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Progress in genetics of breeding for maize transformation ability

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Progress in genetics of breeding for maize transformation ability

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ABSTRACT

Maize (Zea mays) is the most widely grown crop grown and produced crop in the world. It is a foundational model for genomics and genetics. To meet the need of a growing population and climate change the current and future crop improvement efforts will comprise of the utilization of biotechnology-based approaches. This includes the functional analysis and discovery of agriculturally important genes for crop research and product development. Today, most crop genetic engineering systems use the transformation process. But there are limitations to the transformation process. The labor of creating transgenic events is the largest cost associated with the transformation production system. The culture process is the most time-consuming and has the highest labor cost of the system. Then once the trait is verified to be present the trait must be backcrossed into an elite line with better agronomic traits and good yield. The backcrossing process is time consuming and requires a large amount of greenhouse space. The major limitation of plant transformation is genotype dependency. To get more widely adoption of biotechnology and the transformation process for breeding it needs to become less expensive and time-consuming to produce transgenic lines. This can be achieved through discovery of the genetics behind callus formation and regeneration, so any elite line can be made transformable. The discovery of morphological genes like Bbm and Wus2 have made it possible to develop a genotype-independent transformation system in maize and other monocots.

Chapter 1. INTRODUCTION

The cereal crops are major crops that feed the world and are grown in more quantities on more acreage across more different ecosystems than any other crop. Maize is the most widely grown and produced crop in the world. It is used in various industries as raw materials and animal feed. It is also a model plant for genomics and genetics. To meet the needs of a growing population and climate change the current and future crop improvement efforts will comprise of the utilization of biotechnology-based approaches. This includes the functional analysis and discovery of agriculturally important genes for crop research and product development. Today, most of the crop genetic engineering systems use the transformation process. The transformation process includes the critical parts of embryogenic and regenerable tissue cultures (Mookkan et al., 2017; Salvo et al., 2014; Jiao et al., 2017; Yadava et al., 2017).

The term transformation is used to define the insertion of foreign molecules into plant cells, bacteria, and fungi. Transformation of maize cells was first reported in 1986 when the direct uptake of naked DNA into protoplasts occurred. Stable transgene integration was seen in calli derived from electroporated protoplasts of the Black Mexican Sweet maize suspension cell line. Two years later, in 1986, the first transgenic maize plant was reported because there was no reliable plant regeneration system (Rhodes et al., 1988). A reliable and simple regeneration system is essential for establishing an effective transformation system. Several studies were done in the early 1980s testing different starting materials consisting of stem and immature embryos. Only a few studies reported success in regenerating maize plants from protoplasts

(Rhodes et al., 1988; Shillito et al., 1989; Que et al., 2014; Hooykaas et al., 2010). Immature embryos have been shown to be an exceptional source material for creating embryogenic callus and suspension cell cultures for preparing protoplasts for direct gene delivery. After cell suspension and regeneration, plants did not produce viable seeds (Rhodes et al., 1988). Maize embryos can be induced to form one of two types of embryogenic calli: Type I and Type II. Type I calli have a more compact structure with a group of embryos fused together. Type II calli have clusters of "friable" discrete single embryos. To resolve the viable seed issue reported in other studies Type II calli were used (Que et al., 2014; Rosati et al., 1994; Armstrong et al., 1992; Rivera et al., 2012; Krakowsky et al., 2006).

After the first reports of successful maize transformation using protoplasts were published microparticle bombardment (also known as biolistic transformation) was positively confirmed to generate highly fertile maize transformants using calli or embryogenic suspension cell cultures as target tissue. The selectable genes used as markers were ALS (acetolactate synthase), BAR (bialaphos resistance), or HPT (hygromycin phosphotransferase) (Fromm et al., 1990; Gordon-Kamm et al., 1990; Walters et al., 1992; Vain et al., 1993). Events attained from embryogenic calli using biolistic transformation had better fertility than events created by using protoplast transformation. In 1993 immature embryos from an elite maize inbred was used as the target for biolistic transformation to induce the *Bacillus thuringiensis* (Bt) *Cry1Ab,* an insecticidal gene, with use of the BAR selectable marker into the plant tissue (Que et al., 2014). After this occurred, in 1996, one of these events was launched as the first Bt

maize product by Ciba-Geigy (Koziel et al., 1993). Also, in 1996 a protocol for biolistic transformation using H-II germplasm and the ALS selectable marker was successful (Songstad et al., 1996; Que et al., 2014; Yadava et al., 2017). These scientists found pre-culturing the immature embryos before bombardment greatly improved transformation efficiency and survival comparable to the results obtained from the suspension cultures as targets. Other scientists have also reported using conditioned immature embryos as targets of gene delivery for transformation greatly increases the transformation frequency when immature embryos were cultured before and after bombardment on media with high osmolarity (Que et al., 2014; Armstrong et al., 1992; Yadava et al., 2017). Using immature embryos as transformation targets simplifies the target tissue preparation effort and shortens the transformation timeline in comparison to using embryogenic callus cultures or suspended cells. As a result of the plant transformation timeline being shortened plant production is improved by the callus culture period being shortened which lowers somaclonal variation. But there is a need for more greenhouse space to grow more stock plants for immature embryos. Also, isolation of immature embryos is more time-consuming than callus subculture (Que et al., 2014; Hooykaas et al., 2010).

There are other physical gene delivery methods developed for producing transgenic maize plants including aerosol beam injector, electroporation, and silicon carbide whisker-mediated transformation. The two more popular physical transformation methods are silicon carbide whisker-mediated transformation and electroporation. Whisker-mediated transformation was developed in 1994 which used

silicon carbide whiskers to deliver plasmid DNA and embryogenic suspension cultures as the target tissues (Frame et. al., 1994; Que et al., 2014; Hooykaas et al., 2010). The mixture of DNA, whiskers and suspension culture cells are shaken forcefully allowing the whiskers to make tiny holes in the cells allowing the DNA entry. This method has been done using embryogenic cell cultures from Hi-II (Que et al., 2014). Electroporation is a transformation method that uses electric pulses to induce membrane permeabilization providing a local driving force for iconic and molecular transport through the pores (Que et al., 2014; Rivera et al., 2012; Hooykaas et al., 2010).

Agrobacterium-mediated DNA delivery, also called indirect transformation, into maize cells first occurred in the mid-1980s (Graves and Goldman, 1986; Grimsley et al., 1987). Then in 1991 the first successful maize transformation was reported using isolated shoot apices as target tissues. In 1996, a routine method for *Agrobacterium*-mediated maize transformation was developed. In this method immature embryos were used as the target tissue with an Agrobacterium strain containing a "super-binary" vector. Since the introduction of this method it has been the method of choice for large scale event generation for commercial development and trait research. The selection and regeneration conditions were already established for biolistic transformation systems and were easily transferred for use for *Agrobacterium*-mediated transformation. Many transgenic maize events produced by *Agrobacterium*-mediated transformation have become products and released to market (Que et al., 2014; Hooykaas et al., 2010).

Many companies have released maize products containing improved traits since the first transgenic maize cultivars were released 20 years ago. The improvement of the genetic engineering system for maize is important because it allows the expected future challenges of the world to be overcome by genetic engineering of maize cultivars with improved traits. There are many limitations for the transformation production system, for maize, including having a dedicated greenhouse for supplying zygotic embryos, space for marker assisted backcrossing, and genotype dependency. The ideal maize line for maize transformation and for trait breeding is an inbred line with exceptional agronomic characteristics. The best tissue to start with should be highly transformable, available in large quantities year-round, easy to produce, and inexpensive. The current lines available for transformation are far from perfect. Many labs use immature embryos for either *Agrobacterium*-mediated or biolistic transformation. Some elite inbred lines have become the preferred genotypes for trait development including Hi-II, H99, and A188. High efficacy *Agrobacterium*-mediated transformation is only attained in a limited number of elite lines. This genotype dependency is contributed to embryogenic callus formation, competency of immature embryos to *Agrobacterium* infection, and the ability to regenerate plants. Callus formation is critical for transformation. Many transformation production systems use Type II callus because it is fast growing, highly regenerable, and friable. These characteristics favor transgenic plant selection and regeneration. But the formation of Type II callus is limited to very few genotypes such as Hi-II, A188, B73, and B104 which makes it highly genotype-dependent. Hi-II is the most widely used genotype for transformation because it has

high transformation efficiency in laboratories and is reliable. But Hi-II has poor agronomic traits and has a non-uniform genetic background (Que et al., 2014; Yadava et al., 2017, Armstrong et al., 1991).

The genotype dependency is only one of the reasons transformation and biotechnology processes are not used as preferred breeding methods. The transformation process for production is labor intensive and time consuming. The labor of creating transgenic events is the largest cost associated with the transformation production system. The culture process is the most time-consuming and has the highest labor cost of the system. The high cost is associated with needing to isolate large numbers of immature embryos to create a large number of transgenic events. There is a bottleneck that exists with embryo supply and excision. Then once the trait is verified to be present the trait must be backcrossed into an elite line with better agronomic traits and good yield. The backcrossing process is time consuming and requires a large amount of greenhouse space. Also, vector design for commercial transformation is essential to ensure efficient transgenic event production. It must have desirable trait efficacy and timely product regulatory registration and approval. To get more widely adoption of biotechnology and the transformation process for breeding it needs to become less expensive and time-consuming to produce transgenic lines. This can be achieved through discovery of the genetics behind callus formation and regeneration, so any elite line can be made transformable. Being able to do this would cut down on time and resources needed for the transformation system and would open the door for more breeding systems to use a transformation system (Que et al. 2014; Lowe et al. 2006;

Lowe et al., 2016; Lowe et al., 2018; Rivera et al., 2012; Armstrong et al., 1992; Salvo et al., 2014).

This literature review summarizes the recent advancements in the understanding of the genetics of culturability and regeneration for the use in the maize transformation. How these advancements in genetics have helped advance the transformation system and breeding for transformability. The advancements for breeding with a better maize transformation system will be discussed in enough detail for others to advance their own maize transformation systems. The recent advancements made makes transformation and gene editing more sustainable as breeding methods.

CHAPTER 2. Genetics of Culturability and Regeneration

There have been many recent advancements made in the genetics and genomics of callus formation and regeneration, which helps to advance the transformation system. Tissue culture response and regeneration in plants has been shown to be under genetic control in maize. Several developmental, biological, and molecular studies have shown that numerous morphological genes are involved in organogenesis, plant cell division, plant regeneration, and somatic embryogenesis. Expression of leafy cotyledon 1 (LEC1) and LEC2, Maize oval development protein 2, WUSCHEL (WUS2), Baby Boom (BBM), Agamous-like15, and somatic embryogenesis receptor-like kinase1 (SERK1) shows morphogenic control of plant development (Mookkan et al., 2017). Quantitative trait locus (QTL) mapping technology has made it possible to identify QTL and estimate the number of loci controlling genetic variation. Recent advancements in mapping approaches and DNA based molecular marker systems has allowed complex traits to be resolved into several single Mendelian components, understand gene action, phenotypic effects, and characterize their map position (Bolibok and Rakoczy-Trojanowska, 2006; Salvo et al., 2014).

QTL mapping strategies have been used in several plant species to successfully identify genomic regions associated with tissue culture response in crops such has cotton, soybean, rice, and maize (Salvo et al., 2018). The first paper reporting regions associated with tissue culture response was from Armstrong et al. (1992). They intended to improve tissue culture response by backcross breeding. RFLP analysis was used to map chromosomal segments important in culture response in A188. The regions

identified where located on chromosomes 1, 3, and 9 (Armstrong et al., 1992). Wan et al. (1992) identified six in part overlapping regions on chromosomes 1, 2, 3, 6, and 8 in maize involved in regenerable callus formation and embryo-like structures (ELS). Many other studies have found QTL on chromosomes 1, 3, 4, 5, 8 and 9 for tissue culture response and embryo-like structures (Bolibok 2006; Krakowsky et al., 2006; Lowe et al., 2006). A summary of QTL mapping studies of maize tissue culture response is shown in Table 1 (Salvo et al., 2018).

Table 1. Summary of QTL mapping studies on maize culturability (Salvo et al., 2018)

Source	QTL	Marker Type	QTL detection method
Armstrong et al. 1992	1S, 1L, 2, 3, 4, 9L	RFLP	Multiple regression
Lowe et al. 2006	1S, 1L, 2L, 3L, 6S, 10S	RFLP, SSR, SNP	Segregation distortion
Krokowsky et al. 2006	1L, 2L, 3L, 5S, 6S, 8L	RFLP, SSR	PlabQTL with cofactor selection
Zhang et al. 2006	2, 3, 5, 6, 8, 9	SSR	Composite and mapping

QTL on Chromosome 3 Involved in Improved Tissue Culture

A fine mapping study was done by Salvo et al. (2018) to build on this research. They chose to focus on the QTL identified on chromosome 3 in maize that are associated with improved tissue culture and regeneration. Their goal was to advance research in identifying genes involved in regeneration ability and embryogenic capacity in maize. Identification and characterization of these genes will increase the understanding of the genetic mechanisms involved in somatic embryogenesis and will help in the development of germplasm with increased tissue culture response or even

genotype-dependent tissue culture systems. Their mapping identified a 3053 kb region significantly associated with tissue culture response. This region was found between markers PZE-103122471 and SYN29001 with a significant Chi-square test ($P<0.0001$) associated with all tissue culture phenotypes and A188 markers PZE-103135061 and PZE-103133772 also had significant association with tissue culture traits. The region between PZE-103122471 and SYN29001 is a large region and could have hundred genes in this region (Salvo et al., 2018). Other studies on somatic embryogenesis suggest that multiple genes are involved in the process and transcription factors could activate the type of cellular dedifferentiation needed to regenerate somatic embryos into plants (Salvo et al., 2014).

Known Genes Involved in Somatic Embryogenesis

Few genes have been characterized as having a direct role in the induction of somatic embryogenesis in tissue culture in maize have been identified even with the importance it would give to agricultural production. These genes were discovered by using overexpression and gene knockout studies to understand how the genes were behaving. The initiation of somatic embryogenesis involves a complex coordination of multiple pathways. Previous studies have described genes involved in cellular reorganization, hormone response, stress response, signal transduction, and transcriptional regulation. Many of these gene actions have been found in Arabidopsis. Salvo et al. (2014) found similar genes, in maize genotype A188, using a specific developmental window study and sequencing of the genes using B73 as a reference genome (Salvo et al., 2014).

Known genes involved in stress response important to somatic embryogenesis are germin like-proteins (GLP) and GST. Salvo et al. (2014) discovered two stress response genes chitinase A1 and WIP1 which were up regulated 1500-fold in the first hours of embryogenesis. Chitinase proteins are known to promote somatic embryogenesis (Omid et al., 2010). GST family of genes are also involved in plant defense (Galland et al., 2007). They found 15 GST genes had large expression changes during early somatic embryogenesis, which includes ZmGST 8, ZmGST 24, and ZmGST 40. The GST genes were found to be co-expressed with WUS, SERK, PIN, and BBM. GLPs are proteins that affect the plant redox status and are involved in developmental regulation. GLPs are normally only detected in embryogenic tissue. It is possibly used as a secondary signal to promote somatic embryogenesis. One GLP gene Salvo et al. (2014) detected in the early embryogenesis process was GRMZM2G045809 and was co-expressed with the BBM transcription factor. Genes involved in embryogenic pathway initiations include Leafy Cotyledon (LEC) and Baby Boom (BBM) genes. The initiation of the embryogenic pathway is seen when somatic cells acquire embryogenic competence and proliferate as embryogenic cells capable of forming somatic embryos. In studying the available research and BBM being shown to be co-expressed with LEC2, AGL15, GLP, PIN, GST, and WOX it can be concluded BBM as a stimulator of plant hormone production which triggers and signals pathways important for somatic embryogenesis. LEC genes are also important in stimulating somatic embryogenesis. These genes play an important role in regulation and directly interacting with hormone response genes.

Known somatic embryogenesis receptor-like kinase genes called SERK genes are also important for initiating the embryogenic pathway (Salvo et al., 2014).

Known genes involved in embryo formation and development are the Wox, Clavata (CLV), Wuschel (WUS), and Agamus (AGL) genes. These genes are involved in meristem formation and development and have been found to be important for somatic embryo formation as well. The WUS gene is a homeodomain transcription factor involved in floral and meristem development specifically as a regulator of organ identity and stem cell fate (Klaus et al. 1998). It has an important role in activating and regulating pluripotent stem cells by promoting proliferation genes and repressing developmental regulators. Salvo et al. (2014) found the WUS genes had lower expression during early somatic embryogenesis. But they were co-expressed with GST and SERK genes. They explained the lower expression of WUS as maybe being tied to the developmental window they were looking at in their study. They may have captured a time when the organization center was just starting to develop and the transcripts they saw represented a small amount of cells presenting WUS activity during the early stages of stem cell development. Their findings did show the WOX genes had increased in expression in early embryogenesis. Examples of WOX genes found to increase in expression were ZmWOX5A/5B, ZmWOX2A, and ZmWOX11/12B. The WOX genes were co-expressed with AGL15, BBM, and PIN. Salvo et al. (2014) found the expression of CLAVATA or CLV1-like gene (GRMZM2G141517) had a steady increase in their study. CLV is a receptor-like kinase also involved in floral and shoot development and acts upstream of WUS. CLV represses the WUS genes activity by interacting in a regulatory

loop with WUS to promote callus maintenance and initiation. Another known meristem-related gene is AGAMOUS. AGAMOUS is a MADS box transcription factor involved in organ differentiation and flower development. An AGL15-like gene, GRMZM2G139073, had differential expression during early embryogenesis. This gene is co-expressed with PIN, WOX, and GLP. AGL15 has been shown to be preferentially localized in embryonic tissues and promote somatic embryo development in Arabidopsis. It is also known AGL15 interacts with LEC2 and SERK1 genes. Thus AGL15-like genes are important for callus formation in maize (Salvo et al., 2014).

Model of Known Genes Involved in Somatic Embryogenesis

Salvo et al. (2014) purposed a model based on coordinated expression of somatic embryogenesis-related genes looked at in their study and the genes relative expression in early embryogenic tissue culture response. The model was somatic embryogenesis-related gene expression networks as determined by coexpression with a correlation coefficient greater or equal to 0.9 between genes expressed during the early stages of somatic embryogenesis. The relative expression of transgenes in A188 immature embryo explant tissue in tissue culture was reported at 0 h, 24 h, 36 h, 48 h, and 72h. Based on these finds the model highlighted somatic-embryogenesis related genes that were up- and down-regulated during the time period of the study (Salvo et al., 2014).

Their study provides essential information describing the underlying genetic mechanisms controlling somatic embryogenesis in tissue culture for the understanding

of the basic processes involved in somatic embryo formation. They were able to offer information on transcripts detected for major genes formerly described with a role in embryogenesis. This information can be used to help us better understand expression networks and gene functions involved in the initiation of somatic embryogenesis in culture. Advancements in understanding the underlying genetic systems help improve our understanding of genetic mechanisms and biological processes that confer efficient tissue culture response such as somatic embryogenesis in vitro will help advance crop improvement strategies to increase agricultural productivity (Salvo et al., 2014).

The importance of Reference Genomes for Research

Reference genomes for crop plants have been produced over the past decade. These genomes can often be missing complex repeat regions and often fragmented. Accurate and complete annotations and genomes offer essential tools for characterization of functional and genetic variation. These resources allow the determination of biological processes and support translation of research findings into sustainable and improved agricultural technologies. An accurate and complete genome assembly for maize is critical for basic and applied research (Jiao et al., 2017).

There have now been updates made to the assembly and annotation of the reference genome of B73 and interest in a B104 reference genome for the advancement of agricultural research. The B73 genome was assembled using contig sequencing and high-resolution optical mapping. This new reference genome for maize, Zm-B73-Reference-Reference-Gramene-4.0, has allowed transposable elements to be identified

and aided in the identification of transposable element lineage expansions that are unique to maize. Their improved B73 reference genome made improvements to the B73 genome published by Schnable et al (2009) by making improvements in the gene space by correcting misassembles, orientation and order of genes, and resolution of gaps (Jiao et al., 2017). B104 is in the process of being assembled and annotated as a reference genome. B104 is very similar to B73 (98%) but B104 is readily transformable. The availability of B104 as a reference genome would allow other researchers to use the genomic data to work with their own transformants more easily (Manchanda et al., 2016). It gives researchers the genomic data about a transformable line and specific genes involved in transformability, so the information can be used to put together vector cassettes for transformation more easily. The information for this reference genome will be openly shared, which makes the information more easily used by researchers unlike patented research.

CHAPTER 3. Breeding for Transformability

Many companies have used transformation as part of their breeding programs because it has allowed the transfer of specific genes into crops allowing many crops to have enhanced productivity due to their resistance to herbicides or insects. The interest in using the transformation system in a breeding program has sparked an interest in breeding for increased tissue culture response and regeneration ability for transformability. The beginning studies for breeding for improved tissue culture response and transformability in maize included having to screen for tissue culture response and backcrossing (Armstrong et al., 1992; Lowe et al., 2006).

Backcross Breeding for Tissue Culture Response

Armstrong et al. (1992) did two different crosses A188/B73 and A188/Mo17. They took half an ear and put immature embryos on media and the other half ear was left on the plant for seed. Cultures were scored 14-28 days after embryo isolation for the presence of somatic embryos. The plants were regenerated and the regenerated plants with the best tissue culture response were backcrossed to B73 to produce the BC1 generation. BC1 plants were selfed and halve ears were placed in tissue culture and plants regenerated. The plant with the highest embryogenetic frequency was crossed to B73. This continued for six generations of backcrossing with selection at each generation for high frequency initiation of embryogenic cultures. BC6 plants were selfed for four generations and then inbred lines were selected. Their breeding scheme, total number

of ears tested, total number of embryos tested, and percentage of embryos forming regenerable cultures is found in Table 2 (Armstrong et al., 1992).

Table 2. Tissue culture response of B73 and B73/A188 backcrosses (Armstrong et al., 1992)

Generation	Number of Ears Tested	Total Number of Embryos Tested	Mean of all Ears Producing Regenerable Cultures	Lowest Response	Highest Response
B73	21	2248	0.1	0	2
FRB73	10	1462	0.1	0	1.4
BC_1	18	1441	4.7	0	18.3
BC_2	17	1904	13.5	0	37
BC_3	18	1603	1.3	0	6.7
BC_4	17	1800	9.9	0	31.2
BC_5	10	2477	10.2	0	39.3
BC_6	4	1001	0.7	0	3
BC_6S_1	9	384	11	0	40
BC_6S_2	13	1153	84	0	35.4
BC_6S_3	9	1289	23.5	0.7	75.7
BC_6S_4	9	561	45.6	0	91

Armstrong et al. (1992) found the average percentage of embryos forming regenerable cultures varied considerably across ears for each of the six backcross generations. There was a low of 0.7% for BC6 to a high of 13.5% for BC2 (Table 2). The percentage of immature embryos forming regenerable cultures was 75% averaged across all 90 ears and is a 75-fold higher response then the average response of the current parent. The percentage of embryos forming regenerable cultures seen in Table 2 of the BC6S1 through S4 generations also show there were no favorable alleles lost during the later backcross generations. Out of the plants cultured from BC6S4 about half

(45.6%) of all the embryos formed regenerable cultures. Embryos from one BC6S4 plant (91.0%) produced almost all regenerable cultures. This plant was homozygous for all favorable culture alleles transferred from A188 into B73. These data show that regions of the A188 genome can be introgressed into B73 by backcross breeding, resulting in up to a 91.0% increase in Type II culture initiation frequencies from immature embryos on a standard N6-based medium (Armstrong et al., 1992).

Marker Assisted Breeding for Transformability

Lowe et al. (2006) built upon the research of Armstrong et al. (1992) and used a marker assisted breeding (MAB) program to breed for transformability in maize. They used MAB to introgress specific regions of the Hi-II genome into the elite line FBLL. They also screened FBLL-MAB lines for transformation to select highly transformable lines. In their study they first screened for culturability and then for transformability. Then they developed and used markers for breeding with an emphasis on screening for transformability. Their breeding scheme included an initial cross of FBLL X Hi-II to make the F1. Then the F1 was backcrossed to the recurrent parent FBLL, BC1. Selection for cultures that regenerated plants was performed. They performed a full genome scan and segregation distortion analysis to find RFLP markers associated with culturability/transformability. The following RFLP markers showed linkage with those traits: npi234a on chromosomal bin 1.03, npi625 in bin 1.09, npi212b (3.08), npi223a (6.04), and umc44a (10.06). Those markers were used for backcrossing to the recurrent parent FBLL. They combined BC3 lines with the highest number of culturability/transformability regions to form an BC3F1. Self-pollination for several

generations were used to fix selected regions and form inbred lines. The lines were tested for transformability and transformable lines were selected (Lowe et al., 2006). Their breeding scheme can be seen in Table 3 (Lowe et al., 2006).

Table 3. Breeding scheme to transfer Culturability/Transformability from Hi-II into the elite female line, FBLL (Lowe et al., 2006)

Cross	Generation
FBLL X Hi-II	F1
(FBLL X Hi-II)X FBLL	BC1
Backcross to FBLL	BC2
Backcross to FBLL	BC3
BC3 X BC3	F1
Self	F2-4

Their inbred lines were >93% homozygous for the five culturability/transformability regions and the recovered percentage of the recurrent parent was 81-87% confirmed by 179 polymorphic SNP markers. Transformation frequencies of these lines ranged from 2.4 to 11.8% (Lowe et al., 2006).

Table 4. Transformation frequencies of FBLL-MAB Lines (Lowe et al., 2006)

FBLL-MAB Line	Number of Embryos inoculated	Number of Events	Transformation Frequency (%) Mean ± SD
178-178-20	821	70	7.5 ± 6.5
178-74-25	1604	128	7.3 ± 4.6
178-74-39	1439	46	3.9 ± 6.6
178-178-8	485	57	11.8 ± 0.4
178-270-46	124	3	2.4

All the regions transferred into the FBLL lines were A188 segments from Hi-II similar to what Armstrong et al. (1992) found. The improved transformation frequencies of their five inbreds shows MAB can be used to improve culturability/transformability in maize

(Table 4). In using markers, they were able to select for culturable lines without having to culture each line before backcrossing. Markers were used for culturability because transformation relies on culturability. Not all cultural lines are transformable. But transformability must have culturability to be able to select transformed cells. They demonstrate the development of a relatively fixed and elite line with the ability for tissue culture and improved transformation frequencies by using marker assisted breeding (Lowe et al., 2006).

These studies are only two examples out of many showing that culturability, regeneration, and transformability can be improved by the introgression of A188 into an elite line. Plant regeneration can also be improved by using recurrent selection. But these breeding schemes are labor intensive with having to do tissue culture and backcrossing into the recurrent parent. There is an extra step with having to screen for transformability of each line, so the best line for transformability can be selected. This process is labor intensive, time consuming, and costly. However, advancements in the genetics and understanding of culturability, regeneration, and transformability in maize enabled new processes for creating a transformable maize line.

CHAPTER 4. Alternative Approaches to Overcome Genotype-Dependency

Today many advancements have been made in genetics furthering the understanding of how culturability, regeneration, and transformability in maize. There have been various studies using this recently discovered genetics information and have applied it to the transformation processes. Boutilier et al. (2002) used subtractive hybridization to identify genes that are up-regulated during the invitro induction of embryo development from immature pollen grains of *Brassica napus*. The team found two genes from genomic clones from Brassica and one ortholog in Arabidopsis. They did a genome wide search in sequence databases and found the BBM transcription products showed similarity to the AP2/ERF family of proteins. The proteins are a plant-specific class of putative transcription factors that regulate a large range of developmental processes. The BBM proteins from Bassica and Arabidopsis matched the amino acid sequences of related AP2/ERF domain-containing proteins (Boutilier et al., 2002). BBM belongs to the AP2/ERF transcription family. BBM initiates pathway LC1-AB13-FUS3-LC2 to induce somatic embryogenesis. Many studies have chosen to focus on using morphogenic genes like Baby Boom (BBM) and WUSCHEL2 (WUS2) in expression cassettes and use transformation to insert the genes into maize to make a recalcitrant line transformable (Mookkan et al., 2017; Lowe et al., 2016; Lowe et al., 2018).

Many companies are still using *Agrobacterium tumefaciens* transformation, which is an indirect transformation method. This method is proven to give a low copy number, genome integration is precise, consistent gene expression over generations, simple transgene insertions with defined ends, and stable integration and inheritance. It

also has efficient protocols and high transformation efficiency (Rivera et al., 2012). Transformation is typically done on immature embryos. Immature embryos are normally harvested between 9-12 days. Then embryos are induced by plating or mixing the embryos with *Agrobacterium*. The vector constructs commonly used in plant transformation processes are binary and superbinary vectors. Superbinary vectors are improved versions of binary vectors and include the virB, virC, and VirG genes from pTiBo542 which are responsible for the supervirulence phenotype of an *A. tumefaciens* strain. Many of the vectors include a combination of these genes in the cassettes, using promoters from: 35S and Umbi, loxP target sites, and CRE recombinase and multiple cassettes (Figure 1) (Lowe et al., 2017; Lowe et al., 2018; Horstman et al., 2017; Mookan et al., 2017; Heidmann et al., 2011; Boutilier et al., 2018).

Figure 1.

Figure 1. Example of a vector cassette. RB, right border; LoxP, loxP target sites; P_{RAB17}::CRE, promoter Rab17 to drive CRE; P_{ubi}::BBM, promotor Ubi to drive BBM; P_{nos}::WUS2, promotor Nos to drive WUS2, LoxP, loxP target sites, LB, left boarder

Use of Morphological Regulators

There have been many studies showing binary and superbinary vectors being able to make plants transformable. Lowe et al. (2016) used a superbinary vector construct and three different strains of *A. tumefaciens* in their study. The three strains of *A. tumefaciens* used were thymidine auxotrophic (THY-) versions of LBA4404, LBA440, and AG 1. Vectors used to construct the superbinary vectors were psB11 and pSB1 with

T-DNA components. They harvested embryos from inbreds PHN46, PH581, PHP38, and PHH5G, and transformed with the Agrobacterium strain LBA4404. The vector T-DNA included Bbm alone (pPHP24955), Bbm and Wus2 (pPHP35648), and no Bbm or Wus2 (pPHP24600). Vector pHP24600 was used as control vector construct. pPHP24600 contained two expression cassettes in opposite orientations. It had a CaMV 35S promoter driving expression of a phosphinothricin acetyltransferase (PAT) gene followed in the 3′ to 5′ orientation going toward the T-DNA right border with a CaMV 35S 3′ regulatory sequence. A maize Ubiquitin (Ubi) promotor and Ubi intron is driving the expression of DsRED coding sequence with an introduced potato LS1 intron. The marker expression cassette included CaMV $35S_{pro}$:PAT:35S 3′ + Ubi_{pro}:DsRED:pinll. The other vectors contained cassettes encoding BBM and WUS2. Plasmids encoding BbM and Wus2 all had three expression cassettes. The expression cassettes contained a recombinase (moCRE or moFLP) and Bbm and Wus2 genes between recombination target sites (FRT or LoxP sites, respectively) (Lowe et al., 2016).

Transformation data were calculated as the number of callus transformation events for the embryos from each ear. For all inbred lines the base line, or control vector, had low or nonexistent transformation frequencies. It ranged from 0% for inbred PHHG to 2.0% for inbred PHP38. Each inbred line responded differently to either Ubi_{pro}:Bbm + nospro:Wus2 or Ubi_{pro}:Bbm alone. There was a substantial increase in callus transformation frequency for inbred PHN46 from 1.7% in the control treatment to 34.9% for Bbm alone. When Wus2 was added to the cassette with Bbm there was a modest increase in frequency to 38.0%. Inbred PH581 behaved similarly to inbred

PHN46 with increases in callus formation frequency with Bbm alone and Bbm and Wus2 expression cassettes. There was an increase in callus transformation frequency in inbred PH581 from 0.4% in the control treatment to 16.9% for Bbm alone. When Wus2 was added to the cassette with Bbm the frequency increased to 25.3%. Inbred PHP38 followed the same trends, as the other two inbreds, in increased callus transformation frequency changes. It had the lowest change in frequency for Bbm alone and the highest frequency change for Bbm and Wus2 for all the four inbreds. There was an increase in callus transformation frequency in inbred PHP38 from 0% in the control treatment to 10.1% for Bbm alone. When Wus2 was added to the cassette with Bbm there was an increase in frequency to 51.7%. Inbred PHH5G had a different response to Bbm and Wus2 then the other three inbreds had. There was no change or still no transformed callus was produced when the cassette with Bbm alone was used. When Wus2 was added to the cassette with Bbm there was an increase in callus transformation frequency to 45.7%. The transgenic callus grew vigorously and showed a mix of morphology of type I and type II embryo genic callus. PHN46, PH581, and PHP38 produced low levels of compact type I callus. Callus transformation frequencies were increased in all four inbreds by the addition of morphological markers like Bbm and/or Wus2. Excised and single copy plants were obtained for all four inbred lines. The plants were transferred to the greenhouse and the plants were both male and female fertile and were healthy. This study also tested the combination of the Ubi_{pro}:Bbm + nos_{pro}:Wus2 expression in sorghum, rice, and sugarcane. For each of these crops an

increase in the transformation frequency using the same expression cassettes used for maize (Lowe et al., 2016).

Use of Morphological Regulators Without an Exogenous Selective Agent

The study done by Mookkan et al. (2017) was meant to confirm and extend the previous studies using morphogenic regulators to establish genetic transformation of sorghum P898012 and B73 by co-expressing maize WUS2 and BBM genes without the use of an exogenous selective agent. The vector PHP78891 was comprised of four expression cassettes. The first cassette was $RAB17_M$:CRE. The second cassette was NOS_{At}:WUS2. The third cassette was UBI_M:BBM. The last cassette was UBI_M:GFP. The CRE:WUS2:BBM cassette was bracketed by lox P sites. One lox P site flanks the UBI_M:GFP cassette within the left Agrobacterium boarder and the other lox P site flanks the right *Agrobacterium* border. The desiccation-induced expression of CRE resulted in the excision of the CRE:WUS2:BBM cassette allowing plant regeneration to take place (Mookkan et al., 2017).

Transient expression of GFP was observed 3-13 days after inoculation in B73 and P898012 immature embryos. Transient expression analysis was done in B73 using *Agrobacterium* strain AGL1 with or without a superbinary vector. GFP expression was analyzed on day 3 after inoculation. The frequency of transformation was analyzed by seeing the GFP expression. B73 treated with AGL1 alone, their control, had no GFP expression. The transient expression efficiencies were greater with PHP78891 and with the superbinary vector added. The transient expression of GFP of AGL1 with PHP78891

alone was 68%. There was an increase in transient expression of GFP of 98% when PHP78891 had the superbinary vector included than without. The results are seen in Table 5 with the frequency of B73 embryogenic callus recovery without a selection agent using *Agrobacterium* AGL1, AGL1 with PHP78891, and AGL1 with PHP78891-SVB. These results showed the use of *Agrobacterium* AGL1 superbinary vector with PHP78891 was the better delivery method for stable transformation in B73 (Mookkan et al., 2017).

Table 5. Somatic Embryogenic Response and Transformation Frequency in maize B73 using AGL1 with Vectors PHP78891 and PHP78891-SBV (Mookkan et al., 2017)

Experiment	***Agrobacterium* and constructs**	**Number of Immature Embryos Infected**	**Number of Embryogenic Calli**	**Percent of Embryogenic Calli**	**Transformation Frequency**
1	AGL1	75	0	0	
2	AGL1	70	0	0	
3	AGL1	80	0	0	
1	AGL1 PHP78891	60	41	68.3	
2	AGL1 PHP78891	50	36	72	
3	AGL1 PHP78891	65	43	66.1	
1	AGL1 PHP78891-SBV	55	53	96.3	
2	AGL1 PHP78891-SBV	60	59	98.3	
3	AGL1 PHP78891-SBV	80	78	97.5	
1	AGL1 PHP78891-SBV	55	53		14.5
2	AGL1 PHP78891-SBV	60	59		15
3	AGL1 PHP78891-SBV	80	78		15

Vector PHP78891-SBV had higher frequencies of somatic embryo induction at 98% than PHP78891 alone which was 68% as seen in Table 5. These results recommend

that all calli seen to have GFP positive foci present turned out to be embryogenic. No somatic embryogenic response and no formation of embryogenic calli was seen in AGL1 without the PHP78891 vector. There was some increase in the level of embryo response and somatic embryogenic calli at 66.1-72%. But the AGL1 with the PHP78891-SBV vector in B73 showed a high increase in embryo response and somatic embryogenic calli with a percentage of over 96%. The superbinary vector, PHP78891-SBV, had an overall transformation frequency of 14.5-15% in Table 5. Molecular maker analysis was conducted and confirmed the regenerated B73 plants were transgenic. The GFP positive calli were embryogenic type II and had and organization of type I calli (Mookkan et al., 2017).

Genotype Independent Transformation System

Lowe et al. (2018) addressed some of the problems they had seen in their 2016 study. Transformation efficiency in maize can be significantly increased using the Zea mays L. (maize) morphogenic transcription factors Baby Boom (Bbm) and Wuschel2 (Wus2) but can also cause sterility and phenotypic abnormalities. In their 2016 study they described a transformation method using the maize transcription factors Bbm and Wus2 to induce the proliferation of transformed cells. The cells were directly encouraged to form callus by the transgenes rather than transforming cells that had been encouraged to divide by conventional tissue culture. When using this method, they observed pleiotropic effects in the transgenic plants making the excision of Bbm and Wus2 necessary to give normal transgenic plants. They used a desiccation inducible promoter called Rab17 to control CRE-mediated excision and was a method compatible

with embryogenic callus. But this method still took 3 months for callus selection before transferring callus to dry filter papers for 3 days to encourage excision. In this recently published study, they try to alleviate the pleiotropic effects of constitutive expression of the transformation enhancing genes Wus2 and Bbm by doing a genome wide search to find suitable maize promotors to drive tissue and timing specific expression of the transformation enhancing genes. A promotor from a maize phospholipid transferase protein gene (Zm-$PLTP_{pro}$) was identified based on its expression in callus, leaves, and embryos and was found to be downregulated in meristems, reproductive tissues, and roots. They changed the promotor in the cassette from their 2016 study to have Zm-$PLTP_{pro}$ drive Bbm in the cassette and transformed immature embryos along with the Wus2 expression cassette driven still by the nopaline synthase promotor (Nos_{pro}::Wus2). They observed that plentiful somatic embryos quickly formed on the scutella. The embryos were uniformly transformed and could be easily regenerated into plants without a callus phase. These plants showed poor germination. Nos_{pro} was replaced with a maize auxin-inducible promoter (Zm-$Axig1_{pro}$) in combination with Zm-PLP_{pro}::Bbm permitting fertile and healthy plants to be regenerated. These plants germinated normally and had a wild type phenotype (Lowe et al., 2018).

Lowe et al. (2018) used Mini-Maze (FFM line A), B73, Mo17, and five Dupont Pioneer inbred (PHH5G, PH1V69, PHR03, PH184C, and Ph1V5T) lines for their experiment. For all immature embryo transformations (PHP79065, PHP79066, and PHP79094) an auxotrophic (THY-) version of *A. tumefaciens* strain LBA4404 containing pVir9. pVir9 is a separate accessory plasmid containing Bo542 virulence genes. The

promotors they used were Ubi_{pro}, Sb-$ALS_{pro,}$ $PLTP_{pro}$, $LTP2_{pro}$, nos_{pro}, $Axig1_{pro}$, $rab17_{pro}$. 3′sequencies used were PNI, Os-T28 3′, and Sb-PEPC 3′. Marker genes they used were PMI, HRA, moPAT, ZsYELLOW, and ZsGREEN. The maize morphological markers used were Zm-Wus2 and Zm-Bbm (Lowe et al., 2018).

The PHP79094 vector contained three expression cassettes. The first cassette had the maize PLTP promoter driving ZsGREEN. The second cassette had the maize Ubi promoter and intron driving PMI. The last cassette had the Actin promoter, from rice, driving MoPAT. PHP79065 vector had four expression cassettes. The first cassette had the Nos promoter driving Wus2. The second cassette had the Zm-PLTP promoter driving the Bbm gene. The third cassette had the Sorghum ALS promoter driving the maize Hra gene. Hra is a mutant of the ALS gene which confirms resistance to sulfonylureas and imidazolinones. The last cassette had the barley LTP2 promotor driving ZsYELLOW. PHP79066 vector was identical to PHP79065 but the maize Axig1 promotor was used in place of the Nos promoter to drive Wus2 (Lowe et al., 2018).

Lowe et al. (2018) found their rapid transformation method successfully produced transgenic T0 plants from all eight inbred lines used in their experiments shown in Table 6.

Table 6. Transformation frequency of five inbred lines, Mini-Maize, B73, and Mo17 (Lowe et al., 2018)

Genotype	Plasmid	Number of Embryos	Number of Plants Sampled	Transformation Frequency (%)
PHR03		72	32	44
PHR03		41	27	66
PHR03		36	27	75
PHR03		39	30	77
PH184C		188	57	30
PHHG5		75	168	224
PH1V5T		90	26	29
Mo17		133	46	35
Mo17		172	25	15
Mo17		51	16	31
B73		36	18	50
B73		46	4	9
B73		61	8	13
Mini-Maize "A"		105	101	96
Mini-Maize "A"		150	132	88
PHR03	79065	210	60	28.6
PHR03	79066	200	125	62.5
PHH5G	79065	162	41	25.3
PHH5G	79066	165	15	9.1
PHIV69	79065	148	37	25
PHIV69	79066	155	47	30.3

Transformation frequency was calculated as the number of T0 plants ÷ the number of starting immature embryos x 100. The plant transformation frequencies ranged from 8.7 to 96% based on the number of starting embryos shown in Table 6. In a second set of experiments more immature embryos were used from Pioneer inbred lines PH03, PHH5G, and PH1V69 shown in Table 6. These inbreds had transformation frequencies ranging from 9.1 to 62.5%. Transformation frequencies differed between inbred lines from 9% for one ear of B73 to 224% for one ear of PHH5G. Transformation

frequencies also differed between individual ears from the same inbred line shown in Table 6. An example is B73 which had transformation frequencies of 9, 13, and 50% for immature embryos isolated from the three ears tested (Lowe et al., 2018).

Somatic embryo formation happened within the first week after *A*. *tumefaciens* infection. Immature embryos were transformed with one of three of the vectors (PHP7066, PHP79065, or PH79094). The embryos were cultured over night at 21° C with the Agrobacterium. The immature embryos were transferred to cytokinin-free somatic initiation medium containing 0.8 mg L-1, 2, 4-D, and 1.2 mgL-1 dicamba. Dicamba is an inducer of the Zm-Axig1pro. After 4 days they observed the surfaces of most of the zygotic embryos were covered with well-formed somatic embryos. The production of the somatic embryos seemed to be genotype-independent. The embryos were also uniformly transformed conformed by ZsGREEN expression signifying these embryos were of single cell origin. Then somatic embryos were transferred onto maturation medium and rapid germination started 7 days after infection. In using this callus free method, they got many events on a single plate without the need for separating them and every plant was a separate event. Germinating plantlets were then transferred to medium with reduced auxin levels to promote root elongation. One week later the plants were ready to be transplanted in the greenhouse which was 24 days after infection (Lowe et al., 2018).

Chapter 5. Conclusions and Outlook

Many companies have used transformation as part of their breeding programs because it has allowed the transfer of specific genes into crops allowing many crops to have enhanced productivity due to their resistance to herbicides or insects. The major problem of transformation is trying to get the new genes into plant cells. So, plant transformation relies on totipotency of plant cells. Totipotency is defined as gametophytic cells or somatic plant cells that can be regenerated from in vitro-cultured explants into complete and fertile plants by treatment. The basic steps involved in Agrobacterium in vitro method of transformation that includes plant tissues or cell suspensions as targets for Agrobacterium inoculation and a selective agent is added to the medium after transformation to cause morphogenesis of transformed cells, and a whole plant is regenerated. The regeneration phase is the most time-consuming and expensive part of the transformation process. It also requires dedicated equipment and highly qualified staff. Therefore, many companies have not adopted or fully adopted transformation as part of their breeding programs. To have more companies adopt genetic transformation with a reproducible methodology there are several requirements that would have to be considered: easy procedures and low cost that would lead to a large number of transformants per event, small number of genetic copies introduced into each cell, facility to regenerate transgenic plants from single transformed cells, technical simplicity involving the minimum manipulations, operator safety avoiding dangerous procedures or substances, and capability to introduce in a stable way the desired DNA without vector sequences which are not required for gene

integration or expression (Rivera et al., 2012; Hooykaas et al., 2010; Chumakov and Moiseeva, 2012).

Advancements in molecular genetics advanced understanding of callus formation and regeneration to advance the transformation system. The known genes involved in stress response important to somatic embryogenesis are germin like-proteins (GLP) and GST. They found 15 GST genes had large expression changes during early somatic embryogenesis, which includes ZmGST 8, ZmGST 24, and ZmGST 40. The GST genes were found to be co-expressed with WUS, SERK, PIN, and BBM. GLPs are proteins that affect the plant redox status and are involved in developmental regulation. Genes involved in embryogenic pathway initiations include Leafy Cotyledon (LEC) and Baby Boom (BBM) genes. The initiation of the embryogenic pathway is seen when somatic cells acquire embryogenic competence and proliferate as embryogenic cells capable of forming somatic embryos. In studying the available research and BBM being shown to be co-expressed with LEC2, AGL15, GLP, PIN, GST, and WOX it can be concluded BBM as a stimulator of plant hormone production which triggers and signals pathways important for somatic embryogenesis. Known genes involved in embryo formation and development are the Wox, Clavata (CLV), Wuschel (WUS), and Agamus (AGL) genes. These genes are involved in meristem formation, development and have been found to be important for somatic embryo formation as well. The WUS gene is known to be a homeodomain transcription factor involved in floral and meristem development specifically as a regulator of organ identity and stem cell fate. It has an important role in activating and regulating pluripotent stem cells by promoting

proliferation genes and repressing developmental regulators (Salvo et al., 2014). The genes Bbm and Wus2 have been used as morphological genes to make recalcitrant maize lines transformable.

Breeding to make reluctant maize lines transformable have evolved over the last several years. It started with traditional backcrossing of A188 into elite maize lines which included having to screen for tissue culture. Then markers were developed that would aid in marker assisted breeding for transformability. Which meant screening for culturability each generation didn't have to be done, but screening for transformability did. More recently morphological genes have been discovered like Bbm and Wus2 which are involved in the initiation of the embryogenic pathway, embryo formation and development. Lowe et al. 2016 and 2018 showed *Agrobacterium*-mediated transformation with expression cassettes including Bbm and Wus2 can be used to make a reluctant maize line transformable. They made a gigantic break through with their rapid genotype independent transformation method. Using this method transformation frequencies ranged from 9%-224%. Lowe et al. (2018) found using their rapid method of transformation, somatic embryo formation happened within the first week after *A. tumefaciens* infection. In using this callus free method, they got many events on a single plate without the need for separating them and every plant was a separate event. Versions of this transformation method, especially expression cassettes, have been used for other corps besides maize successfully. Transformation of other recalcitrant species including pepper, wheat, rice, sorghum, sugarcane, and coca have been transformed

using morphological genes (Lowe et al., 2018; Heidmann et al., 2011; Florez et al., 2015; Bilichak et al., 2018).

A rapid and easy transformation process is essential for new breeding techniques including gene editing, cisgenesis, DNA methylation, and intrafgenesis. These techniques are evolving rapidly and gaining in popularity. They have been given the name "New Breeding Techniques." Gene editing is targeted genome modifications with the help of site-specific nucleases (SSNs) (Yadava et al., 2017). The rapid method of transformation by Lowe et al. 2018 provides fertile plants without the use of excision which supports genome editing. Edits are not linked to the addition of morphogenic genes, so segregation can be used to separate edits form the transgenes in the following generation. It reduces the importance of obtaining single-copy transformants. Currently they are using variations of this method for CRISPR/CAS9 genome editing and so far, these methods have worked in every genotype tested. The genotype-independent, simple, and fast transformation method, by Lowe et al. 2018, could provide any laboratory with tissue culture experience the ability to produce transgenic maize plants from any genotype by the traditional *Agrobacterium* method or even gene editing (Lowe et al. 2018). Companies being able to use gene editing have the potential to avoid many regulatory issues regarding transgenes because gene editing, by SSNs, modifications achieved in an organisms' original genes to develop desired traits do not employ transgenes of another species. So targeted modified products are basically mutation based and plants modified with these technologies could be regulated as such (Yadava et al., 2017; Lowe et al., 2018).

The independent genotype transformation method by Lowe et al. (2018) would be cost effective for many companies because plants could be regenerated without a callus phase making this method faster than the traditional method, it's a simple, many single copy transformants are created, and it's efficient. The plants can be transferred to the greenhouse 24 days after infection which is about the same amount of time as embryo rescue (Lowe et al., 2018). This method could be used with the traditional *Agrobacterium* method or gene editing methods making it a diverse or universal transformation system, which makes it a good method for companies to adopt. Hopefully making it more feasible for more companies to adopt more biotechnology tools for their breeding programs.

References

Armstrong, C. L., Romero-Severson, J., & Hodges, T.K. (1992). Improved tissue culture response of an elite maize inbred through backcross breeding, and identification of chromosomal regions important for regeneration by RFLP analysis. *Theoretical Applied Genetics*, *84*, 55-762. Retrieved from https://doiorg.proxy.lib.iastate.edu/10.1007/BF00224181

Bilichak, A., Luu, J., Jiang, F., & Eudes, F. (2018). Identification of baby boom homolog in bread wheat. *Agri Gene*, *7*, 43-51. Retrieved from https://doi.org/10.1016/j.aggene.2017.11.002

Bolibok, H., & Rakoczy-Trojanawska, M. (2006). Genetic mapping of QTLs for tissue culture response in plants. *Plant Cell*, *149*, 73-83. doi:10.1007/s10681-005-9055-6

Boutiliter, K., Offringa, R., Sharma, V. K., Kieft, H., Ouellet, T., Zhang, L., Hattori, J., Lui, C. M., Lammeren, A. A. M., Miki, B. L. A., Custers, J. B. M., & Campagne, M. M. L. (2002). Ectopic expression of baby boom triggers a conversion from vegetative to embryonic growth. *Plant Cell*, *14*, 1737-1749. doi:https://doi.org/10.1105/tpc.001941

Chumakov, M. I., & Moiseeva, E. M. (2012). Technologies of agrobacterium plant transformation in planta. *Applied Biochemistry and Microbiology*, *48(*8), 657-666. doi:10.1134/S0003683812080017

Florez, S., Erwin, R., Maximova, S. N., Guiltinan, M. J., & Curtis, W. R. (2015). Enhanced somatic embryogenesis in theobroma cacao using the homologous baby boom transcription factor. *BMC Plant Biology*, *15*, 121-133. doi:10.1186/s12870-015-0479-4

Frame, B. R., Drayton, P. R., Bagnall, S.V., Lewnau, C.J., Bullock, W.P., Wilson, H.M., et al. (1994). Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation. *Plant J, 6*, 941–948. doi:10.1046/j.1365-313X.1994.6060941.x

Fromm, M.E., Taylor, L.P., & Walbot, V. (1986). Stable transformation of maize after gene transfer by electroporation. *Nature*, *319*, 791–793. doi:10.1038/319791a0

Galland, R. Blervacq, A.S., Blassiau, C., Smagghe, B., Decottignies, J.P., Hilbert, & J.L. (2007). Glutathione-S-Transferase is detected during somatic embryogenesis in chicory. *Plant Signaling & Behavior*, *2*(5), 343-348. Retrieved from http://www.landesbioscience.com/journals/psb/article/4652

Gordon-Kamm, W., Dilkes, B.P., Lowe, K., Hoerster, G., Sun, X., Ross, M., et al. (2002). Stimulation of the cell cycle and maize transformation by disruption of the plant retinoblastoma pathway. *Proc. Natl. Acad. Sci. U.S.A., 99*, 11975–11980. doi:10.1073/pnas.142409899

Graves, A.C.F., and Goldman, S. L. (1986). The transformation of Zea mays seedlings with agrobacterium tumefaciens. *Plant Mol. Biol.*, *7*, 43–50. doi:10.1007/BF00020130

Grimsley, N., Hohn, T., Davies, J.W., & Hohn, B. (1987). Agrobacterium mediated delivery of infectious maize streak virus into maize plants. *Nature*, *325*, 177–179. doi:10.1038/325177a0

Hooykaas, P. J. (2010). Plant Transformation. In eLS, (Ed.). doi:10.1002/9780470015902.a0003070.pub2

Heidmann, I., de Lange, B., Lambalk, J., Angenent, G. C., & Boutilier, K. (2011). Efficient sweet pepper transformation mediated by the baby boom transcription factor. *Plant Cell Reports*, *30*, 1107-1115. doi:10.1007/s00299-011-1018-x

Jiao, Y., Peluso, P., shi, J., Liang, T., Stilzer, M. C., Wang, B., Campbell, M. S., C. Stein, J. C., Wei, X., Chin, C., Guill, K., Regulski, M., Kumari, S., Olson, A., Gent, J., Schneider, K. L., Wolfgruber, T. K., McMullen, M. R., Ross-Ibarra, J., Dawe, R. K., Hastie, A., Rank, D. R., & Ware, D. (2017). Improved maize reference genome with single-molecule technologies. *Nature*, *000*, 1-6. doi:10.1038/nature22971

Karami, O., & Saidi, A. The molecular basis for stress-induced acquisition of somatic embryogenesis. *Molecular Biology Reports*, *37*(5), 2493-2507. doi:10.1007/s11033-009-9764-3

Koziel, M.G., Beland, G.L., Bowman, C., Carozzi, N.B., Crenshaw, R., Crossland, L., et al. (1993). Field performance of elite transgenic maize plants expressing an insecticidal protein derived from bacillus thuringiensis. *Biotechnology*, *11*, 194–200. doi:10.1038/nbt0293-194

Krakowsky, M.D., Lee, M., Garay, L., Woodman-Clikeman, W., Long, M.J., Sharopova, N., Frame, B., & Wang, K. (2006). Quantitative trait loci for callus initiation and totipotency in maize (Zea mays L.). *Theoretical Applied Genetics*, *113*(5), 821-830. doi:10.1007/s00122-006-0334-y

Lowe, B. A., Way, M. M., Kumf, J. M., Rout, J., Warner, D., Johnson, R., Armstrong, C. L., Spencer, M. T., & Chomet, P.S. (2006). Marker assisted breeding for transformability in maize. *Molecular Breeding*, *18*, 229-239. doi:10.1007/s11032-006-9031-4

Lowe, K., Wu, E., Wang, N., Hoester, G., Hastings, C., Cho, M., Scelonge, C., Lenderts, B., Chamberlin, M., Cushatt, J., Wang, L., Ryan, L., Khan, T., Chow-Yiu, J., Hua, W., Yu, M., Banh, J., Bao, Z., Brink, K., Igo, E., Rudrappa, B., Shamseer, PM., Bruce, W., Newman, L., Shen, B., Zheng, P., Bidney, D., Falco, C., Register, J., Zhao, Z., Xu, D., Jones, T., & Kamm, W.G. (2016). Morphogenic regulators baby boom and wuschel improve monocot transformation. *Plant Cell*, *28*, 1998-2015. doi:10.1105/tpc.16.00124

Lowe, K., La Rota, M., Hoerster, G., Hastings, C., Wang, N., Chamberlin, M., Wu, E., Jones, T., Gordon-Kamm, W. Rapid genotype "independent" Zea mays L. (maize) transformation via direct somatic embryogenesis. (2018). *In Vitro Cellular & Developmental Biology, 54*, 240-252. Retrieved from https://doi.org/10.1007/s11627-018-9905-2

Manchanda, N., Andorf, C.M., Ye, L., Wimalanathan, K., Rounsley, S., Wang, K., & Lawrence-Dill, C. (2016). Sequencing, assembly, and annotation of maize B104: a maize transformation resource [Abstract]. *Maize Genetics Conference Abstracts, 58*, 52. Retrieved from https://www.maizegdb.org/data_center/reference?id=3172642

Mayer, K. F. X., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G., & Laux, T. (1998). Role of wuschel in regulating stem cell fate in the arabidopsis shoot meristem. *Cell*, *95*(6), 808-815. doi:10.1016/S0092-8674(00)81703-1

Mookkan, M., Nelson-Vasilehik, K., Hague, J., Zhang, Z.J., & Kausch, A.P. (2017). Selectable marker independent transformation of recalcitrant maize inbred B73 and sorghum P898012 mediated by morphogenic regulators baby boom and wuschel2. *Plant Cell*, *36*, 1477-1491. doi:10.1007/s00299-017-2169-1

Que, Q., Elumalai, S., Li, X., Zhong, H., Nalapalii, S., Schweiner, M., Fei, X., Nuccio, M., Keliher, T., Gu, W., Chen, Z, & Chilton, M.M. (2014). Maize transformation technology development for commercial event generation. *Frontiers in Plant Science*, *56*, 1-19. doi:10.3389/fpls.2014.00379

Rivera, A. L., Gomeze-Lim, M., Fernandez, F., & Loske, A. M. (2012). Physical methods for genetic plant transformation. *Physics of Life Reviews*, *9*, 308-345. Retrieved from www.sciencedirect.com

Rhodes, C., Pierce, D., Mettler, I., Mascarenhas, D., & Detmer, J. (1988). Genetically transformed maize plants from protoplasts. *Science, 240*(4849), 204-207. Retrieved from http://www.jstor.org/stable/1701236

Rosati, C., Landi, P., & Tuberosa, R. (1994). Recurrent selection for reservation capacity from immature embryo-derived calli in maize. *Crop Science*, *34*, 343-347. doi:10.2135/cropsci1994.0011183X003400020006x

Salvo S. A. G. D., Hirsch C.N., Buell C.R., Kaeppler S.M., & Kaeppler H.F. (2014). Whole transcriptome profiling of maize during early somatic embryogenesis reveals altered expression of stress factors and embryogenesis-related genes. *PLoS ONE*, *9*(10), e111407. doi:10.1371/journal.pone.0111407

Salvo, S., Cook, J., Carlson, A. R., Hirsch, C. N., Kaeppler, S. M., & Kaeppler, H.F. (2018). Genetic fine-mapping of a quantitative trait locus (QTL) associated with embryogenic tissue culture response and plant regeneration ability in maize. *The Plant Genome*, *11*(2), 1-11. doi:10.3835/plantgenome2017.12.0111

Shillito, R.D., Carswell, G.K., Johnson, C.M., DiMaio, J.J., & Harms, C.T. (1989). Regeneration of fertile plants from protoplasts of elite inbred maize. *Biotechnology*, *7*, 581–587. doi:10.1038/nbt0689-581

Songstad, D.D., Armstrong, C.L., Peterson, W.L., Hairston, B., & Hinchee, M. A. W. (1996). Production of transgenic maize plants and progeny by bombardment of Hi-II immature embryos. *In Vitro Cell Dev. Biol. Plant*, *32*, 179–183. doi:10.1007/BF02822763

Vain, P., McMullen, M.D., & Finer, J.J. (1993). Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Rep.*, *12*, 84–88. doi:10.1007/BF00241940

Walters, D., Vetsch, C., Potts, D., & Lundquist, R. (1992). Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants. *Plant Mol. Biol*., *18*, 189–200. doi:10.1007/BF00034948

Yadava, P., Abhishek, A., Singh R., Singh, I., Kaul, T., Pattanayak, A., & Agrawal, P. K. (2017). Advances in maize transformation technologies and development of transgenic maize. *Frontiers in Plant Science*, *7*, 1-12. doi:10.3389/fpls.2016.01949