Chapter 12
Maize Somatic Embryogenesis: Agronomic Features for Improving Crop Productivity

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Abstract Somatic embryogenesis (SE) systems in maize, have proved to be a useful tool for basic research on embryo development that lately have turned into applied research on the establishment of commercial crops. This review discusses recent findings on maize SE on basic research to reveal fundamental aspects of embryo development, its use as a biotechnological tool, and its application in the development of isogenic crops.

12.1 Introduction

Current development of maize somatic embryogenesis (SE) systems ranges from fundamental aspects of embryo development to the description of genes necessary for embryogenic callus initiation. SE research has been lately directed to optimize its use as an efficient regeneration system for improved transgenic crops. However, the main concern of these improved crops is that transgenes used to over-express the desired characteristic, as the selection marker, or the gene that provides the improvement, come from the genome of others organisms usually virus or bacteria. Although these foreign elements provide a considerable improvement in grain yield to transgenic crops, they are not widely accepted, due to the genome contamination with foreign DNA. Even though there is no convincing evidence for a negative effect on human or animal health by these foreign genes; there is strong opposition to adopt these crops. Particularly in Mexico, the culture of transgenic maize represents a concern, because Mexico is the center of origin and diversification of this highly domesticated crop (Biosafety Law on Genetically Modified Organisms, Mexico (2005)). Therefore, in Mexico as in other countries, it has been implemented a precaution policy for the use

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V.M. Loyola-Vargas and N. Ochoa-Alejo (eds.), *Somatic Embryogenesis: Fundamental Aspects and Applications*, DOI 10.1007/978-3-319-33705-0_12

of genetically modified organisms (Cartagena Protocol 2000). Actually, there are reports of approximately 60 maize varieties that are cultivated in Mexico and Central America, adapted to a wide variety of environmental conditions, ranging from high altitude and lower temperatures to lower lands and high temperature, thus providing a rich germoplasm source that needs to be preserved and improved. In Europe, there is also open rejection for transgenic crops, due to the lack of information about possible health damage. Therefore, development of safe technologies for the improvement of cultivated species has come to the scenario as cisgenic, intragenic, and isogenic models of genetic engineering. These models imply the modification of a gene expression using only elements from the genome of the species to be improved or sexually relate species. Recently, our research group has been focused on developing improved isogenic crops using Mexican maize varieties. Current efforts are directed to improve productivity through isogenic gene over-expression. This review discusses the maize SE process with emphasis on recent findings on basic research to reveal fundamental aspects of embryo development trough SE process. Further, it is discussed the use of SE as a biotechnological tool and finally it is described SE use in the development of the isogenic model for crop improvement.

12.2 Basic Research: Fundamental Aspects of Embryo Development

Currently, basic SE research in maize has been oriented toward its use as a biotechnological tool. However, reports on basic aspects of the process are still being developed. These reports share the goal to overcome the genotype-dependent response of SE by revealing genes that are fundamental to establish SE cultures. In this regard, Salvo et al. (2014) report the transcriptome profile of early SE cultures on the highly embryogenic genotype A188. Authors use enrichment analysis of differentially expressed genes to reveal altered expression of stress factors and embryogenesis-related genes. They conclude that coordinated expression of genes related to stress response, transmembrane transport, and hormone metabolism is essential for SE. They propose a model network of SE-related gene expression, where stress response genes, glutathione-S-transferases (GSTs), and germin-like proteins (GLPs), trigger early somatic embryogenesis, influencing transcription factors that promote SE such as SERK, LEC1, LEC2, BBM, and hormone related such as PIN. Further, they found that this process also affects WUS and WOX genes that regulate stem cell fate.

Following the same approach of global analysis but at the proteomic level, Varhanikova et al. (2014) studied the proteomes of embryogenic (EC) and nonembryogenic (NEC) callus of inbred line A19. They found that increased expression of pyruvate biosynthesis genes in EC callus is in contrast with NEC callus genes expression, which in turn presented suppression of embryogenic genes

by the retinoblastoma related protein (RBR). Further, ascorbate peroxidase (APX), a stress-related antioxidant enzyme was found to help in detoxifying hydrogen peroxide on EC callus, therefore providing the embryogenic characteristics. Furthermore, these authors point out that oxylipin gene determines totipotency on EC callus.

Regarding the function of specific genes in the maize SE process, new gene has come to the scenario. In this regard, Sosso et al. (2012) reported that lack of expression of the pentatricopeptide repeat protein PPR8522 is embryo lethal. Its absence disrupts normal embryo and chloroplast development causing an albino phenotype. On other report, Huang et al. (2014) showed that maize hemoglobins (ZmHbs) define the developmental fate of the embryogenic tissue on SE, since the combined expression and distribution of ZmHb1 and ZmHb2 regulate nitric oxide and Zn^{2+} levels that finally prevents programmed cell death during SE. Further, Liu et al. (2015) reported that ZmDRP3A and ZmSUF4 genes have regulatory roles on intact somatic embryos of the maize Indian inbred line Y423. These reports complement the knowledge on the basic SE process.

12.2.1 Applications of Maize SE as a Biotechnological Tool

(a) Development of transgenic modified maize cultivars.

Classic agronomic engineering is based on adding or altering phenotypic traits conferred by single genes. Traits are delivered to the host plant in vectors that contains the single gene. Current efforts are directed to staking several genes that provide the desired characteristics in a more complex construct design. Que et al. (2010) describe trait stacking of several genes that are inserted into a transgene single locus. They also report the use of molecular stacks (large transgene arrays assembled in vitro) in their transformation experiments. Furthermore, a complex form of vectors is presented as minichromosomes, which offer the potential for simultaneous transfer and stably express multiple genes (Carlson et al. 2007; Ananiev et al. 2009). In this regard, the constructed minichromosomes segregate independently of their host chromosomes. Therefore, they represent a suitable option for functional plant genomics and breeding through biotechnology procedures (Houben et al. 2013).

(b) The tendency for selected genes to improve maize crops

The new tendency on this matter is the use of genes that provide stress tolerance, such as, heat, drought, or salinity. These genes are used alone or in combination with genes that provide resistance to insects attack (*cry1a* and *cry1b* genes from *Bacillus thuringensis*) or resistance to herbicide glifosate (Que et al. 2014). For example, Gullì et al. (2015) designed a vector that included a heat shock gene staked with the gene of resistance to insects attack (*cry1a*). As a result, the transgenic plants showed drought stress tolerance with no alteration in the expression of *cry1a* gene.

(c) Optimization of transformation efficiency

These emerging technologies for plant transformation, allow genomic plant modification. However, the efficiencies and characteristics of the existing methods are under constant improvement. The transformation efficiency is one of the main concerns in the development of high-throughput protocols to improved maize elite inbreeds. Currently, the main characteristics to be improved include Agrobacterium strains, incrementing the plasmid copy number, modifications in media composition, and the type of explants (mature embryos and shoot meristems). Cho et al. (2014) developed an improved Agrobacterium-mediated transformation protocol for recalcitrant maize varieties, such as inbred PHR03. They optimized culture media using a combination of glucose, copper, and cytokinin. Authors proposed that this method is suitable for the propagation of recalcitrant commercial maize inbreeds. Zhi et al. (2015) improved the number and quality of transformation events, using a combination of Agrobacterium strain and incrementing the binary plasmid copy number. This resulted in increase of the transformation frequency. In regard to the tissue source to establish the regeneration system through SE and organogenesis, Sairam et al. (2003) reported a high-frequency somatic embryogenesis protocol from shoot meristem using R23 Pioneer, Hi-Bred. In this work, regeneration frequency of transformed plants was independent of the Agrobacterium strain used. Recently Pathi et al. (2013) developed a standard regeneration protocol for the tropical Indian maize HQPM-1 inbreed cultivar, using mature embryo through embryogenic and organogenic callus cultures to regenerate Agrobacterium transformed plants. They shortened the regeneration time and obtained stable transformants from mature seeds.

(d) Emerging technologies for efficient gene targeting

Commonly transformation techniques use vectors design, that may be delivered to the host plant by Agrobacterium and biobalistic, thus causing random insertion of the transgene into the genome. This often results in variable expression due to the genomic environment of the insertion site, and also can lead to mutations or production of undesired products. Therefore, current efforts have been directed to insert the desired transgene at predetermined positions in the plant genome. Zhang et al. (2003) presented a strategy for marker gene excision in transgenic plants. To this end, the authors used the Cre/lox system from bacteriophage P1 in two forms, the first by crossing plants expressing the Cre recombinase with plants that have a transgene construct with the selectable marker gene flanked by lox sites; the second by auto-excision by activating the recombinase using a heath-shock promoter. Both approaches allowed to remove the selectable marker gene, from callus in the second approach, and from embryos and kernels in the first approach.

D'Halluin et al. (2008) achieved the insertion of a targeted sequence at a pre-engineered I*Sce* I site in the maize genome. They used homologous recombination and targeted DNA double-strand break upon the induction of the I*Sce* I endonuclease gene. Shukla et al. (2009) designed a zinc-finger nuclease (ZFNs) that induced a double-stranded break at their target locus, therefore allowing the precise insertion of an herbicide tolerance gene at the selected locus.

Ayar et al. (2013) developed a strategy for remobilization of a transgene randomly inserted, its excision and insertion into a defined genomic site by using of rare-cutting endonucleases such as I*Sce* I and ectopic somatic recombination. This protocol allows transformation of plant species for which efficiencies are limiting. For a deeper review, readers should consult Mumm (2013), he discusses the development of transgenic products, ranging from the design of the transgenic crop with the desired traits trough the evaluation of commercial liberation of the seed.

12.2.2 Perspectives for Generating Improved Maize Cultivar Using Biobalistic and Isogenic Construction, Considering Elements Only from the Maize Genome to Improve Specific Gene Expression

Alternative genetic engineering technologies have been developed in the last decade, where cisgenic and/or intragenic plants were obtained by bombarding two linear DNA sequences: the desired gene and a marker gene which may be from different genomes since it is used only for the recuperation of successful transformation events. There after directed segregation of progeny plants eliminate the marker gene, and the new plants were called cisgenic, due to the inheritance of the desired gene without marker gene (Romano et al. 2003; Yao et al. 2006). To describe this new genetic engineering process, Schouten et al. (2006) defines a cisgenic plant as a genetically modified organism with genes from the same or sexually related species. The implication is that genes should have the naturally order of the promoter, terminator, introns, and flanking regions. This definition poses restrictions for a versatile use of the plant genome, therefore, to provide better expression results, the intragenic term was proposed as the use of sequences in a different order and orientations than those naturally occurring, (Nielsen 2003; Rommens et al. 2004; Conner et al. 2007). In a similar way, the Food and Agriculture Organization of the United Nations (2000) defined the term "isogenic" as a group of organism that bares the same chromosomic construction with the independence of its homozygous or heterozygous condition. This applies to sequences genetically identical, originated from the same organism or from an inbred strain. Then the isogenic term could be considered similar to the "intragenic" definition. In this regard, the term "isogenic" describes the use of a construction not naturally found in a species in which the expression of a gene of interest is modified. Currently, there is a higher public acceptance of intragenic/cisgenic crops compared to transgenic crops. It is necessary to indicate that cisgenesis and intragenesis, are supported by existing tools for genetic modification such as transformation by *Agrobacterium* infiltration or by biolistics, where the genetic information is delivered into the cell through particles coated with a genetic material (Lusser et al. 2012). Thus, for legal considerations, they are considered as transgenics. Even though, these plants are regulated as genetically modified

organisms (GMO), research on the subject has grown, and different crops have been modified according to these concepts. Actually, cisgenic or intragenic crops are in field trials and others have applications for deregulation (Holme et al. 2013).

Several examples of cisgenic and intragenic species are available in the literature; they use several strategies to modify gene expression. Gene over-expression is one of these examples, such as barley gene *HvPAPhy_a,* used to improve grain phosphate bioavailability that provides better nutrition properties as feed for pigs and chickens (Holme et al. 2012). Another example involves gene silencing, such as genes *Ppo*, *R1*, *PhL* in potato, to prevent the black spot bruise, to reduce starch degradation, and limit acrylamide accumulation in French fries (Rommens et al. 2006). Also gene expression, such as in Durum wheat, where they produce the 1Dy10 gene to improve the flour baking quality (Gadaleta et al. 2008). Other examples, Holme et al. (2013) present a detailed review of the subject.

It should be considered that the aim of isogenic and cisgenic models for crop improvement, particularly in maize, is to obtain plants that express genes that provide an agronomic advantage without including any transgenes (either marker genes or vector components such as backbone sequences). The strategy to obtain isogenic cultivars contemplates the design of a construct comprising the gene of interest, a promoter, that might be constitutive or tissue specific and a terminator. The sequences must come from the same species to be improved. A selectable gene should be considered in the construct design. Stress response genes are the most suitable candidates, since a simple heat or drought stress will allow isogenic plants selection.

In this scenario Rubisco activase (*Rca*), the molecular chaperone that improves the efficiency of Rubisco to fix CO_2 is a suitable candidate gene to improve through the isogenic model. The traditional breeding technique, have demonstrated that by masal stratification trough 23 selection cycles, taller plants and increased grain yield corresponded with high expression levels of *Rca* (Morales et al. 1999). In fact, Schouten et al. (2006) assume that a transferred construction, bearing cisgene or intragen, it incorporated into the genome of the species to be improved, similarly to the natural processes or even to the traditional breeding techniques. Unpublished data from our research group suggest that isogenic over-expression of *Rca* resembles traditional breeding within a significantly shorter time (Almeraya et al. 2016 unpublished data). In this research, construct design considered a tissue specific promoter, the open reading frame of *Rca* and a terminator, all these elements from the maize genome. This construct was delivered into somatic embryos by the biolistic system, and whole plants were regenerated, acclimatized, and maintained in a green house. A general scheme of this method will be presented in the next section. Overall, in maize, this approach will provide a suitable strategy to improved plants using varieties originated and cultivated in México without the problem that poses inclusion of transgenes for this crop.

12.3 Illustration of the Method

12.3.1 Isogenic Construct Design

The following method describes the transformation and regeneration of an isogenic maize variety over-expressing *Rca* gene (Almeraya 2016).

Rca cDNA sequence was obtained from the gene data available at NCBI and maize sequence data bases. The sequence of Rubisco small subunit gene was selected as a promoter, since this region was already characterized as a tissue specific promoter that ensures the protein expression only in specialized photosynthetic cells in the leaves. Finally, a terminator sequence from Rubisco small subunit was included (Fig. 12.1). These sequences were synthesized. Ligation of the three sequences was performed, and the construct was cloned into the vector (puc57) only for the production of the construct in *E. coli* DH5α. To obtain the construct, the plasmid was digested with restriction enzymes. Only the construct containing maize elements was used for the biolistics procedure.

12.3.2 Embryogenic Cultures and Biolistics Conditions

Embryogenic (E) cultures of Tuxpeño raze were established from immature embryos (15–18 days after pollination) following the procedure described in Garrocho-Villegas et al. (2012). Type-I embryonic callus was incubated on hyperosmotic N6 medium containing 3 g/L sucrose and gelsan 1.5 g L^{-1} for approximately 2 h before particle bombardment with PDS1000/He delivery system (Bio-Rad). The construct was deposited onto tungsten particles according to manufacturer instructions (Sanford et al. 1993). After assembling the Bio-Rad PDS-1000/He, microprojectiles were fired to E callus (Helios pressure of 1,300 psi). Two shoots were done for each calli set. Controls were bombarded with nude particles without DNA.

Bombarded calli were kept in the hyperosmotic N6 medium for other 2 h. Then, the calli were transferred onto normal N6 medium and incubated at 25 ± 1 °C in the dark for four weeks with a subculture at week two.

Fig. 12.1 Schematic representation of isogenic over-expression cassette

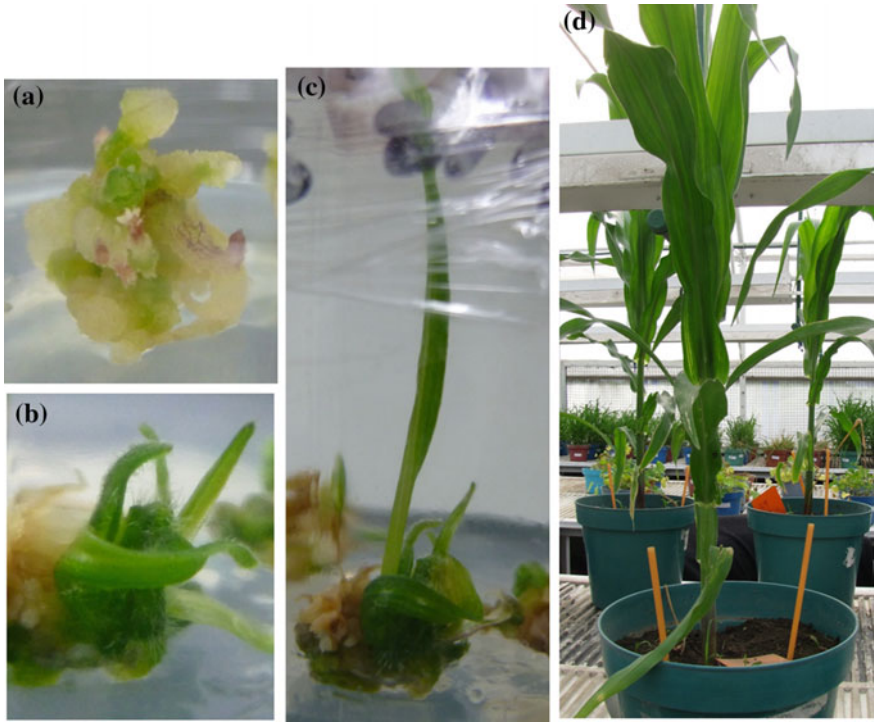

Fig. 12.2 Regeneration and acclimatization of maize plants from SE system. **a** E callus showing somatic embryos as green spots. **b** Plantlet of approximately 2 cm in height. **c** Plantlet of approximately 8 cm ready for transplant to soil. **d** Acclimatized plant

12.3.3 Plant Regeneration

Calli were transferred onto hormone-free MS medium and incubated at 25 ± 1 °C for 16 h illumination/8 h dark for plant regeneration. Subcultures to fresh media were performed every two weeks. After one week of culture, green spots were visible on the callus (Fig. 12.2a). After eight weeks, regenerated plantlets over 2 cm in height (Fig. 12.2b) were transferred to individual culture vessels containing hormone-free MS medium. Plantlets with strong roots and approximately 8 cm in height were transplanted to soil substrate mix Sunshine 3 contained in pots of 1 L capacity. Acclimatization was done by covering pots and plantlets with plastic bags to prevent plant dehydration. After growing for one week in the green house, bags were gradually opened and removed after two weeks. Plants were grown in green house (Fig. 12.2d). Isogenic maize plants were selected after amplification of the construct by PCR using genomic DNA extracted from leaves. mRNA and protein over-expression confirmed transformed isogenic plants. Further agronomic characterization is currently in process.

12.4 Note

The maize isogenic crop improvement presented in this work is an approach that offers the better of conventional breeding and genetic engineering techniques. The desired agronomic traits are taken from conventional crop breeding, and the lessons learned from transgenic engineering techniques are used to modify gene expression, all to obtain a plant without foreign DNA.

The mechanism to achieve this modification depends on the physiological, agronomic, and life cycle characteristics of the species to improve. In maize, the preferred propagation system has been the embryogenic callus transformation. In this report with the use of an isogenic over-expression construct. The maize plants obtained with this strategy, are similar to maize plants improved by classical agronomic methods, since the procedure excludes the use of selection genes, providing an improved final product that maintains the integrity and purity of their DNA. Indeed, this methodology is pending for patent approval (MX/E/2014/088655).

Acknowledgments This research was financed by CONACyT project 213872.

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