# Chapter 26

# Cocoa

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## 1. Introduction

*Theobroma cacao* L. (cocoa), a member of the family Sterculiaceae, is a tropical sub-canopy tree which has its origins in the rain forests of the Amazon basin (Hurst *et al.*, 2002). Cocoa derives its economic importance due to fat-rich seeds that are sole source of cocoa powder and cocoa butter which are raw materials for chocolate, cosmetic and pharmaceutical industries. Livelihood of more than two million growers spread over 50 countries worldwide is dependent on cocoa cultivation (Motamayor *et al.*, 2008).

Incidence of a number of serious pests and pathogens cause severe damage to the cultivation and production of cocoa thereby potential yields of cocoa plantations are reduced to great extent (Fulton, 1989). Traditional approaches of plant protection measures are being utilized to manage pest and diseases incidence in cocoa; however, practical difficulty in applying plant protection chemicals to cocoa plantations, remoteness of plantings and expensive application methods limits their use. Genetic improvement of cocoa to obtain strong resistance against pests and diseases have not yielded any significant outcome because of narrow genetic base and extended life cycle. Moreover, cocoa germplasm pool has not been explored extensively for novel genetic sources to confer disease and pest resistance. Notwithstanding serious impediments, some sources of partial resistance or tolerance to pests and diseases have been identified and utilized in crop improvement programmes (Eskes and Lanaud, 1997). However, till date, cocoa breeding programmes, which suffer from a long life cycle, funding constraints, and other limitations, have made limited progress with respect to the incorporation of durable, horizontal resistance to cocoa pests and diseases into widely used cocoa germplasm. Long-term endeavors towards breeding for disease resistance in cocoa have generated only limited success (Lopes *et al.*, 2011). The long generation time of cocoa plants, coupled with its large size, makes conducting requisite long-term and multi-location field trials cumbersome (Kennedy *et al.*, 1987). Moreover, as many of the cocoa pathogens are opportunistic, sources of resistance have not co-evolved for some of the most devastating of the major cocoa diseases (Maximova *et al.*, 2006).

Modern biotechnology based approaches equips researchers with an array of tools to complement and hasten conventional plant breeding programmes. Techniques and protocols have been standardized in many crops for incorporating novel sources of resistance and value-added traits to develop invaluable genetic resources that help accelerate breeding efforts. Among various techniques, genetic transformation is a very powerful tool in the hands of biotechnologists to alter genetic make-up of an organism not only to develop improved crop phenotype but also to perform gene-structure-function analysis. In the recent years, researchers have begun to apply the tools of genetic modification and molecular genetics to facilitate speeding up of cocoa breeding programmes (Maximova *et al.*, 2009).

## 2. Genetic Transformation Studies in Cocoa

Genetic transformation is a potential tool for performing basic research on functional genomics, and also provides means to introduce novel traits into cacao genome that are otherwise difficult to achieve through conventional or molecular breeding approaches. In the field of cocoa genetic transformation, Purdy and Dickstein (1989) made the first successful attempt by demonstrating the susceptibility of cocoa cells to *Agrobacterium*.

A major break-through was made when successful transformation of cocoa callus cells was established by Sain *et al.* (1994). Leaf strips from two cocoa clones *viz.* ICS-16 and SIC-5 were co-cultivated using the supervirulent *Agrobacterium* strain A281-kan. Accordingly, transformed cells were selected on a callusing medium containing 100 μg ml $^{-1}$ kanamycin. In addition, callus cultures of both the clones growing on kanamycin containing medium were analyzed for marker *npt II* (neomycin phosphotransferase II) gene expression by performing *npt II* assays and stable single site integration of *npt II* gene was demonstrated through Southern blotting studies.

Alongside *Agrobacterium*-mediated transformation, particle bombardment technique was also employed for introduction of foreign genes into cocoa (Perry *et al.*, 2000; Santos *et al.*, 2002). In both instances, reporter genes could be introduced into cocoa cells and visualized. In order to improve the transformation frequencies, optimization of osmotic adjustment was provided as a pre-treatment. Despite the successful regeneration of cacao plants through somatic embryogenesis, and introduction of alien DNA in to cocoa genome through *Agrobacterium*-mediated transformation or particle bombardment methodology, the development of transgenic cacao remained intangible. A highly efficient cocoa regeneration protocol was developed by Maximova *et al.* (2002) that allowed regeneration of secondary somatic embryos. The primary somatic embryo explants amenable for regeneration into secondary somatic embryos were originally initiated from floral tissue explants. The system developed has many advantages as more embryos per explants were produced than what was obtained in primary somatic embryo system. Further, greater proportion of embryos displayed morphological conformity and a significant leap was attained in percentage of conversion to plantlets. A set of stable transgenic cacao plants characterized with proper DNA integration and expression of visible marker green fluorescent protein (GFP), were obtained by Maximova *et al.* (2003). Furthermore, cacao transgenics were grown to maturity and efficient transmission of transgene to next generation was also proved. Co-cultivation of cocoa tissue with *Agrobacterium* was an issue in earlier attempts because growth of *Agrobacterium* overpowers cocoa explants and frequently damages the plant tissue. Furthermore, the commonly used antibiotic cefotaxime in tissue culture media to remove *Agrobacterium* cells post infection caused a severe reduction in cocoa somatic embryo production by 86 per cent. This situation prompted de Mayolo *et al.* (2003) to study and define suitable antibiotic systems and concentrations for cocoa transformation so that sufficient suppression of *Agrobacterium* is achieved without interfering cocoa somatic embryogenesis. Among the four antibiotics evaluated, moxalactam was not only very effective for *A. tumefaciens* counter-selection but also increased the regeneration frequency of secondary embryos.

Ultimately transgenic cocoa plants have been developed by Maximova *et al.* (2003) by combining the secondary somatic embryogenesis system developed earlier (Maximova *et al.*, 2002) with *A. tumefaciens* infection and co-cultivation (de Mayolo *et al.*, 2003). Three different plant transformation compatible binary vectors were constructed on the Bin19 T-DNA vector backbone system, with each plasmid containing *nptII* and *GFP* genes under the transcriptional control of the CaMV 35S promoter derivative E12-Ω. Successful regeneration of transformed secondary embryos results in transgenic cocoa plants carrying the visible marker gene green fluorescent protein (*EGFP*), the selectable marker gene neomycin phosphotransferase II (*nptII*), the class I chitinase gene from cocoa (*Chi*), and tobacco nuclear matrix attachment regions (MARs) in different combinations. Transgene, per se, did not influence number of transgenic plants produced as number of transgenics developed with marker gene and other transgenes such as *Chi* gene or MARs were found to be same. Nevertheless, incorporation of MARs gene in vector construct enhanced mean GFP expression in the transgenics and reduced the incidence of transgene induced gene silencing phenomenon in transgenic lines multiplied though reiterative somatic embryogenesis. The study was a milestone in cocoa genetic transformation as it yielded 94 transgenic plants and their growth and development are comparable to that of untransformed, control plants. Genetic crosses and segregation analysis of transgenic EGFP expression showed a near-perfect 1:1 thus unequivocally proving that the transgenic lines resulted from the insertion of a single locus of T-DNA.

Cocoa transgenic plants over expressing a cocoa class I chitinase gene (*TcChi1*) were developed with a view to obtain resistance against fungal pathogen *Colletotrichum gloeosporioides* causing anthracnose employing *Agrobacterium*-mediated transformation of somatic embryo cotyledons (Maximova *et al.*, 2006). Genetic transformation was done in genotype PSU-Scavina 6. The binary vector pGAM00.0511 (Maximova *et al.*, 2003) was modified to harbor cocoa *TcChi1* chitinase gene, EGFP, and the neomycin phosphotransferase II (NPTII) marker genes, under the control of constitutive E12-Ω CaMV-35S promoter. Southern blot studies revealed stable integration of transgene in eight independent cocoa lines. Further enhanced levels of expression of *TcChi1* transgene expression in genetically modified lines were confirmed by Northern blot analysis. *In vitro* fluorometric and quantified chitinase activity assays indicated that the expression of transgene varied in different transgenic lines; upto six fold increase of endochitinase activity was documented. In order to prove the utility of cocoa transgenic lines, leaf disc bioassay was carried out by evaluating antifungal activity of the transgene against the foliar pathogen *Colletotrichum gloeosporioides*. Bioassay revealed that expression of *TcChi1* in transgenic cocoa leaves significantly impeded the growth of *Colletotrichum* fungus and the development of leaf necrosis was also found to be reduced. These results established for the first time the value of the cocoa transformation system as a tool for gene functional analysis and the potential utility of the cocoa chitinase gene as a means of increasing resistance against fungal pathogens in cocoa.

Despite the availability of technique to genetically transform cacao, poor regeneration and transformation efficiencies hampered the progress. In order to refine the transformation protocol proposed by Maximova *et al.* (2003), various factors affecting somatic mebryogenesis and transformation efficiency *viz.*, concentration of hygromycin, β-lactam antibiotics, polyamines and composition of co-cultivation medium were evaluated. Besides these factors, concentration of *Agrobacterium* and sonication-assisted *Agrobacterium*-mediated transformation (SAAT) were also investigated (Silva *et al.*, 2009). Among the polyamines, spermine at 1,000 μM was found to improve embryogenic callus and increase the number of embryos per embryogenic callus. The study suggested use of β-lactam antibiotics such as timentin and meropenem because of its neutral effect on secondary somatic embryogenesis whereas the commonly used cefotaxime irrespective of the concentration used inhibits somatic embryogenesis. Authors also suggested sonication of explants and explants co-cultivation on tobacco feeder layers (Silva *et al.*, 2009). Thus this study is a comprehensive evaluation of factors affecting successful cacao genetic transformation and regeneration.

Understanding the molecular mechanism underlying somatic embryogenesis in cacao would help devise a better tool to enhance its regeneration potential and find ways to overcome major impediments in cocoa regeneration. *Arabidopsis thaliana* leafy cotyledon gene (*AtLEC2*) has been shown to enhance somatic embryogenic competency of plant cells (Karami *et al.*, 2009) hence, its functional ortholog *Theobroma cacao* leafy cotyledon gene *TcLEC2* was studied. Investigations exploring molecular role of *TcLEC2* gene in somatic embryogenesis and seed fatty acid biosynthesis revealed that *TcLEC2* is consistently expressed in cacao endosperm and cotyledon. Ectopic transient expression of *TcLEC2* activates expression of seed specific TF genes such as *TcAGL15, TcABI3* and *TcLEC1* in cacao. Further, expression levels were high in embryogenic calli implying that *TcLEC2* greatly enhances embryogenic competency. Thus constitutive over-expression of *TcLEC2* dramatically enhanced development of somatic embryos in cacao (Zhang *et al.*, 2014). Hence, it was suggested that expression of *TcLEC2* could be used as biomarker associated with efficient somatic embryogenesis and would help screening cacao genotypes for high embryogenic capacity (Zhang *et al.*, 2014). Along the similar lines, cacao baby boom transcription factor (*TcBBM*) an *Arabidopsis* BBM ortholog, was investigated for its potential in enhancing somatic embryogenesis (Florez *et al.*, 2015). Transient and constitutive expression analysis of *TcBBM* greatly improved proliferation of somatic embryos independent of exogenous hormone application (Florez *et al.*, 2015). These studies underlined the potential of transcriptional factors (TFs) expression based somatic cell reprogramming required to enhance regeneration of an elite cultivar and development of transgenic cacao. Embryogenic potential of cacao cultivars are highly genotypic dependent, hence somatic embryogenesis has not been employed as a tool of mass multiplication of elite cultivars. Hence, *Arabidopsis* ortholog TFs identified and characterized in cacao genotypes would not only serve as biomarker for somatic embryogenesis, but also help enhance its genetic transformation. In the present context, the methodology available for cacao genetic transformation is reproducible yet is marred by low recovery of transgenic embryo (Maximova *et al.*, 2003). Hence, TF expression based induction of somatic embryogenesis coupled with genetic transformation would serve as an invaluable tool in developing transgenic cacao genotypes.

Of late, a transient assay system has been developed for functional genomics study in cocoa genotypes (Fister *et al.*, 2016). The study utilizes, vacuum infiltration of induced *Agrobacterium* cells harboring binary vector into C stage cocoa leaf sections to transiently express visual marker gene. In addition, utility of the system for studying transgene cacao chitinase overexpression was demonstrated by performing bioassays for *Phytophthora tropicalis*. Thus it is is rapid method for gene function analysis in cacao.

## 3. Conclusion

Successful development of genetically modified cacao genotypes and developments of somatic embryogenesis-based regeneration techniques offers plethora of possibilities to obtain improved crop phenotypes. Experiments have established that the importance of inclusion of matrix attachment regions (MAR) in transformation vectors for stabilizing transgene expression during somatic embryogenesis and transgenic line establishment in cocoa. Future research which incorporates genes of interest into vectors containing MAR would further establish the applicability of the system for all alien genes. Such a system could then be used as a basis for analyzing gene structure and function in cocoa and also as a model system for testing the effectiveness of transgenes for enhancement of desired traits such as disease resistance or quality improvement. Developments in the field of cacao molecular biology and identification of cacao transcription factors, involved in somatic embryogenesis, offers a much needed fillip to develop efficient genetic transformation system. Exploration of role of such TFs, and demonstration of their utility in obtaining improved SE and genetic transformation could help bridge major crop improvement objectives in cacao.

Among the various objectives of improved crop phenotypes, development of resistance to pests and diseases is foremost important. In this context, the feasibility of expressing *Bt* toxins to confer cacao pod borer resistance could be explored. Developments in the field of transcriptomics, with the aid of next generation sequencing technologies, would help in deciphering genes responsible for fatty acid composition, cacao flavor that are potential target genes for genetic manipulation to develop specialty cacao products. The utility of genetic transformation system for crop improvement in cocoa, however, remains to be seen keeping in view the continued opposition by the public to crops which have been genetically modified. Transgenic cocoa material has not yet been released and its scope will depend upon economic, social, environmental, and political factors of the country in which transgenic cocoa is introduced. Nonetheless, the efficient tissue culture based somatic embryogenesis system on its own contributes to clonal propagation of elite cocoa germplasm. Genetic transformation protocol available also could contribute to functional genomics studies in cacao by identification of candidate resistance genes that in turn could accelerate molecular breeding of cacao. A recent potent addition to this arena of research is cacao transient assay system for functional genomics analysis (Fister *et al.*, 2016). It is anticipated that such a rapid system of gene-function analysis would be of immense help to accelerate functional genomics studies in cocoa as it precludes development of transgenic cocoa lines for analyzing gene functions.

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