first being demonstrated in readily transformable tobacco by Puchta et al. (1999). However, it required another 10 years until both maize transformation and nuclease-mediated double-stranded break formation permitted this accomplishment in maize (Shukla et al. 2009). More recently, precise homology-dependent integration using Cas9 has been demonstrated (Svitashev et al. 2015). This now includes a more elegant intragenic example, where Cas9-mediated HDR was used to introduce an endogenous maize promoter to replace the promoter in the ARGOS8 gene locus to improve drought resistance directly in a Corteva maize inbred (Shi et al. 2017). All three accomplishments represent important scientific benchmarks for maize genome modification, but all came from large agricultural companies. Where does this leave the average academic research lab that has need of this technology? In addition, despite the substantial progress recently made in maize transformation, this remains a formidable bottleneck for some inbreds (and varieties in other cereals). Developing the next generation of transformation methods, for example, by incorporating new morphogenic/growth-stimulating strategies extending across all inbreds, remains a high priority in order to reach the full potential of genome editing applications in agriculture.

In a nutshell, the full potential of this technology can only be realized when our baseline transformation methods are efficient and accessible to the broad research community. It is tempting to pronounce the problem of maize transformation solved and research can now shift entirely to Cas9-mediated editing. However, we cannot proclaim complete success in maize transformation until all maize genotypes can be genetically modified by any research lab needing this technology. This means that transformation methods must be very robust, simple, and easily adopted by non-experts.

The efficiency of maize transformation system also depends on the complexity of the genomic modification desired. Stated another way, the type of Cas9 genomic modification dictates the requisite supporting transformation efficiency. For example, Cas9-mediated mutagenesis is highly efficient in maize for many laboratories; thus, if a researcher needs to perform a single-base (or small random) targeted change in a recalcitrant genotype, they could reasonably produce an agronomically valuable edit even though the baseline transformation frequency is low (i.e., < 2%). At the other extreme, precisely targeted integration requires homologous recombination (i.e., for creating an otherwise non-transgenic, intragenic promoter swap to create an important trait) which occur at frequencies typically less than 1%. Thus, a low baseline transformation level (again using 2% as our estimated efficiency) becomes a critical limitation, in this example requiring transformation of ~ 5000 explants to recover one successful HDR event, putting this experiment well beyond the average capacity of graduate student or post-doc. Thus, despite the substantial progress recently made in maize transformation, this remains a formidable bottleneck for some inbreds (and varieties in other cereals).

Opportunities ahead

Until recently, standard plant transformation protocols have allowed for the ability to conduct expression analyses, knockout (down) gene expression, make specific adjustments in protein structure and function, and observe overexpression and ectopic characteristics as an enabling technology in basic plant biology. However, the limitations of current standard plant transformation protocols have created a daunting bottleneck for functional genomic analyses and genome editing (Altpeter et al. 2016; Songstad et al. 2017). Ideally, plant transformation should be species, genotype, and explant independent, nearly tissue culture free (i.e., without somaclonal variation introduced through callus intermediates), relatively rapid with high efficiencies, and, perhaps, DNA free. These technologies would present the ability to alter any sequence in any variety, without genetic disruption, in stably heritable events (Kausch et al. 2019). It is now clear that the future focus will be on the development of "near tissue culture free" transformation invoking morphogenic regulators for direct somatic embryogenesis and direct organogenesis without callus intermediates. The development of transformation protocols that are "near tissue culture free" and genotype independent in inbred cereal lines permits direct gene editing of elite inbreds, and this alleviates the need to transform and then systematically backcross into elite germplasm, eliminating associated variation introduced by somaclonal variation or linkage drag.

The recent development of MRT of cereals (Lowe et al. 2016; 2018; Debernardi et al. 2020; Kong et al. 2020) is a major breakthrough toward this ideal, enabling transformation technology for the introduction of genome editing reagents specific toward their identified targets in many maize, sorghum, wheat, and rice varieties. These technologies are a great first step; however, it must be realized that further development is needed for this approach to be broadly applied and widely available to public research.

Achieving this goal will require the coordination of independent disciplines, as well as collaborative sub-disciplines including sequencing, data analysis, computational biology, genomics, target identification, efficient and precise synthesis of reagents, genome editing, target validation, plant transformation, phenotypic analysis, and whole genome sequencing for detection of off-target affects or inclusion of foreign DNA. The routine recovery of heritable events from the transfer of DNA, RNA, proteins, and/or ribonucleoproteins (RNPs) into plants will require a fertile collaboration of these disciplines. The overarching goal of this synergistic triad of technologies is to actualize the fundamental aspects of the technologies that bear on basic plant research and cereal crop improvement.

The ultimate goal is to meet the challenges going forward for world food security and environmental sustainability, and to widely functionalize these abilities to the broader scientific community across cereal species.

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Data availability The composite biological illustrations of Figs. 3, 4, and 5 and all of the individual illustrations they comprise are copyrighted by the author Albert P. Kausch (APK) who provides Springer the permission to publish them in this paper.

Code availability N/A

Declarations

Conflict of interest WGK is an employee for Corteva Agriscience, Johnston, IA 50131, USA.

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