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Somatic Embryogenesis: Fundamental Aspects and Applications

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ISBN 978-3-319-33704-3 ISBN 978-3-319-33705-0 (eBook)
DOI 10.1007/978-3-319-33705-0

Library of Congress Control Number: 2016937943

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland

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Chapter 1
Somatic Embryogenesis. An Overview

Víctor M. Loyola-Vargas and Neftalí Ochoa-Alejo

Abstract Somatic embryogenesis is one of the most powerful tools in plant biotechnology. It can be used to produce plants commercially, or to carry out basic studies of cell differentiation, gene expression, molecular genetics, and many others. We present here a compilation of the different chapters of this book.

1.1 Introduction

Initially, the necessity of solving important fundamental questions in plant biology, such as the cell theory and totipotency, led to the development of plant cell, tissue, and organ culture (PTC). However, nowadays PTC represents a set of very powerful biotechnological tools. The applications of PTC include commercial micropropagation of agronomically important plant species, production of haploid and double-haploid plants and disease-free plants, rescue of hybrid embryos or somatic cell fusion from intra- or inter-generic sources for the production of novel hybrid plants, induction of genetic or epigenetic variation for the production of variant plants, and more recently the genetic engineering of plants to produce new varieties, resistant to pests and diseases, as well to improve the quality and quantity of a particular product obtained from a plant. Other applications include genetic modification to produce plants that can remove toxic compounds or test its toxicity (bioremediation) (Hannink et al. 2001; Krämer and Chardonnens 2001), and 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_1

of micropropagated shoots to maintain plant viruses. Root cultures can be used to study the interaction of roots with nematodes or mycorrhizas. Recently, plants have been modified by genetic engineers with the objective to increase the yield of cellulose or oil for the production of biofuels (Gressel 2008; Stokstad 2012; Takahashi and Takamizo 2012). Somatic embryogenesis is at the core of some of these biotechnological applications and is the focus of this book.

Gottlieb Haberlandt in 1902 (Haberlandt 1902) set the theoretical basis for plant tissue culture. He proposed that a single cell should eventually be capable of giving origin to a complete and functional plant. This theory has been proved to be right. At the core of this proof is the somatic embryogenesis.

1.2 Somatic Embryogenesis

Plant somatic embryogenesis (SE) is a biological process with both amazing basic and applied aspects. SE occurs in nature. At the edges of the leaves of several species of Kalanchoë appear small bipolar structures, some of them produce these structures constitutively, others by the action of environmental stress, and a third group is a combination of both (Garces and Sinha 2009).

In vitro plant cells can undergo dedifferentiation or redifferentiation to enter a new biological program that gives rise to somatic embryos. This process has raised one of the most important biological questions: which signals change the genetic program of a somatic cell and make it an embryogenic cell?

Numerous factors that affect SE have been investigated in order to understand the basis of this process and manipulate it to develop and establish efficient plant regeneration protocols as a fundamental step for its biotechnological application (Loyola-Vargas et al. 2008). A differentiated and specialized somatic plant cell or a group of somatic cells with specific functions must receive a stimulus from a set of plant growth regulators (PGRs), mainly auxins, perceive it, and then initiate the transduction to the nucleus where the specific regulatory and structural genes will be transcribed and subsequently will be translated into proteins involved in the differentiation that ultimately will lead to the formation of a new somatic embryo. All these changes can be followed at morphological, ultrastructural, genetic, physiological, biochemical, and molecular levels.

The idea of this book is to look somatic embryogenesis in an integrative way covering from the historical aspects of somatic embryogenesis to its applications. It is important to know about the history of those researchers whose contributions led to the development of this field. In Chap. 2 we describe the main facts that led to the historical first papers on SE (Miettinen and Waris 1958; Reinert 1959; Steward et al. 1958).

There are several pathways to initiate somatic embryogenesis (Chap. 3). Unlike the initial belief that all plant cells are totipotents, it has been seen that it is necessary to create appropriate conditions for some of them to regain totipotency. Among the several factors that play a role in the induction of SE are the plant

growth regulators, mainly auxins (Altamura et al. 2016). It is interesting that many species require an initial shot of auxins, but thereafter the auxin must be degraded for SE to proceed (Chap. 10) (Altamura et al. 2016). Clearly the onset of SE depends on a complex network of interactions among plant growth regulators, mainly auxins and cytokinins, during the early proembryogenic stages. Ethylene and gibberellic and abscisic acids pass to play a major role during the late stages of development. Together, the PGRs regulate multiple genes temporally and spatially which release the changes in the genetic program of somatic cells, as well as regulating the transition between each embryonic developmental stage.

In addition to phenotype, the origin of the explant, and genetic background of the plant, several stress treatments such as heavy metals, low or high temperature, osmotic shock, among others, might play a crucial role in SE induction, even in the absence of exogenous PGRs (Chap. 9) (Cabrera-Ponce et al. 2015; Ochatt and Revilla 2016; Salo et al. 2016).

An important concern is the fidelity of the somatic embryogenesis-regenerated plants (Chap. 8). There is an epigenetic reprogramming during the SE and the presence of somaclonal variation among the regenerated plants (De-la-Peña et al. 2015; Mahdavi-Darvari et al. 2015; Nic-Can et al. 2015; Solís et al. 2015). This variation can be the result of chromosomal aberrations, genetic alterations, epigenetic regulations, and transposable elements. The variation can be exploited for good, as selecting stress-tolerant somaclones (Bobadilla Landey et al. 2013; Us-Camas et al. 2014).

Beyond the biotechnological application of SE, it can be used to study the very different aspects of its induction and the development of somatic embryos. An aspect that is central to the study of SE is histology. SE has become an appropriate method for studying the morphophysiological and molecular aspects of cell differentiation (Chap. 26). The understanding of the developmental events during the induction phase as well as the development of somatic embryos is essential to regulate and improve each stage of the SE program efficiently. Anatomical and ultrastructural studies may be useful for the development of protocols more efficient for SE induction, as well as for the cellular mechanisms involved in the acquisition of competence for SE (Konieczny et al. 2012; Quiroz-Figueroa et al. 2002).

The molecular aspects of SE have been studied extensively. In this book, several authors have revised the most recent advances in the field. Transcriptomics of several species has been carried out during the induction of SE and the development of the somatic embryos (Chap. 4). Cotton is the species most studied, but the number of species investigated by this technique is growing every day. The pattern that is emerging from these studies suggests a predominant role of auxins during the induction of SE, as well as for genes like *LEC*, *WUS*, *FUS*, and a set of transcription factors (Shi et al. 2016; Tao et al. 2016; Trontin et al. 2016). The Next Generation Sequencing platforms of nucleic acids can be used together with techniques that allow the isolation of a specific cellular type, such as laser-assisted microdissection. Together these two techniques give us a closer approach to the state of the cell in determined space and time (Chap. 27).

The extreme changes required for the transcription of the genome during the change of a somatic cell to an embryogenic cell need a very active participation of transcription factors. In Chap. 5, authors made an extensive analysis of the state of the art in relation to the participation of transcription factors in this process. An interesting finding is that the most frequent transcription factors found active during the induction of SE belong to the pathways of the metabolism of growth regulators, stress, and flower development (El Ouakfaoui et al. 2010; Guan et al. 2009).

Among all the different mechanisms that regulate the expression of the genes, epigenetics also plays an important role (Chap. 6). Different reports suggest that auxins, in conjunction with the in vitro conditions modify the DNA methylation status in the embryogenic cells. These changes in DNA methylation patterns are associated with the regulation of several genes involved in SE, such as *WUS*, *BBM1*, *LEC,* and several others (De-la-Peña et al. 2015).

After the genes are expressed, all the weight of the process is on the proteins. Posttranslational modifications, protein turnover, and protein–protein interactions are common processes associated with the regulation of proteins. All of them are present during the induction of SE and development of somatic embryos. Proteomic studies carried out while the SE has begun to show the deep mechanism that works during the induction of SE (Chap. 7). One key question is if there is a common protein pattern among different species during the induction of SE (Campos et al. 2016; Mukul-López et al. 2012; Tchorbadjieva 2016).

SE has been induced in many different species; many of them crops of commercial interest. In the second part of the book, the SE of several important crops is analyzed: *Agave* spp., *Bixa orellana*, *Capsicum* spp., *Coffea* spp., *Curcuma*, *Musa* spp., *Zea mays*, lipid-producing plants like *Cocus nucifera* and *Jathropha curcas*, conifers such as *Pinus* spp., and model plants as *Arabidopsis thaliana* (Chaps. 11–22).

The two major applications of SE are scale-up propagation (Chap. 24) and genetic engineering (Chap. 23). Among the different systems to scale up the process of SE, the temporary immersion system has some advantages. It can be automatized to reduce labor and costs, and at the same time to produce high-quality plantlets (Fei and Weathers 2014, 2016; Ibaraki and Kurata 2001). The SE process is a very efficient method to regenerate transgenic plants. SE has been used in conjunction with *Agrobacterium* spp., particle bombardment, and chemical-mediated genetic transformation protocols. All the major annual and perennial crops, as well as model plants, have been transformed using efficient SE systems (Arroyo-Herrera et al. 2008; Bouchabké-Coussa et al. 2013; Canché-Moor et al. 2006; Palomo-Ríos et al. 2012).

Another application analyzed is the use of SE to produce secondary metabolites. The production of secondary metabolites by plants requires highly specialized tissues and a fine regulation and coordination in time and development by the plant (De Luca and St Pierre 2000). In nature, several plant species synthesize and store secondary metabolites in the zygotic seed, suggesting that somatic embryos can do it. In Chap. 25 the most recent discoveries related to the accumulation of secondary metabolites by somatic embryos are presented (Aslam et al. 2010, 2011; Sharma et al. 2015).

1.3 Concluding Remarks

PTC in general and SE in particular have turned into an invaluable tool to plant scientists. PTC has been commercialized around the world, and different companies are using plant tissue culture techniques for the massive propagation of plants. The use of PTC and the development of the omics and epigenetics have allowed the understanding of the basic biological process.

The use of SE leads to the understanding of differentiation, as well as to the genetic mechanisms involved in the transition from one stage to the next. Also it has led to the isolation of genes, proteins, and metabolites involved in the cell differentiation process. The combination of SE and genetic engineering will accelerate the discovery, isolation, and characterization of genes involved in different cellular processes.

From the agronomy side, the most important challenges ahead are the generation of resistant plants to pathogens and pests, as well as to abiotic stresses, increments

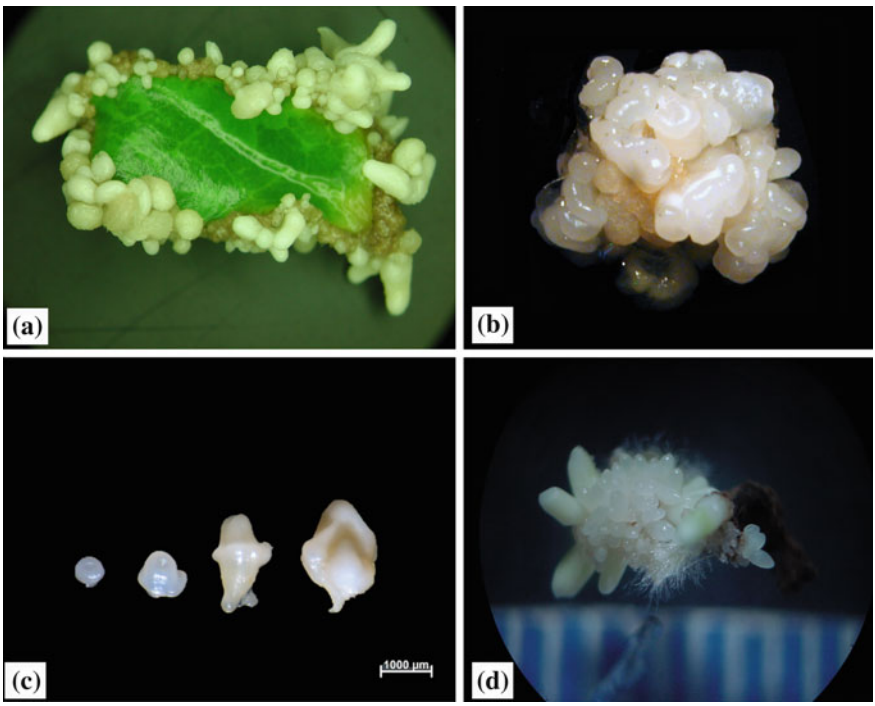

Fig. 1.1 Somatic embryogenesis process in different species. **a** Somatic embryogenesis in a leaf of *Coffea canephora*. **b** Embryogenic mass of *Cocus nucifera*. **c** Different developmental stages of *Musa acuminata* x *Musa balbisiana* genome AAB, subgroup Plantain. **d** Embryogenic mass of *Agave tequilana*. Picture **a** is from the laboratory of Víctor M. Loyola-Vargas. Pictures **b–d** are gifts from the laboratories of Carlos Oropeza-Salín, Rosa M. GraciaMedrano from Centro de Investigación Científica de Yucatán and Benjamín Rodríguez-Garay from Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco, respectively

in yields in commercial crops, the production of better raw material for biofuel production, as well as the generation of plants capable of absorbing toxic compounds from the environment. In all these cases, SE will play an important role (Fig. 1.1).

Acknowledgments The work of the laboratory of VMLV was supported by a grant received from the National Council for Science and Technology (CONACyT 157014).

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Part I
Basic Aspects

Chapter 2
The History of Somatic Embryogenesis

Víctor M. Loyola-Vargas

Abstract Somatic embryogenesis is used currently as a powerful tool in biotechnology. It is also used to study the development of the embryo. Somatic embryogenesis is a natural phenomenon that was moved from nature to the laboratory by man. The history of the study of somatic embryogenesis is plenty of discoveries of very different natures: from the role of growth regulators, mainly auxins, to the function of the components of the media of culture. In this chapter, a revision of the major contribution to the advance of knowledge of somatic embryogenesis is made.

2.1 Introduction

Embryos, essentials for the reproduction of higher plants, were an important improvement introduced by land plants, even before the development of seeds. This sequence of events probably leads to the production of embryos from different kinds of cells (Radoeva and Weijers 2014). (1) Zygotic embryos originate from gametic cells. (2) Somatic cells give origin to somatic embryos. (3) Cells from the seed primordium can develop into an embryo and subsequently in a seed without fertilization in a process known as apomixis. (4) There is also embryo formation from microspores; in the process called androgenesis, the microspores can form haploid or doubled embryos after the use of different kinds of stresses. (5) In the earliest steps of zygotic embryogenesis, there is a connection between the proper embryo and the maternal tissue, this assembly is known as suspensor. This structure is usually formed by a few cells; however, in some cases suspensor can be generated by thousands of cells and be able to form an embryo (Yeung and Meinke 1993).

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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_2

From these different ways to produce embryos, somatic embryogenesis (SE) has become a powerful tool to study and understand the mechanisms of the development of the embryo. In combination with traditional agricultural techniques, SE is used for the propagation of vegetable species of commercial interest and their genetic improvement (Loyola-Vargas et al. 2008). The onset of SE requires a set of interactions between auxins, ethylene, cytokinins, and several other growth regulators to change the genetic program of the cells (Jiménez 2005). Also, other factors can influence the induction and development of somatic embryos, such as the origin of the explant, the physiology of the mother plant, the environmental conditions of the incubation, and the composition of the culture medium (Loyola-Vargas et al. 2008).

A scientific discovery is the result of different efforts, many times from diverse lines of research or thinking. Since the landmark paper by Haberlandt (1902) proposed the theory of totipotency, several research groups were working on the development of plant tissue culture. Almost 60 years later, the discovery of SE was a consequence of the level of development of plant tissue culture at the end of the 50s of the last century. Calli and suspension cultures were well established, and the study of cytokinins (Miller et al. 1955) and auxins (Skoog 1947) was under development. Before the classical papers by Waris (Krikorian and Simola 1999; Miettinen and Waris 1958), Steward et al. (1958b), and Reinert (1959) there were some discoveries that point in the direction of asexual embryogenesis (Levine 1950; Wiggans 1954).

Levine (1950) found that, when indole-3-acetic acid (IAA) was removed from the culture medium, carrot callus regenerated roots and shoots. On the other hand, Wiggans (1954) also found that the carrot cultures were able to produce plantlets when the tissue was transferred to a medium without adenine sulfate, generating buds first and later the roots. Thus, Levine concluded that the removal of auxin led to root formation, followed by whole plantlets, whereas Wiggans deduced that reducing the auxin content of the tissue led to bud formation, followed by plantlets. Both authors concluded that decreasing the IAA in the tissues led to the formation of plantlets through two different routes, root bud in the first case, and viceversa in the second. Its accidental discovery opened an area of research, plenty of challenges, and wonders that have their roots and parallels in the development of plant tissue culture.

During the 30s of the last century, several research groups used *Bryophyllum calycinum* (Crassulaceae), actually known as *Kalanchoë pinnata* Lam. Pers., as a model to study regeneration in plants (Freeland 1933; Howe 1931; Naylor 1932; Yarbrough 1932, 1934). It was observed that at the edges of the leaves of *Kalanchoë* appear small bipolar structures. Several species of this genus can produce somatic embryos constitutively (*K. daigremontiana*). Other species produce plantlets by the action of environmental stress (*K. pinnata*, *K. fedstchenkoi*, *K. strepthantha*, *K. prolifera*, and *K. crenata*) and semi constitutive plantlet-forming species, which produce plantlets both constitutively as well as upon stress induction (*K. gastonis-bonnieri*) (Garces and Sinha 2009). Some additional information of SE in nature was generated from *Tolmiea menziesii* (Yarbrough 1936) and *Crassula multicava* (McVeigh 1938). In all the cases, somatic embryos were found at the edge of the leaves.

SE is a case of accidental codiscovery; in the space of only 15 months, three independent groups published seminal papers on the subject, and a fundamental part of plant tissue culture began its spectacular development. The first of these papers was submitted at the end of 1957 by Harry Waris' laboratory, to the journal Physiologia Plantarum (Miettinen and Waris 1958). Waris worked at the Botanical Institute of the University of Helsinki. The interest of Waris was on the effect of amino acids on the germination of various seeds. He was using amino acids such as alanine, arginine, leucine, α-aminobutyric acid, valine, asparagine, and others. He worked on the hypothesis that the use of different amino acids should change the balance of the proteins synthesis during the development of the new plantlets, and have morphogenetic consequences (Miettinen and Waris 1958; Waris 1957).

Waris found that after 3–4 months in the presence of 13.32 or 53.28 mM glycine the original plantlets almost died. However, new green plants emerged from "minute grains [and were] formed by some root tips of the original, morbid plant" This report was a presentation at the meeting of the Biochemical, Biophysical and Microbiological Society of Finland in March of 1957 (Waris 1957). Later, on November, Waris made a presentation to the Finnish Academy of Sciences entitled "A chemically-induced change in the morphogenesis of a flowering plant"; this talk was published in the Proceedings of the Finnish Academy of Science and Letters. In this talk, Waris presented data of the effect of glycine in carrot. This effect was the production of somatic embryos of carrot (Krikorian and Simola 1999). The next month, and with the help of Dr. Jorma K. Miettinen, a biochemist, submitted a paper to the journal Physiologia Plantarum entitled "A chemical study of the neomorphosis induced by glycine in *Oenanthe aquatica*" (Miettinen and Waris 1958). Waris did not name the structures as "embryos" or something like this, he used the term neomorph and neomorphosis for somatic embryo and SE, respectively. During the next 4 years, Waris published other two papers related to the neomorphosis of the genus *Oenanthe*. The Waris papers not only were among the first papers in SE but also they were the first papers that documented the effect of the nitrogen source on the morphogenesis of plant cells.

At the same time Frederick C. Steward was working at the Department of Botany at Cornell University, on the different ways suspension cultures can grow and multiply. He submitted two papers on June 1, 1958 and they were published back-to-back in the American Journal of Botany (Steward et al. 1958a, b). In the first paper, Steward et al. (1958b) reported the characterization, in particular, the growth, and shape of freely suspended carrot, peanut, and potato cells. The second paper describes the easy formation of roots in the liquid medium, containing coconut milk, from the small aggregates of the suspended cells. After the roots had been cultivated on semisolid medium, they were able to develop shoots opposite to the position of the roots and developed into complete plantlets. Steward et al. (1958a) documented a very ordered process from the sheath of cambium-like cells, to the embryo-like structure. This process is reminiscent of the formation of plantlets from zygotic embryos. Steward observed correctly the formation of vascular tissue before the formation of the root and inferred that such development was characteristic of a "proembryo."

The third paper of this history was published by Jakob Reinert, who was working at the Botanisches Institut der Universität in Tübingen, Germany (Reinert 1959). The paper was submitted on February 12, 1959 to the journal Planta. Reinert was able to induce the formation of carrot shoots, by transferring the callus tissue with roots into a white medium with several organic compounds including inositol, choline, riboflavin, biotin, ca-pantothenate, ascorbic acid, hypoxanthine, and a plethora of amino acids in addition to IAA and 2,4-dichlorophenoxy acetic (2,4-D). The complexity of this "synthetic medium" was antecessor of the complete media published in the next decade (Linsmaier and Skoog 1965; Loyola-Vargas 2012; Murashige and Skoog 1962). Altering this synthetic medium by changing the culture medium, Reinert was able to manipulate the formation of roots and shoots. He deduced, correctly, that the shoots come from "adventitious bipolar embryos."

Confirmations of these landmark papers began to appear in the literature just a couple years later (Kato and Takeuchi 1963; Wetherell and Halperin 1963). At the same time, the first pictures of somatic embryos were published (Kato and Takeuchi 1963; Wetherell and Halperin 1963). The source of the explants extended to *Datura innoxia* anthers (Guha and Maheshwari 1964). In the following years, more species and more different explants were used to study the SE process (Table 1.1). However, *Daucus carota*, had remained the most studied, and become The Model for the study of SE (Fujimura 2014).

Table 1.1 Summary of successful somatic embryogenesis during the first years after its discovery

Species	Explant	Year	Reference
Oenanthe aquatica	Somatic embryos	1958	Miettinen and Waris (1958), Waris (1959)
Daucus carota	Suspension cultures	1958	Steward et al. (1958a)
Daucus carota	Callus	1959	Reinert (1959)
Hordeum vulgare	Zygotic embryos	1961	Norstog (1961)
Cuscuta reflexa	Zygotic embryos	1961, 1962	Maheshwari and Baldev (1961, 1962)
Dendrophthoe falcata	Zygotic embryos	1962	Johri and Bajaj (1962)
Daucus carota	Callus	1963	Kato and Takeuchi (1963)
Daucus carota	Callus	1963	Wetherell and Halperin (1963)
Daucus carota	Seeds	1964	Steward et al. (1964)
Datura innoxia	Anthers	1964	Guha and Maheshwari (1964)
Ranunculus sceIeratus	Suspension cultures/stem	1965	Konar and Nataraja (1965a, b)
Nicotiana tabacum	Callus	1965	Haccius and Laksmanan (1965)
Daucus carota Apium graveolens	Callus	1966	Reinert et al. (1966)
Cichorium endivia	Callus	1966	Vasil and Hildebrandt (1966a)
Petroselinurn hortense	Callus	1966	Vasil and Hildebrandt (1966b)
Solanum melongena	Callus	1967	Yamada et al. (1967)

2.2 The Effect of Different Factors on the Induction of the Somatic Embryogenesis

A set of various factors such as the genotype and physiology of the donor plant, the explant, the composition and pH of the culture medium, the cell density, the incubation conditions, and the growth regulators, among others, affect the embryogenic response of tissues.

Three important discoveries, few years later, were: (a) the inhibitory effect on the induction of SE by 2,4-D, (b) the necessity to dilute the cell suspension culture, and (c) the effect of ammonium in the induction of SE (Halperin and Wetherell 1965). When the cultures derived from root phloem grew without ammonium, they did not form somatic embryos when the cultures were transferred to the proper conditions for the SE induction. These calli grew very well in the presence of only nitrate, but they were not able to form any embryogenic structure. However, the addition of small amounts of ammonium initiated the SE process (Halperin 1966). It is known that the amount of nitrogen in the medium is important. The relationship between nitrate and ammonium is part of the drive force leading the process of cell differentiation, in particularly SE (Fuentes-Cerda et al. 2001; Reinert et al. 1967; Tazawa and Reinert 1969). Casein hydrolysate was also tested successfully as nitrogen source during the induction of SE (Halperin 1995). However, there was controversy over the role of the nitrogen source on the SE. Reinert et al. (1967) argued that ammonium was no necessary, and that the important thing was the amount of the nitrogen source and not the form of nitrogen. However, the same group found that the *D. carota* cells cultivated in high levels of ammonium, the induction medium for SE, contained increasing levels of this compound; the ammonium was scarcely detected into the cells cultivated in the absence of ammonium which did not induce SE (Tazawa and Reinert 1969). It was speculated that the intracellular ammonium was necessary for the induction of SE. In a more systematic study carried out by Wetherell and Dougall (1976), it was found that glutamine, glutamic acid, urea, and alanine could, each one of them, partially replace ammonium as a supplement to nitrate. Other explanation for the effect of ammonium was that this compound could change the pH of the medium (Smith and Krikorian 1990). The Krikorian's laboratory (Smith and Krikorian 1990) was able to culture a *D. carota* proembryogenic mass on a growth regulators-free semisolid medium containing one mM of ammonium as the only nitrogen source. The titrated of the medium to pH 4 produced the best growth and multiplication and the culture stayed as proembryogenic mass. If the pH was increased to 4.5 or higher the proembryos developed into complete somatic embryos (Smith and Krikorian 1990). However, it was not possible to discard the effect of other factors, such as the potassium present in the medium culture (Brown et al. 1976).

Ammonium is not the only factor-driven SE. The size of the clump seems to be important. Clusters of 20–100 cells (47–81 μm) are more suitable to produce somatic embryos (Fujimura 2014; Halperin 1966). However, Kato and Takeuchi in

Japan (Kato and Takeuchi 1963) were able to regenerate plantlets from somatic embryos originating from friable small callus clumps. When single cells from these clumps were cultivated in fresh medium, no result was obtained. Nevertheless, the presence of a clump of growing cells promotes the complete development of the single cell into a plantlet. We know this process as a nurse culture. There is (are) a factor or factors that are produced by the clump of cells, which is (are) necessary for the growth of the single cell and is (are) diffused into the culture medium.

During the late 90s and earliest 20s, the laboratory of Kobayashi published a series of papers showing that, to induce the SE in *D. carota* suspension cultures was necessary to dilute the cell concentration. After several days of culture, the spent medium contained compounds secreted by the cells. Some of these compounds, such as the 4-hydroxybenzyl alcohol, inhibited the SE (Higashi et al. 1998; Kobayashi et al. 1999, 2000a, b, 2001). Other compounds, with similar inhibitory effect were found during SE induction in *Larix leptolepis* (Umehara et al. 2004). These inhibitory molecules were identified as vanillyl benzyl ether (Umehara et al. 2005) and 4-[(phenylmethoxy) methyl] phenol (Umehara et al. 2007). In our laboratory, we found that several phenolic compounds secreted during the induction of SE in *Coffea arabica*, can inhibit the SE of several species (Nic-Can et al. 2015).

"The auxin factor" or the "auxin paradox" was known very early in the research of the SE. Exposition of the embryogenic callus to concentrations of 2,4-D higher than 0.45 μM did not allow the formation of the somatic embryos (Halperin 1964; Halperin and Wetherell 1965; Reinert and Backs 1968). On the other hand, the presence of auxin was necessary to induce the change in the genetic program of somatic cells (Fujimura and Komamine 1979). Auxin removal was essential to allow the induction of SE and the further development of somatic embryos (Halperin 1964). An important problem of the production of somatic embryos is their heterogeneity. In a callus tissue, it is possible to find a mixture of proembryogenic tissue and somatic embryos at different developmental stages. Nevertheless, it is conceivable to "synchronize" the production of somatic embryos. An early method was the sieving of suspension cultures. The best size range to produce synchronized cultures was between 45–75 μm (Halperin 1964). Other factors, in addition to the size of the clumps, to produce synchronized cultures are the species, a low ammonium content in the induction medium, and the frequent renewing of stock culture (Fujimura 2014).

An early observation during the induction of SE was the changes in the morphology of the cells that occur during the process. Most of the cultures possess two kinds of cells: cells highly vacuolated, which in general do not divide, and meristematic small cells. These cells are spherical, and densely packed with starch (Quiroz-Figueroa et al. 2002). During the induction of SE, there are several ultrastructural changes, including a remarkable increase in free ribosomal content, decrease in endoplasmic reticulum, loss of polyribosomal aggregates, and appearance of microtubules (Halperin and Jensen 1967).

2.3 Molecular Biology of Somatic Embryogenesis

Since a very rapid cell division occurred just before the formation of the globular embryo (Fujimura and Komamine 1982), it is expected a high metabolic activity, including the biosynthesis of macromolecules. The first papers related to the role of protein synthesis, and transcription of genes began to appear at the beginning of the 80s. The laboratory of Raghavan published several articles related to the biosynthesis of proteins and nucleic acids. They found that the rate of RNA synthesis increased in carrot embryogenic cells following their transfer to fresh medium (Sengupta and Raghavan 1980a). The rate of rRNA synthesis in the embryogenic cells was lower than that in the nonembryogenic cells. However, embryogenic cells synthesized mRNA at a higher rate than the nonembryogenic cells during the first 96 h (Fujimura and Komamine 1982; Sengupta and Raghavan 1980b). The ratio of RNA to DNA of both cultures increased in the early stage of the culture. The ratio increase was higher in the embryogenic tissue, suggesting that the embryogenic culture was metabolically more active (Masuda et al. 1984) probably because the replicon size was much reduced in the embryogenic samples (Fujimura and Komamine 1982). The use of wheat germ system to translate the mRNA extracted from embryogenic and nonembryogenic cells, in combination with two-dimensional gel electrophoresis and autoradiograms, was let to determinate that there were small, but clear differences between both systems (Fujimura and Komamine 1982), suggesting a regulation at the transcriptional level.

The group of Dr. Z. Renee Sung at Berkeley made seminal contributions to understand the macromolecular processes during the induction of SE. The comparison of the protein profile, between carrot callus and somatic embryos derived from them, showed the presence of two proteins present only in the embryogenic tissues (Sung 1983; Sung and Okimoto 1981). The switch to turn on and off the biosynthesis of these two proteins was the absence or presence of 2,4-D, respectively. At the beginning of these studies, it was fascinating the fact the proteic profiles were very similar in undifferentiated and nonembryonic cells (Choi and Sung 1984). Using antibodies against an extract of carrot somatic embryos, Choi et al. (1987) was able to detect some proteins produced in the onset of SE. This technique lead to the isolation of three cDNA clones, one of them codified for a 50 kDa protein that was present also in the embryogenic tissues of cassava, peach, and maize (Choi et al. 1987).

Suspension cultures of carrot secreted considerable amounts of protein into the culture medium, most of them glycoproteins. This secretion can depend on the culture conditions and the initial source of the explant, among other factors. Most of the first studies were carried out in carrot. The electrophoretic analysis of the secreted proteins showed the presence of two glycoproteins of 65 (GP65) and 57 (GP57) kDa. The presence of 2,4-D, which inhibits the induction of SE, led to the secretion of GP57 in both embryogenic and nonembryogenic cultures. In the absence of 2,4-D embryogenic cultures formed somatic embryos and secreted GP65

(Satoh et al. 1986). In other study, proteins of 13, 17, 29, 38, and 46 kDa were found in the culture medium during the induction of SE in carrot (De Vries et al. 1988). The secretion of these proteins was reduced or absent in the presence of 2,4-D. On the other hand, the presence of 2,4-D promoted the secretion of a complete different set of proteins with molecular masses of 27, 36, 40, 44, 48, and 72 kDa. The proteins of 29, 46, 72, as well as a 52/54 kDa were high-mannose type glycoproteins (De Vries et al. 1988). Other particular protein secreted by embryogenic cultures was a protease inhibitor with a molecular mass of about 12.8 kDa (Carlberg et al. 1987). This protease inhibitor inhibited trypsin activity. The inhibitor was present in both, nonembryogenic and embryogenic cultures, but was released into the culture medium only by the embryogenic cultures (Carlberg et al. 1987).

SE is completely blocked by inhibitors of the glycosylation, such as tunicamycin and deoxynojirimycin. Tunicamycin treatment resulted in the presence of deglycosylated forms of the 46- and 52/54-kD (De Vries et al. 1988) and the inhibition of SE. The inhibition of SE by tunicamycin can be reverted by addition of extracellular proteins from untreated embryogenic cultures.

The qualitative differences between the electrophoretic protein patterns from nonembryogenic and embryogenic cultures, have also been found in calli from *Oryza sativa* (Chen and Luthe 1987) and embryogenic calli of *Pisum sativum* where two proteins of 45 and 70 kDa were found (Stirn and Jacobsen 1987).

Other proteins, such as acid phosphatase or with α-mannosidase activity, were found in the culture filtrate of both embryogenic and nonembryogenic lines, which suggest that the release of the inhibitor from embryogenic lines was not due to cell lysis.

2.4 Concluding Remarks

SE is a very important and interesting biological process. Scientists have been able to carry this process from nature to the laboratory. The history of this development shows how great discoveries are the result of multiple areas of knowledge, and the necessity of a conceptual framework.

The basic knowledge of the SE process was initially established empirically. The role of growth regulators, majorly auxins, was firmly settled very soon. The role of the nitrogen source, as well as the presence of compounds that are secreted into the culture medium, showed the importance to study all the factors possible involved in the induction of SE. The molecular aspect of SE was later recognized, and showed the importance of studying the biochemical and molecular aspects of the induction of SE.

Today, it is known that there are several factors that can induce SE. Most of these factors are related to stress (De-la-Peña et al. 2015). Among the different kinds of stress that can induce SE are cold, heat, osmotic shock, water deficit, heavy

metals, nutrient starvation, wounding, medium culture dehydration, ultraviolet radiation, and pH (De-la-Peña et al. 2015). The question that raises this knowledge is: what is the common factor that leads to the induction of SE?

Acknowledgments The work from VMLV laboratory was supported by a grant received from the National Council for Science and Technology (CONACyT, 157014).

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Chapter 3
The Many Ways of Somatic Embryo Initiation

Attila Fehér, Dóra Bernula and Katalin Gémes

Abstract It is widely believed that all cells of a plant are totipotent and can regenerate the whole organism. This view is supported by uncountable experimental observations demonstrating the regrowth of whole plants from various explants, even from single cells. However, recent investigations have demonstrated that plant regeneration may proceed via transdifferentiation of meristems or root meristem-like callus tissues due to adult stem cells present all over the plants. These pathways do not start from single totipotent cells. There is a strong argument for plant cell totipotency, however, and that is somatic embryogenesis. During this process, differentiated somatic cells change their fate to develop into an embryo. Animal embryos can develop only from the totipotent zygote and its direct descendants (this cell state can also be artificially produced by injecting a somatic cell nucleus into an egg cell cytoplasm during cloning). Plant cells have to be induced to start somatic embryogenesis and not all of them are competent to respond properly to the induction. In conclusion, plant cells cannot be considered as totipotent per se, but some of them can regain totipotency under appropriate conditions. In addition, accumulating evidence supports the view that even somatic embryo development can follow various initial steps not necessarily requiring cellular totipotency. Although, there are experimental observations to support the progression of somatic embryogenesis through a zygote-like state in certain experimental systems, in other instances the reorganization of several cells into an embryo was described. The direct release/induction of the embryo development program in vegetative plant cells may represent a third pathway of somatic embryo development. In this chapter, a brief literature review is provided to support the above view on plant cell totipotency as well as on the various ways to start somatic embryogenesis.

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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_3

3.1 Introduction

An intriguing feature of plant embryogenesis is that embryo initiation is not restricted to the fertilized egg cell, the zygote (zygotic embryogenesis) (Radoeva and Weijers 2014). Embryos may form in nature from unfertilized diploid egg cells (gametophytic apomixis) (Koltunow 2012), from maternal cells surrounding the embryo sac (sporophytic apomixis) (Koltunow 2012), from cells of the suspensor (suspensor-derived embryogenesis (Liu et al. 2015), or somatic cells at leaf margins (pseudovivipary) (Garcês et al. 2007). In addition, embryo development can be induced from microspores/pollen grains (microspore embryogenesis) (Soriano et al. 2013) as well as from various somatic cells/tissues under appropriate in vitro conditions (somatic embryogenesis) (Fehér 2015). It has been also observed in many cases that influencing the expression of various regulatory genes controlling cell division and differentiation can also lead to ectopic embryo development (Radoeva and Weijers 2014). In other cases, mutations in chromatin remodelling factors resulted in ectopic embryo or embryogenic callus formation (Fehér 2015). Although there is a clear genetic background defining the capabilities of plant species/genotypes for non-zygotic embryogenesis (Rose et al. 2010), our knowledge about the underlying molecular processes is rather scarce. Even, we cannot answer the central question: how much these pathways converge on the same molecular mechanisms. It is obvious that as soon as the embryos are formed their development follows default pathways independent of their origin. However, the initial conditions that can trigger embryo formation in planta or in vitro are strikingly different (Radoeva and Weijers 2014; Fehér 2015). In this chapter, we compare the general features of various embryogenic systems with the aim of answering the question: how many ways may exist to initiate embryo development in somatic plant cells? At first, however, we discuss the link between cellular totipotency and embryo development.

3.2 The Totipotency of Plant Cells

The default embryo development pathway starts with the fertilization of the egg cell and the formation of the zygote that exhibits developmental totipotency. Totipotency means that the zygote can autonomously develop into all cells of the mature plant. The zygote, at least in certain plant species including Arabidopsis, divides asymmetrically resulting in two cells, one of which forms the embryo and the other the suspensor. It needs to be mentioned that the cells of the suspensor are also embryogenic and may form embryos spontaneously (in certain species) or if the embryo is non-functional or removed. The developmental potential of the suspensor has been shown to be controlled by the embryo via auxin distribution (Liu et al. 2015). The embryogenic capability of suspensor cells is maintained until the globular embryo stage.

The question is, whether any other cells of the plant can be considered being totipotent? One can frequently meet with the statement in the popular as well as scientific literature that "all plant cells are totipotent". This overstatement can be considered as one of the central dogmas of plant biology. In theory, the single cell that can autonomously form an embryo (e.g. the zygote) can only be considered being totipotent, since it is only the embryo that has the capability to develop directly into a whole organism (Verdeil et al. 2007). In contrast, only pluripotency can be ascribed to single cells regenerating plants in two steps, regenerating the shoot followed by adventitious root development, under two separate conditions. Therefore, if all plant cells are totipotent, each should have the capability to form an embryo autonomously.

In plants, embryo development can be achieved starting from various cell types including differentiated somatic cells. Therefore, the view that all or at least many plant cells are totipotent seems to be well supported by observations. However, cellular totipotency is difficult to consider in systems where plant regeneration starts from several cells instead of single ones. This is true for shoot regeneration followed by adventitious root development (Su and Zhang 2014) as well as for indirect embryo formation through the reorganization of cell masses (Su et al. 2015). Even if embryogenesis starts from a single non-zygotic cell, there is a need for harsh changes in the in planta or in vitro conditions to deviate the cell from its default developmental pathway. This means that these cells are not inherently embryogenic but become embryogenic in response to external or internal cues. Therefore, plant cells cannot be considered as totipotent per se, but some of them can regain totipotency (the capability to form an embryo) under appropriate conditions (for reviews see Laux 2003; Verdeil et al. 2007; Fehér 2015).

In theory, plant cells can regain totipotency (the capability to initiate embryo development) in two main ways: regaining embryogenic cell identity via induction or losing vegetative/somatic cell identity via reversion (Fig. 3.1). The possibility that induction or reversion progress through a zygote-like or directly through the embryonic state needs consideration.

3.3 The Direct Ways of Somatic Embryo Development

3.3.1 Embryogenesis Through a Totipotent Zygote-Like State

An interesting question of developmental biology is whether we can also consider the germ cells or only the zygote to be totipotent (Cinalli et al. 2008; Feng et al. 2013). In animals, totipotency is maintained during the first few divisions of the zygote. The germ lines separate from the soma very early during embryo development and there is a view that these cells inherit the developmental potency of the zygote/young embryo (Seydoux and Braun 2006). This potency is kept during egg

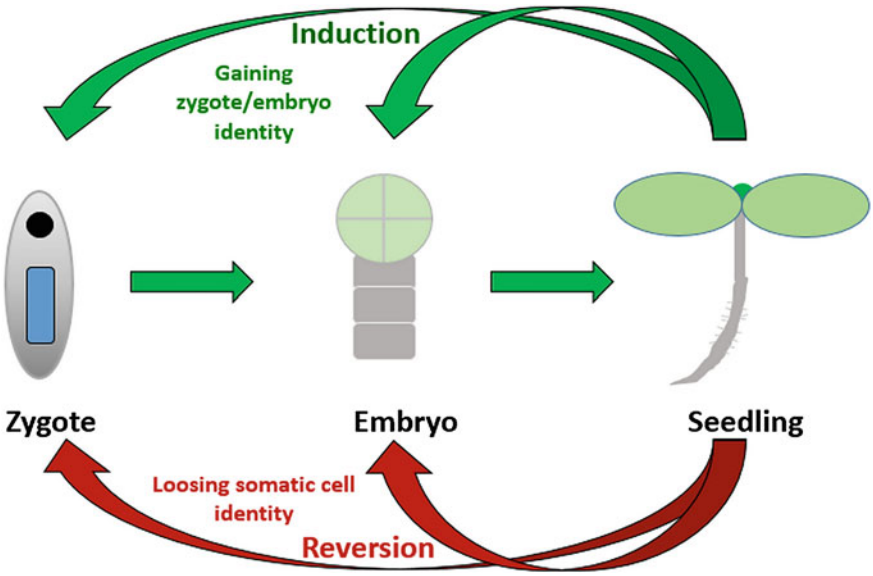

Fig. 3.1 Theoretical ways of somatic embryo formation

cell differentiation but suppressed by epigenetic mechanisms until fertilization takes place (Seydoux and Braun 2006; Cinalli et al. 2008). In contrast, the plant germ lines develop from well-defined differentiated somatic cells of the adult organism during flower formation (Yang et al. 2010; Twell 2011). Nevertheless, diploid egg cells formed during gametophytic apomixis can directly develop into zygotes and embryos indicating the inherent totipotency of plant egg cells. This totipotency is normally suppressed until fertilization by similar epigenetic mechanisms as in animals (Wuest et al. 2010; Feng et al. 2013). Initiation of female germ line development in vegetative tissues means that plants have at least one inherent developmental pathway to convert somatic cells towards totipotent cells (Feng et al. 2013). This pathway starts with the differentiation of the archeospore followed by megasporogenesis and embryo sac development. At present, it seems that embryo sac development is the step when cellular totipotency is re-established. Our knowledge about the molecular background of egg cell totipotency is rather limited. It is clear, however, that egg cell fate is determined by the auxin gradient within the embryo sac (Pagnussat et al. 2009). Manipulation of auxin content and distribution in the embryo sac alters cell fates (Pagnussat et al. 2009). Moreover, the analysis of various mutants demonstrated that cells mispositioned within the embryo sac change their fate depending on the auxin gradient (Sundaresan and Alandete-Saez 2010). Normally, the egg cell forms close to the micropylar end of the embryo sac where there is a relatively high local auxin concentration. High concentration of exogenous and/or endogenous auxin is required to initiate in vitro embryo development from the somatic cells of various explants (Fehér et al. 2003). Therefore,

it is tempting to speculate that somatic embryo development commences with an "egg cell/zygote-like totipotent state". The expression of egg cell/zygote markers in non-zygotic embryogenic cells could strengthen this hypothesis.

The Arabidopsis egg cell and the zygote express the WUSCHEL-like homeotic transcription factors WOX2 and WOX8, which following the asymmetric division of the zygote segregates into the apical and basal cells, respectively (Haecker et al. 2004). The polarity of the zygote is specified by the pollen-derived SHORT SUSPENSOR protein which activates the YODA/MAPK3,6 kinase cascade as well as by the WRKY2 and the RWP-RK-type GROUNDED(GRD)/RKD4 transcription factors (Ueda and Laux 2012). The WOX 2, 8 and 9 transcription factors determining basal and apical cell fates in the dividing Arabidopsis zygote have been implicated in somatic embryogenesis by gene expression data (Palovaara and Hakman 2008; Palovaara et al. 2010; Gambino et al. 2011). However, the exact temporal and spatial expression profile of these genes is yet unknown during this process, especially in the earliest phases.

The indication towards the possible role of these factors in non-zygotic embryogenesis comes from the experiment where the Arabidopsis *RKD4* transcription factor gene was overexpressed in Arabidopsis roots using a dexamethasone-inducible promoter (Waki et al. 2011). Ectopic *RKD4* expression caused overproliferation of root cells. However, if *RKD4* expression was ceased due to dexamethasone removal, somatic embryos appeared on the root surface. This indicates that the transient *RKD4* expression could trigger embryogenesis even in somatic cells. One may hypothesize that *RKD4* expression resulted in an egg cell/zygote-like cell state that favoured the embryogenic pathway. Indeed, ectopic expression of *AtRKD4* in the seedlings resulted in the transcription of genes associated with early embryo development. In a similar study, overexpression of the egg cell-specific *AtRKD1* or *2* transcription factors genes induced the expression of egg cell markers in somatic cells, which were induced to proliferate (Kőszegi et al. 2011). These cells, however, which expressed the *AtRKD1* or *2* transcription factors genes under the control of a constitutive promoter, did not develop into embryos. The expression and role of RKD-type transcriptional regulators during non-zygotic embryogenesis awaits further experimental validation. Detecting the transient expression of these factors may serve as a tool to identify the initial cells starting a zygote-like embryogenic program. This may, however, require a very high sensitivity of detection. The *RKD4* expression could only be detected in Arabidopsis due to a two-component system where the RKD4 promoter was linked to a transcriptional activator regulating the expression of a GFP reporter construct (Waki et al. 2011). In this way, the expression of the *RKD4* gene could be detected in the fertilized zygote and the early embryo (Waki et al. 2011).

Although the expression of zygotic molecular markers during the acquisition of the embryogenic cell fate by somatic cells is unclear, these cells often undergo asymmetric cell division resembling that of the zygote (Rose et al. 2010). Besides the morphological asymmetry, however, not much is known at the molecular level about the two daughter cells. Asymmetric segregation of an arabinogalactan protein epitope specifying cell fate has been described in carrot cell cultures long ago

(Souter and Lindsey 2000). This epitope is recognized by the JIM8 antibody and marks in vitro cultured embryogenic cells in carrot as well as zygotic embryos in Brassica (Pennell et al. 1991). In Brassica, this epitope did not label the zygote only the embryos from the 8-cell stage till protoderm formation as well as the suspensor. This expression pattern is consistent with the expression pattern in the carrot cell culture, where following the asymmetric division of a JIM8-positive cell, the JIM8-positive daughter cell (suspensor-like function?) support the development of the JIM8-negative one (embryogenic fate).

In certain somatic embryogenesis systems starting with an asymmetric cell division, the development of suspensor-like structures from the larger vacuolated cell was reported further supporting the resemblance with zygotic embryogenesis (Smertenko and Bozhkov 2014). These structures, however, often degenerate. The asymmetric divisions of single embryogenic cells can take place even in liquid media indicating that the division asymmetry is defined by intrinsic mechanisms. Only the analysis of asymmetrically dividing single cells devoted to the embryogenic pathway could answer the question on how much the first steps of somatic and zygotic embryogenesis converge. This kind of approaches are now feasible due to recent advances in the sequencing of single cell transcriptomes.

3.4 Release/Induction of the Embryo Maturation Program

Zygotic embryo development is governed among others by a group of transcription factors also implicated in seed maturation (LEAFY COTYLEDON1 (LEC1), LEAFY COTYLEDON2 (LEC2), FUSCA (FUS), ABSCISIC ACID INSENSITIVE3 (ABI3), AGAMOUS-LIKE15 (AGL15)) (Radoeva and Weijers 2014). Mutant phenotypes of these factors are mostly associated with late stages of embryo and seed development including cotyledon differentiation, embryo quiescence, and desiccation. Nevertheless, the overexpression of these genes can result in ectopic embryo development in vegetative tissues, such as in leaves, or at least can promote somatic embryo formation in response to appropriate signals. Considering this feature as well as the fact that they are also expressed during early stages of zygotic and somatic embryogenesis, they are often considered not only as seed maturation but also as embryo identity factors. These transcription factors have to be suppressed during germination to allow seedling development (Holdsworth et al. 2008). This suppression is established at the chromatin level, among others by the chromatin remodelling ATPase, PICKLE (PKL) (Rider et al. 2003; Henderson et al. 2004). Interestingly, the *pickle* mutation, which results in the ectopic expression of some of the above embryo identity factors (e.g. LEC1), may also result in ectopic embryo development (Henderson et al. 2004). Several other chromatin regulators have been shown to repress the embryonic cell fate in vegetative tissues including the POLYCOMB REPRESSOR COMPLEX 1 and 2 or the HISTONE DEACETYLASE 6 and 19 enzymes affecting histone posttranslational modifications (methylation and

acetylation, respectively) (Tanaka et al. 2008; Tang et al. 2012; Fehér 2015; Ikeuchi et al. 2015). Mutations in these genes have also been shown to result in ectopic expression of genes implicated in embryo development and the formation of embryogenic calli, embryo-like structures, or embryos. This supports the view that embryo development is a default pathway that is, however, efficiently suppressed in vegetative tissues at the chromatin level. Factors inducing (or rather releasing) somatic embryogenesis via this pathway therefore, should also act at the chromatin level. These factors might include exogenous plant hormones or stress factors altering the overall gene expression pattern (Fehér 2015).

Not only the released, but the induced expression of the above mentioned and other seed maturation/embryo development factors can result in somatic embryogenesis, including LEC1 (Lotan et al. 1998). *LEC1* gene expression could be detected in zygotic embryogenesis as early as the eight-celled stage. The earliest roles of LEC1 were hypothesized to be at the globular stage of zygotic embryogenesis when the *lec1* mutants exhibit aberrant cell divisions in the suspensor (Harada 2001). Although these observations indicate the role of LEC1 during early embryogenesis, it is still unclear how the overexpression of *LEC1* gene triggers the formation of embryo-like structures on leaves. LEC1 seems to induce embryonic gene expression programs only in seedlings and not in mature plants and therefore, it may need other co-factors to act (Lotan et al. 1998).

The action of the overexpressed *LEAFY COTYLEDON 2* (*LEC2*) gene is better revealed (Stone et al. 2001). This transcription factor also induces somatic embryo formation if ectopically expressed in seedlings. In addition to positively regulating the expression of embryo and seed maturation genes, LEC2 was shown to activate auxin synthesis through the expression of *YUCCA* genes (Stone et al. 2008). It was hypothesized that in *LEC2* overexpressing transgenic plants auxin synthesis is triggered in the genetic environment similar to that of maturating embryos resulting in the ectopic embryo development (Stone et al. 2008). This hypothesis is in agreement with the wide use of immature Arabidopsis embryos as explants for efficient auxin (2,4-D)-induced somatic embryogenesis (Gaj 2001; Ikeda-Iwai 2002). In general, it can be stated that the manifestation of the embryo maturation program (or at least part of it) in vegetative plant cells/tissues favours the ectopic initiation of embryo (or embryogenic callus) development. This favourable genetic environment can be achieved either via the released (e.g. chromatin remodelling mutants) or the induced expression of at least one of the several transcription factors related to embryo development (Ikeda et al. 2006; Radoeva and Weijers 2014; Fehér 2015). The inducer might be exogenous auxin or other factors triggering local auxin synthesis/accumulation (Fehér 2015). Seed and zygotic embryo maturation is dependent on the proper ratio of the plant hormones abscisic acid and gibberellic acid (Holdsworth et al. 2008). Not surprisingly, both hormones have been strongly implicated in somatic embryogenesis (Fehér 2015) further supporting the view that the conditions favouring zygotic and somatic embryo development are the same.

3.5 Indirect Embryogenesis from Embryogenic Cell Clusters

3.5.1 Callus Formation: Dedifferentiation or Transdifferentiation?

In many experimental systems, somatic embryos are not directly formed from somatic cells but there is in between an intervening callus phase. Callus is generally considered to be an unorganized mass of dividing dedifferentiated cells, which are capable of switching cell fate in response to hormonal signals. However, recent investigations suggest that there are various types of calli exhibiting different degrees of differentiation (Ikeuchi et al. 2013). For example, calli formed on Arabidopsis roots cultured in a high auxin/low cytokinin medium (callus-inducing medium, CIM) have a characteristic gene expression pattern reminiscent of partly organized root tip meristems (Sugimoto et al. 2010). This type of callus originates from pericycle or pericycle-like cells surrounding the veins in roots or aerial organs, respectively (Atta et al. 2009). Its development follows the initial steps of lateral root formation (Atta et al. 2009). Therefore, this callus tissue cannot be considered as a dedifferentiated but rather a misdifferentiated root meristem. Subsequent organogenesis from this type of callus, in response to cytokinin or auxin, respectively, can be regarded as transdifferentiation of the root meristem-like tissue into shoot or root meristem (Sugimoto et al. 2011). Embryogenesis has not been linked yet to root meristem-like callus, although initiation of embryos from cells surrounding the veins (procambial cells) was frequently observed (Guzzo et al. 1994; Rose et al. 2010; de Almeida et al. 2012; Fehér 2015). Whether in these cases embryogenesis shares the initial steps of lateral root formation still needs to be experimentally addressed.

Elaboration of a somatic embryogenic system from Arabidopsis roots would represent an excellent experimental system to answer this question due to the availability of mutants and cellular markers associated with lateral root initiation and callus formation.

In carrot, somatic embryo formation could be tracked back to single cells or small cell clusters of provascular origin in the fresh liquid culture of hypocotyl explants (Schmidt et al. 1997). In established cultures, proembryogenic cell masses (PEMs) form as a transitional stage towards embryogenesis in the presence of auxin (2,4-D). Whether PEMs can be regarded as misdifferentiated root meristems or embryos, is an interesting question to be answered.

There are, however, cases where callus and indirect embryo development seems to be preceded by dedifferentiation. Callus development in response to wounding follows a pathway independent of the pericycle or pericycle-like stem cells (Ikeuchi et al. 2013). This type of callus does not express root tip markers but is dependent on the WIND1 (WOUND-INDUCED DEDIFFERENTIATION1) transcription factor (Iwase et al. 2011a, b). Callus development from leaf protoplasts suffering a kind of wounding during the isolation process involves a transient dedifferentiated

stage (Grafi 2004; Grafi et al. 2011a, b). Wound-induced callus might also exhibit the capability for indirect embryo formation. Moreover, experimental observations demonstrate that embryogenic callus formation frequently initiates in epidermal or other highly differentiated cell types instead of procambial or perivascular cells (Nishiwaki et al. 2000; Yamamoto et al. 2005; Wang et al. 2011).

3.6 Embryo Initiation: Single Cell Origin or Reorganization of Cell Clusters?

In many indirect somatic embryogenesis systems, embryogenic calli or PEMs are formed in the presence of high concentration of auxin (especially 2,4-D) and removal of auxin triggers embryo formation on their surfaces (de Vries et al. 1988; Rose et al. 2010). In these cases, embryos may form through the reorganization of cell clusters instead of developing from single totipotent cells. Recent data obtained in the case of indirect Arabidopsis somatic embryogenesis support this view.

In this system, several molecular steps associated with initiation of the somatic embryogenesis pathway have been revealed (Su et al. 2009, 2015; for review see Fehér 2015). Arabidopsis calli (PEMs) formed in the presence of 2,4-D were used as explants. Using fluorescent markers of gene expression and confocal laser scanning microscopy (Su et al. 2009; Bai et al. 2013; Su et al. 2015), resulted in the following model (for a comprehensive figure see Fehér 2015). When the embryogenic calli are moved to auxin-free medium, endogenous auxin synthesis is induced via the expression of *YUCCA* (*YUC*) genes (Bai et al. 2013). The subsequently induced expression of *PINFORMED1* (*PIN1*) gene and the orientation of auxin transport proteins results in local auxin accumulations (Su et al. 2009). This is required to induce the expression of the *WUSCHEL* (*WUS*) meristem identity regulator gene in regions with auxin minima (Su et al. 2009). Interestingly, during auxiliary meristem formation in Arabidopsis, *WUS* expression also appears in cells with low auxin levels (Wang et al. 2014a, b), indicating the possibility that the regulation of the two pathways is similar. Redistribution of PIN1 proteins and auxin, as well as the expression of *WUS* ultimately, lead to the establishment of a shoot meristem organizing centre. The expression of *WUS-RELATED HOMEOBOX 5* (*WOX5*), a master regulator gene of root meristem organization, appears in parallel and almost overlapping with that of *WUS* in response to auxin removal (Su et al. 2015). However, later its expression is correlated with cytokinin rich regions and root meristem emergence. The apical-basal axis of the embryo is established before the formation of somatic embryos would be visible (Su et al. 2009, 2015). These observations indicate that indirect somatic embryogenesis proceeds via the reorganization of hormone synthesis, distribution, and gene expression within groups of callus cells. Formation of shoot and root meristems is followed by the organization of the cells into an embryo-like structure. These somatic embryos are often much larger than the zygotic ones and have no properly formed protoderm, which might be the

consequence of their multicellular origin (Su et al. 2009). The whole process can rather be considered as transdifferentiation of a partly differentiated callus tissue than redifferentiation of single cells within a dedifferentiated cell mass. The observations are also in line with the view that the various types of calli are not merely homogenous masses of fully dedifferentiated cells (Sugimoto et al. 2011). Moreover, this model argues that the regression to a fully dedifferentiated (totipotent) state is not an absolute prerequisite for embryo regeneration from vegetative tissues.

3.7 Transdifferentiation of the Shoot Meristem

A similar mechanism takes place during the formation of embryo-like propagules on the leaf margins of viviparous *Kalanchoe* species. In these species, the SHOOT MERISTEMLESS (STM) transcription factor, which serves to maintain shoot meristem function in cooperation with WUS, is required for the initiation of plantlet regeneration at the leaf margins (Garcês et al. 2007). This is, however, followed by the recruitment of an embryogenic pathway indicated by the expression of *FUS3* and *LEC1*. Interestingly, the *LEC1* expression is not indispensable for plantlet formation, as several species of the genus carry a non-functional *LEC1* allele preventing seed production and making the vegetative plantlet formation the default reproductive pathway. The embryo-like structures of *Kalanchoe* have no root poles but the plantlets form adventitious roots. This may be the consequence of their shoot meristem origin. In certain *Kalanchoe* species, vegetative propagation requires stress induction that is in line with the important role of stress in somatic embryogenesis (Fehér 2015). Arabidopsis shoot meristems have also been shown to be efficiently transformed into somatic embryos in response to stress (Ikeda-Iwai et al. 2003). The ectopic expression of the *STM* gene has been shown to enhance somatic embryogenesis in *Brassica* (Elhiti et al. 2010). Moreover, mutations enlarging shoot meristems have been shown to enhance somatic embryogenesis (Mordhorst et al. 1998). All these observations indicate that the shoot meristem to embryo conversion is generally feasible.

Transient *WUS* overexpression is sufficient to trigger embryo development in various vegetative tissues (Zuo et al. 2002). *WUS*-triggered embryogenesis was demonstrated to start with an asymmetric cell division indicating direct embryo formation. *WUS* itself was actually shown to repress *LEC1* expression suggesting that *WUS* does not directly act through the *LEC1* pathway, which is activated in this system only when the embryos appear on the explants (Zuo et al. 2002). *WUS* is the central regulator of shoot meristem identity, and its expression is detected in the Arabidopsis embryo from the dermatogen stage onward. Therefore, it is unclear how *WUS* overexpression triggers embryo development in a single “zygote-like” cell. One can suppose that *WUS* overexpression initially induces genes required for shoot meristem identity, but the inappropriate signals from the surrounding tissues result in embryo development that might be considered as a kind of transdifferentiation.

3.8 Conclusions

In plants, the gametophytes producing germ cells develop from well-defined somatic cells of the adult organism in response to developmental as well as environmental signals. The formed germ cells are considered to convey developmental totipotency for the zygote following their fusion. It is hypothesized that totipotency is already present in the egg cell but suppressed by various epigenetic mechanisms until fertilization takes place. In agreement, the existence of defined developmental and environmental conditions resulting in the establishment of totipotency in somatic plant cells cannot be questioned. This does not mean, however, that every plant cells is totipotent, as generally thought. Only those single cells, which have the capability to develop directly into an embryo can be considered to possess developmental totipotency. Embryo formation, however, not necessarily progresses through this zygote-like totipotent state. Accumulating evidence supports the view that the development of embryos from somatic cells can follow several pathways as far as the initial steps are considered. At present, at least there main models can be suggested: (1) direct embryogenesis from single cells through a zygote-like stage; (2) direct embryogenesis dependent on seed/embryo identity factors (LEC1 and other related embryo identity transcription factors); and (3) indirect embryogenesis dependent on WUS and WOX5 (as well as related auxin and cytokinin transport, synthesis and signalling pathways). The existence of the two latter pathways is in agreement with the observation that the direct formation of Arabidopsis somatic embryos is *LEC1* dependent while the *lec1* mutants can follow the indirect (WUS-centred) pathway for embryo formation (Gaj et al. 2005). Indirect somatic embryo development has been reported for *stm* and *wus* mutants (Mordhorst et al. 2002). However, the function of these proteins may be redundant considering somatic embryo induction (see above). It cannot be excluded either that in these mutants the *LEC1*-dependent pathway was followed.

Comparison of the various systems using modern experimental techniques that make possible to follow the development of single cells at the genome and epigenome scale might give the final answer on how much the above embryo development pathways are indeed separated or interrelated.

Acknowledgments The authors acknowledge the support of the Hungarian Scientific Research Fund (OTKA K108802).

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Chapter 4
Transcriptome Profile of Somatic Embryogenesis

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Abstract In plants, the formation of somatic embryos from a somatic cell is one of the most studied developmental processes due to its applications. However, the molecular mechanism underlying the control of SE is poorly understood. The development of massive techniques for nucleic acids sequencing has led to the study of the transcriptome. Among the most frequently observed results is the presence of transcription factors. These transcription factors are involved in the later steps of embryogenesis and these can induce dedifferentiation or increase the production of somatic embryos when they are ectopically expressed. Similarly, the overexpression of *BABY BOOM* (*BBM*) and *WUSHEL* (*WUS*), and homologs of other transcription factors is sufficient to induce somatic embryogenesis (SE) in vegetative cells. The use of the high throughput sequencing technology improves the number of genes uncovered during the early steps of embryogenesis. In this chapter, we present a summary of transcriptomes of the SE induction and somatic embryos maturation.

4.1 Introduction

To uncover the regulation in the control of one somatic cell to form a new independent clonal organism is the goal of many scientists in in vitro technologies. In plants, the formation of somatic embryos from a somatic cell is the most studied developmental process due to its application to fields such as germplasm conservation, plants micropropagation, and plant transformation mediated by the callus in vitro formation (Basiran et al. 1987; Capron et al. 2009; Debergh and Zimmerman 1993; Redenbaugh 1991; Sarker et al. 2007).

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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_4

The main studies on SE have uncovered a tight control by several plant grown regulators (PGRs) in the process, being auxins accumulation, the initial and main step of the process (Loiseau et al. 1995; Ribnicky et al. 1996; Su and Zhang 2009). The induction of SE by a treatment with the auxin analog 2,4-dichlorophenoxy acetic acid (2,4-D) has showed to be necessary for many plants to establish the undifferentiated condition letting the morphological reprogrammation to take place. However, in most of the cases, degradation of this PGR is necessary to complete the process (Ayil-Gutiérrez et al. 2013; Fujimura 2014). The addition of a cytokinin, such as benzyladenine (BA), increases the number of somatic embryos. This suggests a close cross talk between auxins and cytokinins to trigger the morphological transition of somatic cells into somatic embryos.

SE has been reported in many different species, including conifer plants (Germanà and Lambardi 2016; Mujib 2016; Trontin et al. 2016). The initial samples used to initiate the in vitro culture can be seeds, hypocotyls and leaves, and the addition of auxins, and cytokinins in many of the cases is important to induce the formation of somatic embryos. The structure of somatic embryos formed during its development resembles the morphological stages of the zygotic embryos: globular, heart, torpedo and cotyledonary stages (Hand et al. 2016; Leljak-Levanic et al. 2015; Trontin et al. 2016; Winkelmann 2016). However, some physiological behavior of plantlets derived from somatic embryos is quite different from that found in the zygotic embryos (Etienne et al. 2013; Jin et al. 2014). This could be a problem for the application of this technique. There is not an easy path to resolve these problems. They can only be solved by a deep mechanistic understanding of the conversion of a somatic tissue into an embryogenic tissue using cutting edge technologies.

4.2 The Transcriptome of Somatic Embryogenesis

The molecular mechanism underlying the control of SE is poorly understood. However, knowledge of the roles of genes involved in the embryogenic response has uncovered the secrets on this process (Elhiti et al. 2013; Karami et al. 2009; Li 2010; Pandey and Chaudhary 2015).

Global regulators for embryo development such as *LEAFY COTYLEDON* (*LEC*) and FUSCA3 (FUS3) transcription factors are required for embryo maturation and SE (Braybrook and Harada 2008; Meinke et al. 1994; West et al. 1994). Mutation or overexpression of *LEC* genes either inhibit or produce somatic embryos in normal conditions, while LEC1 transcription factor regulates *FUS3* gene in maturating embryos (Gaj et al. 2005; Meinke et al. 1994; West et al. 1994). Similarly, the overexpression of *BABY BOOM* (*BBM*) (Boutilier et al. 2002) and *WUSHEL* (*WUS*) genes (Arroyo-Herrera et al. 2008) and homologs of transcription factors (Florez et al. 2015) are sufficient to induce SE in vegetative cells.

Transcription factors involved in the later steps of embryogenesis can induce dedifferentiation or increase the production of somatic embryos when they are

ectopically expressed (Radoeva and Weijers 2014). The *AGAMOUS-15* (*AGL15*) MADS box transcription factor, expressed during embryo development, increases the competence of the explant through the regulation of stress-related genes in soybean and *Arabidopsis thaliana* (Zheng et al. 2013; Zheng and Perry 2014). Similarly, the *RWP-RK DOMAIN CONTAINING* (*RKD*) transcription factor and the receptor-like kinase *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*SERK1*) increase competence of the cell to induce embryo formation in somatic cells (Hecht et al. 2001; Közsegui et al. 2011; Radoeva and Weijers 2014).

The control of auxin biosynthesis by the LEC transcription factor integrates the PGRs control with the genetic regulation in the absence of an exogenous auxin. However, there is lack of studies about how auxins can modulate the fate of the cell competence into the SE at the molecular level. It is well known that the addition of 2,4-D to the explant is a requirement to obtain somatic embryos. However, how the auxin signals are recognized and transmitted to gain competence for the embryo development is not clear yet.

Initial megadata and massive works trying to identify genes related to SE were done in suspension of *Medicago sativa*. This suspension was induced with 2,4-D to produce SE calli after seven days of auxin treatment. Using cDNA cross-hybridization technique (Giroux and Pauls 1997) several differential genes were identified as potential embryo specific ones. However, three alfalfa SE-specific transcripts (*ASET1, ASET2,* and *ASET3*) genes were expressed at early embryo stages in *Medicago sativa* (Giroux and Pauls 1997). These genes were related to signaling processes at early stages of the embryogenesis. The development of new techniques such as subtractive hybridization and macroarrays led to the identification of more than 1,800 and 600 genes regulated from pooled samples of somatic embryo compared to non-embryogenic cells in *Elaeis guinensis* and *Gossypium hirsutum*, respectively (Lin et al. 2009; Zeng et al. 2006). The SE analysis by subtractive hybridization in *E. guinensis* showed a total of 1,867 EST with 1,567 unigenes. Functional gene ontology (GO) annotation was assigned to 928 ESTs, showing metabolism, transcription and post-transcription, protein destination and storage, and signal transduction as the most represented categories.

Further analysis by microarrays of embryogenic cell suspensions induced with auxin and not induced, were evaluated from 0 to 16-day post-induction. A total of 191 transcripts were differentially expressed during SE, from them, 91 transcripts were related to known proteins. Early SE associated transcripts (from 0 to 4 days after SE induction) were related to disease and defense as well as to transcription and posttranscriptional processes although many of them lay on unknown proteins. Later SE (8 and 16 days, somatic embryo) stages transcripts were related to signal transduction, disease and defense (metallothionein-like and peroxidase-related proteins, glutathione-*S*-transferase), energy, protein destination, storage, transcription and posttranscription, and cell structure (Lin et al. 2009).

Similar studies of SE were done in *G. hirsutum*, using non-embryogenic callus (7, 14, 21 days after induction of pooled RNA sample) and preglobular somatic embryos (1, 2, 3, 7, 14, 21 days after induction of pooled RNA sample). The suppression subtractive hybridization (SSH) method used rendered 671 differentially

EST, from them 242 unigenes were highly up-regulated in SE (Zeng et al. 2006). Functional GO annotation of differentially SE genes was termed in transport, transcription, posttranscription, and metabolism categories. The main induced genes found in embryogenic callus/preglobular were involved in lipid metabolism and wax biosynthesis, also transcription and posttranscription factors like ZF-HD homeobox protein genes, putative RNA helicase, PPR-containing protein, nonsense-mediated RNA-protein, splicing factor, SCARECROW regulator-like protein, zinc finger-like (Ran-binding) family, and ethylene-responsive transcriptional coactivator-like protein. Besides signaling proteins like Ser/Thr protein kinases and the *ARF* (ADP-ribosylation factor) gene were positively regulated during SE. cDNA analysis of both SE of *G. hirsutum* and *E. guinensis* showed a large number of newly discovered transcripts related to embryo formation, such as ARF, SCARECROW regulator-like protein, metallothionein-like protein, high-mobility group protein, senescence-associated protein, epoxide hydrolase, among others. However, the particular function of these genes was not clear, and the auxin-related genes were poorly observed (Zeng et al. 2006). This could be due to the limited number of cDNAs evaluated by the SSH method in the comparison or to the tissue used for the experimental embryogenesis test. Nevertheless, these studies marked the complexity of the somatic embryo development and the requirement of massive identification of SE genes. Nowadays, many different sequencing platforms, for the transcriptome analysis, have been conducted for the study of SE (Table 4.1).

The use of the high through put sequencing technology improved the number of genes uncovered during the early steps of embryogenesis. In cotton (*Gossypium hirsutum*), the Illumina digital gene expression platform was used to analyze the transcriptional profile during SE. This platform generated 32,108,458 clean tags of 21 bp long from all RNA samples evaluated (Yang et al. 2012). The existence of the cotton unigene (20,671) database from the NCBI allowed to map this tags and identified more differential expressed genes during SE. A total of 5,076 differentially expressed genes (log2Ratio ≥ 2) were obtained from differentiation (hypocotyl of 6, 24 and 48 h after induction), transition (non-embryogenic and embryogenic calli), and embryo development (globular embryos, torpedo embryos, cotyledonary embryos) compared to hypocotyl explants. More down-regulated genes were found across SE development, maybe involved in the old cell identity. A significant number of genes responsive only in dedifferentiation (588), or the transition from non-embryogenic calli (NEC) to embryogenic calli (EC) (137) and embryo development (813) were observed. GO annotation of the 5,976 genes by biological process showed that cellular and metabolic processes were more represented. Furthermore, 466 (9.18 % of the total) genes were related to transcription factors. De novo transcriptomes from *Larix leptolepis*, *Dimocarpus longan* Lour., *Cinnamomum camphora*, *Cocus nucifera*, *Araucaria angustifolia*, *Zea mays,* and *G. hirsitum* showed an increasing number of genes involved at the early or late stages of SE (Elbl et al. 2015; Lai and Lin 2013; Rajesh et al. 2015; Salvo et al. 2014; Shi et al. 2016; Xu et al. 2013; Zhang et al. 2012). All this data uncover a global view of the differences between the zygotic and somatic embryos development.

Table 4.1 Transcriptomes of the somatic embryogenesis process

Species	Initial tissue	PGR treatment	Control tissue	Test tissue (Time)	Platform	References
Medicago sativa	Calli	5.4 μM 2,4-D[a]	Non-embryogenic cell masses	Embryogenic cell masses	cDNA library	Giroux and Pauls (1997)
	Calli	0.045 μM 2,4-D, 0.46 Kinetin	Non-embryogenic callus and pre-embryogenic callus (7, 14, 24 dai)	Embryogenic callus (1, 2, 3, 7, 14, 21 dai)	Suppression subtractive hybridization/macroarray	Zeng et al. (2006, 2007)
	Calli	0.456 μM IAA, 0.74 μM kinetin, 25 g L^{-1} glucose	Recalcitrant embryogenic cell line	Highly somatic embryogenic cell line	Illumina Hiseq 2000	Xu et al. (2013)
Gossypium hirsutum	Hypocotyls	4.92 μM IBA, 0.46 μM Kinetin	Hypocotyl explants	Hypocotyls (6, 24, 48 h), none embryogenic calli (40 dpi), embryogenic calli, globular embryos, torpedo embryos, cotyledon embryos	Illumina digital gene expression technology	Yang et al. (2012)
	Hypocotyls	4.92 μM IBA, 0.46 μM Kinetin	Zygotic embryos, Somatic embryos	Globular, Torpedo, cotyledonary	Illumina digital gene expression technology	Jin et al. (2014)
Elaeis Guineensis	Embryogenic cell suspension	3.4 μM 2,4-D	Embryogenic cell suspension (with 2, 4-D)	Embryogenic cell suspension (without 2, 4-D; 0, 1, 2, 4, 8, 16 dpi)	Suppression subtractive hybridization	Lin et al. (2009)
Larix leptolepis	Pro-embryogenic mass	Natural	Embryogenic callus	Proembryogenic cell mass	454 GS-FLX titanium	Zhang et al. (2012)

(continued)

Table 4.1 (continued)

Species	Initial tissue	PGR treatment	Control tissue	Test tissue (Time)	Platform	References
Liriodendron tulipifera x L. Chinense	Pre-embryogenic mass	9.04–13.56 μM 2,4-D, 45.6–182.4 μM thidiazuron	Pre-embryogenic mass	Somatic embryos	Illumina	Li et al. (2012)
Dimocarpus logam	Friable embryogenic callus	9.04–18.08 μM 2,4-D	Embryogenic callus	Embryogenic callus	Illumina Hiseq 2000	Lai and Lin (2013); Lin and Lai (2013)
Zea mays	Zygotic embryos	2 ml L^{-1} of 4.52 μM 2,4-D, 2.875 g L^{-1} L-proline, 30 g L^{-1} sucrose	Zygotic embryos	Zygotic induced embryos (24, 36, 48, 72 hpi)	Illumina Hiseq 000	Salvo et al. (2014)
Glycine max	Cotyledon	180.8 μM 2,4-D, 3 % sucrose	Young embryos	Transgenic line 8981	Microarray	Zheng and Perry (2014)
Picea abies	Zygotic embryos	10 μM 2,4-D, 5 μM benzyladenine, 1 % sucrose	Embryogenic callus	Embryogenic callus	Illumina GAII	Yakovlev et al. (2014)
Arabidopsis thaliana	Immature zygotic embryos	5 μM 2,4-D, 20 g L^{-1} sucrose	Leaf tissues	Somatic embryos (5, 10, 15 d)	Illumina Hiseq 2000	Wickramasuriya and Dunwell (2015)
Araucaria angustifolia	Seeds	Abscisic acid, maltose	Zygotic embryos, megagametophyte, none responsive embryogenic	Globular zygotic embryo, cotyledonal stage, ABA-responsive	Illumina HiScanSQ	Elbl et al. (2015)

(continued)

Table 4.1 (continued)

Species	Initial tissue	PGR treatment	Control tissue	Test tissue (Time)	Platform	References
				embryogenesis, globular somatic embryo		
Cocus nucifera	Scooped plumular explant	74.6 µM 2,4-D, 0.1 % activated charcoal, 3 % sucrose, 50 µM spermine, 4.54 µM thidiazuron	Embryogenic callus	Embryogenic callus	Illumina Hiseq 2000	Rajesh et al. (2015)
Cinnamomum camphora	Zygotic embryos	0.5 M Sucrose	Immature zygotic embryos	Somatic embryos (5 w)	Illumina Hiseq 2000	Shi et al. (2016)
Eleutherococcus senticosus	Zygotic embryos	4.4 µM 2,4-D	Embryogenic callus	Yellow embryogenic callus (1 w), global embryo (3 w)	Illumina Hiseq 2000	Tao et al. (2016)

[a]*2,4-D* 2,4-Dichlorophenoxyacetic acid; *IAA* Indole-3-acetic acid; *IBA* Indole-3-acetic acid; *dai* Days after induction; *dpi* Days post induction; *hpi* Hours post induction; *w* weeks

More detailed information about SE process in cotton was obtained by comparing the transcriptional profile of somatic embryogenesis and zygotic embryogenesis using Illumina HiSeq 2000 platform at three developmental stages (globular, torpedo, and cotyledonar). More than 11 million read tags were obtained in each library, from them 5.5 million reads mapped to the unigene cotton database, rendering the transcriptional activity of 20,220 genes. This platform showed 9,103 genes highly expressed (RPKM [Reads per Kilobase per Million] ≥ 25) during the three stages. The SE process showed 4,242 differentially expressed (log2Ratio ≥ 2) genes compared to ZE (Jin et al. 2014). Interestingly, the somatic embryos showed increased transcriptional activity. The SE globular stage showed 337 genes, 300 genes for torpedo and 735 genes for cotyledonary stage, which were at basal levels in ZE. The GO annotation of the differentially expressed genes was mainly related to cellular process, response to stimulus and metabolism.

Even though the development of somatic embryos shares the same morphological stages with zygotic embryo development, in cotton some differences have been observed. The apical cells of somatic embryos were more vacuolated, with numerous lipid droplets and starch grains than the same cells of zygotic embryos, suggesting the involvement of environmental stress during the somatic embryo development (Jin et al. 2014). Actually, 20.1 % of the GO annotated genes belonged to stress responses (Jin et al. 2014). An increased number of genes in response to stimulus were comprised by the responses to chemical stimulus, to stress, and to abiotic stimulus. Therefore, SE development showed an increased stress response compared to ZE. The *ABA2* (short-chain alcohol dehydrogenase) gene was highly induced in SE, besides eight genes involved in abscisic acid (ABA) signaling showed increased expression as well as jasmonic acid related genes. Moreover, transcriptional factors (WRKY and MYB) and downstream stress responsive genes, such as LEA proteins were induced in SE development, suggesting that somatic embryos formation is a response to the stress perceived by the cell. In fact, embryogenic callus stressed with NaCl or ABA, at low concentration, promoted SE development but at high concentration inhibited EC proliferation, indicating a signaling function of stress related genes to produce somatic embryos (Jin et al. 2014).

The abscisic acid (ABA) signaling and biosynthesis genes were the most abundant transcripts detected in SE compared to ZE of *G. hirsutum*. The *ABA2* gene, involved in the biosynthesis of abscisic aldehyde, was induced during SE, as well as the ABA-mediated signaling genes *PYL, PP2CA, PP2C, SnRK2, ABF1, ABF2,* and *ABR1* during the SE when their expression was compared with the development of zygotic embryos (Jin et al. 2014). Also, three *NAC* genes (*NAC002, NAC072,* and *NAC090*), 15 *WRKY* and several *LATE EMBRYOGENESIS ABUNDANT* (*LEA1, LEA6, LEA14,* and *LEA5*) related to downstream stress response were expressed (Jin et al. 2014).

The stress response of SE development was also observed in *A. thaliana.* The transcriptional response of immature somatic embryos induced by 2,4-D showed more than 2,500 genes regulated in early stages of SE compared to leaf tissues (Wickramasuriya and Dunwell 2015). RNA samples of *A. thaliana* embryogenic

cultures from 5, 10, and 15 days showed genes related to osmotic and oxidative stress more active in SE development than in leaf tissues. Interestingly, the genes related to stress were enriched in the transcription factor family genes *AP2-EREBP*. The *RAP2.6* and *RAP2.6 L* genes, involved in ethylene response, showed a higher expression in somatic embryos than in leaf.

In *Z. mays*, at early times (0, 24, 36, 48 and 72 h) of SE induction, stress-related genes were expressed (Salvo et al. 2014). During acquisition of cell dedifferentiation, immature embryos of *Z. mays* showed an increased expression of genes related to stress. Initially, 24 h after the induction of SE the *WIP1* gene, involved in hypersensitive defense response, and *chitinase A1* gene were up-regulated 1,500 fold. Besides, 15 glutathione-*S*-transferase (*GST*) related genes were highly expressed, and some of them showed a 8-fold increment compared with their expression in immature embryos. Interestingly, the genes *BBM, WUS, PIN,* and *SERK*, involved in the somatic embryo development, presented similar expression profile with *GST* genes during SE induction (Salvo et al. 2014). Moreover, one germin-like protein (*GLP*) gene, involved in redox homeostasis of the cell, was co-expressed with *BBM*, showing a more complex regulation. In fact, *AGL15* (*AGAMOUS15*) overexpression in soybean increased the number of genes involved in osmotic and oxidative stress during SE (Zheng and Perry 2014). These stress responsive genes have been observed in different systems during SE development, turning into the question whether the stress response is a consequence of the morphological transition or this stress is involved in the signaling to direct the morphological change (Karami and Saidi 2010).

Auxin homeostasis and signaling seem to be more important during zygotic embryos in *Araucaria angustifolia*, where the Auxin Response Factors (*ARF*) and indoleacetic acid-induced protein, as well as polar auxin transport (*PAT*) genes, were much more induced in globular zygotic embryogenesis (Elbl et al. 2015). Additionally, globular somatic embryos of *A. angustifolia* showed induced expression of *WUSCHEL* gene, involved in the repression of ARF3, which is related to polar auxin accumulation, suggesting that the loss of polar auxin distribution promotes embryo initiation in early stages of the embryos development (Elbl et al. 2015).

An internal indoleacetic-3-acid (IAA) reduction (from 5 to 1 ng/g FW) in cotton hypocotyl explants was observed before the formation of embryogenic callus. Nevertheless, the amount of IAA (48 ng/g FW) content in embryogenic callus was several times higher than in any of the somatic embryo stages (9–15 ng/g FW); thus, the SE development requires an internal mobilization of auxin. This internal dynamic of auxin during SE was also observed in carrot (Newton and Shea 2006), coffee (Ayil-Gutiérrez et al. 2013), and *Abies alba* (Vondráková et al. 2011). Moreover, the internal increase of IAA by the exogenous application of 2,4-D is required to stimulate SE development in many plant models. Unless many transcriptional regulators of SE have been uncovered, many more auxin players have been accounted by transcriptional studies. Several investigations in cotton revealed auxin-related genes during somatic embryo development, which were mainly related to its synthesis, transport, metabolism, and signaling process. Also in other

systems, such as *Elaeis guinensis*, *Dimocarpus longan*, *Cocos nucifera* L., *A. thaliana*, *Z. mays* transcriptomes analysis during SE development at early and late stages showed auxin-related genes up or down-regulated, even in *Cinnamomum camphora* somatic embryos induced by sucrose stress.

Transcriptional profile of cotton SE revealed a complex auxin transcript expression observed in hypocotyl explants induced with 2,4-D to promote somatic embryos formation (Yang et al. 2012). Most of the 86 auxin-related genes found were down-regulated during explant dedifferentiation and SE development. From the eight IAA biosynthetic genes, tryptophan biosynthesis 1 (*TRP1*) and anthranilate synthase (*ASB1*) ankyrin repeat and SOCS box-containing protein genes were up-regulated while nitrilase (*NIT4*) gene was down-regulated at all stages, pointing to IAA production by the tryptophan pathway. Also, the IAA conjugates-related genes were repressed; however, during dedifferentiation stages the IAA-hydrolases GH3, amidohydrolases ILL and ILR genes were induced, but repressed in embryogenic cells and embryo development. Only *GH3.6* and *IAMT1* genes were induced in the dedifferentiation and re-differentiation states. From the ten genes involved in auxin transport, only *PIN3* was expressed at 0 and 24 h after induction.

Interestingly, most *AUX/IAA* genes (13) were down-regulated, and only the *IAA19, IAA14-1*, and *AUX2-11-2* showed up-regulation at the beginning of SE or during embryo formation. *ARF* detected genes (6) were all repressed, except for *ARF2* and *ARF6* that showed induction at embryo formation and hypocotyl induction, respectively. Interestingly, cotton cell lines recalcitrant or responsive to auxin-dependent SE showed a great number of auxin-related genes regulated in undifferentiated cell stage but also a lower number in the pro-embryogenic cell mass (Xu et al. 2013). The presence of the synthetic auxin 2,4-D in immature embryos of *Z. mays* or *A. thaliana* triggered the induction of auxin transporters like *PIN1* genes (Salvo et al. 2014) at early induction and up-regulation of stress and auxin-related genes through the time (Wickramasuriya and Dunwell 2015), possibly to promote accumulation of endogenous auxin to induce somatic embryo development.

In other models such as the oil palm *E. guinensis*, SE formation required the absence of exogenous auxin to allow visible somatic embryos (Lin et al. 2009); similarly, in *C. nucifera* low 2,4-D was required to stimulate SE. SSH studies in *E. guinensis* or RNA-seq in *C. nucifera* showed a reduced number of auxin genes, the presence of stress-related genes like *GST* linked the stress response and auxin regulation. It might be that the stress response in this monocot was the first step, and the auxin was used as a signal. How the stress response and the auxin signaling communicate is still an open field for investigation. However, there is a correlation between the dedifferentiation state of the cell and the presence of genes involved in both processes in many transcriptomes.

Another interesting player in SE formation that is not accounted in the analysis is cytokinins signaling. There is a tight relationship between auxins and cytokinins homeostasis. During the induction of SE, in *G. hirsutum,* zeatin biosynthesis related genes were found mainly expressed in a pro-embryogenic cell, as well as cytokinin

signal transduction genes in agreement with the increase in the amount of zeatin (Xu et al. 2013). The transcriptome data from the induction of several species suggest that the homeostasis of auxins y cytokinins, as well as their cross talk, play a central role in the induction of SE.

4.3 Concluding Remarks

Currently, the new sequencing technologies of DNA and RNA can give a closer approach to the state of the cell in a specific space and time. The transcriptome sequencing of a single cell, avoiding bias due to non-responsive tissue, will yield very important results to understand how the somatic cell changes its genetic program and become an embryogenic cell.

The potential and uses of the sequencing methods vary in both cost and coverage. De novo transcriptome sequencing by 454 pyrosequencing is commonly used for non-model species, whereas Illumina sequencing is used for comparative transcriptomes in species with reported genome or transcriptome annotated.

Deep RNA-seq sequencing at early stages of the SE may underlie the set of genes responsible for the control of the differentiation stage. However, several reports have been focused on the comparison of late events or stages for a global view of the SE. The expression of genes like *LEC*, *WUS*, *FUS* transcription factors and auxin and cytokinins related genes are the master regulators of the development of each stage. The global vision of the somatic or zygotic development is marked by differences in stress response and auxin signaling genes as well as by the transcription factor involved in the maturation of embryos. However, it is necessary to visualize early events of embryo induction to detect genes involved in the reset of the vegetative cell to initiate the complex embryo transition.

Acknowledgments The work from VMLV laboratory was supported by a grant received from the National Council for Science and Technology (CONACyT, 157014).

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Chapter 5
Transcription Factors in the Regulation of Somatic Embryogenesis

Katarzyna Nowak and Małgorzata D. Gaj

Abstract Somatic embryogenesis (SE), the process through which already differentiated cells reverse their developmental programme and become embryogenic, requires drastic changes in the transcriptome of the explant cells. Among the various factors that underlie this developmental switch, genes encoding transcription factors (TFs), which constitute the sequence-specific DNA-binding proteins, are widely accepted as playing a central function in the gene expression regulation. In recent years, intensive analysis of the global transcriptomes of plant cells that are undergoing embryogenic transition and the use of Arabidopsis (a model in plant genomics) in studies on the genetic control of SE have substantially contributed to the identification of SE regulators. A survey of SE-associated transcriptomes illustrated the combinational effects of stress and hormone signalling that are related to the in vitro environment that is imposed during a culture. Accordingly, among the TFs that are considered to be essential in SE induction, those that are involved in stress and hormone plant responses and especially flower development were found to be most frequent. This chapter provides a comprehensive review of the current knowledge about the TFs that are involved in the induction of SE in plant explants that are cultured in vitro. In addition to a general characterisation of the TF transcriptomes that are associated with SE induction in different plants, the individual TF genes with documented functions in the regulation of SE are presented with a special reference to their possible targets and the TF-controlled molecular mechanisms that underlie SE induction.

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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_5

5.1 Global Characteristics of the SE-Associated TF Transcriptomes

The reprogramming of already differentiated somatic cells towards embryonic development requires a substantial modification of a cell's transcriptomes. Embryogenic transition induced in somatic cells involves the repression or activation of numerous genes and thus transcription factors (TFs) that have a key function in the regulation of gene expression seem to play a crucial role in this process. The large number (6–10 %) of TF-coding genes that have been found in plant genomes imply the transcriptional regulation to play an even more important role in plant than in animal development (Riechmann et al. 2000).

Most of the available data on SE-involved transcriptomes was provided by global analytical approaches and among these microarray analysis has been intensively applied to investigate the embryogenic cultures of different plants including oil palm (Low et al. 2008), *Medicago truncatula* (Mantiri et al. 2008a), potato (Sharma et al. 2008), rice (Chakrabarty et al. 2010) and cucumber (Wiśniewska et al. 2012). Besides microarrays, EST sequencing in wheat (Singla et al. 2007) and RNA-seq in Arabidopsis (Wickramasuriya and Dunwell 2015) and cotton (Yang et al. 2012) have been applied in order to reveal SE-related transcriptomes. The microarray-based data showed that 1–12 % of all of the genes that were significantly modulated during embryogenic induction in different plants were found to encode TFs. However, the number of SE-involved TF genes based on microarray data appears to be seriously underestimated. A much more accurate evaluation of the TF genes that are involved in SE might provide approaches that are focused specifically on the analysis of TF transcriptomes. Accordingly, a multi-parallel qRT-PCR analysis of almost 1,900 TF genes of Arabidopsis showed that 1,768 (94 %) TF genes were expressed during SE induction and a large fraction of these (41 %) was found to have undergone a significant modulation of transcription (Gliwicka et al. 2013).

The examination of SE-related transcriptomes indicated that a common set of TF genes that encode the proteins representing MYB, MADS, AP2/ERF, bHLH, C2H2, WRKY, NAC and HB families is engaged in SE induction in different plants. Members of SE-involved TF families belong to various functional groups and the TF genes that are engaged in the transcriptional regulation of hormone and stress responses as well as those that control plant developmental processes, predominantly embryo and flower development, have been found to be the most frequent (Thibaud-Nissen et al. 2003; Che et al. 2006; Hosp et al. 2007; Sharma et al. 2008; Gliwicka et al. 2013; Wickramasuriya and Dunwell 2015). Interestingly, the *TF*s that control the development of the plant generative organs were found to be especially frequent among SE-modulated transcripts, thus suggesting that there are some similarities in the genetic regulation of generative and embryogenic transitions (Thibaud-Nissen et al. 2003; Sharma et al. 2008; Gliwicka et al. 2013).

The TFs that control zygotic embryogenesis (ZE) constitute the obvious candidates for SE regulators due to the anticipated similarities of an SE to its zygotic counterpart (Dodeman et al. 1997). Expression of at least 500 genes has been reported to control ZE in Arabidopsis and among the genes-encoded TFs, the members of ABI3VP1, AP2/ERF, ARF, C3H and Dof families have been indicated (Tzafrir et al. 2004), but these data also appear to be underestimated. A recent study on the ZE transcriptome of Arabidopsis showed that at least 60 % of Arabidopsis genes are expressed during seed development and up to 5 % of these were identified as TF-encoded (Belmonte et al. 2013). In support of the expected similarities in SE- and ZE-related transcriptomes, an analysis of an embryogenic culture in rice revealed that out of 242 rice homologues of the genes that are essential for ZE in Arabidopsis, 87 % were expressed during SE (Su et al. 2007). Further support for some type of convergence of the genetic determinants that control SE and ZE was provided by the observation that most of the genes that are differentially regulated during SE in various plants were revealed to represent the major TF families that are engaged in ZE (Singla et al. 2007; Imin et al. 2008; Chakrabarty et al. 2010; Wisniewska et al. 2012; Gliwicka et al. 2013; Wickramasuriya and Dunwell 2015).

In contrast to the numerous TF genes that have a differential expression in the embryogenic cultures of different plants and are thus assumed to contribute to SE induction (Singla et al. 2007; Sharma et al. 2008; Mantiri et al. 2008a; Chakrabarty et al. 2010; Wiśniewska et al. 2012), only a small number has been experimentally proven to control SE transition. Conclusively for the mechanism that is involved in SE induction, among the TF genes that have validated functions in SE, those that are related to hormone and stress responses have been indicted to be prevalent (Zavattieri et al. 2010; Fehér 2015).

The overrepresentation of the TFs that is related to hormone responses among the SE-modulated genes that have been reported in different plants reflects the common belief about the essential role of plant growth regulators in the control of the morphogenic pathways, including SE induced in plants in vitro (Jiménez 2005; Fehér et al. 2003). In Arabidopsis, 43 % of SE-modulated TF genes have been annotated to be hormone-related (Gliwicka et al. 2013). Besides the TFs that are involved in the metabolism and signalling of auxin, the genes that are related to cytokinin (CK), abscisic acid (ABA), jasmonic acid (JA), ethylene (ET), gibberellin (GA) and brassinosteroids (BR) have been reported in Arabidopsis and other plants (Singla et al. 2007; Imin et al. 2008; Chakrabarty et al. 2010; Wisniewska et al. 2012; Gliwicka et al. 2013; Wickramasuriya and Dunwell 2015). In support of the essential role of the genetic regulation of hormone metabolism and signalling in embryogenic transition, the mutations affected the level and sensitivity of different hormones (IAA, ABA, GA and ethylene) and the inhibitors of hormone metabolism or signalling have been shown to negatively impact SE induction in Arabidopsis (Gaj et al. 2006; Bai et al. 2013; Nowak et al. 2015).

Relevant to the common use of auxin treatment in SE induction in different plants (Gaj 2004) and the key role of IAA in the control of plant development (reviewed in Vanneste and Friml 2009), auxin signalling and metabolism have been postulated as being crucial for in vitro induced SE (Jiménez 2005; Fehér et al.

2003). In accordance, auxin-related TFs have been found to be the most frequent among the hormone-related genes that are involved in SE induction (Yang et al. 2012; Gliwicka et al. 2013; Wickramasuriya and Dunwell 2015). The TF genes that have a regulatory role in SE include the core regulators of auxin signalling, *AUX/IAA* and *ARF* genes (Rensing et al. 2005; Su et al. 2007; Wu et al. 2009; Yang et al. 2012; Gliwicka et al. 2013). A differential expression of *IAA16*, *IAA29*, *IAA30*, *IAA31* and *ARF1*, *ARF2*, *ARF3*, *ARF5*, *ARF6*, *ARF8* and *ARF11* was observed during SE induction in Arabidopsis and the mutants in these genes were substantially defective in the embryogenic response (Gliwicka et al. 2013; Wójcikowska and MDG, in preparation for publication). AUX/IAA–ARF-mediated auxin responses were also assumed to operate in the embryogenic cultures of other plants including cotton, rice, *Cyclamen persicum* and *Gossypium hirsutum* (Rensing et al. 2005; Su et al. 2007; Wu et al. 2009; Yang et al. 2012). This induction of *AUX/IAA* and *ARF* expression that is commonly associated with SE parallels a substantial function of auxin signalling in the control of zygotic embryo development (Sato and Yamamoto 2008; Rademacher et al. 2011, 2012).

In spite of the notable progress that has recently been made in deciphering the auxin-mediated regulation of plant development, our knowledge about the functions of *ARFs* in different developmental processes is still fragmentary and the results that have been obtained in various experimental approaches are frequently inconclusive (Rademacher et al. 2011). Within *ARF*s, the *ARF5* encoded so-called MONOPTEROS (MP) protein is functionally the best characterised and the role of MP in the mediation of auxin signal has been documented in a number of developmental processes including ZE (reviewed in Möller and Weijers 2009). In addition to auxin signalling, ARF5 was also reported to control the polar auxin transport by targeting the auxin efflux carrier, *PIN1* (*PIN-FORMED1*) (Wenzel et al. 2007). Our recent results provided some evidence about the engagement of *ARF5* in SE induction including the auxin-stimulated, strong accumulation of its transcripts in the explant parts that are involved in SE and the significantly impaired embryogenic potential of the *afr5* mutant and the overexpressor line (Wójcikowska and MDG, in preparation for publication). However, the mechanism of *ARF5* action in embryogenic transition remains to be demonstrated and a prerequisite for an understanding of the biological function of *ARFs* in somatic cells that are undergoing SE induction is the identification of their target genes, especially those that are directly controlled.

5.2 *LEAFY COTYLEDON* Genes—Master Regulators of the Embryogenic Development in Plants

The *LEC* group of genes includes the *LEC1*, *LEC2* and *FUS3*-encoded TFs that have a major role in the control of the morphogenesis and the maturation phases during ZE (Harada 2001). *LEC1* encodes the CCAAT box-binding factor HAP3 subunit (Lotan et al. 1998) while *LEC2* and *FUS3* encode proteins that have a

plant-specific B3 domain, which binds a highly conserved RY motif and regulates the expression of ZE-specific genes (Stone et al. 2001; Braybrook et al. 2006). The observation that the overexpression of *LEC2* and *LEC1* resulted in developmental disorders in plants that included the formation of callus and somatic embryos on seedlings suggested SE-related functions of *LEC*s (Lotan et al. 1998; Stone et al. 2001). In support of the proposed role of the *LECs* in the embryogenic transition of somatic cells, the *lec* mutants were found to be strongly defective in SE but not in the shoot organogenesis that was induced in vitro (Gaj et al. 2005). In addition, a key role of LEC TFs in the establishment of a cellular environment that promotes embryo development supported the activity of the LEC genes, which has commonly been observed in the embryogenic cultures of different plants (Zuo et al. 2002b; Harding et al. 2003; Yazawa et al. 2004; Ikeda et al. 2006; Fambrini et al. 2006; Guo et al. 2013; Zhang et al. 2014; Zhu et al. 2014a). The complex interactions between LEC genes and hormone metabolism that were revealed to control the maturation phase in zygotic embryo development (reviewed in Jia et al. 2014) provided a clue as to how LEC genes might support SE induction. The LEC-mediated control of auxin, ABA and GA metabolism observed during ZE seemed to especially be of importance for the promotion of SE (Braybrook and Harada 2008).

5.2.1 LEC2

Among the *LEC* genes, the *LEC2*-mediated mechanism of SE induction has been the most intensively investigated. As a result, the auxin-related functions of *LEC2* in the embryogenic transition of somatic cells have been documented. Similar to the regulatory link that was observed between the *LEC2* gene and *YUC* genes involved in auxin biosynthesis in Arabidopsis seedlings (Stone et al. 2008), *LEC2* was found to stimulate *YUC1*, *YUC4* and *YUC10* transcripts in in vitro cultured explants (Wójcikowska et al. 2013). As a result of the LEC2-mediated activation of the *YUC* pathway of auxin biosynthesis, a significant increase of IAA content was demonstrated in explant tissue that was undergoing embryogenic transition (Wójcikowska and Gaj 2015). The activation of *YUC* genes was also reported in the embryogenic callus of Arabidopsis in which somatic embryos were induced in response to the removal of auxin from a medium (Bai et al. 2013). *LEC2* was postulated to directly target *YUC4* in planta (Stone et al. 2008); however, further analyses are necessary to reveal the mode of regulatory interaction that has been observed between *LEC2* and *YUC* genes upon SE induction in vitro. Collectively, *LEC2* contributes to SE induction via an increase in the endogenous auxin levels that in turn results in the activation of the auxin-responsive genes that are operating in the SE-inductive network. Genetic components of this network and their complex interactions remain to be determined. Revealing how similar the molecular pathways that are triggered by endogenous versus exogenous auxin during SE induction will also be challenging.

In addition to the regulation of auxin metabolism, *LEC2* may be involved in the control of auxin signalling. To support this, the *LEC2*-mediated activation of the key components of the auxin-response pathway, members of *AUX/IAA* family (*IAA1*, *IAA17*, *IAA30* and *IAA31*), was reported in Arabidopsis seedlings (Braybrook et al. 2006; Stone et al. 2008). Relevant to these observations and important for the predicted functions of *AUX/IAA* genes in SE, mutations in two of these genes, *iaa30* and *iaa31,* were observed to seriously impair the embryogenic potential of in vitro cultured explants (Gliwicka et al. 2013). In addition to the postulated regulatory interactions with the auxin metabolism and signalling, the possible involvement of *LEC2* in auxin polar transport cannot be ruled out as the upregulation of auxin efflux facilitators, *PIN1* and *PIN2*, was observed in transgenic tobacco plants that overexpressed *LEC2* (Guo et al. 2013). PIN proteins are believed to direct plant developmental responses to environmental and endogenous signals through the control of the polar cell-to-cell transport of auxin (Habets and Offringa 2014), and relevantly, a key function of the auxin efflux carriers in ZE was documented (Friml et al. 2003). The findings that the explants of a *pin1* mutant were unable to undergo embryogenic induction in vitro (Su et al. 2009) and that the inhibitors of the auxin polar transport severely impaired the embryogenic response of explants in different plants (Venkatesh et al. 2009; Palovaara et al. 2010) provided further support for the involvement of *PINs* in SE induction.

The complex *LEC2*-mediated crosstalk between hormones is assumed to be associated with the mechanism of SE induction considering that in tissues that overexpress *LEC2*, the increase of auxin content has been related to the extensive changes in the accumulation of cytokinins, ABA and SA (Wójcikowska and Gaj 2015). Furthermore, a link between *LEC2* and ethylene may be also expected considering the *LEC2*-stimulated expression of the *ACS4* gene that is engaged in the synthesis of an ethylene precursor (Braybrook et al. 2006), and the regulatory relationship that has been indicated between the *LEC2* and *ERF022* genes involved in the ethylene biosynthesis/signalling (Nowak et al. 2015).

The observation that the overexpression of the *YUC* genes alone is not sufficient to induce SE provided some additional insight into the hormone-related functions of *LEC2* in SE (Zhao et al. 2001). This implies that only SE-competent cells can respond to the auxin signal. Thus, relevant to the *LEC2* function in the maturation phase of ZE, the gene was proposed to enable somatic cells to become capable of responding to the SE-inductive signal by lowering the GA content (Braybrook and Harada 2008). In this GA-related regulatory circuit, LEC2 directly activates *AGL15*, which in turn activates *GA2ox6* resulting in a reduced GA level coupled with the enhanced potential for the formation of somatic embryos (Wang et al. 2004). The report on the negative impact of exogenous GA_3 on the embryogenic response of Arabidopsis explants supports the inverse relation between the GA level and a tissue's capacity for SE (Gaj et al. 2006). The *LEC2*-mediated establishment of a proper balance between GA and ABA levels promotes the accumulation of the storage reserves that was proposed to enhance the embryogenic competence in cells (Braybrook and Harada 2008). The fact that the ectopic expression of *AtLEC2* has

been reported to induce the maturation processes in transgenic Arabidopsis, tobacco and *Theobroma cacao* tissue (Stone et al. 2008; Guo et al. 2013; Zhang et al. 2014) and that the high expression of the genes encoding storage proteins, including *CRA1* and *OLEO4*, was found to be associated with the embryogenic potential of Arabidopsis (Stone et al. 2008; Gliwicka et al. 2012) support this speculation.

Considering that *LEC2* positively impacts auxin accumulation and in turn, auxin activates its expression (Ledwoń and Gaj 2009; Wójcikowska et al. 2013), the revealing of the genetic components of a regulatory feedback loop that seems to operate between auxin and *LEC2* is required for the full understanding of the LEC2-controlled mechanism of embryogenic transition. In line with this notion, an auxin-responsive AuxRE element was identified in *LEC2* promoter region, which implies the involvement of ARFs in the regulation of *LEC2* expression. Among the potential regulators of *LEC2*, there are several *ARFs* (*ARF1*, *ARF2*, *ARF3*, *ARF5*, *ARF6*, *ARF8*, *ARF11*) that are differentially expressed in SE of Arabidopsis (Wójcikowska and MDG, in preparation for publication).

In the search for the genetic regulators of *LEC2* in SE, the proteins that are indicated to directly inhibit the *LEC2* expression in planta should be considered including, TT8 (TRANSPARENT TESTA8), ASIL1 (ARABIDOPSIS 6B-INTERACTING PROTEIN1-LIKE1) and PRC (POLYCOMB REPRESSIVE COMPLEXES) (Gao et al. 2009; He et al. 2013; Chen et al. 2014). In addition, miRNA166 was reported to indirectly control *LEC2* expression through the regulation of *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) (Tang et al. 2012). Whether similar regulatory interactions exist during SE induction is as yet unknown.

In summary, a possible model of the LEC2-controlled and hormone-related pathways that seem to underlie the embryogenic transition in somatic cells can be proposed (Fig. 5.1).

5.2.2 LEC1

Expression of *LEC1* has been associated with SE that is induced in vitro and in planta (Lotan et al. 1998; Yazawa et al. 2004; Garcês et al. 2007; Alemanno et al. 2008; Ledwoń and Gaj 2009; Guo et al. 2013; Nic-Can et al. 2013; Zhu et al. 2014a). Similar to *LEC2* and meaningful for the possible role of *LEC1* in the SE-inductive mechanism, the encoded TF is involved in auxin metabolism/signalling. Among the candidate targets of LEC1, the *YUC10* gene, which is involved in auxin biosynthesis and the members of the Aux/IAA family (*IAA5*, *IAA16*, *IAA19*), were postulated (Junker et al. 2012). In addition to auxin, the regulatory relations between *LEC1* and the metabolism/signalling of other hormones including ABA, JA and BR were implicated as underlying the function of *LEC1* in zygotic and somatic embryogenesis (reviewed in Junker and Bäumlein 2012; Junker et al. 2012).

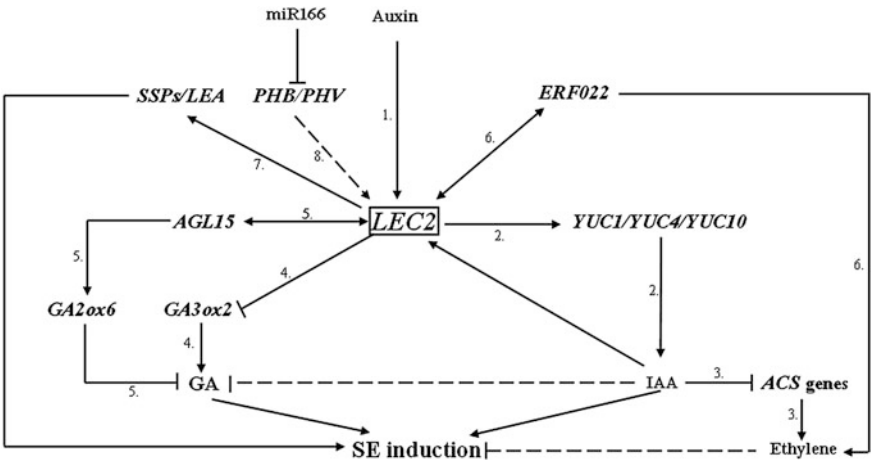

Fig. 5.1 Model for the *LEC2* role in SE induction through gene interactions with hormones. The interactions that need verification are indicated with dashed lines. *1* Ledwoń and Gaj (2009); *2* Wójcikowska et al. (2013); *3* Bai et al. (2013); *4* Curaba et al. (2004); *5* Braybrook et al. (2006); *6* Nowak et al. (2015); *7* Braybrook et al. (2006); *8* Tang et al. (2012). SSP-seed storage protein

In addition to hormones, the sugar-related functions of *LEC1* were reported to promote embryonic cell identity and in support of this, sucrose was demonstrated to modulate the penetrance of embryogenic traits in a *turnip* mutant that ectopically expressed *LEC1* (Casson and Lindsey 2006). Given the fact that sugars are proposed to act as morphogens that provide positional information in plant development, *LEC1* may exert its role in control of SE via the regulation of the sugar metabolism (Rolland et al. 2002). Another clue for the identification of the SE-promoting functions of *LEC1* was provided by the observation about the upregulation of the genes encoding the cell wall associated enzymes—hydrolase xyloglucan (XTH9) and expansine (EXP1B) in response to *LEC1* overexpression (Junker et al. 2012).

LEC1 is a member of the nuclear factor Y (NF-Y) family of TFs, which are highly conserved in all eukaryotic organisms. The NF-Y heterotrimer consists of three subunits NF-YA, NF-YB and NF-YC while *LEC1* represents NF-YB9 TF (Mu et al. 2008). Importantly, for the understanding of the *LEC1*-related regulatory interactions that can operate during SE, the overexpression of *LEC1* upregulates *NF-YA1*, *NF-YA5* and *NF-YA9* and, in turn, the overexpression of *NF-YA1* and *NF-YA9* positively regulates the expression of embryo- or seed-specific genes including *LEC1* (Mu et al. 2008, 2013). Similar to *LEC1*, the overexpression of *NF-YA1*, *NF-YA5*, *NF-YA6* and *NF-YA9* is sufficient to induce the formation of somatic embryos from vegetative tissues (Mu et al. 2013). Moreover, the cooperation of *NF-YA5* and *LEC1* is involved in the regulation of the genes that are responsible for the zygotic embryo development (Zhao et al. 2009) and whether similar interaction occurs during SE induction remains to be determined.

Collectively, some of the evidence presented above indicates the involvement of *LEC1* in SE induction; however, the impact of the encoded TF on the embryogenic response appears to be less pronounced in comparison to *LEC2*.

5.2.3 FUS3

In contrast to the other two *LEC* genes, *FUS3* is not upregulated in Arabidopsis explants induced towards SE, and overexpression of *FUS3* does not lead to the formation of somatic embryos (Ledwoń and Gaj 2011). However, the existence of a pathway that involves an LEC2-induced increase in auxin levels that promotes FUS3 activity was proposed (Braybrook and Harada 2008), thus suggesting that FUS3 might be involved in an LEC2-controlled mechanism of SE induction.

Numerous hormone-related genes are expressed in response to the activation of *FUS3* and among them the *YUC* genes of auxin biosynthesis, *AUX/IAAs* and *ARFs*, which encode the key components of auxin signalling, and the genes that are related to the biosynthesis of ABA, CK and BR were reported (Yamamoto et al. 2010; Wang and Perry 2013). Moreover, the relation of *FUS3* to GA was documented and the encoded TF, similar to two other LEC TFs, may enhance the competence for SE induction via the repression of *AtGA3ox2*, thereby resulting in a reduced level of bioactive GA (Curaba et al. 2004).

FUS3 was also reported to regulate vegetative phase transitions by negatively modulating ethylene-regulated genes in Arabidopsis, and among the downregulated genes those involved in ethylene biosynthesis (*ACS6*) and signalling (*ERF1*, *ERF104*, *ESE3*, *EDF4*) were reported (Lumba et al. 2012). In support of the ethylene-related function of *FUS3*, the ethylene level was found to correlate with the expression of *GmFUS3* in SE of soybean (Zheng et al. 2013). The role of the ethylene-associated activities of *FUS3* in SE induction requires further study.

5.3 SE-Related Functions of *AGL15*

AGL15 encodes one of the MADS domain proteins that it is believed to play key roles in the regulation of the developmental processes in eukaryotes (reviewed in Smaczniak et al. 2012). TF with MADS domain selectively binds to a consensus DNA sequence, the CArG (C-A/T rich-G) motif, to either activate or repress the expression of the targeted genes (West et al. 1997). The SE-related function of *AGL15* was postulated in Arabidopsis due to the somatic embryo-promoting effect of *AGL15* overexpression that was observed in the seedlings and in immature zygotic embryos that were cultured in vitro (Harding et al. 2003; Thakare et al. 2008). The AGL15 protein was found to accumulate during the early stages of ZE in *Brassica napus*, *Zea mays* and *A. thaliana*, in the somatic embryos of *Medicago sativa* and in a microspore culture of *B. napus* (Perry et al. 1999). The role of

AGL15 in the promotion of embryogenic responses was reported as being related to the GA metabolism and as a result, the *GA2ox6* that encodes gibberellin oxidase was identified among the targets of AGL15 (Thakare et al. 2008). It is postulated that AGL15 controls SE via the downregulation of the level of biologically active GA, and the inhibitory effect of GA on cell division may account for the requirement of a low level of this hormone during the early stages of SE (Wang et al. 2004). Besides GA, AGL15 seems to control the metabolism of ethylene. Recently, *At5g61590*, a member of AP2/ERF family and an orthologue of *MtSERF1*, which is involved in SE induction in *M. truncatula,* was identified as being a direct target of AGL15 (Zheng et al. 2013). It was shown that *At5g61590* (*DEWAX–Decrease Wax Biosynthesis*) acts as a repressor of the biosynthesis of cuticular wax (Go et al. 2014). In addition, the ethylene biosynthesis genes, *ACC SYNTHASE* (*ACS*) and *ACC OXIDASE* (*ACO*), are expressed in response to *AGL15* (Zheng et al. 2013). Stress-related functions of *AGL15* were postulated in soybean and the enhanced embryogenic response of explants that was observed upon the overexpression of *GmAGL15* was suggested to be the result of the activation of the genes that are involved in stress response (Zheng and Perry 2014).

AGL15 is believed to be a component of the SERK1 (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1) complex (Karlova et al. 2006), which was proposed to mark the cells that are competent in SE (Hecht et al. 2001). *SERK1* was assumed to interact with BRI1 (BRASINOSTEROID-INSENSITIVE1), and thus the function of AGL15 in BR signalling might be inferred (Aker and de Vries 2008).

AGL15 was also hypothesised to be involved in chromatin repression via its interaction with SIN3/HDAC (SWI-INDEPENDENT3/HISTONE DEACETY LASE), HDA (Hill et al. 2008) and topless (TPL) and topless-related (TPR) proteins (Causier et al. 2012). AGL15 has also been suggested to contribute to chromatin repression by increasing the efficiency of the formation of a repressive complex or the recruitment of a corepressor (Fernandez et al. 2015). Further analysis is needed to identify the targets of the epigenetic repression that is mediated by AGL15 during embryogenic induction.

Besides protein-encoded targets, the genes encoding microRNA156 (*MIR156a* and *MIR156c*), which are involved in the suppression of the SQUAMOSA promoter binding protein-like 3 (SPL3) transcription factor that promotes the floral transition in Arabidopsis, have recently been indicated to be AGL15-controlled (Wu and Poethig 2006; Serivichyaswat et al. 2015). Considering the apparent similarities in the genetic control of floral and embryogenic induction (El Ouakfaoui et al. 2010), a regulatory interaction between AGL15 and the floral suppressor, miR156, may be anticipated during SE induction. In support of this, the significant repression of the miR156 accumulation was observed during SE induction in Arabidopsis (Szyrajew and MDG, data unpublished). However, the existence of a regulatory link between the AGL15 and *MIR156* genes in an embryogenic culture remains to be validated, and the identification of the miR156-regulated mRNAs upon SE induction is required in order to reveal whether the mechanism of embryogenic transition is convergent with floral induction.

5.4 Stress-Related TFs

The embryogenic transition of the somatic cells was postulated to manifest a general response of the cultured tissue to stress conditions imposed in vitro such as wounding or the 2, 4-D treatment of explants (Fehér et al. 2003; Karami and Saidi 2010). In line with this postulate, various stress factors (osmotic, salt, water and heavy metals) were shown to replace or enhance the hormone treatment that was used for SE induction in different plant species (Kamada et al. 1993; Patnaik et al. 2005). Relevant to the assumed role of stress in SE induction, numerous (4–12 %) stress-related mRNAs have been found among those that were differentially expressed during SE induction (Legrand et al. 2007; Chakrabarty et al. 2010; Yang et al. 2012; Jin et al. 2014; Wickramasuriya and Dunwell 2015). In conjunction with the stress-regulated mechanism of SE induction, almost 40 % of the differentially expressed TF genes in an embryogenic culture of Arabidopsis were annotated to stress-related functions (Gliwicka et al. 2013). Some of these genes were subjected to closer inspection including *NTL8*, *DREB2F*, *ATHB-12*, *LBD20* and *MYB74*, and the SE-impaired phenotypes that were observed in the plants that carried mutations in these genes strongly support the notion that SE induction shares a mechanism at the molecular level that is relevant to general stress responses (Gliwicka et al. 2013).

5.4.1 ERF *Genes and the Ethylene Response*

In SE induced in Arabidopsis, *AP2/ERF* genes constituted a substantial part (10 %) of the stress-related TF genes that had a differential expression (Gliwicka et al. 2013). Similar to these data that are based on qRT-PCR analysis, RNA-seq analysis of the SE-related transcriptome of an embryogenic culture in Arabidopsis indicated that almost 40 % of the highly stimulated TF genes represented the genes encoding AP2/ERF TFs (Wickramasuriya and Dunwell 2015). In addition to Arabidopsis, the differential activity of numerous *ERF* genes was described in embryogenic cultures of wheat (Singla et al. 2007), *M. truncatula* (Imin et al. 2008), rice (Chakrabarty et al. 2010), cucumber (Wisniewska et al. 2012) and *Hevea brasiliensis* (Piyatrakul et al. 2012). Given that ERF TFs were assumed to regulate stress responses, and especially ethylene-related pathways (Nakano et al. 2006), the common expression of *ERF*s during SE induction implies the involvement of ethylene in SE induction.

The observed modulation of ethylene-related *ERF* genes during SE induction may result in part from the auxin treatment of explants. To support this assumption, auxin was documented as influencing ethylene signalling and metabolism and the complex interactions between auxin and ethylene were recently demonstrated in the regulation of plant development (Stepanova et al. 2007; reviewed in Muday et al. 2012). In accordance with in planta development, the ethylene level was also shown

to affect auxin biosynthesis and distribution in an embryogenic culture of Arabidopsis (Bai et al. 2013).

In contrast to dozens of the ethylene-related genes that are differentially expressed during embryogenic transition, the functions of only a few of them have been experimentally proven as being related to SE.

5.4.1.1 *MtSERF1*

MtSERF1 gene was identified as promoting SE induction in *M. truncatula* and its high expression, which was observed in globular somatic embryos, was found to be ethylene-induced (Mantiri et al. 2008a). The orthologues of *MtSERF1* were also reported to positively impact the embryogenic responses in cultures of Arabidopsis (Zheng et al. 2013) and *H. brasiliensis* (Piyatrakul et al. 2012). Importantly for the SERF1-mediated mechanism of SE induction, an Arabidopsis orthologue of *MtSERF1* (*At5g61590*) was recently identified as being a direct target of AGL15 (Zheng et al. 2013), which has an essential role in SE induction (see paragraph 3). In addition, interactions between *MtSERF1* and the members of HD-ZIP III family, PHB, PHV and REV (REVOLUTA), which are regulators of early zygotic embryo development in Arabidopsis, were suggested, and the encoded TF was proposed as linking the stress response to development during SE induction (Mantiri et al. 2008b).

5.4.1.2 *ERF022*

ERF022 expression was observed to be drastically inhibited during SE induction in Arabidopsis and a significantly impaired embryogenic response of the *ERF022* mutant was found to be associated with increased ethylene production (Gliwicka et al. 2013; Nowak et al. 2015). Further analysis indicated the negative impact of *ERF022* on the biosynthesis and signalling of ethylene and the candidate target genes including *ACS7* involved in ethylene biosynthesis, and *ERF1*, which is an essential element in the ethylene signal transduction pathway (Nowak et al. 2015). The inhibitory effect of ethylene on SE induction that was observed in Arabidopsis was postulated as resulting from the negative impact of ethylene on biosynthesis and the local distribution of auxin (Bai et al. 2013). In support of this postulate, auxin accumulation following the LEC2-mediated activation of the *YUC*-dependent pathway of IAA production was found to be required for SE induction in an Arabidopsis culture (Wójcikowska et al. 2013). The interactions between *ERF022* and *LEC2*, which is the key regulator of auxin-dependent embryogenic transition in Arabidopsis, were also demonstrated to be important for the role of *ERF022* in the genetic network that underlies SE induction (Nowak et al. 2015).

5.4.2 TFs that Control LEA Accumulation

LEA proteins are accumulated in the late stages of ZE and the increased expression of *LEA* and other genes that encode storage proteins was found to be associated with SE induced in numerous plants including soybean (Thibaud-Nissen et al. 2003), maize (Che et al. 2006), rape (Hosp et al. 2007), potato (Sharma et al. 2008) and Arabidopsis (Gliwicka et al. 2012). As a consequence, it was postulated that the increased tolerance to stress that is caused by an accumulation of storage proteins promotes the induction of embryogenic development (Stone et al. 2008).

Consistent with this hypothesis, several TF genes that are presented below appear to promote SE via accumulation of the storage proteins. Among such genes are those encoding the proteins of the MYB family that regulate the transcription of the target genes through a highly conserved DNA-binding domain, which is homologous to animal c-MYB (Dubos et al. 2010). The functions of two of the *MYB* genes, *MYB118* and *MYB115*, support the proposition that a storage protein-related mechanism might be considered in SE promotion. *MYB118* and *MYB115* were indicated to have stress-related functions, and their SE-promoting activity in the seedlings and root explants of Arabidopsis was reported (Wang et al. 2008). Relevant to the concept on the positive relation between the storage proteins and the embryogenic potential of tissue, *MYB118* and *MYB115* were documented to positively control *LEA* (*LATE EMBRYOGENESIS ABUNDANT*) genes, including *EM1*, *EM6*, *EM10*, *LEA76* and *ECP63* in ZE (Zhang et al. 2009). In addition to the stimulation of LEA production, *MYB118/MYB115* were recently reported to negatively control bezonyloxy glusosinolate biosynthesis, which is a secondary metabolite produced in response to stress (Zhang et al. 2015). This finding provides further support to the stress-related functions of *MYB115/118* in SE induction.

Another TF gene, *bHLH109*, which is a member of bHLH family, is also assumed to promote SE induction in Arabidopsis via the activation of *LEA* genes. The strong activation of *bHLH109* expression was found to be associated with SE induction, and the overexpression of this gene was indicated as enhancing the embryogenic response of Arabidopsis explants (Gliwicka et al. 2013). Recently, it was postulated that *bHLH109* might operate in SE as an activator of the *LEA* gene *ECP63*, and the TF genes that were annotated to stress-related functions (*At5g61620*, *bZIP4* and *bZIP43*) were indicated to be among the potential regulators of *bHLH109* (Nowak and Gaj 2016).

Collectively, the identification of the TF genes that control the LEA proteins of stress protective function among the SE regulators provided new evidence that the cell responses to stress that are imposed under in vitro conditions underlie the promotion of SE.

5.4.3 WIND1

WOUND INDUCED DEDIFFERENTIATION 1 (*WIND1/RAP2.4*) of the AP2/ERF TF superfamily, which positively regulates cell dedifferentiation in Arabidopsis, was found to be induced by wounding (Iwase et al. 2011). An elevated *WIND1* expression was demonstrated to be sufficient to promote unorganised cell proliferation and the redifferentiation of the callus into roots, shoots and embryos on a hormone-free medium. *WIND1*-overexpressing explant cells were demonstrated to reacquire pluripotency and the modulation of the cytokinin biosynthesis/signalling through ARR-dependent signalling pathway was proposed as being associated with the SE-promoting functions of *WIND1* (Iwase et al. 2011). Other molecular elements that link the WIND1-mediated initial wound response to the control of cell dedifferentiation needs to be revealed.

5.5 *PLETHORA* Genes—The Integrators of Hormonal Inputs

The AINTEGUMENTA-LIKE (AIL) family of TF genes, which have an AP2/ERF domain, includes the *AINTEGUMENTA* (*ANT*), *BABY BOOM* (*BBM/PLT4*) and *PLETHORA* genes. All of these are expressed in young, dividing tissue and play central roles in different developmental processes including embryogenesis (Elliott et al. 1996; Klucher et al. 1996; Boutilier et al. 2002; Aida et al. 2004; Galinha et al. 2007). The importance of the *AIL* function and its relation to auxin in zygotic embryo development was indicated (Aida et al. 2004; Blilou et al. 2005). Besides auxin, *AIL* genes were reported to be related to ABA, GA and JA signalling and thus, *PLT/BBM* genes were postulated to integrate multiple hormonal inputs in the plant development and to act as 'hubs in a plethora of networks' (Horstman et al. 2014).

Similar to *BBM/PLT4*, other *PLETHORA* TFs (*PLT1*, *PLT2*, *PLT3*, *EMK/PLT5* and *PLT7*) have also been indicted to exert the SE-related functions because the somatic embryo formation in response to their overexpression was observed (Tsuwamoto et al. 2010; Horstman 2015). Although our knowledge about the *PLT*-mediated induction of SE is rather fragmentary, a recent finding that *PLT3*, *EMK/PLT5* and *PLT7* stimulate auxin biosynthesis through the activation of *YUC* genes (*YUC1* and *YUC4*) to control phyllotaxis (Pinon et al. 2013) implies a possible role of *PLTs* in the auxin-related mechanism of SE induction. An *EMK/PLT5*-controlled induction of SE may also be related to GA and an encoded TF was reported to negatively impact GA biosynthesis in the control of the storage protein accumulation in Arabidopsis seeds (Sundaram et al. 2013).

Regulatory interactions between the *PLT* genes and other TFs that play key roles in SE may be expected. In support for this assumption, the activation of the *LEC*

genes (*LEC1*, *LEC2*, *FUS3*) that have essential functions in SE (Gaj et al. 2005) was found to be associated with the overexpression of *PLT2* and SE induction (Horstman 2015). The effect of *PLT2* overexpression is dose dependent, and its high expression exclusively leads to the formation of a somatic embryo (Horstman 2015). Along with the central role of *PLT2* in the embryonic root development during ZE (Horstman et al. 2014), the gene was recently shown to be involved in the formation of the root stem cell niche in the embryogenic callus (Su et al. 2015).

5.5.1 BBM

BBM/PLT4, the best characterised *PLT* gene, which was identified in the microspore-derived embryogenesis in *B. napus,* was found to produce somatic embryos on a hormone-free medium as a result of its overexpression, and relevant to this observation, it was suggested that the encoded TF may stimulate the production of auxin or increase a cell's sensitivity to this hormone (Boutilier et al. 2002). The identification of BBM-binding sequences during SE in Arabidopsis revealed the targets that are related to the biosynthesis (*YUC3*, *8*, *TAA1*), transport (*PIN1*, *4*) and signalling (*ARF2*, *10*, *IAA2*, *7*, *28*) of auxin. Among the direct targets of BBM during SE, the *LEC* genes that have documented SE-promoting functions were also proposed, which implies a linkage between the BBM- and LEC-mediated SE pathways (Horstman 2015).

The phenotypes that are related to *BBM* overexpression were indicated to be dosage- and context dependent, and accordingly, a model of AIL functions has recently been proposed (Horstman 2015). According to this model, SE induction requires a high level of AIL transcripts and the mode of the embryogenic pathway that is triggered depends on the developmental stage of the seedling. Direct SE is induced when *BBM* is activated before or during seed germination, whereas post-germination activation of the gene leads to the indirect pathway of SE induction. Analysis of BBM targets revealed the gene's involvement in the positive control of cell division, cell wall modification and the differentiation of plant organs (Passarinho et al. 2008; Nic-Can et al. 2013).

The *HD-ZIP IV/HOMEODOMAIN GLABROUS* (*HDG*) TFs, which are expressed in the L1 layer of meristems and specify an epidermis identity, were reported within the potential targets of BBM (Takada et al. 2013). *BBM* and *HDGs* are co-expressed during early ZE and their transcripts were found to promote cell divisions and differentiation, respectively (Horstman et al. 2015). The antagonistic functions of these genes are also observed in SE where the downregulation of *BBM* and the overexpression of *HDGs* result in a reduced embryogenic response of cultured explants. The overexpression of *HDG1* leads to the development of highly differentiated cells along the margin of the cotyledons and leaves due to the downregulation of cell proliferation genes including the D-type cyclin CYCD3;1. In contrast, the cotyledons of 35S::BBM transgenic seedlings consist of small,

undifferentiated cells that are able to produce somatic embryos. BBM and HDG1 have common target genes that might be antagonistically regulated or co-regulated, i.e. *PLT5* is activated by BBM in contrast to HGD1 (Horstman et al. 2015).

5.6 *WOX* Genes

The *WOX* (*WUSCHEL-RELATED HOMEOBOX*) genes form a plant-specific subclade of the eukaryotic homeobox TF superfamily whose members display the specialised functions that are related to either the promotion of cell division and/or the prevention of the premature cell differentiation. Accordingly, WOXs repress or activate their targets depending on the cell type and developmental stage (reviewed in van der Graaff et al. 2009). Fifteen members of *WOX* (*WUS* and *WOX1-14*) family were identified in the Arabidopsis genome, but only a subset of these has yet been characterised in detail. The activity of *WOX* genes that is specific to the tissue and the developmental process was reported. Consequently, in order to maintain stem cells in Arabidopsis, *WOX5* has to be expressed in RAM (Sarkar et al. 2007) and *WOX4* in the cambial meristem (Hirakawa et al. 2010). *WOX2, WOX8* and *WOX9* transcripts accumulate in the early stages of ZE in *Arabidopsis* and *P. abies* to control the polarity of cell divisions (Ueda et al. 2011; Zhu et al. 2014b). *WOX9* regulates cell divisions in SAM and acts upstream of *WUS* (Wu et al. 2005).

Consistent with the *WOX* activity in ZE (Hacker et al. 2004), the expression of the *WOX* family members was also indicated to be associated with somatic embryo development. In an embryogenic culture of *P. abies*, *WOX2*, *WOX8* and *WOX9* were transcribed in the early stage of the somatic embryo and later in the development; the expression of *PaWOX2* was visible in the basal part of the developing embryo while the *PaWOX8/9* transcripts marked the future RAM and the sites of the initiation of the cotyledon (Palovaara et al. 2010). In accordance with this finding, a reduced expression of *WOX8* and *WOX9* was found to result in the aberrant development of somatic embryos because of the deregulation of the cell divisions that were related to the downregulation of the *PaE2F* and *PaCYCBL* genes that control the cell cycle progress (Zhu et al. 2014b).

5.6.1 *WUS and WOX5 in Control of the Apical–Basal Axis of the Embryo*

The *WUS* gene that encodes the WUSCHEL protein was identified as a positive regulator of the stem cells in the SAM formation through the control of the meristematic cell number (Mayer et al. 1998). Parallel to the activation of floral patterning, the encoded TF was also indicated to repress the stem cell regulation and this bifunctional mode of activity placed the WUS TF among the developmental

regulators with unique functions (Ikeda et al. 2009). The role of *WUS* in the promotion of the vegetative-to-embryogenic transition was uncovered in a culture of an Arabidopsis mutant that produced somatic embryos on root explants that were cultured on a hormone-free medium (Zuo et al. 2002a). Moreover, the *WUS* overexpression was indicated to compensate the requirement of auxin treatment in SE induction in *Capsicum chinense* and *Coffea canephora* (Solís-Ramos et al. 2009; Arroyo-Herrera et al. 2008) and to enhance the embryogenic potential in an embryogenic culture of *G. hirsutum* (Zheng et al. 2014).

WUS together with *WOX5* were found to play a key role in the origin of the apical–basal pattern of the shoot–root axis in the zygotic embryos of Arabidopsis and the establishment of SAM and RAM, respectively (Jürgens 2001; Friml et al. 2003). Both genes were also recently demonstrated to specify the establishment of apical–basal polarity during formation of somatic embryos in Arabidopsis; however, some remarkable differences were noticed in comparison to ZE. In contrast to the distinct spatiotemporal separation of the *WUS* and *WOX5* expression that underlies the formation of the opposite embryo poles in early ZE, *WUS* and *WOX5* were simultaneously activated in nearly overlapped callus cells in the embryogenic culture of Arabidopsis, thus implying that the stem cell niches of the SAM and the RAM are developmentally related during SE initiation (Su et al. 2015).

Expression of *WUS* in SAM regeneration in vitro is positively affected by auxin or cytokinin depending on the mode of the morphogenic pathway that is induced. Auxin was found to stimulate *WUS* activity during SE induction (Su et al. 2009) and cytokinin was reported to enhance the gene expression in the regenerating shoots of root-derived cultures (Gordon et al. 2009). Some evidence implies that the mechanism of the WUS-mediated hormonal regulation of SE initiation differs from shoot and root regeneration that is induced separately; however, the genetic interactions that determine this difference need further investigations (Su et al. 2015).

In controlling SAM, WUS directly represses the transcription of the *ARABIDOPSIS RESPONSE REGULATOR* genes (*ARR5*, *ARR6*, *ARR7* and *ARR15*), which act in the negative feedback loop of cytokinin signalling (Leibfried et al. 2005). The differential expression of *ARRs* that is observed during SE of Arabidopsis (Gliwicka et al. 2013) and *M. truncatula* (Imin et al. 2008) provides the possibility for the role of cytokinin signalling in this process; however, the components of the WUS-controlled initiation of embryonic SAM during SE remain unknown. Cytokinin signals and *WUS* were postulated to reinforce each other through multiple feedback loops (Gordon et al. 2009) and a high specificity of these interactions might be expected. The regulatory relation between auxin and *WUS* might also be assumed and in accordance, auxin treatment was found to be essential for the correct regulation of *WUS* expression during somatic embryo induction in Arabidopsis (Su et al. 2009). In addition, *WUS* appears not to interact with the auxin metabolism during SE because the content of IAA was not modulated in response to *WUS* overexpression in the embryogenic callus of cotton (Bouchabké-Coussa et al. 2013). The understanding of the interactions between the endogenous

hormones and *WUS* expression might contribute to the application of this TF in the genetic improvement of plants with a poor capacity for in vitro regeneration, as was demonstrated in *C. chinense* (Solís-Ramos et al. 2009).

5.7 Conclusions

The central role of the transcriptional regulation in the control of the embryogenic transition of somatic cells has been recently documented. However, in contrast to the spectacular progress on the identification of TFs that are decisive for the reprogramming of differentiated cells into totipotent stem cells that has been made in animals, much less is known about these master regulators in plant cells. In the last 10 years intensive analysis of the global transcriptomes of plant cells that are undergoing embryogenic transition and the use of Arabidopsis (a model in plant genomics) in studies on the genetic control of SE have substantially contributed to the identification of the TF regulators of SE. As a result, dozens of TF genes that are differentially expressed in embryogenic cultures have been identified that provide a base in searches for other genetic elements of their decisive roles in SE induction. So far, only a small subset of the potential SE regulators has been verified experimentally. The emerging picture of a TF-controlled process of SE induction shows a complex network of genetic interactions in which the transcriptional regulation of hormone and stress responses appears to play a fundamental role (Fig. 5.2). It is also apparent that the majority of the already identified SE-involved TF genes are also critical for the development of zygotic embryos. Thus, the similarities in the regulatory mechanisms that underlie SE and ZE that were expected from early 1990s have recently become evident at the molecular level.

In addition to ZE-associated regulators, TFs that have less obvious functions in the control of SE induction have been recognised. Among them, TF genes that enhance cell tolerance to stress imposed under in vitro conditions including the activators of storage material accumulation such as LEA proteins were found to be essential for embryogenic transition.

In spite of the apparent progress that has been made, it seems that most of the TF genes that have a decisive role in the reprogramming of somatic cells into embryonic ones still remain uncovered. Special efforts should be focused on the identification of the targets of the SE-involved TF genes that operate at the very early stage of embryogenic transition, which is the most intriguing moment in the reprogramming of somatic cells. In order to recognise the early targets of SE-related TFs, new efficient approaches can be applied including the protein-binding microarray coupled with the analysis of co-regulated genes that was recently recommended for exploring the regulatory networks in plants (Franco-Zorrilla et al. 2014). However, given the fact that many variables determine the cellular and developmental context of TF–DNA interactions (Slattery et al. 2013, 2014), the candidate TF targets that are identified using in vitro approaches should be verified as also acting in vivo during SE induction. To meet this requirement, the versatile

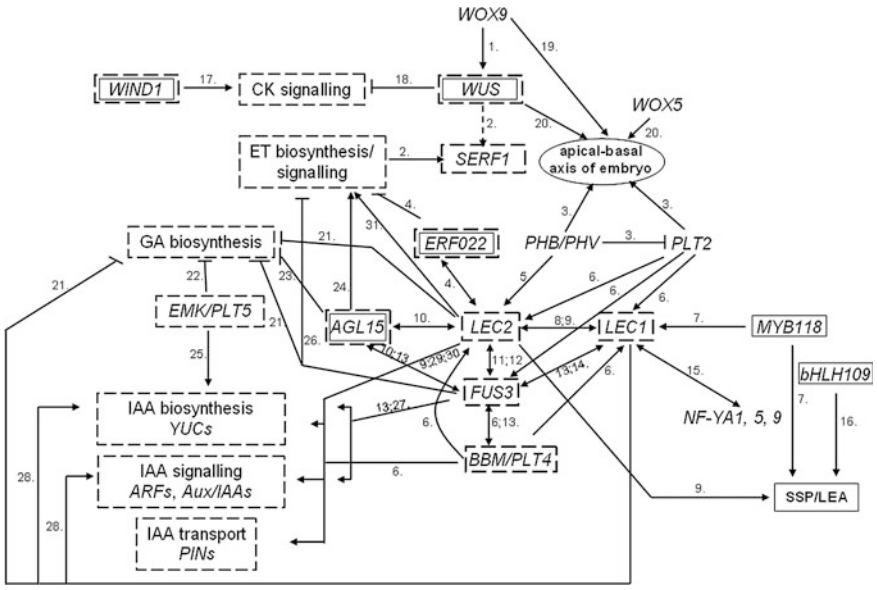

Fig. 5.2 Interaction between SE-related TFs. *Arrows* indicate the activation of the genes expression. The blunt end shows inhibition of gene expression. The *dotted line* indicates the suggested interactions. *Full-line frame* indicates stress-related genes. *Dotted frame* indicates hormone-related genes. *1* Wu et al. (2005); *2* Mantiri et al. (2008b); *3* Smith and Long (2010); *4* Nowak et al. (2015); *5* Tang et al. (2012); *6* Horstman (2015); *7* Zhang et al. (2009); *8* To et al. (2006); *9* Stone et al. (2008); *10* Thakare et al. (2008); *11* Gazzarrini et al. (2004); *12* Yamamoto et al. (2010); *13* Kagaya et al. (2005); *14* Mu et al. (2013); *15* Nowak and Gaj (2016); *16* Iwase et al. (2011); *17* Leibfried et al. (2005); *18* Palovaara et al. (2010); *19* Su et al. (2015); *20* Curaba et al. (2004); *21* Sundaram et al. (2013); *22* Wang et al. (2004); *23* Zheng et al. (2013); *24* Pinon et al. (2013); *25* Lumba et al. (2012); *26* Wang and Perry (2013); *27* Junker et al. (2012); *28* Wójcikowska et al. (2013); *29* Guo et al. (2013); *30* Braybrook et al. (2006). SSP—seed storage protein

molecular tools that are available for functional genomics in Arabidopsis might be helpful. In addition to the dissection of SE-specific TFs and their targets, the recognition of TF regulators, especially the chromatin remodelling factors and miRNAs, is a prerequisite for the full understanding of how already differentiated cells become competent to respond to the embryogenic signal that triggers the developmental switch.

Besides its cognitive value, the efforts that are aimed at the revealing the TF-controlled regulatory network that governs embryonic transition in plants may enable further progress in the genetic improvement of plants (Zuo et al. 2002b). Such perspectives for the use of the TFs that control SE induction in increasing the regeneration potential of some crop species have already been demonstrated for *BBM*, *WUS* and *LEC2* (Arroyo-Herrera et al. 2008; Deng et al. 2009; Solís-Ramos et al. 2009; Belide et al. 2013; Zheng et al. 2014).

Acknowledgements This work was supported by a research grant of the National Centre for Science in Poland (Grant N 2013/09/B//NZ2/03233)

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Chapter 6
Epigenetic Advances in Somatic Embryogenesis in Sequenced Genome Crops

Fátima Duarte-Aké and Clelia De-la-Peña

Abstract Despite the promise of genetic sequencing for the field, more than 100 sequenced plants so far have not shed light on the biological process of somatic embryogenesis. Today it is known that there are differences between SE and its counterpart, zygotic embryogenesis (ZE). SE is impossible to induce in some plants, while others have very reproducible protocols. Advances in molecular biology and the biochemistry behind the SE process indicate that plants share some of the SE-related genes and regulatory pathways. However, the primary difference in response is in the sensing of plant growth regulators. There are plants that need to be exposed to auxins to initiate the SE process, while others do not need any auxin at all. Either way, once the induction has started, gene regulation follows the course and new embryogenic structures start to emerge. One important component of gene regulation is epigenetic modifications, such as DNA methylation and histone modifications. These mechanisms have been studied in different plants, such as monocots and dicots, and differences have been linked to class. In this chapter we will discuss the work done in SE in different sequenced crops of agronomic importance, including sugarcane, maize, coffee, orange, cacao and others.

6.1 Introduction

Somatic embryogenesis (SE) is the process by which a single somatic cell or a group of somatic cells originate a somatic embryo that develops in several distinct stages. These stages are labeled globular, heart-shaped and cotyledon-shaped, in cotyledon species; globular scutellar and coleoptilar stages in monocots; and globular, early cotyledonary and late cotyledonary embryos in conifers. SE is an important tool for clonal propagation of important economical and agronomical species (Loyola-Vargas and Ochoa-Alejo 2012). The success of this technique lies

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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_6

in the high number of embryos produced for explant, which can be converted into functional plants. The switching of development that suffers a somatic cell to regenerate to a complete plant involves several events associated with molecular recognition of internal and external signals (Nic-Can et al. 2015, b; Joshi et al. 2013). This eventually results in specific gene expression of different families of genes that are involved in plant growth perception, endogenous homeostasis of growth regulators, transduction signal and morphogenic response (Yang and Zhang 2010).

Environmental factors and the conditions of the growth culture are inherent factors that control the interactions among epigenetic mechanisms during SE (Nic-Can et al. 2015; Nic-Can and De-la-Peña 2014; De-la-Peña et al. 2015). The epigenetic mechanisms, such as DNA methylation, post-transduction covalent modifications of histones, and small RNAs, act together to regulate gene expression (Wang et al. 2015; Us-Camas et al. 2014; Allis et al. 2015).

We are close to understanding the role of epigenetic mechanisms during SE in different model plants; however, not all findings can be applied to non-model plants, even within the same genus (Nic-Can et al. 2015), or to plants with different metabolisms (Duarte-Aké et al. unpublished data). In this chapter, we discuss the work done in SE to apply epigenetic understanding to sequenced plants of agronomic relevance, such as sugarcane, maize, coffee, orange, cacao, beans and others.

6.2 Genes Involved in Somatic Embryogenesis

In plant biology, zygotic embryogenesis (ZE) is a model to study the expression of genes and translated proteins in the signal response during embryo development. However, SE has been found to be a useful tool as well, and can probe different questions surrounding these phenomena, and in a more controlled way. Due to the advantages of SE for the culturing of thousands of crop plants of economical importance, a field of study has been opened to understand the regulatory processes involved during the initiation and progress of different embryo developmental stages. The most studied genes regarding these processes are listed below.

6.2.1 SERK1

SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (*SERK*) is a transmembrane receptor that has been implicated in triggering embryogenesis. It was isolated for the first time from *Daucus carota* suspension cells, where expression was up regulated in the globular stage of embryogenesis (Schmidt et al. 1997). SERK is activated via autophosphorylation, specifically in the residue threonine 468 (Shah et al. 2001), glutathione S-transferase fusion and in vitro phosphorylation assays. SERK traduces the signal from cell membrane to action site, regulating the

subsequent downstream proteins in the signal transduction pathway. To date, different experiments suggest the pivotal role of this protein in the initiation of embryogenic competence. For instance, the ortholog *SERK* of Arabidopsis enhances the ability to undergo the somatic embryo response (Hecht et al. 2001). The histochemical and immunochemical techniques using *β*-glucuronidase detection have revealed the spatial localization of gene expression during indirect somatic embryogenesis. The expression was detected in four places: in embryogenic callus, where the embryos were developed, in the basal parts of the embryos, in the outer layers of cotyledons and in the provascular and vascular strands of developing somatic embryos.

6.2.2 *LEC*

The *LEAFY COTYLEDON* (*LEC*) genes are classified into two classes. The first class are HAP3- related transcription factors, represented in Arabidopsis by LEC1 and L1L (Lotan et al. 1998; Kwong et al. 2003). The second class encodes B3 domain transcription factors, which are represented by LEC2, FUS3, and ABI3 in Arabidopsis (Stone et al. 2001, 2008; Mönke et al. 2012; Luerßen et al. 1998). Among the *LEC* genes, *LEC1* y *LEC2* have been suggested to have complementary and partial redundancy to induce SE. They have similar but not identical functions, as *lec1lec2* double mutants have synergistic phenotypes (West et al. 1994; Lotan et al. 1998; Stone et al. 2001). Conversely, the overexpressing phenotypes of LEC1 seedlings arrest embryo-like seedlings and fail to develop; cotyledon-like organs sometimes form in place of the first leaves (Lotan et al. 1998). *LEC1* has been associated with the maturation of embryos because in *LEC1* mutants the phenotype is defective and the conversion from cotyledons to leaf-like structures does not take place. In *LEC2*, the embryo-like seedlings continued to proliferate, producing callus, cotyledon-like structures and leaf-like organs in addition to the SE. Transgenic plants expressing *LEC2* ectopically develop somatic embryos, which have cotyledon-like and leaf-like structures (Stone et al. 2001).

6.2.3 *AGL15*

AGAMOUS-LIKE15 (AGL15) belongs to the MAD-box proteins family, which is involved in the control of flowering time, homeotic regulation of floral organogenesis, fruit formation and seed pigmentation (Pařenicová et al. 2003; Rounsley et al. 1995). The role of AGL15 during embryo development was studied in *Arabidopsis* and *Brassica napus*. In Brassica embryos, embryonic tissues, and early and late globular stages, a great accumulation of mRNA of *AGL15* was demonstrated by in situ hybridization techniques, suggesting a regulatory role during embryo development. In other species the presence of *AGL15* has been detected

during embryo development. For instance, in the soybean, GmAGL15 was present in young embryos and the highest expression was detectable in somatic embryo cultures (Thakare et al. 2008). Furthermore, this transcription factor promotes the development of somatic embryos and enhances embryonic tissue production in Arabidopsis (Harding et al. 2003).

6.2.4 *BBM*

BABY BOOM (BBM), which is a member of the APETALA2-Ethylen responsive factor family (AP2-ERF), is a transcription factor involved in cell proliferation and morphogenesis during embryogenesis. BBM was isolated from an androgenic system in *B. napus*, where it is expressed during seed and embryo development (Boutilier et al. 2002). When this gene is overexpressed ectopically in *Arabidopsis* and *B. napus*, it can promote somatic embryos. The ectopic expression of *BBM* leads to spontaneous embryo formation from seedlings, as well as ectopic shoots and calli at lower frequency (Boutilier et al. 2002). In tobacco, the heterologous expression of *BBM* promotes the formation of both callus and shoots (Srinivasan et al. 2007).

6.2.5 *CLV*

There are three *CLAVATA* (*CLV*) genes that are important for meristem development; these are *CLV1*, *CLV2* and *CLV3* (Nikolaev et al. 2007). *CLV1,* which encodes to a transmembrane receptor kinase, promotes the progression of meristem cells toward organ initiation throughout a signal cascade for the initiation of organogenesis (Clark et al. 1996, 1997). *CLV2* encodes to a receptor-like protein and is required for the accumulation of CLV1 with which to form a heterodimer useful for signaling transduction (Jeong et al. 1999). In the case of *CLV3*, this gene is expressed in the central zone cells of the shoot apical meristems and is a key regulator for shoot and meristem development (Clark et al. 1995).

6.2.6 *WUS*

WUSCHELL (*WUS*) encodes to a transcription factor that directly regulates other genes (Lenhard and Laux 1999). WUS is a homeodomain protein that promotes SE when it is ectopically expressed (Zuo et al. 2002). Its principal function is to maintain the undifferentiated state of cells and in response to different stimuli to switch the developmental fate of tissues (Gallois et al. 2004).

During somatic embryogenesis of *Picea abies*, a member of the *WUS* genes, *PaWOX2*, is highly expressed in the early stage and its expression gradually declines when the embryos mature. *PaWOX2* expression can be used as a marker for embryogenic potential in embryogenic cell cultures (Palovaara and Hakman 2008). Furthermore, the expression of *WUS* depends on other factors such as proteins (e.g., CLAVATA) and growth regulators (e.g., auxins). For example, in *Medicago truncatula*, the overexpression of *WOX5* provokes a highly embryogenic callus formation when the explant is cultured in the presence of auxin, whereas without auxin in the medium, the embryo formation is direct. In both cases the expression of *WOX* genes stimulates embryo formation in the presence and absence of auxin (Imin et al. 2007).

6.2.7 GH3

GRETCHEN HAGEN 3 (GH3) genes, found in many plants (Terol et al. 2006), were discovered in *Glycine max* in response to auxins (Hagen et al. 1984). Evolutionary data show that GH3 proteins descend from a common ancestral chromosome before the eudicot/monocot splits (Okrent and Wildermuth 2011). GH3 genes have important roles in the regulation of the stress response in Arabidopsis (Park et al. 2007) and have been classified into three main groups (Chen et al. 2010). Group 1 contains AtGH3.11 (JAR1/FIN219), which adenylates jasmonic acid (JA), and AtGH3.10 (DFL2) (Staswick and Tiryaki 2004). On the other hand, AtGH3.2 (YDK), AtGH3.5 (AtGH3a), AtGH3.6 (DFL1), and AtGH3.17, which belong to group 2, adenylate indolacetic acid (IAA) and conjugate with salicylic acid (SA) (Staswick et al. 2005, 2002). Furthermore, AtGH3.5, AtGH3.6 and AtGH3.17 are suggested to be targets of the auxin response factor 8 (ARF8) (Tian et al. 2004). Group 3 of the GH3 genes contains AtGH3.9, AtGH3.12 and AtGH3.17; both AtGH3.9 and AtGH3.17 are activated by IAA (Okrent and Wildermuth 2011).

6.2.8 ARF

AUXIN RESPONSE FACTOR (ARF) encodes a transcription factor that activates minutes after auxin stimulation (Ulmasov et al. 1999; Smit and Weijers 2015). There are 23 *ARF* genes in Arabidopsis and they have different functions (Okushima et al. 2005; Remington et al. 2004; Guilfoyle and Hagen 2007; Smit and Weijers 2015; Hamann et al. 2002; Weijers et al. 2006; Weijers and Jürgens 2005). For instance, *ARF 1* and *3* are involved in fruit development (de Jong et al. 2009; Guillon et al. 2008; Goetz et al. 2007); *ARF2* in senescence (Lim et al. 2010); *ARF 5*, *17* and *23* in embryogenesis (Weijers et al. 2006; Hamann et al. 2002); and *ARF 6* and *8* in the expansion of leaves (Nagpal et al. 2005; Tian et al. 2004). Moreover,

ARF 7, *10*, *16* and *19* have been shown to be involved in lateral root formation and development (Marin et al. 2010; Okushima et al. 2007; Tatematsu et al. 2004), while *ARF 12*, *13*, *14*, *20*, *21* and *22* respond to other plant hormones such as ethylene (Li et al. 2006), brassino steroids (Vert et al. 2008) and abscisic acid (Yoon et al. 2010; Xie et al. 2015).

6.2.9 PIN1

PIN-FORMED (PIN) proteins are involved in the polar auxin transport across cell membranes (Petrášek et al. 2006; Petrášek and Friml 2009; Křeček et al. 2009). In Arabidopsis, the PIN family is formed by eight genes, which are divided in two subclades. In the first subclade there are the canonical PINs, consisting of *PIN1-4* and *PIN7*. These are localized in the plasma membrane and function as auxin efflux transporters in a direct manner. The second subclade consisting of *PIN5*, *PIN6* and *PIN8*, which are localized in the endomembranes, suggesting their role in auxin distribution and homeostasis in the intracellular compartments (Rodríguez-Sanz et al. 2015). Among the canonical PINs, PIN1 is the protein that has a central role during embryogenesis. PIN1 controls the direction of polar auxin transport in embryo development and the establishment of polarized auxin fluxes, auxin gradients and auxin maxima in the basal and apical regions at defined developmental embryo stages. This protein is accumulated during the earliest developmental stages in the cells that would become embryos. That accumulation moves from nonpolar to polarized and then to the basal side of the provascular cells once the early globular stage is reached (Friml et al. 2003). In addition to *PIN1*, *PIN4* is also expressed in the proembryo at the globular stages and is functionally redundant to *PIN1* in the seedling stage (Vieten et al. 2005; Weijers et al. 2006).

6.2.10 LEA

LATE EMBRYOGENESIS ABUNDANT (*LEA*) genes are expressed only in the later stages of embryo maturation, a fact which has been used as a molecular marker to discriminate between direct and indirect somatic embryogenesis (Corre et al. 1996).

6.2.11 STM

The genes *SHOOTMERISTEMLESS (STM)* and *CLAVATA 1 (CLV1)* were isolated from *Brassica* species, and ectopically expressed in Arabidopsis to understand the embryogenic process (Elhiti et al. 2010). The ectopic expression of these orthologous genes affects embryo production in vitro. The ectopic expression of *BoSTM*,

BrSTM and *BnSTM* increased the number of somatic embryos obtained, whereas in ectopic expression of CLV1, the embryo yield was repressed. Therefore, the antagonistic expression of these genes is necessary for SE coordination.

6.3 Epigenetic Studies on Sequenced Plants

The sequencing of plant crop genomes is a helpful tool to understand the process of SE, and when these genomes are shared in public databases, gene sequence searches become effective ways to analyze differential regulation. The massive sequencing methods applied to generate genome databases of economically important species have helped us to understand the evolutionary aspects of different genes among plants, as well as the loss or gain of certain genes or groups of genes. Although transcriptomics allows the identification of important genes that are up or down regulated in response to different environmental conditions, the mechanism of regulation is unknown at this level.

The study of epigenetics contributes a new level of understanding of the regulation of gene expression throughout chromatin conformation. DNA methylation, histone post-translational modifications and the microRNA (miRNA) mechanism are now enabling us to uncover gene regulation during the embryogenic response. It is known that SE is difficult to attain in some species due to genetic or epigenetic variability (Schaffer 1990; Miguel and Marum 2011; Phillips et al. 1994; Kaeppler et al. 2000; De-la-Peña et al. 2015). The micropropagation of small fruits such as cranberry and blueberries, as well as many non-model plants, traditionally problematic. However, the studies listed below have shown that epigenetic regulation can be used in order to improve the SE response. Here we describe studies on epigenetic and SE done in some important sequenced genome species of agronomical importance. Although almost 100 plants have been sequenced already, few have been the species that are propagated by SE and fewer yet are those for which epigenetic mechanisms have been analyzed.

6.3.1 Barley

Hordeum vulgare L. is one of the world´s earliest domesticated crop species and represents the fourth most abundant cereal (http://faostat.fao.org). Barley is resistant to different environmental conditions and for this reason is cultivated and consumed in arid and marginal regions. The improvement and scale production of this crop is performed by in vitro systems (Jähne et al. 1991; Lührs and Lörz 1987). In particular, microspore embryogenesis is an important tool in breeding to obtain double-haploid plants (Mordhorst and Lörz 1993; Solís et al. 2015). During this process, changes in differentiation and proliferation are regulated by DNA methylation (Solís et al. 2015). The study of the DNA methylation dynamic using 5-Aza

have revealed that this drug, at a concentration of 2.5 μm for 4 days, can induce a major embryo production as a consequence of the DNA hypomethylation. In contrast, the longer the treatment with 5-Aza, the lower the embryo production becomes. These results suggest a key role of DNA methylation in totipotency acquisition and microspore reprograming in barley. This can be used as a powerful tool to improve embryo production not only in barley but in other important crops as well.

6.3.2 Common Bean

Phaseolus vulgaris L., the common bean, is the most consumed legume in México. This species is recalcitrant to both SE and in vitro regeneration. For this reason, the study of SE in the induction process is necessary to understand the process and improve the protocols in order to achieve a better regeneration rate. For instance, Barraza et al. (2015) have used a regeneration-competent callus that was successively transformed. These embryogenic calli were regenerated and transformed with a PvTRX1hRiA construction to down regulate the expression of the *PvTRX1h* gene. This gene is an ortholog of a lysine methyltransferase in plants. The low expression of *PvTRX1h* not only altered the concentration of the hormone content in the calli but also affected the expression of important genes involved in auxin biosynthesis. On the other hand, the down regulation of *PvTRX1h* activated the expression of other histone lysine methyltransferases, such as PvASHH2h. These results suggest a crosstalk among histone methyltransferases, with plant regulators signaling for the generation of somatic embryos.

6.3.3 Brassica

In the *STM* overexpression line of *Brassica oleracea* the expression of genes involved in hormone perception and signaling, as well as genes encoding DNA methyltransferases, were affected (Elhiti et al. 2010). On the other hand, pharmacological experiments performed to confirm some of these results showed that Arabidopsis SE is encouraged by a global DNA hypomethylation during the induction (when in presence of 2, 4-D, the cells acquire the competency to form embryonic cells) (Elhiti et al. 2010).

6.3.4 Cacao

Theobroma cacao L. is endemic to South American rainforests and was domesticated approximately three hundred years ago in Central America. This species is a very important tree crop because it is the source of chocolate. One of the main

problems for chocolate production is that the plant is susceptible to many pests and diseases (Iwaro et al. 2006) and the difficult cultivation via apical microcutting for large-scale production has led to an increasing interest in the application of SE for clonal multiplication (Traore et al. 2003). However, cocoa SE carries an elevated risk for genetic mutations, and as a result the genetic and epigenetic variation has been evaluated in this species (Rodríguez López et al. 2010). The simple sequence repeat (SSR) markers and methyl-sensitive amplification polymorphism (MSAP) analysis have revealed high genetic and epigenetic variation, respectively. In the somatic callus was found a possible interaction of DNA methylation with aberrant recombinant events during the embryogenesis, which might allow de novo mutation (Rodríguez López et al. 2010).

6.3.5 *Coffee*

Every day more than 2.25 billion cups of coffee are consumed around the world. Approximately 11 million hectares are cultivated to supply this demand for coffee (Denoeud et al. 2014). There are two economically important species of coffee: *Coffea arabica* and *Coffea canephora*, which represent 70 and 30 % of the coffee produced in the world, respectively (Mondego et al. 2011). In coffee production, SE is applied industrially for large-scale and rapid dissemination of selected hybrids that provide an increase in the yield of high quality coffee, and promises an efficient system for the multiplication of varieties with modified caffeine content (Etienne et al. 2012; Bertrand et al. 2011; Ogita et al. 2003). Studies on the epigenetics in coffee have attempted to understand the embryogenic capacity (Nic-Can et al. 2015, 2013b) and the somaclonal variations present in coffee multiplication (Bobadilla Landey et al. 2015, 2013).

In *C. canephora*, treatment with 5-azacitidine (5-Aza, an inhibitor of DNA methylation) revealed that DNA methylation is important for embryo development by disturbing the expression of important genes involved in SE, such as *LEC1* and *BBM1* (Nic-Can et al. 2013b). Another recent study (Nic-Can et al. 2015) shows that the treatment of *C. canephora* explants of low molecular mass with the conditioned medium fraction from *C. arabica* not only reduced the number of embryos per explant but also affected the DNA methylation levels, thus revealing the importance of DNA methylation during embryogenic competence for embryo formation. In coffee, SE has also indicated the importance of histone post-translational modifications (Nic-Can et al. 2013a, b). In *C. canephora*, the coordinated expression of genes involved in SE, such as *WOX4*, *LEC1* and *BBM1*, is regulated by the epigenetic marks H3K9me2 and H3K27me3.

6.3.6 Cotton

Gossypium hirsutum is the most important natural textile fiber and its seed is an important source of feed and foodstuff. Cotton breeding is dependent on in vitro systems for scale production (Kim and Triplett 2001). SE is an effective plant regeneration procedure from transgenic cotton propagation (Zhang et al. 2001). Cotton SE is a process that provides an outstanding experimental tool for studying the biochemical and molecular bases of cellular SE in recalcitrant genotypes.

In cotton SE studies there have been identified two phases of chromatin reorganization associated with endogenous auxin/cytokinin dynamic activity that may underlie dedifferentiation and redifferentiation events (Zeng et al. 2007). On the other hand, in studies on miRNAs during the SE of cotton, it was found that 36 differentially expressed known miRNA families and 25 novel microRNAs with 476 genes as targets were involved in the process (Yang et al. 2013). The expression patterns of miRNAs and their targets were validated. For instance, the expression of miRNA 167 and 156 were evaluated and correlated with the expression of the target genes *ARF* and *SPL*, respectively.

6.3.7 Eggplant

Solanum melongena L. is a vegetable crop species, which is genetically important due to its different agronomical qualities, such as extra-large fruit size, high tolerance to biotic and abiotic stresses, and parthenocarpy without any negative pleiotropic effects. These are characteristics that would help to improve other important solanaceum species. Highly effective protocols for in vitro plant regeneration via SE from cotyledon explants are available in eggplant to understand the organogenic and embryogenic process. In the embryogenic process, the changes regulated by epigenetic mechanisms are necessary for embryo generation. One of the most studied mechanisms is DNA methylation. Using isoschizomer restriction enzymes *MspI* and *HpaII,* it was found that the methylation varied widely in the DNA (Bucherna et al. 2001). The sequences that show major changes in DNA methylation levels were in a sequence that has a 91 % similarity to an uncharacterized sequence found in tomato ovaries, so this transcript could have a function in morphogenesis and differentiation. The authors propose that this epigenetic mechanism may play a role in the regulation of gene activity and cell differentiation in embryogenic suspensions.

6.3.8 Grapevine

Vitis vinifera L. is one of the most important cultivars for both fruit and beverage production. Due to the limited natural variability of the cultivars, traditional vine improvement is considered difficult. However, the in vitro embryogenic system is

used as to select improved grapevine phenotypes with specific characteristics, such as vigor, berry size, sugar and acid concentration, and flavor components. The effect of in vitro embryogenesis in the grapevine genotypes was studied in two variants, Chardonnay 96 and Syrah 174 (Schellenbaum et al. 2008). The study reveals that the in vitro conditions cause a few changes in the polymorphism bands. However, the somaclones can conserve their main characteristics, which is a prerequisite for grapevine cultivation. Comparative MSAP analysis between mother plants and somaclones has revealed that the somaclones have slightly higher methylation levels in comparison with mother plants (Baranek et al. 2010).

6.3.9 Maize

Zea mays L. is one of the most important cultivated cereal crops and is used as source of food, livestock feed and raw material for the industry (Huang et al. 2002). Due to its relevant economic importance, maize is a great model for improvement. In maize, the induction of SE starts in a specific balance of environmental conditions, including darkness and growth regulators in the media (Conger et al. 1987).

Recently it was found that the induction process in maize is characterized by an enrichment of small RNA molecules that are involved with SE initiation (Chávez-Hernández et al. 2015). Chávez-Hernández et al. (2015) investigated the accumulation of certain miRNAs, and their predicted targets, during the SE of maize under different environmental conditions. It was found that miRNAs 156, 164, 168, 397, 398, 408, and 528 increased upon growth regulator depletion, while photoperiod conditions increase the expression of the targets *SBP23*, *GA-MYB*, *CUC2*, *AGO1c*, *LAC2*, *SOD9*, *GR1*, *SOD1A* and *PLC*. These results demonstrate that the concentration of growth regulators has an influence on specific miRNA accumulation during SE, while their targets are additionally influenced by the presence of light.

6.3.10 Medicago

The role of DNA methylation in the somatic embryogenesis of legumes was studied using the model plant *M. truncatula*. In a pharmacological study, it was found that the treatment with a Hypermethylation drought 5-Aza in a high embryogenic line provokes a loss of embryogenic capacity, suggesting an important role for DNA methylation in embryogenic capacity (Santos and Fevereiro 2002).

6.3.11 Norway Spruce

Picea abies (L.) Karst is one of the most widespread and ecologically and economically important plants in Europe. In *P. abies*, SE protocols are used to

understand the zygotic process, which is dependent on temperature and regulation by epigenetic mechanisms. For instance, Yakovlev et al. (2014) identify differentially expressed transcripts during the SE of Norway spruce. Their study revealed that there is an epigenetic memory affected by temperature during the embryogenesis process. It was found that the formation of the epigenetic memory induced by warm and cold conditions during SE is accompanied by differential expression of different sets of genes. These included epigenetic machinery-related genes, such as DNA methyltransferases, histone methyltransferases, histone acetylases and histone deacetylases.

6.3.12 Oak

Quercus suber L. is an ecologically and economically important species cultivated in the Mediterranean area. The in vitro culture of *Q. suber* L. via SE is an alternative to conventional methods of reproduction that solve the problem of its long reproductive propagation and irregular seed yield. However, the major bottleneck for its micropropagation is the complete maturation of the embryos. It is known that SE is an event controlled by epigenetic mechanisms additional to the genetic and biochemical regulation. Recently, it was discovered that genes necessary for embryo maturation are under the regulation of histone H3 modifications and chromatin remodeling (Pérez et al. 2015a).

SE in oak is a complex process, where, in addition to epigenetic controls, hormonal pressure plays an important role in embryo maturation (Pérez et al. 2015b). A biochemical and immunohistochemical analysis has revealed that the beginning of embryo maturation is characterized by a peak in the ABA levels, while the acquisition of germinative capacity needs a cold treatment. The germination ability is accompanied by a decrease in ABA levels as well as DNA methylation status in the meristematic areas. This work opens the possibility of using demethylating agents or ABA inhibitors to improve the number and quality of the mature somatic embryos, reducing the time of cultures.

6.3.13 Oil Palm

Elaeis guineensis Jacq., the most efficient African oil palm, has a long life cycle, around 25 years. Because this plant is recalcitrant to vegetative propagation, in vitro propagation methods based on SE have been implemented (Pannetier et al. 1981). However, around 5 % of somatic embryo-derived palms show abnormalities in their floral development, causing a mantled phenotype, which is the feminization of the male parts in flowers of both sexes. This somaclonal variation was observed in different proportion between two types of calli. Five percent of the regenerants from nodular compact calli (NCC) presented mantled phenotype while the fast

growing calli (FGC) produced 100 % of the variant palms. The two types of calli that were used have the same genotype and they were cultured in same conditions. Therefore, this variation was found to be of an epigenetic nature (Jaligot et al. 2000). The epigenetic mechanism involved in this somaclonal variation was recently identified (Ong-Abdullah et al. 2015). The plants with abnormal phenotypes are hypomethylated near the *Karma* transposon splice site, which prevents the correct transcription of the homeotic gene *DEFICIENS,* which is involved in the correct development of the floral parts. This situation was due to embryogenic culturing; however, the discovery of the epigenetic mechanism involved in this somaclonal variation would facilitate the introduction of higher-performing clones and optimize the yield of oil palm production.

6.3.14 Rice

Rice (*Oriza sativa* L.) is one of the most important crop plants of the world, feeding over half of the global population. Due to its economic importance, rice is considered to be a model plant for studies on genomic and epigenetic research. In rice, the analysis of abundances and identification of the differential miRNAs between the non-differentiated state and the differentiated calli has revealed that miRNA 397 is more abundant in the non-differentiated calli, while 156 is found at high levels in the differentiated tissue (Luo et al. 2006). MiRNA 397 has been involved in mature tissues and targeted *LACCASE* genes, which might be vital to maintain the meristematic state in non-differentiated callus. Comparing the sequenced miRNAs between undifferentiated and differentiated calli, it was noticed that around 50 miRNA sequences vary in abundance between the two types of calli, suggesting a differential role in meristem development. Among them, the miRNA 408, 397 and 528, which are strongly expressed in rice seeds, have a significantly higher abundance in undifferentiated calli, in contrast with the differentiated calli. These data suggest that miRNAs would modulate the development of meristems by regulating the expression of crucial target genes involved in the differentiation process (Chen et al. 2011).

6.3.15 Rubber Tree

Havea brasiliensis Willd. is the major commercial source of natural rubber, a latex polymer with high elasticity, flexibility and resilience that is used in the manufacture of over 50,000 products (Nair 2010). The high variation in the plantation of rubber trees is a common problem due to the use of bud grafting for the propagation of the rootstocks (Carron et al. 2009; Omokhafe 2004). Although SE studies have not been performed in this plant, the use of zygotic cleavage polyembryony in immature fruits opens a new avenue for the successful multiplication of rubber

trees. Epigenetic analysis was performed on different samples to determine global DNA methylation using MSAP. However, under these induction conditions no epigenetic variation was found (Karumamkandathil et al. 2015).

6.3.16 Sugar Beet

Beta vulgaris is an important crop of temperate climates. This plant is cultivated on two million hectares worldwide. Sugar beet provides nearly 30 % of the world´s annual sugar production and it is a source for bioethanol and animal feed. Its genome was sequenced in 2014, opening the opportunity for breeding and management of different epigenetic aspects associated with development of this economically important crop (Dohm et al. 2014).

This species is propagated by different protocols of embryogenesis for both multiplication and breeding (Zhang et al. 2008). Among the unexplored elements in the breeding of this plant is the epigenetic changes that occur during in vitro propagation (Kornienko et al. 2014). For instance, in sugar beet, acquisition of competence to generate embryos from an explant, and the subsequent proliferation and morphogenesis events, are dependent on changes in DNA methylation levels. On the other hand, in this species the dynamic connection between plant morphogenesis, cell redox state and the changes in DNA methylation and H3 acetylation marks was evident (Causevic et al. 2005, 2006). Causevic et al. (2006) demonstrated that the distinct morphogenic capacity of tree lines, organogenic (O), non organogenic (NO) and dedifferentiated (DD), are associated with different levels of epigenetic parameters and corresponding enzymes, such as DNMT and HDAC, which catalyze DNA methylation and histone acetylation, respectively. The DD line presents more reactive oxygen species (ROS) and nonenzymatic antioxidant properties. Additionally, this line presented hypermethylation in a key enzyme that is fundamental to oxidative stress, such as the super oxide dismutase (SOD). In the case of the lines O and NO, the level of acetylation in H3 was reduced.

6.3.17 Valencia Sweet Orange

Citrus fruits and juice are the prime human source for vitamin C, an important component of human nutrition. In the aim of improving citrus crops, different biotechnological approaches such as transformation, protoplast fusion, in vitro mutation breeding and SE have been used with success for the regeneration of plants of *Citrus sinensis* (Xu et al. 2013; Gmitter et al. 2012).

Most of the epigenetic studies on SE have been on DNA methylation or histone modifications. However, in the case of SE in citrus, the small RNA has been extensively studied. The abundance and presence of certain micro RNAs (miRNAs)

and the effect on predicted targets have contributed to the understanding of plant cell totipotency as well as embryogenic capacity of somatic cells. For instance, in *C. sinensis* around ten conserved plant miRNAs were detected and sixteen genes were predicted to be targeted by six miRNAs (Wu et al. 2011). Stage- and tissue-specific expressions of miRNAs and their targets suggest the involvement of these small molecules in the modulation of SE. During the induction process, the abundance of miRNA156, 168 and 171 is necessary for embryogenic competence and for embryo formation. In non-embryogenic callus, these microRNAs are not present, or their abundance is low (Wu et al. 2011). Interestingly, miRNA156 has been linked to the control of the change of phase in plant development in Arabidopsis by regulating the expression of the *Squamosa promoter-binding-like* (*SPL*) gene family (Wu and Poethig 2006). In *C. sinensis*, this miRNA regulates the expression of *SPL 2*, *4*, *5* and *9*, allowing embryonic competence acquisition in callus cells (Wu et al. 2011). The miRNAs 159, 164, 390 and 397 were related to globular-shaped embryo formation while miRNAs 166, 167 and 398 were required for cotyledon-shaped embryo morphogenesis (Wu et al. 2011).

The contrasting abundance of differential small RNAs between calli that are competent to form embryos and those that are not is evident in sweet orange. According to the high-throughput sequencing (HTS) analysis of small RNAs and RNA degradome tags, 50 known and 45 novel miRNAs were identified, as well as 203 target genes (Wu et al. 2015). The abundance if miRNAs in embryogenic callus were lower than in non-embryogenic callus, suggesting a possible repression of important transcripts in target genes, activating the biological processes required for differentiation.

6.4 Conclusions

Many crop plants have been sequenced; however, not all have been propagated using SE. In the current crisis of global warming and declining arable soil, it is urgent to secure the future of food availability. The use of SE is a powerful tool that can be exploited in plants used for human consumption. In difference to other methodologies, SE does not need foreign genes or expensive and sophisticated technologies. It takes what the plant is programmed to do already and manipulates it by using epigenetic regulation under specific conditions. The use of drugs such as 5-Aza, which has been found to promote SE and increase the number of embryos produced, could help to solve the problems of multiplication rates in some SE protocols. The three epigenetic mechanisms studied in many of the plants discussed in the present chapter reveal the importance of DNA methylation, histone modifications and miRNAs to gene regulation, morphogenesis and embryo production.

Acknowledgments This work was supported by a grant received from the National Council for Science and Technology (CONACyT 178149 to C.D.P.). F.D.A was supported by a scholarship (255368) from CONACyT.

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Chapter 7
The Current Status of Proteomic Studies in Somatic Embryogenesis

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Abstract Somatic embryogenesis includes the genetic reprogramming of somatic cells to acquire the embryogenic potency necessary to generate an embryo, which can develop into a whole plant. Acquisition of embryogenic capacity requires rigorous biochemical coordination that includes several metabolic and signal transduction pathways. Recent genomic and epigenetic studies in somatic embryogenesis have shown interconnection among signals associated with growth regulators, stress factors, and modulation of the genome structure. A broad range of key proteins, posttranslational modifications, protein turnover, and protein–protein interactions are common factors associated with the establishment of the necessary biochemical status of cells during the acquisition of the embryogenic potential. Recent proteomic studies have begun describing the molecular basis of somatic embryogenesis. However, the diversity of the embryogenic response among plant species makes it difficult to define key protein factors associated with embryogenic cultures or specific stages during the transdifferentiation of somatic embryos. In this chapter, we review the most prominent proteomic studies carried out in the past

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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_7

decade and discuss the contributions of proteomics studies to elucidating the molecular basis of somatic embryogenesis.

7.1 Introduction

The capacity of a somatic cell to acquire embryogenic competence and generate a new plant by somatic embryogenesis (SE) has been studied for several decades. Strategies that have been used to induce SE across plant species include: different types of explant, modification of the culture media (addition, subtraction, and combination of culture media components), plant growth regulators (different types, concentration, and combinations), culture conditions (different wavelength of light, dark culturing, and ventilation), and the application of culture media coming from embryogenic cultures (conditioned media). These studies have provided empirical information, which has identified key physiological cues that lead to embryogenic potential, although specific culture condition requirements vary with species. In addition, microscopy studies in several plant species defined key morphological characteristics associated with embryogenic cultures (Popielarska-Konieczna et al. 2008; Xu et al. 2011; Kikuchi et al. 1995; Sterk et al. 1991; Steinmacher et al. 2012; Pennell et al. 1992). However, the molecular bases of induction, embryo development, maturation, and germination are not well-known. Several studies have shed light on the important roles of growth regulators, protein receptors, and transcription factors (Martin 2004; Zeynali et al. 2010; Cheung and Wu 2011; Wolf and Hofte 2014; Karami et al. 2009). Recently, with the surge of "omics" technologies we have been able to generate a more comprehensive panorama of the genome-wide expression profiles during SE (Silva et al. 2014; Noah et al. 2013; Gomez-Garay et al. 2013; Salvo et al. 2014; Hoenemann et al. 2010; Rode et al. 2011, 2012). These comprehensive studies allow us to identify specific modifications of genome architecture through epigenetic regulation during SE (Feher 2015; Nic-Can et al. 2013; Imin et al. 2005). Furthermore, proteomic studies of SE in several species have created protein profiles of cultures in different conditions during the induction of embryogenic potential (Varhanikova et al. 2014; Correia et al. 2012; Marsoni et al. 2008; Sharifi et al. 2012; Almeida et al. 2012; Guzman-Garcia et al. 2013). These studies have been followed up with the focused identification of key proteins associated with each stage of the transdifferentiation of somatic embryos (Rode et al. 2012; Bian et al. 2010; Vale Ede et al. 2014). Moreover, the morphological and physiological similarities between zygotic and somatic embryogenesis have suggested similar biochemical status for cells undergoing both of these processes, and protein profiles for zygotic and somatic embryogenesis have been compared in a few plants species (Sghaier-Hammami et al. 2009; Winkelmann et al. 2006; Noah et al. 2013). Altogether, the proteins identified in several conditions and stages of SE reflect the biochemical status of cells. These biochemical statuses suggest that cells need to overcome stress conditions during genome reprogramming and the development of SE (Smertenko and Bozhkov 2014; Feher 2015; Zavattieri et al. 2009). However, the technical difficulties of establishing somatic embryogenesis,

such as the differential response among plant species, the asynchronous transdifferentiation of the somatic embryos and the participation of several types of cells, increase the complexity of proteomics studies. Moreover, the key proteins associated with cellular division, expansion, and differentiation are expressed in very narrow windows of time in each stage of SE. Regulatory proteins such as receptors and transcription factors are mostly present in low abundance in a specific subcellular compartment (Gupta et al. 2015; Qi and Katagiri 2009; Chen et al. 2001; Van Leene et al. 2007; Smaczniak et al. 2012).

In this chapter, we review the important proteomics studies associated with somatic embryogenesis during induction, embryo transdifferentiation, and maturation. We mention the major contribution of proteomic technology to understanding the basis of the molecular foundation of SE and we discuss the current proteomics tools suitable for overcoming the hurdles of analyzing the proteome of SE.

7.2 Induction Stage: The Starting Point

The starting point of somatic embryogenesis in plant systems is the induction of embryogenic competency. This allows somatic cells to be reprogrammed and develop into mature embryos capable of germination. Due to of the broad diversity of embryogenic response in the plant kingdom, several culture conditions have been proposed. Many include the addition of growth regulators (auxin and/or cytokinin) during the induction stage (Imin et al. 2005; Nolan et al. 2003; Schmidt et al. 1997). Several types of explants have been used for the establishment of SE including leaflets, stems, cambium, immature zygotic embryos, cell suspensions, and callus (Imin et al. 2005; Varhanikova et al. 2014; Correia et al. 2012; Sharifi et al. 2012; Guzmán-García et al. 2013). SE can be induced directly from the explant (direct embryogenesis) or indirectly through the formation of somatic embryos from callus or suspension cultures (George et al. 2008). Pioneering microscopy observations distinguished the generation of embryogenic and non-embryogenic tissues from the same explants. Both of these tissues can be maintained in culture for long periods of time, maintaining their particular identities (Varhanikova et al. 2014; Correia et al. 2012; Marsoni et al. 2008; Sharifi et al. 2012; Nomura and Komamine 1985; Pennell et al. 1992). In general embryogenic cultures comprise compact globular masses with clusters of meristematic cells (small, highly cytoplasmic, and mostly containing starch) while in non-embryogenic cultures it is common to observe fast growing large parenchymal cells (vacuolated, amorphous, and translucent in appearance). However, the morphological and physiological features of embryogenic and non-embryogenic tissues are very specific to the plant species studied. Although, embryogenic cultures in most species develop into somatic embryos while non-embryogenic cultures remain undifferentiated (Fig. 7.1). These pairs of distinct cultures have been analyzed using proteomics tools with the goal of identifying proteins markers associated with embryogenic potency (Varhanikova et al. 2014; Correia et al. 2012; Marsoni et al. 2008; Sharifi et al. 2012; Almeida

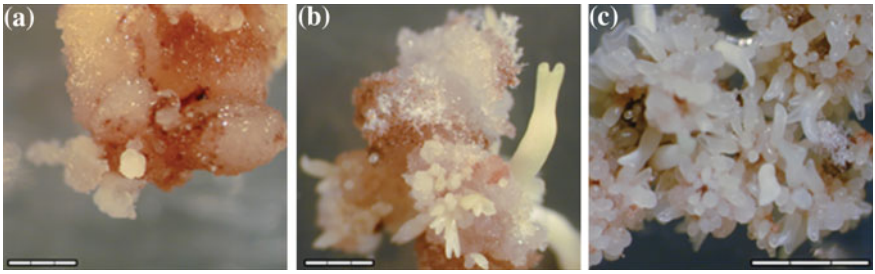

Fig. 7.1 Different responses can be obtained from zygotic embryos of *Magnolia dealbata,* cultured on WP media supplemented with 2.26 μM 2,4-D. **a** Non-embryogenic callus. **b** Using a zygotic embryo it is possible to induce non-embryogenic and embryogenic calluses. The growth of somatic embryos in some cases is observed in specific areas of the explant. **c** Somatic embryos at different stages of development after induction of direct secondary somatic embryogenesis from embryogenic callus. Scale bar: 3 mm

et al. 2012; Guzmán-García et al. 2013). However, these studies have not used sufficiently robust proteomics pipelines and therefore have not covered a comprehensive proportion of the proteome during initiation of the acquisition of the embryogenic potency (Rode et al. 2011, 2012). In most of the proteomics studies of somatic embryogenesis, proteins have been analyzed with 2D-SDS-PAGE and mass spectrometry tools (Rode et al. 2011, 2012; Varhanikova et al. 2014; Correia et al. 2012; Marsoni et al. 2008; Sharifi et al. 2012; Almeida et al. 2012; Guzmán-García et al. 2013; Vale Ede et al. 2014).

In this chapter, we compile the most relevant proteomic information by considering prominent proteomic studies in embryogenic and non-embryogenic cultures from different plant species (Varhanikova et al. 2014; Correia et al. 2012; Marsoni et al. 2008; Sharifi et al. 2012; Almeida et al. 2012; Guzmán-García et al. 2013). We group the identified proteins from each plant species by functional category. Our analysis indicates that the two most prominent differences between embryogenic and non-embryogenic cultures are proteins associated with oxidation–reduction processes and proteins associated with protein folding and binding domains (Fig. 7.2). In fact, several proteomic studies have shown over-accumulation of reactive oxygen species (ROS), reactive nitrogen species (RNS) (Zavattieri et al. 2009; Ötvös et al. 2005), and ROS-scavenging enzymes including extracellular peroxidases, superoxide dismutase (SOD), catalases, thioredoxins, and isoflavone reductases occur in embryogenic cultures (Sharifi et al. 2012; Marsoni et al. 2008; Nomura and Komamine 1985). Interestingly, an outburst of constant oxidative stress can lead to irreversible post-translational modifications in proteins inducing oligomerization, fragmentation, destabilization, aggregation, and degradation of unfolded proteins (Meriin et al. 2010; Nystrom 2005; Lindermayr et al. 2005; Spadaro et al. 2010). Furthermore, these oxidative conditions overload the protein quality control (PQC) system, which can cause a second major wave of stress conditions able to destabilize the proteostasis system of the cell (Tu and Weissman 2004; Dahl et al. 2015). This reduction in protein integrity is reflected in proteomic studies, where several proteins associated with a

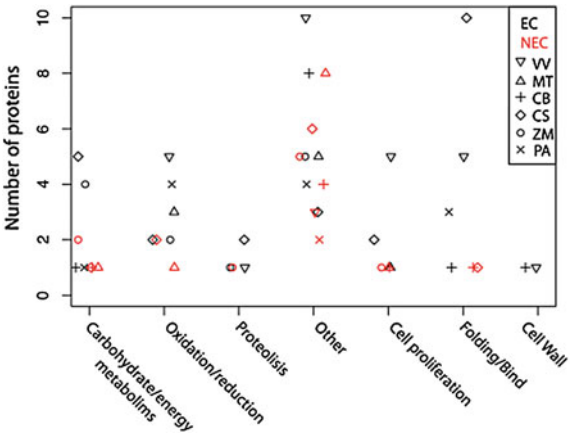

Fig. 7.2 Comparison of proteomic data from embryogenic and non-embryogenic cultures. We considered the most recent and representative proteomic data. Proteins identified in each study were grouped in function families both in embryogenic (*EC*) and non-embryogenic cultures (*NEC*). Species depicted are *VV Vitis vinifera*; *MT Medicago truncatula*; *CB Cyphomandra betacea*; *CS Crocus sativus*; *ZM Zea mays*; *PA Persea americana*

folding and binging activity such as heat shock proteins 60 (HSP60) and 70 kDa (HSP70), were identified more frequently in embryogenic than non-embryogenic cultures (Fig. 7.2). Therefore, it seems likely that fine scale regulation of gene expression and the over-accumulation of ROS/RNS scavenging enzymes and folding associated proteins at the induction stage of SE are key factors for overcoming stress conditions and continuing the acquisition of embryogenic potency in embryogenic cultures. This interpretation is consistent with the pattern of identification of proteins associated with cellular proliferation, which mirrors the induction and development of somatic embryos in embryogenic cultures (Fig. 7.1). It is noteworthy that this oxidation–reduction regulation may be part of global master regulation and contribute to crosstalk among several regulatory networks (Feher 2015).

7.3 Transdifferentiation of Somatic Embryos Resembles Zygotic Embryogenesis

One of the main goals of the application of somatic embryogenesis is the large-scale production of homogeneous plants with defined characteristics. This task has been difficult to accomplish in all species in which it has been tried. The first challenge, described above, is establishing optimal conditions for the induction of SE. After the induction of SE, involving a global shift in gene expression and the rigorous coordination of several metabolic pathways, each embryo undergoes transdifferentiation. In some cases, this process includes multiple stages with unique morphological, molecular, and physiological conditions. Transdifferentiation of somatic embryos can be very different across species depending on the genomic background. In some species, it is possible to observe the globular, heart, torpedo, and cotyledon stage for dicotyledons, and globular, elongated, scutelar, coleoptilar

stages for monocotyledons (von Arnold et al. 2002; Zimmerman 1993). However, the asynchronous nature of the embryo transdifferentiation gives rise to a mix of embryos at different morphogenetic stages (Gray and Purohit 1991). Moreover, somatic embryos in several plant species show abnormal development during transdifferentiation. In most of the cases, aberrant embryos cannot be completely transdifferentiated, halting at some point in SE. There is even variation in somatic embryogenesis between species of the same plant genus. For instance, although generation of *Coffea canephora* through SE is straightforward, the induction of SE in *C. arabica* is a very difficult task (Nic-Can et al. 2015; Tonietto et al. 2012). Recent studies have suggested that molecular factors secreted by *C. arabica* cultures including the phenolic compounds, caffeine and chromogenic acid, act as repressors of somatic embryogenesis (Nic-Can et al. 2015). Caffeine and chromogenic acid are known to affect DNA methylation (Nic-Can et al. 2015) and it is possible that they affect the proteome and secretome of the cultures of *C. arabica* as well. Altogether, the molecular basis of the physiological, morphological and biochemical differences during asynchronous transdifferentiation is not well understood and difficult to control. Since somatic embryogenesis (SE) resembles zygotic embryogenesis (ZE) in several aspects, molecular, and microscopy studies have analyzed the two processes simultaneously (Rode et al. 2011; Takac et al. 2011; Sghaier-Hammami et al. 2009; Rodríguez-Sanz et al. 2014; Dobrowolska et al. 2012). Several studies have suggested that zygotic embryos may provide important clues about the physiological and biochemical preconditions necessary for proper transdifferentiation, maturation, and germination of somatic embryos. Unfortunately, few massive proteomic studies have broadly analyzed the proteome at each stage of SE and ZE (Rode et al. 2011, 2012; Balbuena et al. 2009).

In the absence of a single comprehensive study, we resort to a meta-analysis of public data across studies. However, this approach is non ideal because of the high variability of transdifferentiation in somatic embryos which makes it very difficult to compare proteomics data even within plant species. In addition, proteomic studies have used a broad range of extraction procedures, mass spectrometers, and databases. Future comparison of data would be facilitated by the establishment of universals procedures, proteomics pipelines, and more compatible bioinformatic platforms among laboratories around the world studying SE. We examined the most representative and recent proteomic studies, where either somatic or zygotic embryogenesis have been analyzed at least in three different stages of development, by classifying cellular stages and functions in broad groups and comparing counts of uniquely identified proteins (Fig. 7.3). We can clearly observe a wide variety of proteins identified in the proliferation stage for SE, as opposed to ZE. However, at the globular and torpedo stages, the proteins identified in both somatic and zygotic embryogenesis belong to similar functional categories (Fig. 7.3). Similar proteins were identified in proteomic studies at the torpedo stage of SE and ZE in *Theobroma cacao*. In both cases, proteins associated with stress and folding/sorting/degradation were more prominent in SE, while proteins associated with carbohydrate metabolism were more prominent in ZE (Noah et al. 2013).

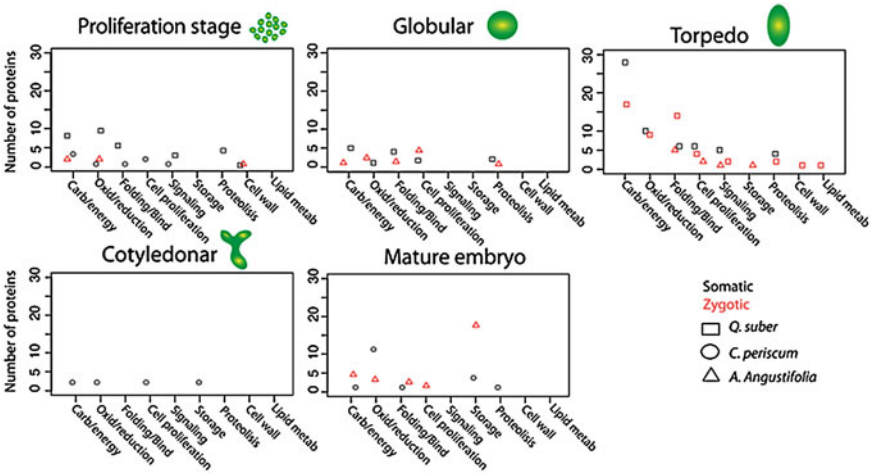

Fig. 7.3 Comparison of proteomics studies carried out during the transdifferentiation of somatic and zygotic embryos. Proteins identified in each study were grouped in functional families for both somatic (*SE*) and zygotic embryogenesis (*ZE*)

7.4 Maturation of Somatic Embryos

Maturation of the somatic embryos is a critical step, which is defined by the conversion of a mature somatic embryo to a plantlet with a functional shoot and root system (Stasolla and Yeung 2003). Maturation is characterized by active cellular expansion and differentiation and accumulation of storage proteins such as globulins and small enolases (Rode et al. 2011, 2012; Vale Ede et al. 2014). Although the function of these small enolases is unknown, they have a lower than theoretically predicted molecular weight (Rode et al. 2011) and, therefore, may be the result of proteolysis of larger inactive enolases, which are then repurposed as storage proteins (Rode et al. 2011). During recent decades great effort has been devoted to understanding and improving the maturation of somatic embryos and preventing precocious germination, because these processes define the efficiency of plant regeneration through SE (Stasolla and Yeung 2003; Gutmann et al. 1996; Merkle et al. 1990; Bapat et al. 1988). There are several factors which alter maturation of somatic embryos including mineral salts, carbohydrates, gelling agent, coconut water, polyethylene glycol, amino acids, cytokinins, and abscisic acid (Rode et al. 2011, 2012; Vale Ede et al. 2014; Sghaier-Hammami et al. 2010). These treatments have been mainly associated with the over-accumulation of storage proteins and proteins involved in carbohydrate/energy metabolism (Rode et al. 2011, 2012; Vale Ede et al. 2014). The accumulation of storage molecules is a common feature during the maturation of zygotic embryos; this accumulation can serve as a marker to compare quality and fidelity of somatic embryogenesis. This has most notably been accomplished using small enolases in *Cyclamen persicum* (Thorpe 1995; Rode et al.

2011). Additionally, findings across proteomic studies indicate that the main difference between matured somatic and zygotic embryos is the availability of storage compounds (carbohydrates, lipids, and proteins). This is clear in *Phoenix dactylifera* (date palm), where comparative proteomic studies between SE and ZE of showed an over-accumulation of storage proteins in zygotic embryos (Sghaier-Hammami et al. 2009). Furthermore, proteomic studies during ZE of *Agave angustifolia* found large accumulations of storage proteins at the maturation stage (Fig. 7.3) (Balbuena et al. 2009; Thorpe 1995). Clearly, in ZE, storage proteins and other storage molecules (small enolases, oligosaccharides, and lipids) are key factors that provide energy to the seedling until the establishment of photosynthesis (Winkelmann et al. 2006; Rode et al. 2011). Therefore, increasing the availability of storage molecules in somatic embryos could improve conditions and promote the generation of high quality in vitro plantlets.

In order to move beyond identifying necessary protein categories (e.g., storage) and toward identifying the specific protein requirements for efficient SE, a standardized approach across labs is necessary. A universal proteomics approach with powerful technologies applied to each stage of somatic embryogenesis in a variety of plant species will lead to a better understanding of the molecular nature of each embryo stage during SE. Identifying and characterizing key proteins, such as the small enolases, can provide invaluable molecular markers necessary to the establishment of efficient and effective protocols for somatic embryogenesis and massive propagation.

7.5 New Technologies for Proteomics Studies in Somatic Embryogenesis

The goal of proteomic studies is the global analysis of the proteome of a cell, tissue, or organ at a specific time under defined conditions. Recently, with the establishment and application of new pipelines extensive progress has been made in proteomics studies, resulting in the in-depth characterization of bacteria and yeast proteomes (Zielinska et al. 2012; Nothaft and Szymanski 2010). Moreover, studies in a multicellular model organism such as *Mus musculus*, *Homo sapiens*, *Arabidopsis thaliana* and *Solanum lycopersicum* have demonstrated the application of new proteomic tools in a broad range of organisms (Lopez-Casado et al. 2012; Zielinska et al. 2012; Kim et al. 2014). This may be possible even when whole genome sequence is not available. However, these techniques have not been applied systematically or in sufficient detail to somatic embryogenesis. Studies are needed to create detailed profiles of each stage of embryo transdifferentiation (Rode et al. 2012). Moreover, considering the intercommunication among several tissue layers, specific cell types, and subcellular compartments during cellular differentiation, division, and expansion, profiles of individual tissues are necessary as well.

The major hurdle for proteomic studies in SE is asynchronous transdifferentiation and the limited amount of biological material available in each stage. Specifically, the bottleneck for proteomics studies of SE is obtaining sufficiently large samples of protein (Gupta et al. 2015; Zawadzka et al. 2014). Even when samples are available in large quantity, in most cases more than 50 % of the proteins present are at low concentration, falling below the limit of detention for even the most powerful mass spectrometry instrumentation available. As mentioned above, most of the proteomics studies in somatic embryogenesis have been conducted with 2D-SDS-PAGE and mass spectrometry tools. However, considering the limitations of 2D-SDS–PAGE (poor reproducibility, the narrow dynamic range of identification, limitations in detecting membrane and low abundance proteins) (Rabilloud and Lelong 2011), proteomic studies in SE should be conducted using alternative proteomic tools. In fact, several approaches have been used to isolate and analyze low abundance proteins including, precipitations, depletion of abundant proteins, and affinity chromatographic tools (Hage and Matsuda 2015; Polaskova et al. 2010; Jiang et al. 2004). We suggest, that affinity chromatography, combinatorial peptide ligands library (CPLL), and powerful mass spectrometers will be key to digging deeper into the proteome of somatic embryogenesis in several plant species.

Affinity chromatography has been the favorite approach for low abundant protein enrichment, including for studies emphasizing identification of proteins with posttranslational modifications. This versatile technique utilizes a broad range of ligands covalently linked to different types of matrixes (e.g., Sepharose, agarose, and others) (Zielinska et al. 2012; Ruiz-May et al. 2014). A wide variety of ligands is now commercially available including: antibodies used to enrich specific antigens, substrates to trap enzymes, ligands to isolate receptors, and lectins to enrich and characterize low abundance glycoproteins (Wilchek and Chaiken 2000; Steen et al. 2006; Hage and Matsuda 2015). Pioneering work in carrot somatic embryogenesis indicated glycosylated secreted proteins as the main factor associated with the activation of the somatic embryogenesis (Lo Schiavo et al. 1990; Cordewener et al. 1991; Sterk et al. 1991; van Engelen et al. 1991). However, follow-up studies are needed. Combining affinity chromatography with powerful mass spectrometers could provide important clues about low abundant glycoproteins with key roles during the induction of somatic embryogenesis and the transdifferentiation of the SE and ZE. Recently, several pipelines including affinity chromatography with lectins with multiple affinities to broad types of sugar structures in glycoproteins have been reported as effective approaches for the enrichment of a comprehensive population of glycopeptides and glycoproteins (Ruiz-May et al. 2014; Zielinska et al. 2012).

Another important consideration for future work in understanding signal transduction associated with embryogenic potency is the proteomic analysis of redox-based posttranslational modifications (PTM). This is especially relevant because of the association between the induction of somatic embryogenesis and the oxidative status of embryogenic cultures. For instance, protein carbonylation is an irreversible PTM that marks proteins under oxidative stress conditions (Lounifi

et al. 2013; Madian and Regnier 2010; Moller et al. 2011). Protein carbonylation can be direct, through the oxidation of amino acid residues (proline, lysine, arginine, and threonine), or indirect, through the formation of adducts with lipid peroxidation products or glycation products (Madian and Regnier 2010). In addition, it has become increasingly clear that protein *S*-nitrosylation is an important PTM in plant biological processes (Spadaro et al. 2010; Corpas et al. 2008). Recent studies have shown that cysteine residues are the major site of action for ROS/RNS species leading to the formation of *S*-nitrosylation and *S*-glutathionylation, and sulphenic acid, sulphinic acid, and disulphide formation (Spadaro et al. 2010). Both carbonylation and *S*-nitrosylation affect the structure and function of several proteins (Moller et al. 2011; Tada et al. 2008; Lindermayr and Durner 2009; Davies 2005). Therefore, great effort has been focused on establishing proteomic pipelines for the isolation, enrichment, and characterization of carbonylated and *S*-nitrosylated proteins (Lindermayr et al. 2005; Lindermayr and Durner 2009). The implementation of new technologies of affinity chromatography and powerful mass spectrometers will underpin the new era of proteomics studies in somatic embryogenesis.

The CPLL is another fascinating approach that diminishes the dynamic range of protein extracts, thereby, allowing analysis of very diluted proteins, which are often invisible due to highly abundant proteins. This technology consists of several million hexapeptides, which are covalently linked to porous beads, capable of binding to a great number of proteins (both high and low abundance) in all cases tested thus far (Boschetti and Giorgio Righetti 2008; Boschetti et al. 2009; Antonioli et al. 2007; Fortis et al. 2006; Guerrier et al. 2007; Sennels et al. 2007; Castagna et al. 2005). This approach drastically reduces the presence of highly abundant proteins, because the most abundant proteins saturate the ligands for which they have affinity faster than low abundance proteins. Consequently, continuous overloading of the ligand libraries will allow the enrichment of very dilute proteins while the unbound highly abundant proteins are discarded in the flow through. Therefore, using CPLL coupled with mass spectrometry during the transdifferentiation of somatic or zygotic embryos will allow the identification of key protein factors present at very low concentration either in a specific layer of tissue or extracellular culture media. Furthermore, CPLL may serve to increase the effectiveness of other technologies. For instance, in studies of the tomato pericarp *N*-glycoproteome, a high dynamic range of identified *N*-glycoproteins was found even after enrichment with lectin affinity chromatography (Ruiz-May et al. 2014). Combining an affinity chromatography approach with CPLL may provide an alternative pipeline to reduce the high dynamic range of proteins with posttranslational modifications.

The above-mentioned chromatography tools, combined with peptide fractionation and cutting edge mass spectrometry technology will provide the means to a better understanding of somatic embryogenesis. For several decades manufacturers of mass spectrometers and scientists around the globe have been working to overcome the challenges posed by the size and heterogeneity of peptides and proteins, and solubility of protein complexes (Marcoux and Cianferani 2015;

Tsiatsiani and Heck 2015; Hu et al. 2005; Hardman and Makarov 2003; Eliuk and Makarov 2015). Recently, several excellent reviews of improvements in mass spectrometry and bioinformatics tools have been published (Larance and Lamond 2015; Marcoux and Cianferani 2015; Tsiatsiani and Heck 2015; Yates et al. 2009; Vowinckel et al. 2014). The new advances in proteomics pipelines include: the improvement of peptide fractionation with nano-HPLC/nano-UPLC systems, the combination of mass analyzers, alternative fragmentation methods (CID, ECD, HCD, and ETD), database search algorithms, and new implementation of data independent analysis (SWATH), which have allowed the identification and characterization of several thousands of glycoproteins and phosphoproteins (Hu et al. 2005; Liu et al. 2014; Vowinckel et al. 2014; Yates et al. 2009; Choudhary et al. 2015). Furthermore, today it is possible to identify and quantify proteins with less than 100 copies per cell within short period of time (Picotti et al. 2009). In addition, bottom-up, middle-down and top-down proteomics have emerged as integrative tools for the characterization of posttranslational modifications and the structural analysis of complex isoforms (Rosati et al. 2012; Moradian et al. 2014). The coming years will bring exciting discoveries in somatic embryogenesis with the application of cutting-edge proteomics approaches.

7.6 Conclusions

SE is a fascinating biological process that consists of a series of complex molecular mechanisms precisely located spatially and temporally both within particular cells and within specific tissue layers in those cells. The major players associated with the activation and regulation of embryogenic response is still unknown. Several cellular aspects of SE are still poorly studied including the molecular and structural modifications associated with the cell wall and cytoskeleton of various cell types during early stage SE, embryo transdifferentiation, maturation, and germination (Šamaj et al. 2006). There is clear interconnection among the cytoskeleton, plasma membrane, and cell wall which appears to play a key role during cellular expansion and division (Šamaj et al. 2006; Thomas and Staiger 2014). In fact, microscopy and immunocytochemistry data have strongly suggested differences between embryogenic and non-embryogenic cultures in the methyl-esterification of pectic epitopes in the cell wall (Sala et al. 2013). However, the characterization of the cell wall and membrane proteome during SE is still needed in a broad range of plant species. In addition, embryogenic cells differ from non-embryogenic cells in several other aspects including subcellular compartments and the ultrastructure of organelles (Šamaj et al. 2006). However, massive proteomic characterization of subcellular compartments such as nucleus, mitochondria, chloroplast, and extracellular space (culture media) has not yet been carried out. The next frontier in proteomics studies in plants systems will be the simultaneous characterization of multiple PTM, the crosstalk between PTM, and massive profiling of protein–protein interaction in single cells and specific tissues. Proteomics studies in SE are still in their infancy.

Nevertheless, with the increasing number of new and accessible proteomic technologies, the application of proteomics tool to SE is easier than ever before.

Acknowledgments We thank Dr. Irene Perea Arango and M.C. Jose Miguel Elizalde Contreras for helpful discussion and input. Our research is funded by a grant (CB-2014-01, 242990) from the National Council of Science and Technology (CONACYT).

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Chapter 8
Plant Fidelity in Somatic Embryogenesis-Regenerated Plants

Hervé Etienne, Romain Guyot, Thierry Beulé, Jean-Christophe Breitler and Estelle Jaligot

Abstract This chapter reviews the literature on somaclonal variation affecting the micropropagation through somatic embryogenesis. The first part covers the following aspects: (i) principle, protocols, and applications of somatic embryogenesis and (ii) epigenetic reprogramming and changes in cell fate that underlie somatic embryogenesis. The second part addresses the problem of somaclonal variation in somatic embryogenesis by first (i) assessing their impact on somatic embryo-derived plant production and (ii) describing the multiple origins of somaclonal variation (chromosomal aberrations, genetic alterations, epigenetic regulations, and transposable elements). The last part focuses on how to manage somaclonal variation in commercial productions of SE-derived plants by covering different aspects: (i) detection of undesirable phenotypes: screening out the variants, (ii) strategies of avoidance and incidence limitation, (iii) generation and exploitation of desirable phenotypes in plant breeding, (iv) beyond the induction of stress-tolerant somaclonal variants: a plant breeder's perspective.

Abbreviations

AFLP	Amplified fragment length polymorphism
6-BA	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
LTR	Long terminal repeats
PGRs	Plant growth regulators

The original version of this chapter was revised: The incorrect tagging of the given names and the family names of the authors was corrected to H. Etienne, R. Guyot, T. Beulé, J.-C. Breitler, E. Jaligot. The erratum to this chapter is available at DOI 10.1007/978-3-319-33705-0_28

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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_8

RAPD Random amplified polymorphic DNA
SE Somatic embryogenesis
SSR Simple sequence repeat
SV Somaclonal variation
TEI Transgenerational epigenetic inheritance
TEs Transposable elements

8.1 Somatic Embryogenesis: Principle, Protocols, and Applications

Somatic embryogenesis (SE) is the process through which a whole individual is regenerated from somatic tissues via the differentiation of an embryo. This process occurs either spontaneously in vivo or can be induced in vitro—a property which is unique to plant organisms and is most strongly displayed in explants sampled from immature tissues and relatively young individuals (Baurens et al. 2004; Monteuuis et al. 2008; Smulders and de Klerk 2011). The process of SE is made possible because of the ability of many differentiated plant tissues to revert back to the undifferentiated, totipotent state, thereby restoring their full organogenic potentialities. An alternative, albeit controversial, hypothesis has suggested that the extent of the dedifferentiation is, in fact, milder than is commonly assumed and that the process also includes the developmental switch of the explant cells to a root stem cell-like functioning (Sugimoto et al. 2010). Whichever of these two mechanisms is involved, a variable number of individual plants that are (in principle) genetically identical to the explant donor can be regenerated from a single explant, depending on the efficiency of the procedure and on the nature of the protocol (Pierik 1997).

Overall, the initial step of dedifferentiation leading to the acquisition of the embryogenic competence is common to most plant species, whereas the following step of the proliferation of the embryogenic material may be performed according to two distinct strategies. The first is the proliferation through secondary embryogenesis which involves first differentiating the somatic embryos before enhancing their proliferation through adventitious budding (Fig. 8.1). The second consists of establishing embryogenic suspensions (ESP) to favor large-scale embryogenic cell proliferation before the subsequent embryo differentiation step. In the perspective of developing an industrial-scale propagation process, the development of ESP represents the best option to ensure synchronous and massive somatic embryo production (Etienne et al. 2006). Moreover, the embryogenic suspension allows the production of large numbers of embryogenic-competent cells and this process can be easily scaled up. Among micropropagation methods, SE has the best potential for rapid and large-scale multiplication of selected varieties in a wide range of economically important species such as coffee (*Coffea arabica*), oil palm (*Elaeis guineensis*), date palm (*Phoenix dactylifera*), banana (*Musa acuminata*), cocoa (*Theobroma cacao*),

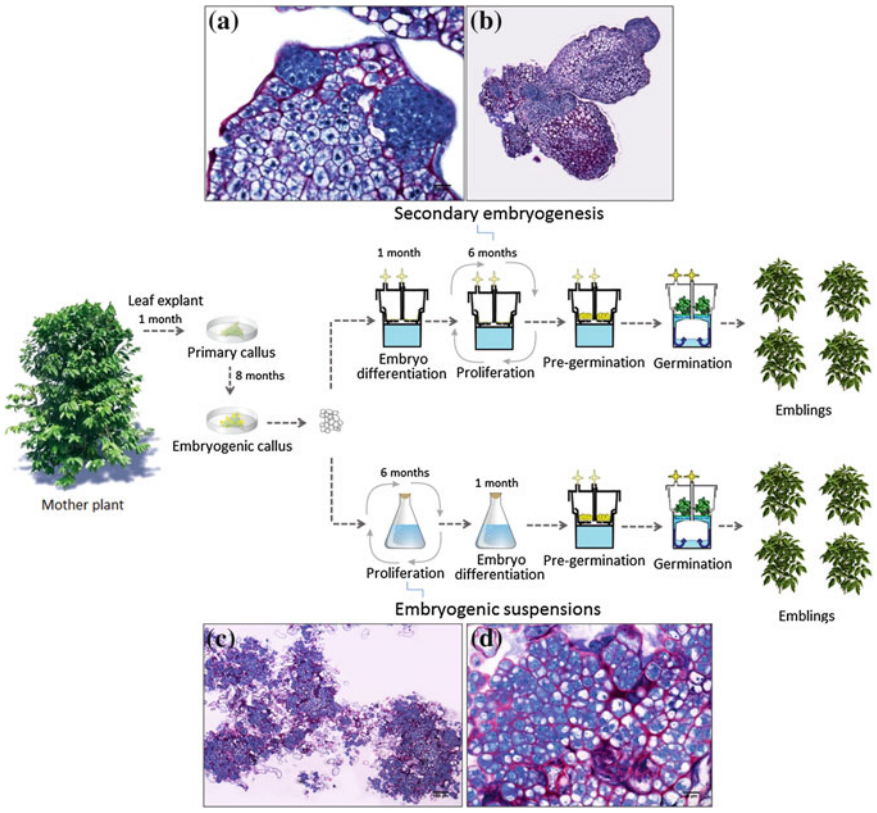

Fig. 8.1 Schematic representation of two types of somatic embryogenesis processes applied at the industrial level. The first somatic embryogenesis process (*upper section* of the flow diagram) involved a proliferation step based on secondary embryogenesis in RITA® temporary immersion bioreactors (photos 1A, 1B). The second process (*lower section* of flow diagram) included a proliferation step based on embryogenic suspensions (photos 1C, 1D). 1A, initial developmental stages of secondary embryos at the root pole of primary somatic embryos; 1B, clusters of primary and secondary embryos; 1C, clusters of embryogenic cells in suspension; 1D, embryogenic cells in suspension. (From Bobadilla Landey et al. 2013)

grapevine (*Vitis vinifera*), and conifers. It is also increasingly used for the ex situ conservation of germplasms and endangered species (Reed et al. 2011).

8.2 Epigenetic Reprogramming and Changes in Cell Fate Underlie SE

The molecular basis of the SE phenomenon lies in the implementation of development programs through the coordinated regulation of gene expression by epigenetic mechanisms (Fig. 8.2). In most eukaryotic organisms, epigenetic regulations

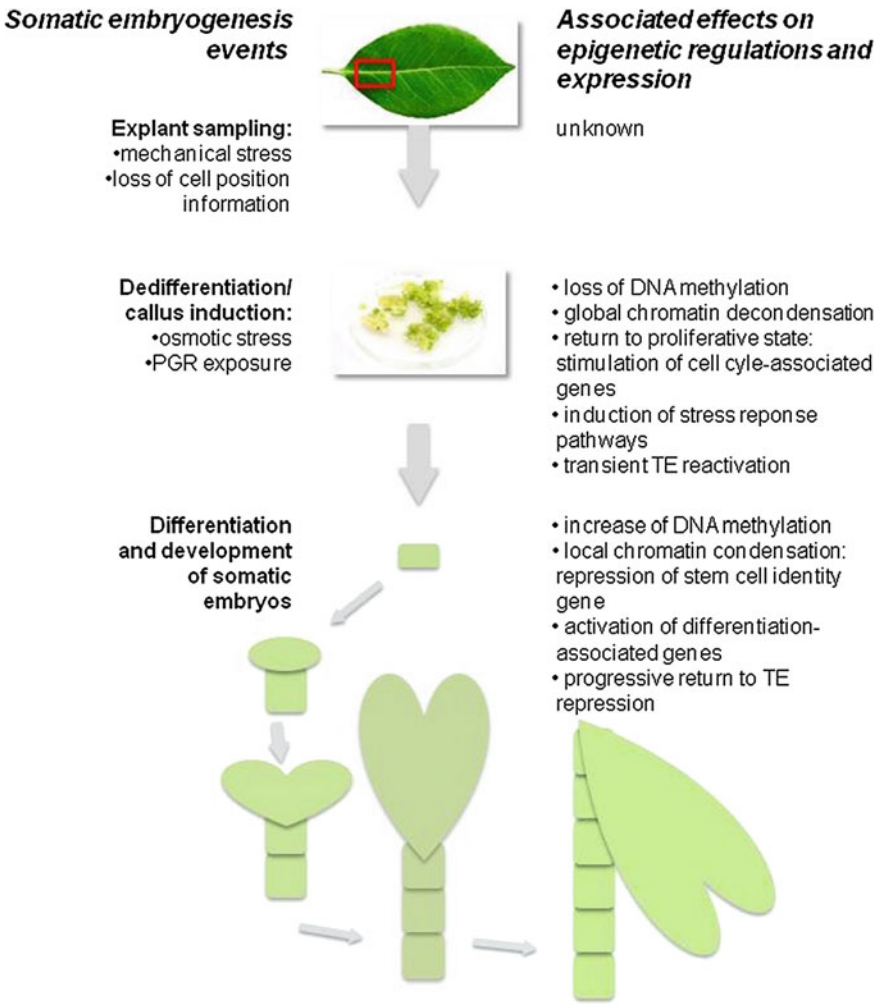

Fig. 8.2 Events affecting epigenetic regulations and gene expression during the somatic embryogenesis process. *Left-hand column*: steps of the somatic embryogenesis protocol and corresponding sources of stress; *right-hand column*: documented effects on regulatory processes. See text for details. Photo credit: stock photos and Wikipedia

include both DNA methylation on cytosine residues, which has a globally repressive effect on transcription, and histone posttranslational modifications, which can have either a repressive or an enhancing effect depending on their nature and location on a given histone protein. Together, these modifications are aimed at ensuring the timely and quantitatively adequate expression of specific genes in the different tissues (Hemberger et al. 2009; de Vega-Bartol et al. 2013) and they are highly susceptible to change, both qualitatively and quantitatively, in response to a variety of stresses.

During the dedifferentiation or reprogrammation phase of SE, the competent cells of the explant undergo a genome-wide resetting of epigenetic patterns which translates into the combined loss of both genomic DNA methylation and chromatin condensation. This phase is generally induced through culture of the explant on a medium enriched in plant growth regulators (PGRs) in order to induce callus formation (Ikeuchi et al. 2013). However with a few Gymnosperm species such as *Pinus sylvestris* and *Araucaria angustifolia*, the use of PGR supplementation is not compulsory for either the induction of SE or the maturation of somatic embryos (Lelu et al. 1999; Elbl et al. 2014).The most widely used are the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), which is either applied alone or in combination with a cytokinin (LoSchiavo et al. 1989; Exner and Hennig 2008; Miguel and Marum 2011). Fehér (2015) has suggested that the differences in responsiveness of plant tissues to the dedifferentiating stimulus could be related to their different balances in endogenous auxins and/or to their varying abilities to respond to auxins (auxin sensitivity). Indeed, transcriptome analysis of Brazilian pine (*Araucaria angustifolia*) during SE points out to defects in auxin signaling as the origin of the loss in embryogenic ability in the suspensions (Elbl et al. 2014). The downstream molecular targets of endogenous and exogenous auxins in this reprogramming process are still largely unknown with the exception of auxin response factor (ARF) family of transcription factors. However, the change in cell fate and the reentry into the cell cycle that are promoted after auxin treatment are associated with the combined activity of genes encoding components of the repressive Polycomb-Group (PcG) and activating Trithorax-Group (TrxG) complexes determining either maintenance of the stem cell-like status or differentiation, respectively (de la Paz et al. 2015), and to the enhanced expression of both mitogen-activated protein kinases (MAPKs) (Zhang et al. 2012) and cyclin-dependent kinases (CDKs) (Fehér 2015).

Most of our knowledge of the molecular mechanisms behind epigenetic resetting in plants are derived from the study of sexual reproduction, when two such events take place successively during meiosis and fertilization, respectively. Although little evidence has been gathered on the reprogramming step in tissue cultures, it seems to indicate the occurrence of strong similarities between both processes in these aspects.

With these precautions in mind, inhibition of methylation maintenance mechanisms targeted at genic and repetitive sequences by the products of the *METHYLTRANSFERASE1* (*MET1*) and *CHROMOMETHYLASE3* (*CMT3*) genes, respectively, seems to be required. However, this passive mechanism alone is insufficient to account for the magnitude of the decrease in genomic DNA methylation within a very limited number of cell divisions (Calarco and Martienssen 2011). In addition to this, DNA glycosylases-lyases encoded by genes such as *DEMETER*, *REPRESSOR OF SILENCING1* (*ROS1*) and *DEMETER-LIKE3* (*DML3*) (Gong et al. 2002; Ono et al. 2012; Ibarra et al. 2012) are instrumental in actively removing DNA methylation through the excision of methylated cytosines from the DNA molecule. Another mechanism, involving the enzymatic oxidization of methylcytosines and their physical replacement by unmethylated cytosines, has

been observed in mammals during gametogenesis and early embryogenesis, and it might be that it also occurs in plants (Cantone and Fisher 2013).

As for chromatin modifications, the frequent superimposition of several different regulatory marks and associated cofactors on the same histone protein makes it unlikely that this resetting mechanism could be efficient enough at the genome-wide scale. Indeed, it has been demonstrated that modified histones are replaced by unmodified ones in the zygote and that this process is independent of DNA replication (Feng et al. 2010b; Ingouff et al. 2010; Feng and Jacobsen 2011; Wollmann and Berger 2012). In addition to the targeted resetting of either DNA methylation or histone modification marks, more generic mechanisms involving chromatin remodeling proteins such as those encoded by *DECREASE IN DNA METHYLATION1* (*DDM1*) and *MORPHEUS' MOLECULE1* (*MOM1*) are also required (Iwasaki and Paszkowski 2014), although their mode of action is still to be elucidated.

Parallels have been drawn between the epigenetic chain of events leading to the formation of a zygotic embryo and that preceding the formation of a somatic embryo. Indeed, in both processes the erasing of preexisting epigenetic modifications occurring as a consequence of either fertilization or dedifferentiation, respectively, is a prerequisite before new cell fates and embryonic development programs can be determined. In this resetting step lies what is probably the major difference between animal and plant organisms, because in the latter the newly formed zygote undergoes a genome-wide « demethylation wave » which removes almost all parentally transmitted DNA methylation marks (Feng et al. 2010b; Cantone and Fisher 2013). Under the artificial conditions of in vitro reproductive cloning procedures, in which the nucleus of a somatic cell is transferred to an enucleated egg, such an exhaustive resetting has proved impossible to achieve and severe, often lethal developmental abnormalities have resulted. By contrast, it seems that the formation of a functional plant zygote does not necessitate a complete erasure of epigenetic marks (Hajkova et al. 2008; Slotkin et al. 2009; Pillot et al. 2010; Feng et al. 2010a; Ingouff et al. 2010; Baroux et al. 2011). If this results holds true for the dedifferentiation occurring prior to somatic embryo development, as seems to be the case in coffee (Nic-Can et al. 2013), it might help explain why most plants can be cloned in vitro. These observations might indicate that, in plants, the threshold of epigenetic changes required to achieve reprogramming is comparatively lower than it is in animals. This relatively lesser cost of resetting the epigenome could be the reason why, in plants, germ cells can be generated from fully differentiated floral tissues whereas in animals a very early segregation of the germinal cell lineage is observed (Bourc'his and Voinnet 2010; Feng et al. 2010b; Jacob and Martienssen 2011; Heard and Martienssen 2014).

Overall, most of the studies on the role of epigenetic regulations in SE focus on changes taking place during somatic embryo development, which follows globular-, heart-, torpedo-, and cotyledon stages that are similar to those observed in zygotic embryo development. After the genome-wide decrease in DNA methylation characterizing the initial reprogramming, the onset and expression of the embryogenic capacity are correlated with increased genomic methylation (Santos and Fevereiro

2002; Fraga et al. 2012; Nic-Can et al. 2013). However, depending on the plant system the relationship between genomic methylation and embryogenic capacity is variable, and variations of the former can appear to have antagonistic effects on the latter. LoSchiavo et al. (1989) observed that both 2,4-D-induced DNA hypermethylation and 5-azacytidine-induced hypomethylation abolished the embryogenic capacity of carrot cell suspensions. Similarly, Teyssier et al. (2014) showed a decrease in the embryogenic capacity in hybrid larch (*Larix* × *eurolepis*) somatic embryos treated by either hypermethylating or hypomethylating drugs. In their study of DNA methylation in embryogenic suspensions of *Pinus nigra*, Noceda et al. (2009) noticed that the ability to produce somatic embryos was negatively correlated with the initial genomic methylation rate of the proliferating cell suspension, whereas Rival et al. (2013) reported an opposite trend in embryogenic suspensions of oil palm.

Concomitantly to the imposition of new DNA methylation patterns, the imposition of histone modifications through the coordinated action of PcG (repression of genes involved in the proliferative state) and TrxG (induction of genes involved in differentiation) complexes is observed (Nic-Can et al. 2013; Fehér 2015). These chromatin modifications ensure the successive activation of genes involved in stem cell/meristematic status such as *WUSCHEL* (WUS), then those with a role in embryo maturation and organ primordia differentiation such as the *LEAFY COTYLEDON* genes (*LECs*), *BABY BOOM1* (*BBM1*) and a variety of other transcription factor-coding genes such as those belonging to the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) and *MADS* families (Gliwicka et al. 2013; Mahdavi-Darvari et al. 2014; Fehér 2015). In a number of plant species (hybrid yellow poplar, sweet orange, rice, cotton…), it has been observed that the expression of these stage-specific transcription factors was itself targeted by microRNAs displaying differential accumulation throughout somatic embryo development (Wu et al. 2010; Li et al. 2012; Mahdavi-Darvari et al. 2014; Wu et al. 2015). Downstream targets of the induced transcription factors include genes involved in plant development, stress response, and PGR signaling (Gliwicka et al. 2013).

8.3 Impact of Somaclonal Variation on SE-Derived Plant Production

The term “somaclonal variation” (SV) has been coined in order to encompass phenotypic variations of all nature arising within a somaclone, i.e. a (theoretically) clonal progeny generated from a single mother plant through in vitro tissue culture (Larkin and Scowcroft 1981). SV presents a broad range of phenotypic changes easily observable, sometimes affecting important agronomic traits. Trait alterations have been described for both qualitative and quantitative features (Bairu

Fig. 8.3 Aspect of some phenotypic variations observed in *Coffea arabica*. The arrows indicate the variant plant material. A, plant exhibiting a normal phenotype in the nursery B, Angustifolia variant with elongated leaves in the nursery; C, Variegata variant in the field; D, plant showing a normal phenotype in the field; E, Dwarf variant in the field characterized by a compact phenotype and small leaves; F, Bullata variant in nursery with round and small leaves

et al. 2011). The modification of qualitative traits is widespread and not restricted to any culture system, some of them like variegation or leaf abnormalities not seriously affecting the agronomic performances (Nehra et al. 1992; Kaeppler and Phillips 1993; Etienne and Bertrand 2003; Sato et al. 2011). However, some phenotypic changes can be detrimental or lethal by generating plants that are unable to adapt and develop in greenhouse or field conditions like the multistem *C. arabica* variant (Etienne and Bertrand 2003) or the hooked stem phenotype found in white spruce (Tremblay et al. 1999). In *Coffea arabica,* eight distinct qualitative variant phenotypes (Fig. 8.3) have been described among SE-derived nursery and field plants (Etienne and Bertrand, 2001, 2003; Bobadilla Landey et al. 2015). It has been shown that their frequency directly depend on the in vitro culture conditions.

Quantitative trait variations were frequently described in variants, affecting positively or negatively the agronomic performances. These alterations could affect plant height, plant biomass, grain yield and weight, soluble solid content (SSC) in fruit, and essential oils production. Although this type of variation is more difficult to measure and desirable phenotypes might have been overlooked, documented examples show a tendency towards poorer agronomic performances and a general instability of aberrant phenotypes (Nehra et al. 1992; Kaeppler and Phillips 1993; Biswas et al. 2009).

8.4 The Multiple Origins of SV

At the molecular level, the sources of SV are multiple, but they essentially fall into two main categories, namely (i) changes in DNA structure, either at the sequence or at the genome level and (ii) changes in epigenetic patterns or regulatory pathways. Of course, these different defects are not mutually exclusive, and several different sources of SV can be observed within the same regenerant population.

8.4.1 Chromosomal Aberrations Enhanced by Long-Term Embryogenic Cultures

The use of a callus or embryogenic suspension phase in the SE process has frequently been associated with an increased risk of genetic instability and SV in the regenerated plants (Jähne et al. 1991; Rival et al. 1997; Etienne and Bertrand 2003; Roux et al. 2004; Lu et al. 2006). Although embryogenic suspensions have been developed for some important crops, it has therefore not been widely applied for commercial purposes, with the exception of coffee (Bobadilla et al. 2015). Somaclonal variation in embryogenic suspension-derived plants is probably related to the presence of 2,4-dichlorophenoxyacetic acid (2,4-D), which is often essential for maintaining proliferating cells in an embryogenic, undifferentiated state (Lambé et al. 1997; Von Aderkas and Bonga 2000). This auxin analog could enhance SV through the stimulation of rapid, disorganized growth that can influence the mitotic process, resulting in chromosomal aberrations (Karp 1994, Bukowska 2006).

The balance in gene dosage is essential for normal function of most eukaryotic genomes. Imbalance can cause severe phenotypic syndromes in both plants and animals (Birchler and Veitia 2007). Among the heritable types of variation, gross changes, such as variation in ploidy level, number of chromosomes and structural changes are mitotic aberrations that represent major genomic alterations of in vitro plants often generated during cell proliferation and differentiation (D'Amato 1985; Lee and Phillips 1988; Kaeppler et al. 2000; Neelakandan and Wang 2012). The most reported abnormalities were chromosomal rearrangements (deletions, duplications, inversions, and translocations) whereas the occurrence of aneuploidy and polyploidy was less common. Aneuploidy involves the loss (monosomy) or gains (trisomy) of one or more specific chromosomes or large chromosomal segments (segmental aneuploidy) and results in a dosage imbalance of genes on the affected chromosome (Makarevitch and Harris 2010). Aneuploidy is found in vitro, rather frequently during the first phases of embryogenic callus and suspension cultures in barley (*Hordeum vulgare*), sweet orange (*Citrus sinensis*), and pea (*Pisum sativum*) (Gözükirmizi et al. 1990; Hao and Deng 2002; Kumar and Mathur 2004; Giorgetti et al. 2011). It is produced through nuclear fragmentation (amitosis) followed by mitosis or by defective chromosome behavior during mitosis (D'Amato 1985). Variations in chromosome number and structure have been described among

SE-derived plants for several species (Al Zahim et al. 1999; Hao and Deng 2002; Mujib et al. 2007; Leal et al. 2006). Gross changes occur during coffee SE and are directly related to SV, whereas the rate of genetic and epigenetic (methylation) alterations is very weak (Bobadilla-Landey et al. 2013, 2015). The presence of mitotic aberrations, including double prophase, lagging chromosomes, aneuploids, and polyploid cells, has previously been described in leaves and embryogenic calli of *C. arabica* (Ménendez Yuffa et al. 2000; Zoriniants et al. 2003).

The production of cytogenetic abnormalities was mainly found in long-term callus cultures (Hao and Deng 2002; Smýkal et al. 2007; Rodriguez-López et al. 2010) and in derived regenerants in a lot of species including strawberry (*Fragaria x ananassa*), wheat (*Triticum aestivum*), citrus, black spruce (*Picea mariana*), banana, and coffee (Nehra et al. 1992; Henry et al. 1996; Tremblay et al. 1999; Hao and Deng 2002; Jambhale et al. 2001; Bobadilla Landey et al. 2015). The use of high concentrations of PGRs can enhance the frequency of ploidy changes. Polyploidy in tissue culture is generally produced by endoreduplication or nuclear fusion. Endoreduplication is produced when the nuclear genome continues to replicate without the normally succeeding cell division, leading to elevated nuclear gene content and polyploidy (Palomino et al. 2008). Unequal chromosome distribution, involving the distribution of replicated chromosomes into only one daughter cell also results in polyploidy (Lee and Phillips 1988).

8.4.2 Genetic Alterations Associated to SV

Genetic changes were frequently associated with in vitro-regenerated plants (Neelakandan and Wang 2012). Mutations affect the primary sequence of DNA and include numerical and structural chromosome changes, somatic recombination, point mutations, deletion, and transpositions also occurring in mitochondrial and chloroplast genomes (Orton 1983; Karp 1991; Duncan 1997; Kaeppler et al. 2000; Neelakandan and Wang 2012), including those of SE-derived plants (Rani et al. 2000). These authors used different DNA markers (RAPD, random amplified polymorphic DNA, and SSR, simple sequence repeat) to assess the genetic integrity of *C. arabica* SE-derived plant, and they found a higher polymorphism level (4 %) in the nuclear genome. By performing RAPD analyses on Norway spruce somatic seedlings, Heinze and Schmidt (1995) concluded that gross SV was absent in their plant regeneration system. In contrast, RAPD and SSR markers allowed the detection of high genetic variation in somatic cotton seedlings regenerated in the presence of 2,4-D (Jin et al. 2008). AFLP analysis of 24 rye somatic seedlings led to the scoring of 887 AFLP markers, among which 8.8 % identified the same polymorphism in plants obtained independently, revealing putative mutational hot spots (De la Puente et al. 2008).

All of these classes of mutations would be expected to give rise to stable, sexually heritable variation with the possible exception of transposable elements. These mechanisms are also involved in allele inactivation (Larkin and Scowcroft

1981; D'Amato 1985; Duncan 1997; Kaeppler et al. 2000; Neelakandan and Wang 2012). DNA sequence variations such as single base substitutions (SBS) and small InDels (i.e. base insertions or deletions) are predominant in progenies generated by tissue culture (Jiang et al. 2011; Carrier et al. 2012). Single base mutation can alter genes if they correspond to nonsynonymous mutations, therefore altering the final protein. Genetic mutations can then generate a great diversity of abnormal phenotypic traits. For example, in Arabidopsis SE-derived plants SBS and InDels resulted in a different array of phenotypes including bleached and long hypocotyls, dwarfish plants, late flowering, and large flower plants (Jiang et al. 2011).

8.4.3 *Role of Epigenetic Regulations in the Emergence of SV*

As we have pointed out previously, the developmental reprogramming of tissues underlying SE is based on the correct implementation of epigenetic regulatory mechanisms. Because of their extreme sensitivity to the stressful environment that surrounds the SE process, the disturbance of these regulations may result in altered patterns of DNA and chromatin modifications. While a proportion of epigenetic changes are "silent" with respect to gene expression, some actually result in the emergence of altered phenotypes in the clonal progeny (Smulders and de Klerk 2011; Miguel and Marum 2011).

A now-classical example of such an epigenetic somaclonal phenotype is the *mantled* variant of oil palm (Fig. 8.4). The most widely used protocol to date for oil palm SE starts from immature leaf tissues as explants and, while all the protocols

Fig. 8.4 Oil palm fruits with normal (*left*) and mantled (*right*) phenotypes

include a callus induction stage on solid (agar-based), auxin-supplemented medium, the propagation stage can either be performed in solid or in liquid medium, as embryogenic suspensions (de Touchet et al. 1991; Teixeira et al. 1995; Gorret et al. 2004), thereby allowing to achieve higher multiplication rates.

The transfer of the first somatic embryo-derived oil palm clonal progenies from the production units to the pilot plantations made apparent the occurrence of several somaclonal variant phenotypes, among which one has a detrimental effect on oil production. In the so-called *mantled* variant (Corley et al. 1986), apparent feminization of the male floral organs (stamens in male flowers, staminodes in female flowers) is observed in the inflorescences from both sexes and results in supernumerary carpel-like structures. The severity of the phenotype is variable: less affected flowers still retain some fertility, and the resulting fruits accumulate reduced amounts of palm oil, whereas in more extreme cases of organ alterations the flowers are sterile (Rao and Donough 1990). This variability is perceptible at different scales: between flowers or fruits within the same inflorescence or bunch and between different regenerant palms from the same clonal progeny. In addition to this, the frequency of the *mantled* phenotype was also found to vary widely between genotypes, with an overall average incidence of 3–5 % of the regenerated palms and up to 20 % in certain clonal lines (Rival et al. 1998; Durand-Gasselin et al. 2010). Through time, a spontaneous, gradual reversion occurs, with 100 % of the "slightly *mantled*" variants and 50 % of the "severely *mantled*" ones having reverted to the normal floral phenotype within 9 years after planting (Rival 2000).

In the absence of a detectable alteration in genome sequence or structure in connection with the *mantled* variation, and considering the observed characteristics of the abnormal phenotype in the field (i.e., heterogeneity, unstability), the hypothesis of an epigenetic misregulation prompted by the SE process was proposed (Rival et al. 1998). This assumption was soon provided support by the demonstration of a highly significant deficit in DNA methylation in both in vitro (embryogenic calli) and adult tissues (leaves, inflorescences) from *mantled* oil palms, with respect to their true-to-type counterparts (Jaligot et al. 2000, 2002, 2004; Matthes et al. 2001). The sum of these works led to the conclusion that there was no such thing as a "methylation threshold" allowing to discriminate reliably between normal and variant clonal regenerants of oil palm. Also, because of the considerable inter-individual and inter-genotype variation observed in the rates and patterns of DNA methylation, no "universal" phenotype-specific differentially methylated marker could be identified. Consistent with this assumption that the genome-wide disturbance in DNA methylation levels and patterns are a symptom rather than the cause of the *mantled* variation, no clear relationship could be found between the expression of the three principal DNA-methyltransferase genes of oil palm and the variant phenotype in a later study (Rival et al. 2008). Another SV has been associated with genome-wide changes in DNA methylation (Bairu et al. 2011; Smulders and de Klerk 2011; Miguel and Marum 2011). However, in most cases the functional involvement of epigenetic variations in the variant phenotype could not be demonstrated and therefore, which suggests that these changes were merely nonspecific by-products of faulty reprogramming mechanisms.

8.4.4 *Transposable Elements as a Source of Both Genetic and Epigenetic SV*

Transposable elements (TEs) are repetitive sequences that have the capacity to move (to transpose) from one chromosomal location to another one. In certain case, they also have the capacity to increase their copy number within the host genome. Transposition was initially discovered by Barbara McClintock in the 1940s through the analysis of chromosome breakages in maize (McClintock 1984). Now, with the complete sequencing of the maize genome, we know that TEs represent the main part (84 %) of its nuclear DNA content (Schnable et al. 2009), playing a fundamental role in the genome structure and evolution (Bennetzen and Wang 2014). This is also true for numerous plant species with large genome sizes such as in cereals, for which genomes are mainly composed of TEs (for example 85 % for bread wheat (Choulet et al. 2010). TEs are traditionally classified according to their life cycle (Wicker et al. 2007): class I elements (or retrotransposons) are moving via a RNA intermediate and a so-called « copy and paste » mechanism, while class II elements (or transposons) are moving as a DNA molecule as part of a « cut and paste » mechanism. Class I includes particularly LTR (Long Terminal Repeats) retrotransposons and non-LTR retrotransposons such as LINEs (Long Interspersed Nuclear Elements) and SINEs (Short Interspersed Nuclear Elements). Class II contains terminal inverted repeat (TIR) transposons and Helitrons groups of elements (Fig. 8.5).

The most abundant TEs in many plants genomes are LTR retrotransposons because their specific mode of replication can increase their copy numbers. For example, LTR retrotransposons represent 75 % of the maize genome (Schnable et al. 2009), 67 % of wheat (Paux et al. 2006, Daron et al. 2014), 55 % of *Sorghum bicolor* (Paterson et al. 2009), and 42 % of the coffee genome sequences (Denoeud et al. 2014). Because of their mobility, TEs can generate a wide variety of mutations, from point mutations due to the de novo insertion of one element to large-scale modifications via the promotion of genomic rearrangements and chromosome breakages (Slotkin and Martienssen 2007, Casacuberta and Gonzalez 2013). In their default state, however, TEs are targeted by potent and highly specific epigenetic repression mechanisms from the host genome. This repression is based on a combination of chromatin condensation through histone modifications and targeted DNA methylation guided by noncoding small RNAs produced by the TEs themselves (a phenomenon known as RNA-directed DNA methylation, RdDM), which results in the extinction of both the transcriptional and transpositional activities of all copies of a given element (Cao et al. 2003; Lippman et al. 2004; Slotkin and Martienssen 2007; Lisch 2009; Rigal and Mathieu 2011; Mirouze and Vitte 2014). Also, as TE sequences tend to accumulate mutations through time, they gradually lose the ability to be transcribed and to transpose autonomously, although some are still susceptible to be targeted in *trans* by the peptides produced by more recent autonomous copies (Wicker et al. 2007; Feschotte 2008; Lisch 2013).

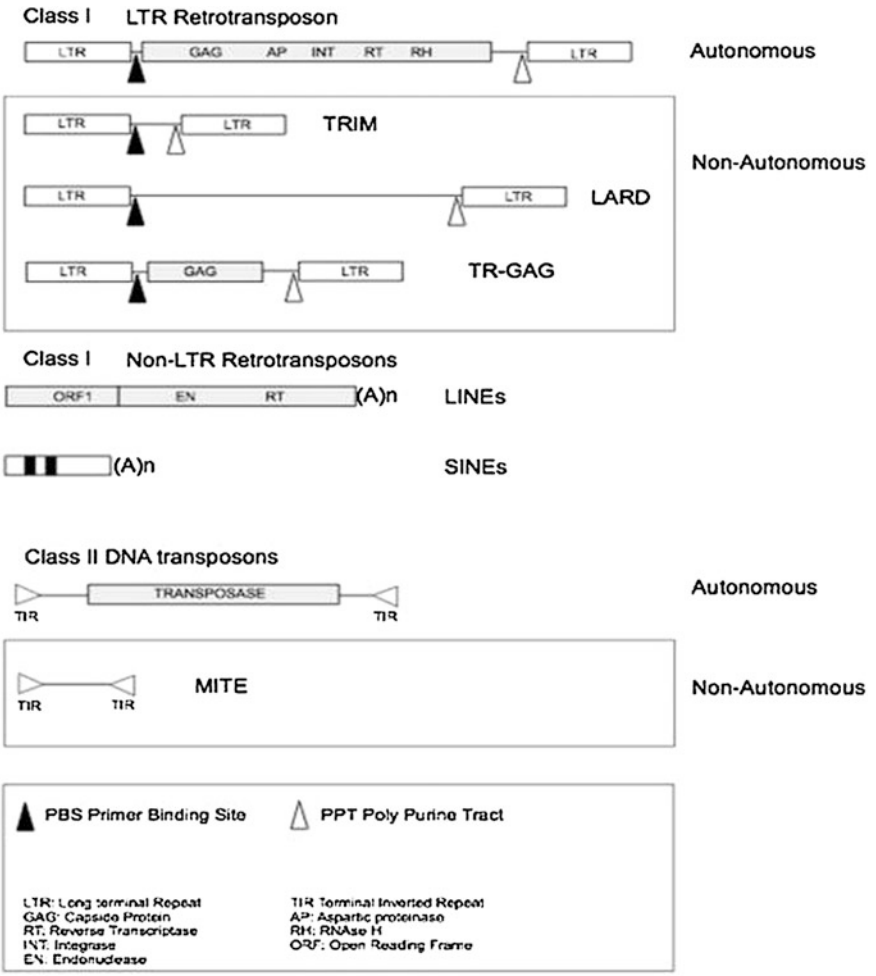

Fig. 8.5 Types of transposable elements in plant genomes. See text for details

The best known triggers of TE transcriptional and transpositional activities are stresses of all nature, biotic as well as abiotic ones (McClintock 1984; Capy et al. 2000; Todorovska 2007; Bui and Grandbastien 2012; Chénais et al. 2012; Wheeler 2013). In vitro tissue culture can be considered as an ensemble of abiotic stresses (Paszkowski 2015) and as such, it has indeed been demonstrated to be a powerful source of TE derepression in plant species such as tobacco with the reactivation of the *Tnt1* (Grandbastien et al. 1989) and *Tto1* (Hirochika 1993) LTR retrotransposons, rice with the *Tos17* LTR-retrotransposon (Hirochika et al. 1996), the *Karma* LINE (Komatsu et al. 2003), and the *mPing* Miniature Inverted–Repeat Transposable Element (MITE) (Jiang et al. 2003), or barley with the *BARE-1*

LTR-retrotransposon (Suoniemi et al. 1996; Kalendar et al. 2000). Exhaustive lists of TEs which activity responds to tissue culture conditions in a wide range of plant species have been published recently (Bairu et al. 2011; Neelakandan and Wang 2012; Grandbastien 2015).

In several instances, remobilization has been correlated with the loss of either DNA methylation or repressive histone modifications or both, in the micropropagated material (Komatsu et al. 2003; Liu et al. 2004; Ding et al. 2007; Ngezahayo et al. 2009; La et al. 2011), a result that is consistent with the global alleviation of epigenetic repressive mechanisms has been observed throughout plant genomes in stress conditions. Ultimately, these reactivated elements can contribute to the phenomenon of SV and promote the emergence of altered phenotypes. This may occur either directly, through structural disruptions induced by their mutagenic capacity, or indirectly, through the modification of epigenetic or transcriptional regulations in neighboring genes (Kaeppler et al. 2000; Smulders and de Klerk 2011; Saze and Kakutani 2011; McCue and Slotkin 2012; Lisch 2013; Le et al. 2015). In the latter case, it is interesting to note that the effect of TEs on proximal gene expression is independent of their ability to be transcribed or remobilized since, it relies on the recognition of structural elements by the host's silencing pathways (Hoen and Bureau 2012; Cui and Cao 2014). Indeed, SE-induced DNA methylation changes of the defective *Karma* element of oil palm have been proposed to be responsible for the *mantled* SV through the production of a nonfunctional splice variant of *EgDEF1*, the floral organ identity gene controlling stamen formation (Ong-Abdullah et al. 2015).

Overall, the different requirements of TE families with respect to the extent of epigenetic modifications that are necessary to achieve stable repression make them also differentially sensitive to stress-induced reactivation conditions (Mirouze et al. 2009; Pélissier and Mathieu 2012). As a consequence, with the exception of extreme conditions using several different stresses or methylation-deficient backgrounds (Reinders et al. 2009; Colomé-Tatché et al. 2012), only a few families of elements are typically reactivated under a given constraint and only for a limited time, whereas most of the TE content in the host genome remains under control.

8.5 Managing SV in Commercial Productions of SE-Derived Plants: A Curse or a Blessing in Disguise?

8.5.1 Detection of Undesirable Phenotypes: Screening Out the Variants

In the perspective of the production of clonal plants with stable and homogeneous genotypic and phenotypic characteristics, SV is considered primarily as an undesirable side effect of the micropropagation process and as such, it is one of the main

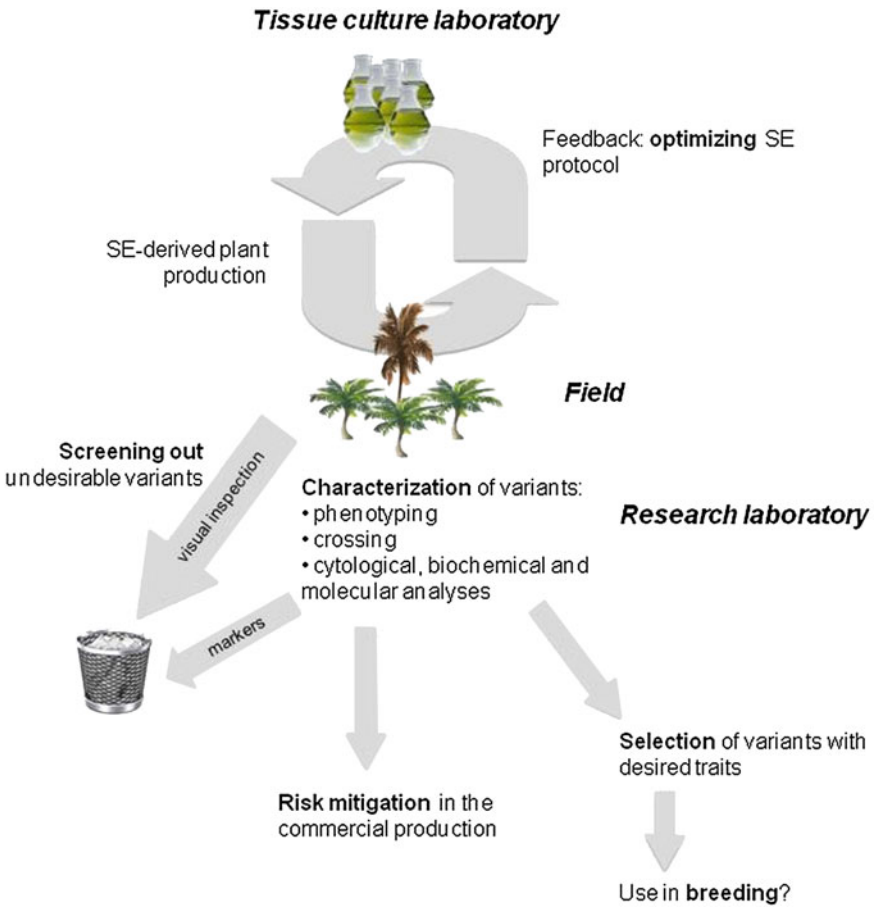

Fig. 8.6 Strategies for the management of SV in SE-derived production. See text for details

bottlenecks to the large-scale commercial development of SE (Kaeppler et al. 2000; Miguel and Marum 2011; Neelakandan and Wang 2012; Wang and Wang 2012). The success of genetic transformation protocols, which include the regeneration of the stably transformed plant from in vitro-cultivated tissues, is also impacted by the occurrence of SV.

The first step towards managing SV in a clonal production is the detailed characterization of the agro-morphological traits of the regenerant plants and their follow-up through time in the field (Fig. 8.6). This evaluation phase is meant to allow the detection of potential variant phenotypes, although it is more time consuming in the case of perennial plant species with long life cycles. Such is the case in conifers because the agronomic trait evaluation cannot be made before 20 years of field development (Lelu-Walters et al. 2013). In oil palm, the visual assessment of the *mantled* floral phenotype in SE-derived individuals can only be made when they

reach reproductive maturity, i.e., 3–5 years after the regenerants have been transferred to the field (Jaligot et al. 2011). The low rate of certain SV phenotypes in plant productions with high potential economical impact may require the implementation of large-scale field trials, so that their frequency can be properly assessed. In coffee, field evaluation of SE-derived plants performed on a massive scale (200,000 plants) showed less than 1 % SV rate (Bobadilla Landey et al. 2013).

In addition to these observations and in view of future molecular analyses, it can also be useful to assess the (mitotic) stability of the variant phenotypes through time, and their meiotic heritability through sexual generations. In oil palm for instance, this kind of evaluations allowed to show that the *mantled* SV undergoes a spontaneous, albeit slow, reversion process towards the normal floral morphogenesis, and that the sexual transmission of the trait is not Mendelian (Rival and Parveez 2005), thereby leading to the hypothesis of an epigenetic origin.

8.5.2 Limitation of the Incidence of SV in SE Processes

Once SV has been evidenced in a clonal progeny, the second step is the optimization of the SE protocol in order to mitigate the incidence of the variant phenotype(s). As previously mentioned, this is generally achieved by changing the type and concentration of PGRs and limiting the duration of tissue exposure to these substances through the decreased number of regeneration cycles/subcultures (i.e., culture age). If a particular explant or genotype has been demonstrated to yield a high percentage of variants, using explant material from different tissues or genotypes can also contribute to lowering SV rates. In commercial coffee micropropagation laboratories, the risk to get a high frequency of SV in a commercial batch is reduced by using very low PGRs levels and short embryogenic culture proliferation periods (6 months), but also by mixing regenerated plants from different cell lines at the end of the nursery phase (Etienne et al. 2012). To ensure this last objective, it was necessary to introduce a system of traceability of production batches derived from independent cell lines. In this way, commercial batches are constituted by a mix of several production batches (set of plants derived from the same in vitro introduction date), themselves constituted by several embryogenic cell lines (set of plants derived from a same embryogenic callus). The maximal number of plants regenerated from an embryogenic coffee cell line is now fixed at 100,000 plants.

Because of the associated economical impact of SV, most research efforts in this field are currently directed at both (i) understanding the origin of SV in order to minimize their impact on plant production, and (ii) providing the industry with discriminating markers enabling quality control of the SE process and assessment of clonal conformity in clonal progenies. Recent reviews have extensively summarized the different cytological, biochemical, and molecular techniques that are available for the characterization of the variants (Bairu et al. 2011; Smulders and de Klerk 2011; Miguel and Marum 2011; Us-Camas et al. 2014). The industrial experience in coffee associated with researches on SV cellular and molecular

mechanisms and potentially inducing culture parameters showed that SE based on embryogenic cell suspensions is efficient and reliable for true-to-type propagation of selected *C. arabica* varieties (Bobadilla-Landey et al. 2013, 2015). Actually around 7 millions of SE-derived coffee plants are planted in Central America fields. The results are clear: over 99 % of coffee trees regenerated, fully conform to the mother plant, both morphologically—they grow, flower and produce normally—genetically, and epigenetically. Hence, strong genetic and epigenetic changes in proliferating embryogenic cells are not a fatality. It also demonstrated the importance of embryogenic culture age on SV and hence the nonrandom nature of this phenomenon. The genetic and epigenetic alterations are particularly limited during SE. These results open the way for the use of the embryogenic cell suspension technique to other plant species: a revolution in the world of plant micropropagation on an industrial scale. True-to-type SE protocols have also been established for oil palm industrial propagation in Indonesia and Malaysia.

8.6 Generation and Exploitation of Desirable Phenotypes in Plant Breeding

With increasing attention being focused on the exploitation of new sources of phenotypic diversity in order to tackle the current challenges facing plant breeding (consequences of climate changes, increased pressure on food and energy supplies), SV can also be regarded as a reservoir of potentially beneficial traits (Wang and Wang 2012; Rival et al. 2013). As a matter of fact, the disruption of cellular correlations and the exposure to high concentrations of growth regulators during in vitro tissue culture protocols are powerful triggers of stress response pathways through their capacity to induce both genome-wide and sequence-specific changes in epigenetic patterns (Finnegan 2002; Van Zanten et al. 2012; Springer 2013). The induction of stress-associated factors during SE and the likely involvement of some of them, such as the somatic embryogenesis receptor-like kinase (SERK) family or genes involved in either oxidative or pathogen stress signaling, in embryogenic competence and development were confirmed experimentally in many studies such as those from Thibaud-Nissen et al. (2003) in soybean, Hu et al. (2005) in rice, Mantiri et al. (2008) in alfalfa, Zhang et al. (2010) in maize, Ma et al. (2012) in pineapple, Jin et al. (2014) in cotton and Elbl et al. (2014) in Brazilian pine.

Among the many stress-induced DNA methylation and chromatin conformation changes affecting gene expression and TE activity as a result of a constraint, a fraction might have a role in either short- or mid-term acclimation processes through the transient enhancement of phenotypic plasticity (Wada et al. 2004; Rapp and Wendel 2005; Lukens and Zhan 2007; Bossdorf et al. 2008; Nicotra et al. 2010; Angers et al. 2010; Bui and Grandbastien 2012; Makarevitch et al. 2015). Grafi et al. (2011) went as far as to propose that in vitro propagation and stress response

could have a common requirement for a transient reversion to the undifferentiated state, with only a partial epigenetic resetting achieved in the latter case.

Clearly, the predisposition of in vitro-propagated tissues to display increased stress response make them the tools of choice in the identification and the selection of novel tolerant phenotypes based on stress-induced epigenetic variations (Rai et al. 2011). The main difficulty in this is the extreme lability of most, if not all, stress-induced epigenetic marks and the corresponding reversibility of the associated tolerant phenotypes once the constraint is no longer in action (Bossdorf et al. 2008; Richards 2011). Nevertheless, such plants could be used for their ability to respond more quickly and efficiently to the future occurrences of the same stress within a generation. This phenomenon is known as "priming" by analogy to the immune response and in certain cases its effects are perceptible over two generations at most (Bruce et al. 2007; Jaskiewicz et al. 2011; Holeski et al. 2012; Slaughter et al. 2012; Ding et al. 2014). Both activating and repressive histone methylation marks controlled by Trithorax and Polycomb complexes, respectively, have been involved in this form of mid-term epigenetic stress memory (de la Paz et al. 2015; Avramova 2015).

8.7 Beyond the Induction of Stress-Tolerant Somaclonal Variants: A Plant Breeder's Perspective

Inducing such ephemeral, targeted stress-adapted phenotypes in order to face transient changes in the environment can in some instances be advantageous. However, the long-term exploitation of SV with stress-induced epigenetic tolerance traits in breeding schemes implies that these traits, once they have been identified among the many seemingly random epigenetic alterations that have been induced (Eichten and Springer 2015), can be stabilized and made meiotically heritable over a predetermined number of generations (Schlichting and Wund 2014). In the current state of our knowledge of transgenerational epigenetic inheritance (TEI), it is still very much unclear what the criteria for stable sexual transmission of epigenetic states are, especially when it comes to those that are triggered in response to stresses. A growing number of studies have been conducted in order to investigate the modalities of this inheritance but so far, they have generated mixed results (Molinier et al. 2006; Pecinka et al. 2009; Verhoeven et al. 2010; Lang-Mladek et al. 2010; Boyko and Kovalchuk 2011; Scoville et al. 2011; Kou et al. 2011; Bilichak et al. 2012; Ou et al. 2012). This relative inconclusiveness is at least partly due to the difficulty to associate a given epigenetic polymorphism with the stress tolerance phenotype, since many unspecific epigenetic changes are also triggered. Another common issue with such works is the experimental burden of following the stable transmission of both the epigenetic patterns and the associated character over more than two generations after stress treatment and within large progenies—a kind of experimental setup that cannot be put in practice easily for long-lived perennial species.

So far, the most compelling evidence about any "rule" TEI might follow in plants comes from studies making use of progenies with little or no genetic variation, i.e., either natural (Schmitz et al. 2011) or artificial (Mirouze et al. 2009; Johannes et al. 2009) inbred lines of the model plant *Arabidopsis thaliana*. Although results obtained in a species with such a small genome and low TE content can hardly be used as a template to infer epigenetic regulations within larger, more redundant genomes, a common pattern seems to emerge. It has been observed that the stable inheritance of both methylation and expression patterns is in many instances facilitated or enhanced by the occurrence of nearby repetitive sequences (including TEs) (Slotkin and Martienssen 2007; Ito et al. 2011). This result is reminiscent of the requirement for repeated sequences in the paramutation phenomenon of maize, in which a metastable epigenetic state is stably inherited over a virtually unlimited number of generations (Arteaga-Vazquez and Chandler 2010; Belele et al. 2013; Giacopelli and Hollick 2015). It has been hypothesized that repetitive and/or TE-derived sequences might ensure both the transmission and the remodeling of epigenetic patterns at neighboring coding sequences through their ability to produce small noncoding RNAs and thereby recruit components of the RNA-dependent DNA methylation pathway (RdDM). A similar mechanism has been demonstrated to occur between parental alleles in the progeny of intraspecific hybrids of Arabidopsis (Greaves et al. 2012, 2014). Obviously, this capacity to produce repeat-derived small RNAs and to modulate this production in stress conditions is directly related to the degree of structural redundancy in the genome. As a consequence, in their stress response polyploid crops with high genomic TE contents are expected to display both a larger reservoir of phenotypic plasticity, through enhanced epigenetic (DNA methylation) and transcriptomic (alternative splicing) variability, and also a greater potential for achieving stable TEI, as demonstrated through the study of polyploid hybrid genomes (Combes et al. 2012; Yoo et al. 2014; Fu et al. 2016).

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Chapter 9
The Relationship Between Stress and Somatic Embryogenesis

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Abstract Plants have a high plasticity for cell differentiation that not only allow them to regenerate damaged parts of their body but under certain conditions, plant cells can regain totipotency and change their developmental fate to re-differentiate into a somatic embryo and finally a whole plant. The ability to change their cell fate and generate somatic embryos is restricted to a discrete group of cells. Somatic embryogenesis (SE) is a complex phenomenon, and it is poorly understood. SE can start in various ways and the differential response among cells can be stimulated by several factors, among them highlight the genotype, the physiological state and the origin of the explants, as well as the medium culture or the plant growth regulators (PGRs) used for in vitro culture. However, several stress treatments such as low or high temperature, heavy metals, osmotic shock, among others, might play a crucial role in SE induction, even in the absence of exogenous PGRs. Here, we will provide an overview about the role of stresses and their influence on SE induction.

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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_9

9.1 Introduction

Somatic embryogenesis (SE) represents a valuable tool for clonal propagation of important crops. However, the SE is a complex process that is not well understood. Even so, we know that somatic cells contain all the essential information to generate a new plant and that SE can proceed without fertilization events. Therefore, the SE forms the basis of cellular totipotency in higher plants, a characteristic absent in mammals. According to the developmental stages, the SE closely resembles zygotic embryos, since exhibits a similar cell polarity and presents almost the same developmental stages (globular, heart, torpedo, and cotyledonary shapes). Unlike zygotic embryogenesis, during SE development, somatic embryos can be easily collected at specific developmental stages, and a large number of embryos can be obtained (Quiroz-Figueroa et al. 2006). Therefore, the SE represents an interesting system to investigate the events of cellular differentiation, from somatic cell to whole embryo, as well as the biochemical and physiological functions that allow the embryo development. Also, SE is the major technique for massive propagation of plants and germplasm conservation. On the other side, SE represents the basis of cellular and genetic engineering allowing the establishment of biotechnological strategies including genetic transformation, somatic hybridization, production of artificial seeds, among others (Basnayake et al. 2011; Grosser and Gmitter 2005; Krishna Kumar and Thomas 2012).

The ability to change the cell fate (from vegetative to embryogenic state) and generate somatic embryos is restricted to a discrete group of cells. This differential response among the cells can be influenced by several factors, such as the genotype, the physiological state of the plant, the origin of the explant, the culture medium, the nitrogen and carbon sources, the exposure time to several plant growth regulators (PGRs) as well as the in vitro culture conditions for each plant species (Fuentes-Cerda et al. 2001; Loyola-Vargas et al. 2008; Quiroz-Figueroa et al. 2001). Also, during the beginning of SE process, the plant tissues receive certain mechanical damage due to the surface-sterilization process, the cut of the explants or sieving and centrifugation of cell suspension before their incubation in artificial environmental conditions for their culture. All these factors exert a considerable stress to the cells, tissue, and organ used as source to SE induction. Hence, it is thought that stress could be the switch that stimulate the embryogenic competence and the cellular reprogramming in the differentiated somatic cells and direct them to the acquisition of cellular totipotency (Karami et al. 2009; Yang and Zhang 2010). Here, we will provide an overview about the role of stresses and their influence on somatic embryogenesis induction in several plant species.

9.2 Somatic Embryogenesis: When the Pressure Cannot Destroy Life

Under appropriate conditions, a variety of cell types such as zygotes, microspore cells, nucellar cells, unfertilized cells, and somatic cells experiment a wide molecular reprogramming that can derive into the generation of a new plant (Ayil-Gutiérrez et al. 2013; Carimi et al. 1998; Touraev et al. 1996; Van Dijk and Van Damme 2000). This fact represent the most extreme characteristic of plant regeneration, because the differentiated plant cells can be reverted into an earlier developmental state and begin a differentiation process before regaining the pluripotency or totipotent capacity. It is known that SE process from different cell types may be influenced by the expression of hundreds of stress-related genes as response to the effects of in vitro culture conditions (Jin et al. 2014; Thibaud-Nissen et al. 2003; Zeng et al. 2007). Thus, it is possible that a common signaling pathway exists, but modulated by different environmental conditions, which stimulate the change in the cell fate and redirect it to become an embryogenic cell.

Under normal conditions, plant cells receive signals and stimuli from adjoining tissues to maintain the normal physiological conditions in the whole plant. However, this fact can be disrupted by the excision of tissue, which induces a physiological shock in the cells. If the explants are subjected to higher stress conditions, generated by the nutrients and PGRs present in the culture medium, the cells initiate alternative regulatory mechanisms for their adaptation. Among these changes are the reprogramming of gene expression, and physiological and metabolic changes that let the explant survive and differentiate (Fehér 2008; Zeng et al. 2007). Among PGRs, it is well known, that auxin plays a key role in the induction of SE, because most species require its presence, either before or during the embryogenic process (Ayil-Gutiérrez et al. 2013). Among auxins, the most used for the SE induction is the 2,4-dichlorophenoxy-acetic acid (2,4-D). This stress-related substance has been used, either alone or combined with others PGRs, in more than 65 % of protocols to induce SE (Karami and Saidi 2010). Their role during SE induction has been extensively reviewed (Fehér 2015; Karami and Saidi 2010; Umehara et al. 2007; Yang and Zhang 2010). However, it is also true that not only the presence of PGRs is determinant for the embryogenic shooting, but other stresses can stimulate the acquisition of embryogenic competence and also increase the embryogenic response and improve the somatic embryos development (see Table 9.1). However, the mechanisms by which these stresses influence the somatic embryo induction are largely unknown. The stress conditions used to stimulate the onset of SE depend on application time (either before or during the culture) as well as on the type of stress (osmotic shock, high or low temperatures, among others) (Fehér 2015; Ikeda-Iwai et al. 2003). The suitable balance among all factors mentioned above and the composition of culture medium, substantially impacts the initiation phase of SE in different plant species.

Table 9.1 Somatic embryogenesis induced by stress

Family	Species	Stress	Explant	Reference
Apiaceae	*Daucus carota*	Osmotic	Apical meristem	(Kamada et al. 1989)
			Cotyledon/apical meristem	(Kamada et al. 1993)
			Apical meristem	(Kiyosue et al. 1989)
		Heavy metal ions	Apical meristem	(Kiyosue et al. 1990)
		Heat	Apical meristem	(Kamada et al. 1994)
		Dry	Somatic/zygotic embryos	(Tetteroo et al. 1998)
		High synthetic auxin concentration	Hypocotyl	(Kitamiya et al. 2000)
		Nutrient starvation	Cotyledon	(Lee et al. 2001)
Apocynaceae	*Catharanthus roseus*	Salinity	Embryogenic callus from hypocotyls	(Fatima et al. 2015)
Araliaceae	*Panax ginseng*	Salinity	Zygotic embryos	(Choi et al. 1998)
Asteraceae	*(Cichorium Intybus x Cichorium)*	Heat	Leaf	(Decout et al. 1994)
Brassicaceae	*Arabidopsis thaliana*	Heavy metal ion/osmotic dehydration	Shoot apical tip/floral buds	(Ikeda-Iwai et al. 2003)
	Arabidopsis thaliana Ws, Cor, and *Ler* ecot.	Osmotic, heavy metal ion, and dehydration stress treatments		(Ikeda-Iwai et al. 2003)
	Brassica napus cv. Topas	Heat stress (32 °C)	Microspores	(Solís et al. 2012)
		Low temperature	Microspores	(Prem et al. 2012)
		Heat stress (32 °C)	Microspores	(Rodríguez-Sanz et al. 2014b)
		Heat stress (32 °C)	Microspore	(Rodríguez-Sanz et al. 2015)
		Heat stress (32 ± 0.2 °C for 1–5 days)	Microspores	(Dubas et al. 2014)

(continued)

Table 9.1 (continued)

Family	Species	Stress	Explant	Reference
Celastraceae	*Euonymus europaeus*	Osmotic	Zygotic embryo	(Biahoua and Bonneau 1999)
Cucurbitaceae	*Curcubita pepo*	Nitrogen source (NH_4Cl, KNO_3, $NaNO_3$) -Influence of pH on embryo development	Mature embryos detached of cotyledons	(Leljak-Levanic et al. 2004)
		1 mM ammonium as the sole source of nitrogen	Mature zygotic embryo	(Mihaljevic et al. 2011)
		Ammonium	Pro-embryogenic cell line and embryogenic callus line	(Pencik et al. 2015)
Euphorbiaceae	*Hevea brasiliensis*	Osmotic	Inner integument	(Linossier et al. 1997)
Fabaceae	*Arachis hypogaea*	Osmotic	Inner integument	(Mhaske et al. 1998)
	Medigaco sativa	High synthetic auxin concentration Heavy metal ion	Leaf protoplast	(Pasternak et al. 2002)
	Medicago truncatula	Salinity	Leaf	(Nolan et al. 2006)
	Phaseolus vulgaris	Use of a cytokinin (BA) coupled with osmotic stress (sucrose)	Zygotic embryo	(Cabrera-Ponce et al. 2015)
Fagaceae	*Quercus suber*	Cold, heat, osmotic, and UV	Somatic embryos	(Puigderrajols et al. 2002)
		Heat stress (32 °C)	Microspores and immature zygotic embryos	(Rodríguez-Sanz et al. 2014a)
Gentianaceae	*Gentiana lutea*	Osmotic stress	Somatic embryos	(Holobiuc 2015)
Malvaceae	*Gossypium hirsutum cv. YZ1*	NaCl and ABA	Embryogenic callus	(Jin et al. 2014)
Pinaceae	*Picea abies*	Temperature (18 °C versus. 30 °C)	Somatic callus tissue derived from zygotic embryos	(Yakovlev et al. 2014)
	Pinus taeda	Osmotic	Zygotic embryo	(Li et al. 1997)

(continued)

Table 9.1 (continued)

Family	Species	Stress	Explant	Reference
Poaceae	*Cymbopogon martinii* (Roxb.)	Salinity	Nodes	(Patnaik and Debata 1997)
	Hordeum vulgare var. disticum cv. Bomi)	Osmotic	Immature embryos	(Hollung et al. 1997)
	Hordeum vulgare	Cold stress (4 °C)	Microspores	(El-Tantawy et al. 2014)
	Oryza sativa	Hydric stress	Seeds	(Meneses et al. 2005)
	Pennisetum americanum	Osmotic	Inflorescences	(Rangan and Vasil 1983)
	Pennisetum purpureum		Leaf	(Chandler and Vasil 1984)
	Triticum aestivum L.		Embryogenic callus	(Galiba and Yamada 1988)
	Triticum aestivum	Cold, heat, and C/N starvation	Microspores	(Indrianto et al. 1999)
	Triticum aestivum	Drought stress and heavy metals	Leaf bases	(Patnaik et al. 2005)
	Zea mays	Osmotic	Zygotic embryo	(Santos et al. 1996)
	Zea maize genotype A188	Culture initiation medium	Immature embryos	(Salvo et al. 2014)
Solanaceae	*Capsicum annuum*	Heat treatment at 35 °C for 8 days	Anthers from buds from the first flowering	(Bárány et al. 2005)
	Capsicum chinense	Salinity	Hypocotyl	(Solís-Marroquín et al. 2011)
	Nicotiana tabacum	Heat/osmotic	Microspores	(Touraev et al. 1996)

9.3 Stress Treatments Commonly Used for the Induction of Somatic Embryogenesis

9.3.1 Osmotic Shock

Abiotic stress, especially high saline concentrations, can stimulate the correct development of somatic embryos. During SE induction in *Triticum aestivum*, the formation of somatic embryos was incomplete due to a precocious germination during the early phases of SE and high cell proliferation. These defects were corrected by adding 40 mM NaCl in the culture medium, which suppressed the precocious germination and increased somatic embryos production (Galiba and Yamada 1988).

Stress treatment has also been applied to plant cells to determine the first changes related to embryogenic induction. In *Daucus carota*, somatic embryos development can be induced by culturing shoot apical meristems on PGRs-free medium with a chemical stressor. Changes in sucrose concentration (0.7 and 0.1 M, respectively) induced an important modification in the cell fate that promoted the generation of numerous somatic embryos directly on the surface of shoot apical meristem explants (Kamada et al. 1989, 1993). In *Arachis hypogaea*, a high concentration of sucrose (0.5–0.7 M) triggered the accumulation of triglycerides, the increase of these lipids did not improve the embryogenic system and the embryos became necrosed. The supply of 20 μM abscisic acid (ABA) to the medium, partially improved the morphology of the embryos. Neither the osmotic, or desiccation treatments, stimulated the conversion of embryos into plantlet (Mhaske et al. 1998). In callus cultures of *Hevea brasiliensis*, application of exogenous ABA (1×10^{-5} M) only stimulated the formation of globular embryos. The use of an osmotic agent, such as polyethylene glycol (PEG; 140 g L^{-1}), improved the conversion of pro-embryogenic mass into torpedo-shaped embryos and allowed the proper establishment of morphological appearance of embryos and contributed to decrease the secondary SE (Linossier et al. 1997).

Application of osmotic treatments with different sources of carbohydrate also impacts on somatic embryo generation. In *Euonymus europaeus*, Biahoua and Bonneau (1999) reported that sucrose did not act as nutrient, instead, sucrose increased the osmotic pressure generating a stress that stimulated the expression of SE. The better embryogenic responses was reached using sucrose 350 mM, followed by glucose 89 mM, being the osmotic potential of –1.42 and –1.30 MPa, respectively, for each osmotic. These data suggest that the frequency of somatic embryos enhanced with the increase of osmotic potential. Unlike *E. europaeus*, the embryogenic aggregates of *Gentiana lutea* required a moderate osmotic stress to produce all embryogenic stages. This stress can be produced by sugar alcohols such as mannitol or sorbitol (0.16 M) in a PGR-free medium (Holobiuc 2015). On the other hand, *Phaseolus vulgaris*, considered as a recalcitrant species, can produce somatic embryos under osmotic stress. The application of 0.5 M sucrose by 48 h to zygotic embryos, used as explants, in combination with benzyladenine

(BA) (44.3 μM) and adenine free base, led to the induction of direct SE (Cabrera-Ponce et al. 2015). These data suggest that SE response could be related to the adaptation to osmotic stress and the internal pool of cytokinins; a mechanism that needs further investigations.

9.3.2 Heavy Metal Ion Treatments

Similar to osmotic stress generated by sucrose in carrot cells, heavy metal ions can act as stress inducers in plants and can also promote SE in the absence of PGRs treatment. In carrot, more than 40 % explants treated with 0.5 mM $CdCl_2$ during two weeks were able to produce healthy embryos (Kamada et al. 1989). Other heavy metal ions, such as cobalt, nickel, zinc, silver, and copper have also been used for SE induction. However, they only induced a low rate of somatic embryos in carrot (Kiyosue et al. 1990) or exhibit SE in *T. aestivum.* Nevertheless, leaf explants of wheat treated with cadmium (0.5 mM) and cultured in the absence of 2,4-D produced somatic embryos (Patnaik et al. 2005). Taken together all these results, it would be interesting to investigate how and when the cadmium ion induce the cell reprograming in the somatic cells and the acquisition of cellular totipotency since these plant species (carrot and wheat) did not require the exogenous stimulus of PGRs. Using similar stress treatments as in carrot, in the model plant *Arabidopsis thaliana* positive results were obtained during SE induction (Ikeda-Iwai et al. 2003). However, shoot apical meristem explants cultured on PGRs-free solid medium and exposed, either to osmotic treatment (0.7 M mannitol, 0.7 M sorbitol, 0.7 M sucrose, or 0.3 NaCl), or heavy metal ion (0.6 mM $CdCl_2$) required an additional treatment with 2,4-D to stimulate embryo development (Ikeda-Iwai et al. 2003).

9.3.3 Temperature Stress Treatments

Another treatment able to induce embryogenic development is temperature shock. It has been observed that exposition of explant tissues for suitable periods, either low or high temperatures can induce the acquisition of cell totipotency and even mimic the early zygotic embryogenesis (ZE) development. *Brassica napus* was one of the first plants in which temperature-treatment was used to stimulate the embryogenic program (Lichter 1982). Before the induction of SE in *B. napus*, the plants were grown at 25 °C day/16 °C night until plants generated the first flower buds. After that, the temperature was decreased 12–15 °C day/7–10 °C night, until bud collection, and only then, the heat shock treatment was imposed (Belmonte et al. 2006; Prem et al. 2012; Seguí-Simarro et al. 2003). In this model system, it is well established that treatments at high temperature (32 °C) during 8–72 h was a necessary step for both anthers and microspores to change their gametophytic program

by an embryogenic pathway (Elhiti et al. 2010; Joosen et al. 2007; Seguí-Simarro et al. 2003). The embryogenic pattern might be different, depending on treatment duration. For instance, microspores pretreated at 32 °C for 24 h tended to develop a suspensor-like structure as occurs during ZE, whereas a prolonged treatment up to five days prevented the formation of suspensor in the embryos (Dubas et al. 2011; Dubas et al. 2014). This response could be due to prolonged heat-treatment that affected the polar auxin distribution in the uninucleate microspores which prevented the proper establishment of apical-basal polarity in the cells (Dubas et al. 2014).

In *Cichorium intybus* x *Cichorium endivia* SE process is also temperature-dependent. When leaf explants were cultured in the presence of PGRs (0.1 μM naphthaleneacetic acid (NAA) and 0.1 μM isopentenyladenine (2iP) and exposed to several temperatures (20–35 °C), the cells in the wounded leaves began to grow and proliferate, regardless the temperature conditions. However, the cells were directed to different type of morphogenesis. It is interesting to observe how temperature can impact the plant cell plasticity. Callus generation was predominant when the explants were incubated at 20 °C, but the shoot formation was stimulated at 25 °C, whereas the incubation at 35 °C targeted the direct SE (Decout et al. 1994).

Direct SE was also induced in carrot when the apical tip explants were pretreated at 37 °C for three weeks before culture them in PGRs-free medium at 25 °C. But, when temperature was decreased at 35 °C, the explants did not generate somatic embryos; instead, apical explants developed into plantlets (Kamada et al. 1994). In microspores of *Nicotiana tabacum*, the embryogenic response at normal temperatures was around 50 %. However, when they were exposed to heat treatment (33–37 °C) for three days, followed by incubation at 25 °C, their embryogenic capacity increased (Touraev et al. 1996). The same authors reported that embryogenic response might increase even more, through the combination of sucrose starvation and heat shock treatment, independent of the heterogeneity of microspores. On the other hand, although the high temperatures can involve important cellular damages, in *Capsicum annuum* the heat shock treatment necessary to induce the embryogenesis was larger than in other species. In this crop, the low yield induction was improved using a pretreatment of anthers at 35 °C for eight days and cultured in the presence of kinetin (4.64 μM) (Bárány et al. 2005). Not all plant species respond to heat treatment, in some cases the high temperatures can lead to an inhibition of SE. In wheat, microspores isolated from tillers, did not show any response to embryogenesis induction when they were exposed to 33 °C; instead that, the microspore embryogenesis could be accomplish without any stress pretreatment, but the rate of conversion into embryos was relatively small. On the other side, when microspores were pretreated at 4 or 25 °C for 48 h, the percentage of induction of SE in the microspores showed a twofold increase respect to the response of microspores isolated from freshly tillers. This response was conditioned by the presence of some ovary´s in the culture medium (Indrianto et al. 1999).

In *B. napus*, high temperature treatments were a prerequisite to induce embryogenesis, but only under suitable exposition time (32 °C, one day) the embryos developed suspensor-like structures (Dubas et al. 2014). It has been also

shown that in this plant, the continue use of low temperature (18 °C) induced microspore embryogenesis. The first division allows the establishment of asymmetric cell identities, early polarity, and the formation of suspensor-like structures, in a process resembling the ZE process (Prem et al. 2012). Even when the number of embryos decreased respect to those induced at 32 °C, there was a synchrony and a homogeneity among the embryos generated. Additionally, more than 50 % embryos developed suspensor-like structure, which was very important to understand the early events that establish the polarity and developmental embryo patterning (Prem et al. 2012).

9.3.4 Nutrient Starvation

It is known that sucrose is frequently used as primary carbon source in the culture medium for several plant species; however, it is also known that high concentrations of sucrose suppress SE response (Mhaske et al. 1998). By contrast, it has been shown that nutrient starvation stress is another important factor for SE induction in some plant species. For instance, the sucrose and nitrogen starvation induced the embryogenic development of tobacco microspores, even better than heat stress (Harada et al. 1988; Touraev et al. 1996). In wheat microspores, the first step for embryogenesis induction might require a nitrogen starvation in a medium with mannitol as the only carbon source for a two-day period, as well as high temperatures followed by a culture in an ovary conditioned medium. Under these conditions, microspores can develop multicellular structures; however, the carbon source must be changed by maltose to embryogenesis proceed. Whereas glucose or sucrose impairs wheat embryogenesis through the increase in size and the accumulation of starch, maltose can be used to induce embryogenesis. The effect of maltose on microspore embryogenesis can be attributed to a slow hydrolysis by plant cells, which exerts early starvation conditions in the medium culture and a stable osmolarity later on (Indrianto et al. 1999). In carrot, the embryo production increased up to 4.5-fold when embryogenic callus was cultured on MS basal medium in the absence of sucrose and reduced humidity respect to MS medium with sucrose. Embryogenic callus without medium for five days (stress starvation) showed a 20-fold increase in the production of somatic embryos. This result suggests that the absence of medium culture inhibited cellular dedifferentiation, but improved the production of somatic embryos (Lee et al. 2001).

Sucrose starvation in embryogenic callus of *Phoenix dactylifera* also caused a significant effect on somatic embryos production. It was found that reduction of MS at half-strength, in a liquid medium, and two weeks without sucrose were the best conditions to obtain embryogenic structures which reached their germination after culturing in 3 % sucrose (Veramendi and Navarro 1996).

Gossypium hirsutum somatic embryogenesis, as for other species, is a genotype-dependent process and shows low frequency of somatic embryos formation. This SE response was modified when callus was transferred from 2,4-D to a

PGRs-free liquid medium without *myo*-inositol by a single cycle of 10 days, followed by three subcultures on MS basal medium. This fact did not only improve the efficiency, but also induced SE synchronization in cotton (Kumar and Tuli 2004). *Myo*-inositol starvation could stimulate the signaling pathways mediated by phosphoinositides through the release of intracellular Ca^{+} and diacylglycerol to activate the protein kinase C (Anderson et al. 1999).

9.3.5 Macro and Micronutrients

Inorganic nitrogen forms such as NO_3^-, and NH_4^+ also affect the SE response (Higashi et al. 1996; Leljak-Levanic et al. 2004). For instance, the carrot callus cultured on medium containing NH_4^+ as sole nitrogen source, improved the formation of embryogenic cells instead of a combination of both NO_3^- and NH_4^+ (Smith and Krikorian 1992). Similar results were also observed in *Cucurbita pepo* when NH_4Cl was used as the sole nitrogen source. NH_4Cl (1 mM) could replace the use of 2,4-D and the conversion of pro-embryos to globular stages was stimulated, while the addition of unreduced nitrogen improved the embryos maturation (Leljak-Levanic et al. 2004; Mihaljevic et al. 2011). These authors also showed that the presence of NH_4^+ in the culture medium culture induced a high activity of glutamine synthetase and phenylalanine ammonia-lyase. This increment of enzymatic activity coincided with a higher activity of stress-related enzymes, such as superoxide dismutase and soluble peroxidase, suggesting that a burst of oxidative stress targeted the tissue when NH_4^+ was the only nitrogen source.

In *Oryza sativa* as in wheat, sucrose stimulated the starch accumulation and cell death; sucrose starvation for three days considerably affected induction of cell division of immature pollen grains and allowed the formation of cellular mass. Also, it was observed that substitution of NaH_2PO_4 by KH_2PO_4 improved the frequency of embryogenic colony formation (Ogawa et al. 1994). Furthermore, it was reported that when zygotic embryos of *Panax ginseng* were cultured on MS PGRs-free medium supplemented with high levels of NH_4NO_3 (61.8 mM), their growth was suppressed and the explants turned brown. A high formation of somatic embryos was observed from these abnormal zygotic explants, whereas the use of KNO_3 as only nitrogen source decreased at half the embryogenic response (Choi et al. 1998).

On the other hand, the micronutrient boron appears to be an important factor in the onset and development of SE. It was shown that the induction of SE from leaf explants of *Cucumis sativus*, only began in the absence of boron and PGRs in the culture medium (Mashayekhi et al. 2008). Also it was found that boron in the medium influenced somatic embryos development of *D. carota*. For instance, without boron the apical shoot was suppressed, whereas that of roots was promoted (Mashayekhi and Neumann 2006). More recently, the importance of boron in the activation of several transcription factors was highlighted, and the impact of these transcription factors on the levels of endogenous PGRs as well as proteins related to the acquisition of embryogenic potential (Pandey et al. 2012).

9.4 Expression Patterns of Stress-Related Genes During Somatic Embryogenesis Induction

Much work has been done to understand the molecular mechanisms by which plant cells retain their plasticity and enter the embryogenic pathway. Several reports indicate that some genes, suggested as indicators of SE, such as *WUSCHEL* (Zuo et al. 2002), *LEAFY COTYLEDON* (*LEC1*, *LEC2*) (Braybrook et al. 2006; Lotan et al. 1998), *AGAMOUS-LIKE15* (Thakare et al. 2008), *BABY BOOM* (Boutilier et al. 2002), and *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1* (Schmidt et al. 1997) are conserved among species and their expression patterns are similar between somatic and zygotic embryogenesis (Jin et al. 2014; Malik et al. 2007). Also, it has been shown that their overexpression trigger the embryo formation from vegetative cells. It is important to note that a high number of stress-related genes show altered expression during the onset of embryogenesis, mainly influenced by both maternal and in vitro culture conditions, as well as by specific cell types (Jin et al. 2014). For instance, in immature cotyledons of *Glycine max*, the development of somatic embryos from the adaxial side of cotyledons was preceded by changes in the transcripts of genes associated to oxidative stress and cell division, suggesting a balance between cell proliferation and cell death (Thibaud-Nissen et al. 2003). It was observed that several genes, related to the oxidative burst were upregulated during the first 14 days in the presence of 2,4-D, specially a large number of *GLUTATIONE-S-TRANSFERASE* genes (*GST*7, *GST8*, *GST11*, *GST*16, *GST*19). During the beginning of SE development there was a coordinate expression of several families of genes. Wound-induced genes (expansin, extensine), cell wall remodeling genes (pectinesterase, glucanase), as well as various genes encoding proteins associated to oxidative stress control (thaumatin, ACC oxidase) or in the programed cell death (adenosine-5′-adenylphosphosulfate reductase, endonucleases) showed an increase in their expression (Thibaud-Nissen et al. 2003).

On the other hand, it was found that some stress-responsive genes are also upregulated during later stages of somatic embryos. For instance, during maturation of maize embryos, the transcript levels of genes encoding heat shock proteins and hydrolytic enzymes, such as nucleases, proteases and glucosidases showed an increase in their expression (Che et al. 2006). In potato, as in soybean, an increase in the level of transcription of stress-related genes was observed during the induction of SE; however, the patterns were different. This suggest a species variation or differences due to the type of explant and the culture medium used for the induction or embryo maturation (Sharma et al. 2008).

Other reports confirmed that the gene expression related to the oxidative stress and redox homeostasis is a common pathway that is triggered, independently of the SE system. For instance, *GSTs* are expressed during the onset of SE on oil palm, soybean, *B. nupus, Pinus Pinaster* and maize (Salvo et al. 2014; Vega-Bartol et al. 2013). A coordinated correlation between gene expression of GST and auxin related genes, such as AUX/AIA was observed (Lin et al. 2009; Singla et al. 2007;

Vega-Bartol et al. 2013). Lin et al. (2009) suggested at least two different contexts for GSTs function during SE in oil palm. First, down regulation of *GST*s has a relation with a reduction in the auxin content due to the elimination of 2,4-D from the medium. Second, by contrast, accumulation of these transcripts can be observed just before the beginning of the development of somatic embryos. These findings correlated with the accumulation of AUX/AIA and showed that *GSTs* in the proliferating cells was critical for plant development through roles in auxin transport and signaling, as well as in somatic embryo generation (Vega-Bartol et al. 2013).

On the other hand, oxidative stress-related genes that encode thioredoxin H, cytosolic ascorbate peroxidase, glutamine peroxidase, among others peroxidases, also showed a high abundance during onset of oil palm somatic embryos (Lin et al. 2009). The same authors also found ESTs encoding several pathogenesis-related proteins (endo-β-1,3-glucanase, γ-thionin, and oil palm defensine EGAD1) that although were expressed in response to pathogen attack, they seemed to play a significant role during embryogenic callus generation and somatic embryo development.

Although SE and ZE development are similar in many aspects, recently it was highlighted an important difference between both processes. According to differential gene expression patterns, cotton SE process showed a high transcriptional activity of stress-related genes in comparison to ZE (Jin et al. 2014). These genes included mainly GRTs-related genes (genes involved in ABA biosynthesis, as well as jasmonic acid). Other genes are members of kinase family and downstream stress-responsive genes, particularly Late Embryogenesis Abundant (LEA), Early Responsive to Dehydration (ERD) and Responsive to Dehydration (RD), as well as at least 15 WRKY transcription factors family members. All these genes also showed an increase in their expression under stress treatment either by NaCl (75 mM) or ABA (0.5 mM). Both treatments were involved in the repression of cell proliferation of embryogenic callus and consequently accelerated the somatic embryos development. These data suggest that stress responses might regulate the balance between cell proliferation and differentiation.

In maize around 2,000 genes were overexpressed 8-fold in only 24 h after SE induction (Salvo et al. 2014). These genes were classified in diverse biological processes, such as oxidation-reduction, metabolic processes, protein phosphorylation, transmembrane transport and stress response, which were consistent with a complex coordination of multiple pathways involved in the transition of somatic cell into SE (Salvo et al. 2014). Two maize genes *WOUND INDUCED PROTEIN 1* (*WIP1*) and *CHITINASE A1* related with plant defense and stress response, respectively, were upregulated over 1,500-fold from 0 to 24 h. *WIP1* is involved in the hypersensitive defense response; however, its role is unknown during SE. The expression of the chitinase gene has an important function during early stages of SE (De Jong et al. 1992). Also about 50 % of *GSTs* family members showed a high transcriptional activity during the onset of SE. Likewise, it was found that *GST*s were coexpressed with *BBM*, *WUS*, *SERK*, *PIN-FORMED* (*PIN*), and *GERMIN LIKE PROTEIN* (*GLP*). Whereas PIN is involved in the auxin transport, GLPs affect the plant redox. These results are consistent with the fact that GST, indirectly

influence the auxin transport, and might also promote the embryogenic environment for the expression of master transcriptional factors associated with SE (Salvo et al. 2014). Recently, the transcriptome dataset obtained from the SE process in *Arabidopsis*, revealed a much higher transcriptional activity in somatic embryos than that in actively dividing callus. These data are consistent with the fact that SE involves a highly stress function than their counterpart, the ZE, particularly during the first days of induction (Salvo et al. 2014). It was observed that SE development exhibited a higher level of expression of several genes encoding for oxidative stress, such as peroxidase superfamily members (AT1G68850, AT2G18980, AT5G14130, AT5G17820, one FAD-binding berberine family protein). Also, genes for salt stress (plant invertase/pectin methylesterase inhibitor superfamily), and genes encoding several LEA proteins (AT3G19430, AT4G27400, AT5G54370, and AT5G60530). Interestingly, these last genes encoding LEA proteins were coexpressed with at least two genes that encode for auxin metabolism (flavin-binding monooxigenase and auxin-responsive GH3). However, further studies must be done to discard whether these results are specific to embryogenic process itself, or is a side effect of the 2,4-D used for the SE induction.

9.5 Conclusions

It is well accepted that SE induction is promoted, particularly after of exposing the cells to a high condition of stress. The fact that different types of stresses, namely osmotic shock, application of high or low temperatures, starvation treatments, among others, can impact the cell fate and begin the embryogenic competence is really interesting for scientific research. For instance, a strong osmotic stress caused by sucrose, promoted the generation of numerous somatic embryos in *D. carota* (Kamada et al. 1993). The exposition to heavy metal ions, such as cadmium also induced cell reprograming and the acquisition of cellular totipotency in carrot, wheat or *Arabidopsis*, even in the absence of PGRs (Ikeda-Iwai et al. 2003; Kiyosue et al. 1990; Patnaik et al. 2005). Interestingly, the capacity of cell reprograming, specifically using continuous lower temperature can induce microspore embryogenesis efficiently in *B. napus*, in a process that resembled the ZE process, due to asymmetric cell identities, early polarity and establishment of suspensor-like structure (Prem et al. 2012). In other plant species, the SE can be induced by sugar alcohols (Holobiuc 2015), or by changing the composition of the nitrogen source (Leljak-Levanic et al. 2004) by substitution of some macroelement (Ogawa et al. 1994), or by the absence of a carbon source (Veramendi and Navarro 1996) and in some cases some microelements (Mashayekhi et al. 2008) in the culture medium. All these results show that stress-related treatments not only can stimulate the acquisition of embryogenic competence, but can also increase the embryogenic response and even improving the somatic embryos development. In this way, the stress treatments could influence the differential reprograming of somatic cells to re-entering the cell cycle and regain totipotency. Therefore, the totipotent pathway

could be reached in response to unique complex combination of molecular and metabolic signals to begin a new developmental window (Chupeau et al. 2013). However, the relationship among these complex networks must to be clarified in more detail. Fortunately, the recent depth molecular studies have begun to unravel the onset of SE. Transcriptome analysis clearly indicates that SE induction is preceded by the transcription of many stress-related genes. Other set of genes highly expressed are related to oxidative stress and cell division, suggesting that there exist a balance between cell proliferation and cell death (Thibaud-Nissen et al. 2003). For instance, several studies are consistent with the detection and accumulation of GSTs transcripts during the first days of SE induction (Salvo et al. 2014; Thibaud-Nissen et al. 2003; Vega-Bartol et al. 2013). Although these genes are responsive to auxin, GSTs can also be induced by H_2O_2, ABA, and jasmonic acid associated with abiotic and biotic stress, suggesting a significant participation of *GSTs* to avoid the harmf caused by oxidative stress. Also, the transcriptional activation of pathogen-related genes, such as kinases-related genes, or downstream stress-responsive genes as *LEA* family members, integrate an important stress-signaling network. This network promotes the expression of master transcriptional factors for the establishment of the embryogenic environment (Jin et al. 2014). The fact that SE development displays a major signaling to stress than ZE development could be interpreted as an adaptation process, may be to compensate the absence of endosperm, and accelerate the somatic embryo development.

It is clear that our knowledge about the early events of SE process has increment exponentially recently. However, there are many areas that need to development to have a complete overview of the SE process.

Acknowledgments The work from VMLV laboratory was supported by a grant received from the National Council for Science and Technology (CONACyT, 157014).

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Chapter 10
The Role of the Auxins During Somatic Embryogenesis

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Abstract It is well known that auxins (Ax) and cytokinins (Ck) are key regulators of plant cell division and differentiation. These growth regulators can induce plant cell reprogramming. The balance between Ax and Ck during their application in the plant tissue culture determines the state of cell di- or dedifferentiation. Other plant growth regulators can also induce callus. However, Ax is the most widely used plant growth regulator to control organ regeneration, callus induction or somatic embryogenesis induction. Ax plays a central role in early and post-embryogenic plant development. Elegant studies have shown that temporal and spatial Ax distribution mediates important steps during the early embryo patterning formation of zygotic embryogenesis (ZE). The application of exogenous Ax into the culture medium can unbalance the endogenous of this growth regulator and modify the IAA metabolism inside the cell. In most of the cultures, it has been demonstrated that the removal of the exogenous Ax from of culture medium promotes the formation of embryogenic structures. The increment in the endogenous Ax regulates the expression of a great number of transcription factors, several of them related to stress. The other aspect about the homeostasis of Ax, which has an effect on the induction of somatic embryogenesis, is its transport. In this chapter, a revision and analysis about the role of Ax in different aspects of somatic embryogenesis is made.

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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_10

10.1 Introduction

Plants display a great cellular plasticity, a special feature that provides them with multiple regeneration mechanisms and by which the in vitro culture was made possible. It is well known that plant growth regulators (PGRs) such as Ax and Ck are key regulators of plant cell division and differentiation. Ax and Ck can induce plant cell reprogramming. Therefore, the balance between Ax and Cx during their application in plant tissue culture determines the state of cell di- or dedifferentiation (Skoog and Miller 1957). For instance, the callus formation is related to a moderate ratio of Ax and Ck, whereas a high ratio of Ax to Ck originates roots and a high ratio of Ck to Ax induces shoots (Skoog and Miller 1957). Other PGRs, such abscisic acid (ABA) or brassinosteroids can also induce callus (Siddiqui et al. 1998). However, the most widely PGRs used to control organ regeneration, callus induction or induce somatic embryogenesis (SE) are Ax and Ck. An optimal amount of Ax is necessary for the induction of SE.

Ax plays a central role in early and post-embryogenic plant development (Cueva-Agila et al. 2016; Elhiti and Stasolla 2016). Elegant studies performed through Ax-inducible promoters have shown that Ax temporal and spatial distribution mediate important steps during the early embryo patterning formation of ZE (Friml et al. 2002; Friml 2003; Smit and Weijers 2015).

The research field on Ax and their relation with development has been extensively studied (Zhu and Geisler 2015). Important clues about their metabolism, transport, degrading and signaling are now known (Benjamins and Scheres 2008; De Smet et al. 2010; Schaller et al. 2015). Ax include the indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) (Sauer et al. 2013); however, it has been determined that the IAA is the most abundant Ax in higher plants. There are five Ax biosynthesis pathways. One of them is the tryptophan-independent and the other is the tryptophan-dependent. In the case of the tryptophan-dependent pathway, the tryptophan is converted to indole-3-piruvate by the TRYPTOPHAN AMINO TRANSFERASE, and then by the action of YUCCA family monooxygenase, the indole-3-piruvate is converted to IAA (Kasahara 2015; Tivendale et al. 2014; Zhao 2014). On the other hand, the Ax biosynthesis tryptophan-independent involves the conversion of IBA to IAA, via β-oxidation in the peroxisomes (Nonhebel 2015; Wang et al. 2015).

Once the Ax has been synthesized, it is distributed through the plant by specialized efflux or influx protein carriers. PINFORMED family members, most of the times referred as PIN and ATP-binding cassette subfamily B (ABCB) transporters act as Ax efflux carriers, whereas AUX1/LIKE AUX1 (AUX/LAX) family members act as influx carriers (Adamowski and Friml 2015; Reemmer and Murphy 2014; Scheuring and Kleine-Vehn 2014). Using Ax-inducible promoters, it was determined that differential Ax distribution plays an essential role in the earliest step of ZE (Petrásek and Friml 2009). However, Ax distribution is complex, because of the highly regulated synthesized IAA in specific environmental conditions. In order to maintain the appropriate levels of Ax in the cells, the IAA is conjugated (Ludwig-Müller 2011;

Novák et al. 2014). The conjugation can be either with sugars or amino acids, and both can lead to the inactivation of IAA. However, the conjugation with the amino acids leucine or alanine can be reversible and lead to increase in IAA availability according to the cellular requirements (Tromas and Perrot-Rechenmann 2010). The conjugation of amino acids to IAA is achieved throughout a process catalyzed by specialized GH3 family members, known as acyl acid amido synthetases (Hagen et al. 1984; Hagen and Guilfoyle 1985; Schaller et al. 2015).

The understanding of Ax action comes from diverse studies such as exogenous application, inhibitors of the Ax transport and from mutants unable to respond normally to Ax. Therefore, the Ax perception is found highly regulated inside the cells. For instance, Ax application induces quick changes at the transcription level (Hagen et al. 1984; Hagen and Guilfoyle 1985). In the presence of high Ax concentration, it promotes the degradation of the protein auxin/indole-3-acetic acid (AUX/IAA), which is a transcriptional repressor (Dinesh et al. 2016; Li et al. 2016). This elevate Ax concentrations activates Auxin Response Factors (ARFs), in this sense the Ax interacts and activates the inhibitory transport factor1 (TIR1), an F-box component of SCF^{TIR} E3 ubiquitin ligase that carried out the ubiquitination of AUX/IAA proteins sending them for destruction to the 26S proteasome (Dinesh et al. 2016; Li et al. 2016). From this mechanism, the dimerization of AUX/IAA with ARF-DNA binding transcriptional activator it has to be disrupted and then, ARF can activate the transcription on the promoter of its target. On the other hand, in the presence of low Ax concentration, the AUX/IAA, with a help of TOPLESS (TPL), a transcriptional repressor, target the ARFs and blocks their function.

The unlocking of ARFs can impact the modulation of expression of a high number of Ax-regulated genes troughout the binding of ARF to the TGTCTC sequence of the auxin response elements (AUXREs) needed to each developmental response. However, it is an important to highlight that the Ax concentrations, as well as their gradients within the cells, must be sensed by specialized receptors. One of this receptor is the AUXIN BINDING PROTEIN1 (ABP1). This has been considered as a putative important receptor since the loss of ABP1 function display an embryo lethal phenotype, particularly by suppressing the development at the globular stage causing cellular dedifferentiation (Chen et al. 2001; Grones et al. 2015). However, this function is still on controversy (Gao et al. 2015).

10.2 Auxin Response During the Somatic Embryogenesis

Since the discovery of cellular totipotency acquisition from carrot cells, it has been shown that the application of exogenous Ax into the culture medium can change the balance of Ax levels in the cells modifying IAA metabolism (Andreae and Good 1955; Ayil-Gutiérrez et al. 2013; Davies 1972; Michalczuk et al. 1992; Pescador et al. 2012), which is one of the determining factors during the induction of SE. Therefore, the Ax homeostasis must be kept at a specific level where the pro-embryogenic mass is able to acquire the embryogenic potential.

In general, pro-embryogenic calli contains more endogenous Ax than non-embryogenic calli. This observation has been documented in a different species, such as *D. carota* (Jiménez and Bangerth 2001a) (Sasaki et al. 1994; Tianran and Neumann 1985), *Medicago falcata* (Ivanova et al. 1994), *Saccharum officinarum* (Guiderdoni et al. 1995), *Zea mays* (Jiménez and Bangerth 2001c), *Triticum aestivum* (Jiménez and Bangerth 2001b), *Coffea canephora* (Quiroz-Figueroa et al. 2006), *Pennisetum purpureum* (Rajasekaran et al. 1987), *Brassica napus* (Rodríguez-Sanz et al. 2015) and *Prunus* spp. (Michalczuk and Druart 1999).

2,4-diclorophenoxyacetic acid (2,4-D) have a strong Ax-like activity and it has been widely applied to induce SE. 2,4-D can act as PGR, but it is more likely to work as a stressor. In carrot, it has been demonstrated that the removal of 2,4-D from a culture medium promotes the formation of embryogenic structures (Fujimura 2014; Halperin 1964). Even when we do not always observe this response, it is believed that 2,4-D facilitates the transition from embryonic cells to somatic embryos. Furthermore, 2,4-D lead the lose of the cell original polarity and interferes with the Ax gradients for a correct polar Ax transport and disrupting the organogenesis program (Karami et al. 2009). In summary, like in zygotic embryos, the polar transport of Ax is indispensable for the correct establishment of polarity in dicotyledonous somatic embryos (Abrahamsson et al. 2012; Hakman et al. 2009; Liu et al. 1993; Schiavone and Cooke 1987).

Unlike IAA, the contribution to the embryogenic efficiency by 2,4-D could be made by the lack of its degradation in the cell, which might influence the transcriptional reprogramming redirecting the developmental program of the cells; particularly in those plants species with higher levels of endogenous Ax. In this sense, treatments with 2,4-D (100 μM) in alfalfa cell cultures, for at least 1 h, induce the generation of somatic embryos after 2 weeks of culture (Dudits et al. 1991), whereas that the treatment with lower concentration of 2,4-D (4.5 μM) in carrot epidermal cells by 12–24 h promotes direct SE after to the transference of the epidermal cell to a free 2,4-D medium culture (Masuda et al. 1995). Later, using *Arabidopsis thaliana*, it was determined that at the molecular level the 2,4-D impacts the expression of a high number of transcription factors, particularly those related to stress (Gliwicka et al. 2013).

The results described above suggest that the exogenous Ax induces the accumulation of endogenous Ax, which appears to be essential for SE induction. In order to induce SE in *C. canephora*, first the plantlets used as a source of the explants, require a pretreatment period with naphthalene acetic acid (ANA) and Kinetin (KIN) for 2 weeks, and then, the leaf explants used to induce SE only need the presence of benzyladenine. During the pretreatment of the plantlets, the total free Ax, as well as some of their conjugates, was determined and it was found an important accumulation of IAA and IBA before to SE induction (Ayil-Gutiérrez et al. 2013). At the same time, it was found an increment in the transcripts of *CcYUC1*, suggesting that ANA/KIN ratio induces the biosynthesis of the endogenous Ax.

Another important fact, related to the endogenous concentration of IAA, is that during the induction of the SE free IAA decreases in just a few hours after the

induction, and *CcGH3.1* transcript increases, which correlates with the rise of IAA-Ala conjugate (Ayil-Gutiérrez et al. 2013). IAA-Ala can be stored and reused later on by the cells (Ludwig-Müller 2011). These results suggest that endogenous Ax accumulation appears to be essential for the changing of cell fate before the beginning of the embryogenic pathway. The results found in *Abies alba* also showed an increase of IAA, particularly in the embryogenic suspensor mass. However, during the first week of maturation, this Ax decreases suggesting that the increase in the level of endogenous IAA is important to start the differentiation process. Later, the IAA levels increase again according to the embryo growth, which can be seen as an important mechanism for the correct establishment of cells division patterns and the organization of both shoot and root meristems (Vondráková et al. 2011).

In oil palm (*Elaeis guineensis*), *EgIAA9*, a gene member of the AUX/IAA family that is rapidly induced by Ax, was selected as a gene marker to measure the embryogenic potential of oil palm explants (Ooi et al. 2012). In oil palm SE, callus is generated before the SE induction. IAA9 exhibits a higher transcription level in embryogenic calli (EC) than in non-embryogenic calli (NEC). As the exogenous 2,4-D increases, the *EgIAA9* transcript decreases, probably due to the increase of endogenous Ax levels.

In a work conducted in cotton, it was observed that IAA accumulation was related to the cellular dedifferentiation process. The concentration of IAA was 90 % more than at the beginning of the SE induction. This IAA concentration dropped by 78 % at the globular stage, followed by a slight increase at the cotyledonar stage (Yang et al. 2012). These data suggest that the endogenous IAA is highly regulated during the differentiation process. The analysis of gene expression profiling revealed that at least 86 genes, related to Ax synthesis, transport, metabolism and signaling were differentially expressed from the onset of dedifferentiation process to the late stages of SE. Several of them, *PIN3, AUX-LIKE 1, AUX/IAA, ARF, YUC, SMALL AUXIN UP RNA (SAUR), GH3.17*, show a high abundance, mainly during the generation of the embryogenic calli and low abundance during the embryo development process (Yang et al. 2012). *PIN3* and *AUX-LIKE1* genes encode to transport proteins to efflux and influx of IAA, respectively, showing high expression levels during the dedifferentiation process (Yang et al. 2012) and suggesting an important mobilization of IAA. Furthermore, the fact that *GH3.17* shows high levels of expression during the cellular dedifferentiation (Yang et al. 2012), it also suggests that this IAA-amido synthetase can help to maintain cell homeostasis by producing IAA-Glu, a conjugate that leads to the IAA degradation (Staswick et al. 2005). Moreover, it can be hypothesized that the relatively low expression of *TIR1* detected during the induction of SE and the increase of *IAA19* (AUX/IAA) expression implicates an important modulation in the expression of some ARF family members during the SE of cotton (Yang et al. 2012).

A recent study showed that one possibility by which SE can be more feasible in one cell line than in other could be related to gene expression rather than absence of genes involved in the SE (Xu et al. 2013). The same authors determined that differential gene expression, mainly those involved in the Ax biosynthesis, might

determine the embryogenic potential among sister cell lines. The establishment of embryogenic callus EC was correlated with at least 185 transcripts related to metabolism, signal transduction and Ax biosynthesis pathway, compared to the 125 transcripts found in the non-embryogenic callus NEC. From all the transcripts, *GH3, AUX/IAA, TIR*, *AUX* and *YUC* were the most abundant in the EC than in the NEC. Interestingly, two transcripts from *YUC*, four from *GH3*, and six from the *SAURs* family were present exclusively in the EC line suggesting that these genes can contribute to the induction of EC and; therefore, to the developing of somatic embryos (Xu et al. 2013). However, it has to be shown whether these genes are directly involved to the SE induction.

On the other hand, PIN has been extensively characterized during the early zygotic embryo development (Aida et al. 2002; Petrásek and Friml 2009). The role of PIN during the onset of SE also seems to be important. Like the SE cotton (Yang et al. 2012), during the SE in maize the expression of *ZmPIN*1, as well as the orthologs *ZmPIN1b* and *ZmPIN1c*, were substantially increased during early SE (Salvo et al. 2014).

Su et al. (2009) showed that in early SE of Arabidopsis, PIN1 is polarized within the cells in just 16 h after SE induction. Twenty hours later, its expression was directly correlated with the beginning of *WUSCHEL* expression, an important regulator of stem cell (Mayer et al. 1998), suggesting that polar transport of Ax, addressed by PIN1, is crucial for somatic embryo development. This possible regulation was proved by blocking the Ax transport with the *N*-1-naphthylphthalamic acid (NPA). NPA prevents several events such as the PIN1 polar distribution, the *WUS* expression, and the formation of somatic embryos. Furthermore, the loss of function of *PIN1* also inhibited the SE process, suggesting that PIN plays a key role in the transport and accumulation of Ax necessary for *WUS* expression (Su et al. 2009). These results are in agreement with the fact that the accumulation and distribution of Ax are very important for the onset of SE.

In *Quercus suber*, a similar pattern of localization of endogenous IAA was observed during the induction of SE from microspore and immature ZE. At early multicellular embryo cells, a differential and significant increase of IAA endogenous levels was found. After the induction of SE, both explants showed low levels of IAA (Rodríguez-Sanz et al. 2014).

During the onset of cellular reprograming to totipotency it has been observed an important correlation between stress and the onset of Ax biosynthesis. The use of low temperatures for the induction of SE in microspores of *B. napus*, leads to a similar process than that to the ZE, with the formation of suspensor-like structure (Lawyer et al. 1989). Heat stress treatment (32 °C) also induces SE in *B. napus* in the absence of PGRs. There is a relationship between different type of stress conditions and Ax homeostasis with downstream factors essential to the acquisition of embryogenic capacity. For example, heat treatment allows the establishment of the first cell divisions and the concomitant expression of *TAA1* and *NITRILASE2* (*NIT2*).

During the stress treatment of *B. napus* spores, a differential distribution of the endogenous concentration of IAA was found. In general, there are low levels of

IAA in the suspensor-like structure and higher levels in the proper embryo. Using mild heat temperature treatment to induce the SE in *B. napus* and GFP as a marker, under control of the synthetic auxin DR5-promoter, Dubas et al. (2014) founded that after the induction of SE and the first asymmetric transverse division, the DR-5 activity was detected in both apical and basal cell. However, the basal cell had a higher activity suggesting that the Ax flux was carried out from the basal cell to the apical cell (Friml et al. 2003; Jenik et al. 2007), the same pattern during the first division of ZE. Another similitude with the ZE pattern was the accumulation of DR-5 activity in the cell that would originate the hypophysis. Later and before the transition to heart stage of the embryo, the DR-5 activity was higher during the establishment of provascular cambium and the cotyledonar leaf primordia, which suggest that Ax distribution gradient is necessary for the correct pattern formation of the development embryo (Dubas et al. 2014).

The overexpression of the orthologue *SHOOTMERISTEMLESS* (*STM*) gene from *B. rapa*, *B. olareacea* or *B. napus* into Arabidopsis (Elhiti et al. 2010) let to the increase in the embryogenic response by twofold than the wild-type line, even at low concentration of 2,4-D (0.75 μM). The constitutive expression of *SMT* altered the expression genes involved in the Ax transport and signaling, as well as some IAA-induced elements such as IAA1, IAA8, IAA9 and IAA28. This result suggests that the improvement in the somatic embryos production was due to an increment in the sensitivity to 2,4-D (Elhiti et al. 2010). The overexpression of *LEAFY COTYLEDON1* (*LEC1*) can induce the generation of somatic embryos from vegetative cells, even without pulses of Ax. It has been suggested that this transcriptional factor can activate the transcription of important genes needed to embryo morphogenesis and cellular dedifferentiation (Lotan et al. 1998). It has also been shown that *LEC1* overexpression can induce the accumulation of Ax, and this can be used as a trigger to SE by modulating the expression of *YUC10* (Junker et al. 2012). In the same context, *LEC2* (another regulator master of embryogenesis) overexpression also induces the activation of *YUC2* and *YUC4* increasing the concentration of IAA. *YUC4* is only expressed in specific cells in the developing embryos, where the higher Ax concentration co-localizes with the activity of LEC2. These findings are consistent with the role of *LEC2* in the establishment of embryogenic patterns during the ES (Stone et al. 2008). Furthermore, it seems to be that both *IAA16* and *IAA19* are found among the genes targeted and regulated by *LEC1* (Junker et al. 2012), whereas *IAA7* is targeted by *LEC2* (Stone et al. 2008). This regulation suggests a strict control between the Ax biosynthesis mediated by YUC family members and the Ax response factors, an attempt to properly regulate the differential growth response during SE.

Recently, the beginning of the cellular totipotency was studied from dedifferentiation and regeneration process using Arabidopsis protoplast (Chupeau et al. 2013). The onset of the regeneration involved the reentry to the cell cycle accompanied by important changes in the expression profiles of auxin-related genes. The Ax biosynthesis pathway was activated, since *TRYPTOPHAN SYNTHASES, NITRILASES AND SPECIALIZED CYTOCHROME* P450 genes were upregulated at the beginning of the dedifferentiation process, and gradually

deactivated according to the redifferentiation process. The genes *GH3.2* and *GH3.3*, involved in the conjugation of Ax, were maintained actively during all the embryogenic process. In addition, the Ax efflux gene members (*PIN1* and *PIN6*) were upregulated together with some genes encoding Ax receptors such as *AFB2* and *AFB5*. Furthermore, *IAA7-9, IAA20, IAA29* and *ARF4-6* were also actively expressed during the regeneration process (Chupeau et al. 2013). The strong regulation of Ax homeostasis during the early steps of regeneration also activated important *WUSCHEL*-related homeobox genes such as *WOX5*, *WOX8* and *WOX13,* which are essential to early embryo development (Gambino et al. 2011) suggesting a complex molecular interaction during the beginning of the cellular redifferentiation.

In addition, the gradual accumulation of IAA is distributed in early stages of the development of the somatic embryos; it accumulated in the apical and basal regions of the embryos in the torpedo stage. It can be hypothesized that this special distribution and accumulation to specific zones along the somatic embryo could be the responsibility of PIN1-like, since there is a gradual increase of their transcripts from early multicellular embryos to torpedo stage (Rodríguez-Sanz et al. 2015). These results clearly suggest that the participation of *PIN1* during the SE development in several plant species, such as maize, cotton, Arabidopsis and *B. napus* is crucial to maintaining the embryogenic potential. (Rodríguez-Sanz et al. 2015; Salvo et al. 2014; Su et al. 2009; Yang et al. 2012) since the disruption of Ax polar transport by NPA modified the auxin distribution and then negatively impact in the embryo development.

In *Curcubita pepo,* the SE can also be induced in the absence of PGRs by using ammonium as the only nitrogen source. As the 2,4-D is removed, the transfer of embryonic pumpkin callus from ammonium containing medium to other with both, ammonium and nitrate, also produced a reduction in the concentration of the endogenous IAA and, in consequence, the development of the embryos (Pencik et al. 2015).

Acknowledgements This work from VMLV laboratory was supported by a grant received from the National Council for Science and Technology (CONACyT, 157014). The authors thank Osvaldo J. Couoh-Dzul and Hugo A. Méndez Hernández for their assistance.

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Part II
Somatic Embryogenesis of Basic Models and Industrial and Agronomical Crops

Chapter 11
Somatic Embryogenesis in Arabidopsis

Barbara Wójcikowska and Małgorzata D. Gaj

Abstract In recent years, studies on Arabidopsis have greatly contributed to the formulation of the universal molecular mechanisms that are involved in the developmental plasticity of somatic cells and, especially, in the identification of the genes that govern the induction of somatic embryogenesis (SE) in an in vitro culture. Various in vitro culture systems have been applied in molecular studies on SE in Arabidopsis, which enable the direct or indirect induction of somatic embryos. In this chapter, the different factors that determine the mode of the embryogenic response of in vitro cultured explants of Arabidopsis are reviewed. In addition to an in vitro culture, the induction of SE *in planta* is also characterised. The different approaches that are used for SE induction in Arabidopsis are presented in relation to studies on the molecular determinants of plant totipotency.

11.1 Why Study SE in Arabidopsis?

In the last 50 years of plant biology, *Arabidopsis thaliana* has become the unquestionable plant leader that has contributed greatly to the spectacular progress that has been made in diverse research areas including studies on cell biology, development, hormones, plant immunity, signalling in response to abiotic stress, transporters, the biosynthesis of cell walls and macromolecules such as starches and lipids, epigenetics and epigenomics, genome-wide association studies and natural variation, gene regulatory networks, modelling and system biology and synthetic biology (Provart et al. 2015). Arabidopsis provides an excellent study system for functional and comparative genomics due to the availability of a plethora of genomic resources such as EST and full-length cDNA databases, huge collections of characterised insertion mutants and a large set of expression data that covers numerous environmental conditions and developmental stages. In addition to their

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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_11

cognitive value, the important discoveries in plant biology that have been made using the powerful model system of Arabidopsis are predicted to also have an important impact on crop species. Although the translatability to crops of the data produced in basic research is rather unsatisfactory at the moment, an increased role of model species, including Arabidopsis, for further improvements in plant breeding is expected (Spannagl et al. 2011; Nelissen et al. 2014).

Studies based on Arabidopsis have become the key driver to elucidate the molecular pathways that underlie the growth and development of plants not only *in planta* but also under in vitro conditions. In the last decade, Arabidopsis has played a pivotal role in revealing the basic molecular mechanisms that control the developmental plasticity of plant somatic cells in vitro. Among the various morphogenic pathways that have been induced in the cultured plant explants, somatic embryogenesis (SE) has attracted the most attention because of the cognitive as well as practical value of this unique process for plant biotechnology. In order to elucidate the mechanisms of SE induced in vitro, questions on the genetic factors that control embryogenic transition have recently been addressed in Arabidopsis and as a result, the complex network of the genetic elements that interact at molecular level has just begun to emerge (see Chap. 5).

11.2 Different Approaches Used to Induce SE in Arabidopsis

The first report on somatic embryo induction in Arabidopsis tissue cultured in vitro was published in 1983 (Huang and Yeoman 1983) and since then seventeen protocols for SE induction in this plant have been elaborated in which different induction media, explants and genotypes have been evaluated (Table 11.1).

11.2.1 Direct Versus Indirect Pathway

In Arabidopsis, two pathways of SE induction have been observed and somatic embryos can develop directly from the explant tissue (direct SE, DSE) or through the establishment of a callus culture that induces the production of somatic embryos (indirect SE, ISE) (Gaj 2004). A survey of seventeen reports on SE induction in Arabidopsis shows that, similar to other plants, the indirect pathway is more frequent and somatic embryos have been regenerated from the callus in 76 % of the protocols (Fig. 11.1a). It was found that a developmental stage of the explants, immature zygotic embryos (IZEs), which are commonly used for SE induction in Arabidopsis, determines the mode of the embryogenic response that is triggered (Gaj 2004). In contrast, IZEs that represent the early stages of development (heart and torpedo), which produce somatic embryos exclusively via callus, IZEs at the advanced green cotyledonary stage are able to efficiently and directly induce SE

Table 11.1 Protocols that have been applied under in vitro culture to induce somatic embryogenesis in Arabidopsis

	Explant type	Age of explant	Indirect (I) or direct (D) SE mode	Solid (S) or liquid (L) basal medium	Light (L) or dark (D) conditions	Plant growth regulators	Conversion into plants	Genotypes	References
1	Seeds	?	I	B5*/S	L	0.5 mg/L 2,4-D + 0.05 mg/L kinetin	+	?	Huang and Yeoman (1983)
2	Roots	35-day-old plants	D	MS**/S	?	3 mg/L IAA 0.15 mg/L 2,4-D 0.6 mg/L BA 0.3 mg/L IPA	+	RLD, Col	Marton and Browse (1991)
3	Immature zygotic embryos	Intermediate and torpedo stage of development	I	B5/L	D	1 mg/L 2,4-D	+	Ler	Wu et al. (1992)
4	Leaf protoplast	21–28-day-old plants	D	B5/L	L/D	1 mg/L DICAMBA or 1 mg/L 2,4-D or 8 mg/L DICAMBA + 0.1 mg/L 2,4D	+	C24, Ler, Est, Col	O'Neill and Mathias (1993)
5	Immature zygotic embryos	Heart to bent torpedo stage of development	D/I	B5/S	?	4.5 µM 2,4-D followed by 9 µM 2,4-D + 0.11 µM BAP	+	Col	Pillon et al. (1996)
6	Immature zygotic embryos	Globular to bent torpedo stage of development	I	B5/L	D	0.125–8 mg/L 2,4-D	+	Nd-0, Ler, C24, Col, No, Ws-2	Luo and Koop (1997)
7	Leaf protoplast	20–30-day-old plants	I	B5/L	D	2 mg/L DICAMBA 0.15 mg/L BA	–	Nd-0, Ler, C24, Col, No,Ws-2	Luo and Koop (1997)

(continued)

Table 11.1 (continued)

	Explant type	Age of explant	Indirect (I) or direct (D) SE mode	Solid (S) or liquid (L) basal medium	Light (L) or dark (D) conditions	Plant growth regulators	Conversion into plants	Genotypes	References
8	Immature zygotic embryos	Early bent cotyledonary stage of development	I	B5/L	L/D	4.5 µM 2,4-D	+	Ler, RLD, Col, En-T, Est, No, Wie-0, Di-G, Cvi-0, Coi	Mordhorst et al. (1998)
9	Seedlings	?	I	MS/L	L/D	4.5 µM 2,4-D	+	Ler	Mordhorst et al. (1998)
10	Suspension culture of hypocotyl and cotyledon	6-day-old seedlings	I	MS/L	L	11.4 µM 2,4-D + 2.3 µM kinetin	–	Ler	Meijer et al. (1999)
11	Immature zygotic embryos	Late cotyledonary stage of development	I	MS/S	L/D	5 µM 2,4-D	+	Col, RLD, Ws, Wilna, C24, Ler	Gaj (2001)
12	Immature zygotic, somatic embryos	Bent cotyledon stage of development	I/D	B5/S + L	L/D	4.5 µM 2,4-D followed by 9 µM 2,4-D	?	Col	Ikeda-Iwai et al. (2002)
13	Immature zygotic, somatic embryos	Early bent cotyledon stage development	I	B5/L	L/D	4.5 µM 2,4-D	+	Ler	Mordhorst et al. (2002)
14	Shoot apical tip (about 1 mm in length)	5-day-old seedlings	I?	B5/S	L	4.5 µM 2,4-D	?	Ws, Col, Ler	Ikeda-Iwai et al. (2003)
15	Floral and axillary buds	56–84-day-old plants	I?	B5/S	L	4.5 µM 2,4-D	?	Col	

(continued)

Table 11.1 (continued)

	Explant type	Age of explant	Indirect (I) or direct (D) SE mode	Solid (S) or liquid (L) basal medium	Light (L) or dark (D) conditions	Plant growth regulators	Conversion into plants	Genotypes	References
16	Immature zygotic, embryos	Bent cotyledon stage of development	I	B5/S + L	L/D	5 µM 2,4-D + followed by 9 µM 2,4-D	?	Col	Su et al. (2009)
17	Germinating embryos	Isolated from seeds 1 day after sowing	I	B5/S	L	4.5 µM 2,4-D	+	Col, Ler, Ws, No, JA2, 8, 126, 192, 240, 262, 335, 347	Kobayashi et al. (2010)

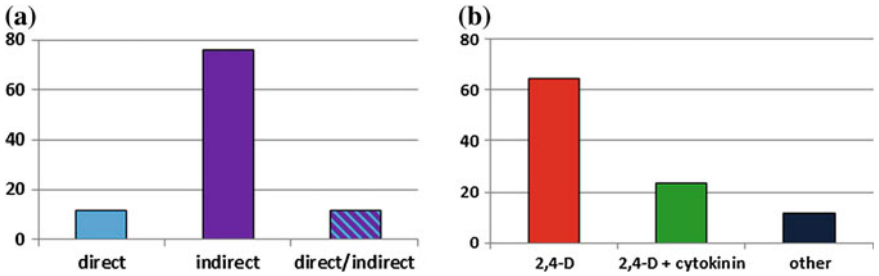

Fig. 11.1 Types of **a** SE pathways induced to produce somatic embryos, **b** plant growth regulators in media applied for SE induction in Arabidopsis in vitro cultures

(Wu et al. 1992; Gaj 2001). In addition to the stage of the IZE, the type of culture medium (liquid vs. solid) appears to influence the mode of SE that is induced in Arabidopsis. A liquid induction medium favours callus formation while an agar medium is recommended to limit callus production (Gaj 2004). It would be of interest to define the molecular determinants of the direct versus indirect mode of SE and their relation to the explant stage and medium type.

11.2.2 Explant Type

Immature zygotic embryos at an advanced developmental stage are recommended for efficiently inducing SE in Arabidopsis (Gaj 2001). Accordingly, a culture of IZEs that were isolated at the 11–13 DAP (days after pollination) stage of development (the so-called late cotyledonary stage) promotes SE induction in over 90 % of the explants in various genotypes, including the most commonly used Col-0 (Gaj 2011). Thus, the IZEs that represent this stage of development have been used successfully for SE induction in various laboratories (Table 11.1). Alternatively, the primary somatic embryos produced in an IZE-culture can be used as the explants to induce secondary SE (SSE) (Ikeda-Iwai et al. 2002; Gaj and Czubin 2004; Su et al. 2009). A callus that was induced in such an SSE culture was reported to maintain embryogenic potential at least for one year (Pillon et al. 1996).

Explants that represent post-embryonic tissue have been used to induce SE much less frequently than IZEs and limited success has been reported. Accordingly, seeds (Huang and Yeoman 1983), germinating seeds (Kobayashi et al. 2010) or young seedlings (Mordhorst et al. 1998) were cultured and an embryogenic callus was formed in response. Moreover, SE induction was reported in culture of roots of 5-week-old plants (Marton and Browse 1991), hypocotyls and cotyledons of 6-day-old seedlings (Meijer et al. 1999) and mesophyll protoplasts were isolated from the leaves of 3- to 4-week-old plants (O'Neill and Mathias 1993; Luo and Koop 1997). However, the efficiency of SE induction in seedling- or plant-derived explants has been found to be very low and varies in the range of 0–50 % of embryogenically responsive explants.

It has also been reported that auxin treatment combined with stress factors enhances the embryogenic response of post-embryonic explants including the shoot apical tip and the floral as well as axillary buds isolated from seedlings and plants (Ikeda-Iwai et al. 2003). This observation implies that some culture conditions, other than hormone treatment, may be required to trigger the embryogenic programme in tissue that is isolated from post-embryonic organs.

In summary, with the current state of knowledge, the use of IZEs, especially those that are advanced in development, guarantees the induction of a productive and repetitive SE response in Arabidopsis. However, because of the per se embryogenic nature of IZEs, these explant types have some limitations in the identification of the genetic and physiological factors that control the reprogramming of fully differentiated somatic cells. Hence, the establishment of the culture conditions that enable an efficient and controlled embryogenic transition in the post-embryonic organs or tissue of Arabidopsis is extremely desirable.

11.2.3 Genotype

The majority of the protocols for SE induction have been optimised for the genotypes that are commonly used in molecular biology research, including Col-0, Ler and WS (Table 11.1). The frequency of SE-responsive explants (IZEs at the late cotyledonary stage) was evaluated at over 90 % and up to 60 % for Col-0 and Ler/WS, respectively (Gaj et al. 2001). In the best SE-responsive Col-0 genotype, more than 90 % of the late cotyledonary-stage IZE explants underwent rapid and efficient SE. A subculture of IZE-derived somatic embryos onto an auxin-free medium resulted in their conversion into plants with an average frequency of 80 %. The Col-0 plants that were obtained using this system were found to be fertile and they displayed a low level of somaclonal variation, which was manifested by sporadically observed chlorophyll mutations. Hence, the IZE-based SE induction system that was established for Col-0 was recommended for wide applications in studies on the mechanisms that govern plant totipotency including the identification of the genetic factors that control the embryogenic transition of somatic plant cells (Gaj 2004).

The strong genotype dependency of SE induction was also confirmed by an analysis of almost 350 natural accessions of Arabidopsis. In that study the germinating embryos, which had been cultured in the presence of 2,4-D, were able to induce SE with an efficiency ranging from 0 to 92 % depending on the genotype (Kobayashi et al. 2010).

11.2.4 Physiological Conditions of Donor Plants

In addition to genetics, the physiological state of the donor plants may also substantially impact the efficiency of the embryogenic response induced in Arabidopsis

explants. Thus, clearly defined and controlled growth conditions are recommended in order to obtain healthy donor plants for the isolation of the explants (IZEs). Among others, the careful protection of the donor plants against pathogens is very important as explants that were isolated from the aphid-invaded plants showed drastically reduced capacity for SE (Gaj 2011).

11.2.5 2,4-D as a Potent SE Inducer

Supplementation of a medium with auxin has been found to be indispensable for SE induction in Arabidopsis and in the majority (94 %) of the protocols 2,4-D was applied as the sole plant growth regulator (65 %) or in combination with cytokinins (24 %) (Fig. 11.1b). Other auxins have sporadically been applied, e.g. DICAMBA, alone or in combination with BAP, was used to induce SE in a culture of Arabidopsis leaf protoplasts but with limited success (O'Neill and Mathias 1993; Luo and Koop 1997).

The successful use of 2,4-D in SE induction in different plant species, including Arabidopsis, has stimulated a debate on the molecular processes that are triggered by this compound in the cultured plant cells. A dual function of 2,4-D in SE induction has been proposed and besides its auxin-like activity, it has been suggested that 2,4-D promotes SE via a stress-related mechanism (Fehér 2015; Karami and Saidi 2010; Zavattieri et al. 2010). In support for the suggested stressor-like activity of 2,4-D, an increased amount of reactive oxygen species (ROS), which are associated with oxidative stress, was detected in plant cells that had been treated with 2,4-D (Pfeiffer and Höftberger 2001). Moreover, in addition to auxin-responsive genes, numerous stress-related genes, including the genes encoding the transcription factors (TFs), have been reported to accompany SE induction in Arabidopsis (Gliwicka et al. 2013; Wickramasuriya and Dunwell 2015; Nowak et al. 2015).

In line with the assumption about the auxin-like activity of 2,4-D, its active AUX-mediated transport to cells and its affinity to the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) protein, which is one of the auxin receptors that operate in the auxin signalling pathway, were indicated (Delbarre et al. 1996; Kepinski and Leyser 2005; Tan et al. 2007). Relevant to these findings, a variety of auxin-responsive genes have been found to be upregulated in 2,4-D-induced explants of Arabidopsis undergoing SE induction, including the key regulators of SE (Ledwoń and Gaj 2009; Gliwicka et al. 2013). However, whether 2,4-D may directly activate the SE-regulatory genes or whether it contributes to the accumulation of endogenous indole-3-acetic acid (IAA) via an as yet undetermined mechanism, which in turn triggers a network of genes that control embryogenic transition remains unclear (Michalczuk et al. 1992; Charrière et al. 1999). Recent studies on the *LEC2* function in SE induction in Arabidopsis have provided some hints to support this hypothesis. An increase in the endogenous IAA level in an embryogenic culture of Arabidopsis was indicated to result from 2,4-D-stimulated transcription of *LEAFY COTYLEDON 2* (*LEC2*), which is a key regulator of SE

induction that positively controls the *YUCCA*-mediated pathway of auxin biosynthesis (Wójcikowska et al. 2013; Wójcikowska and Gaj 2015).

Epigenetic changes might be also assumed to be among the possible mechanisms that activate SE-related genes in response to 2,4-D since an increased level of DNA methylation in 2,4-D-induced embryogenic cultures of different plants has been reported including *Daucus carota*, *Acca sellowiana*, *Cucurbita pepo* and *Coffea canephora* (LoSchiavo et al. 1989; Fraga et al. 2012; Leljak-Levanić et al. 2004; Nic-Can et al. 2013). In further support for a DNA methylation-related function of 2,4-D during SE induction, 5-azacytydin (5-AzaC), an inhibitor of DNA methylation, was documented to affect the embryogenic capacity of *Coffea canephora* and Arabidopsis cultures (Nic-Can et al. 2013; Szurman et al. 2009).

11.3 Histological and Morphological Characteristics of Embryogenic Cultures

The induction of SE in IZEs at an advanced cotyledonary stage of development is cultured on a 2,4-D (5 μM) supplemented agar medium is accompanied by rapid and distinct changes in the explant tissues that are visible at the morphological and histological levels (Fig. 11.2) (Kurczyńska et al. 2007). The morphological changes that accompany SE induction include the straightening and expansion of the previously bent cotyledons of IZEs and swelling of the cotyledon node during the first week of the culture. The first somatic embryos appear on the adaxial side of cotyledons in the area that is proximal to the cotyledon node by the second week of the culture (8–10 days). Somatic embryos are produced asynchronously and by the end of week two the cotyledon part of the IZE is covered with somatic embryos in various stages of development.

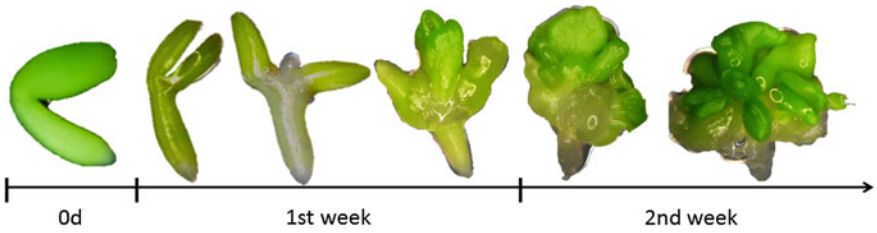

Fig. 11.2 Time-course of SE induction in a culture of the IZE explants of Arabidopsis following the method of Gaj (2001). IZEs at the late cotyledonary stage of development (0d) are cultured on an E5 solid medium with 5 μM of 2,4-D. During the first week, the straightening and expansion of cotyledons and swelling of the cotyledon node were observed. The first somatic embryos begin to emerge by the second week on the adaxial side of the cotyledons in the area proximal to the cotyledon node of the culture (8–10 days). By the end of the second week the cotyledon part of the IZE is covered with somatic embryos

At the histological level, SE begins with the proliferation of the explant cells and embryogenic centres that are composed of small cells with a dense cytoplasm are formed due to the periclinal divisions in the protodermis of the explant. Expression of *LEC2* was observed in the few-celled clusters, thus indicating that these cells had switched to an embryo identity. These embryonic cell clusters can be recognised by the surrounding thick cell walls and cutin deposition in the cell walls of a potential embryonic protodermal cell of an IZE explant. In addition to the cutin, the developing somatic embryos are also symplastically isolated from the explant tissue.

During the first two weeks of a culture most of the somatic embryos develop directly from the explant cells although the indirect formation of some of the embryos (especially those developing later in the culture) is also possible. At the third week of the culture, the callus tissue is formed mostly from the abaxial side of cotyledons and then, somatic embryos develop from the callus following indirect SE. Somatic embryos were reported to originate from a single explant cell and multi-cell embryogenic centres; however, a single-cell origin of somatic embryos appears to be more frequent in direct SE (Kurczyńska et al. 2007).

Determining that the callus formation does not contribute to somatic embryo development can be difficult due to the fact that DSE and ISE may be induced simultaneously in explant tissue under the same in vitro conditions (Omar et al. 2016). Thus, a histological analysis is necessary to exclude the participation of the callus in embryogenic transition. In light of the recent findings on the origin of callus cells, a distinction between the DSE and ISE pathways appears to be even more complicated and the common view on the nature of callus tissue requires revision. Accordingly, the callus was recently indicated to originate from a pre-existing population of the stem cells of an explant and trans-differentiation rather than dedifferentiation of explant cells is postulated to be involved in the regeneration processes that are induced in vitro (Sugimoto et al. 2010). In line with this notion, it was shown that the callus formation is not just the result of the reprogramming of differentiated cells to an undifferentiated state but callus cells were demonstrated to resemble the tip of a root meristem (Sugimoto et al. 2010). These findings imply the need to verify the common belief that dedifferentiation of explants cells into a callus commonly precedes the induction of the indirect pathway of plant regeneration in vitro. Thus, revealing the origin and nature of the callus cells that develop somatic embryos in Arabidopsis appears to be of basic interest for understanding the developmental pathway that underlies ISE. The existence of ISE and DSE pathways in Arabidopsis provides a unique research opportunity for the identification of the cytological and molecular elements that differentiate the direct and indirect development of somatic embryos.

It is expected that upon isolation from the underlying tissue of the explant and transfer onto a hormone-free medium, somatic embryos elongate and undergo apical-basal differentiation to form well-defined shoot and root meristems. However, analysis of the auxin maxima in somatic embryos of Arabidopsis using the DR5::GUS reporter line indicated that the formation of root poles is frequently disturbed. A lack of a properly formed root meristem that was dependent on the auxin concentration and duration of treatment was observed in 35–80 % of in vitro

induced somatic embryos (Nowak et al. 2012). In addition, frequent developmental malformations of somatic embryos such as multiple, trumpet-shaped embryos with fused cotyledons can be observed but these morphological defects do not seem to negatively interfere with the capability of somatic embryos to develop into plants. It was estimated that somatic embryos developed shoots or shoots with roots with an average frequency of 79.5 % (Gaj 2001). Given that the ratio of embryo-like structures converting into plants distinctly surpasses the frequency of true somatic embryos (i.e. those that properly form a root pole), it remains to be determined whether an embryonic root pole that lacks a properly formed auxin maximum is able to elongate and develop into a functional root or whether adventitious roots are formed upon its transfer onto a hormone-free medium.

11.4 SE Systems Commonly Used in Molecular Studies

In studies on the molecular mechanisms that govern embryogenic transition in plants, the IZEs of Arabidopsis at the late cotyledonary stage of development are applied as the explants most frequently. Based on these explants, two alternative methods of SE induction in Arabidopsis that include one-step and direct SE induction are recommended (Gaj 2001) versus a multi-step and callus-involved indirect SE (Ikeda-Iwai et al. 2002). Although IZEs are cultured in the presence of 2,4-D in both of these methods, they distinctly differ in the concentration of auxin, the sequence of the media used, the duration of the culture and as a result, in the mode of SE pathway that is induced (direct vs. indirect).

In the direct, one-step method, the IZEs are cultured on an agar medium containing 5 μM of 2,4-D. A minimal induction time of an eight-day treatment with 2,4-D is required to induce embryogenic response; however, it is recommended the explants be cultured in the presence of auxin for 2 weeks in order to induce efficient direct SE (Gaj 2011).

In the callus-involved method, the IZE explants are cultured onto a liquid medium with 4,5 μM 2,4-D for 3 weeks until the primary somatic embryos are produced. Then, in order to force callus production, the culture is transferred into a liquid medium with an increased concentration of 2,4-D (9 μM). As a result, an embryogenic callus is produced from the primary culture, which can then be maintained on an auxin medium and subcultured every 2 weeks. To induce the massive production of secondary somatic embryos, the callus is transferred onto hormone-free medium (Ikeda-Iwai et al. 2002; Su et al. 2009).

11.4.1 SE Induced in Planta

In Arabidopsis, in addition to the in vitro culture conditions used to induce SE, the development of somatic embryos *in planta* was reported in genotypes that are affected in the genes that control embryogenic transition. Accordingly, a number of

genes encoding transcription factors have been identified that, when overexpressed, induce SE in Arabidopsis seedlings including *LEC1* (Lotan et al. 1998) and *LEC2* (Stone et al. 2001) of *LEAFY COTYLEDON* genes, *WUSCHEL* (*WUS*) (Zuo et al. 2002), *BABY BOOM* (*BBM*) (Boutilier et al. 2002), *AGAMOUS LIKE-15* (*AGL15*) (Harding et al. 2003) and *AINTEGUMENTA-LIKE5*/*PLETHORA5*/*EMBRYOMAKER* (*AIL5*/*PLT5*/*EMK*) (Tsuwamoto et al. 2010). A lack of auxin treatment might account for a greater fraction of true somatic embryos that are produced *in planta* than in in vitro systems and up to 75 % of embryos that formed on the seedlings overexpressing *LEC2* were indicated to have a properly developed root pole (Nowak et al. 2012).

In addition to overexpressor lines, spontaneous promotion of SE was also described in mutants that were affected in SE-related genes. Accordingly, knock-down mutations in genes encoding the VP1/ABI3-LIKE (VAL) proteins thus resulting in the increased expression of *LEC* genes led to the formation of an ectopic somatic embryo on seedlings (Suzuki et al. 2007). Moreover, knock-down mutations in the genes of the POLYCOMB REPRESSIVE COMPLEXES1 (PRC1) complex, which is involved in the epigenetic regulation of SE-regulators including *LEC*, *Atring1a Atring1b* and *Atbmi1a Atbmi1b*, were indicated to de-repress the embryonic traits in somatic plant tissues and to promote the formation of a somatic embryo (Chen et al. 2010; Bratzel et al. 2010). A similar SE-inducing effect was observed in a single *fie* and a double *clf swn* knock-down mutant that was defective in the *CURLY LEAF, SWINGER* and *FERTILISATION-INDEPENDENT ENDOSPERM* gene encoding protein of the POLYCOMB REPRESSIVE COMPLEXES2 (PRC2) complex that is engaged in histone modification (Makarevich et al. 2006; Bouyer et al. 2011).

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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

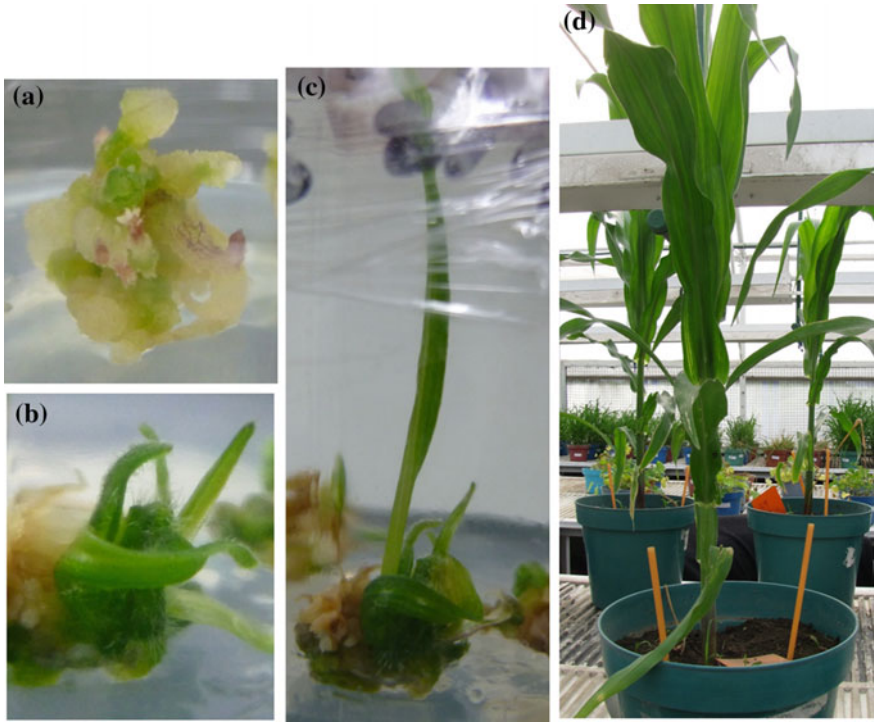

Fig. 12.2 Regeneration and acclimatization of maize plants from SE system. **a** E callus showing somatic embryos as green spots. **b** Plantlet of approximately 2 cm in height. **c** Plantlet of approximately 8 cm ready for transplant to soil. **d** Acclimatized plant

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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Chapter 13
Somatic Embryogenesis in Annatto (*Bixa orellana* L.)

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Abstract Our research group has pioneered the work on somatic embryogenesis of *Bixa orellana* (annatto), and since then we have directed efforts in understanding several aspects of this morphogenic pathway in annatto. Here, we present a synthetic description of such works, emphasizing anatomical analyzes and the characterization of the cellular alterations that occur in the process, and the association of the *SERK* gene expression and somatic embryogenesis. These results are unprecedented and contribute to a better understanding of the processes involving

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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_13

somatic embryogenesis in the species. Advances in this area will facilitate the improvement of the mass propagation, genetic manipulation of the carotenoid biosynthetic pathway, and the overall breeding perspectives of the genus.

13.1 Introduction

Worldwide interest in *Bixa orellana* L. has increased in the past years, because it accumulates two natural dyes at outside the seed coat, named bixin and norbixin carotenoids. Nowadays, these dyes are largely used as substitutes for synthetic dyes in the food and cosmetic industries (Giuliano et al. 2003; Dias et al. 2011; Marcolino et al. 2011; Mala et al. 2015) or as histological plant staining (Akinloye et al. 2010).

There are scientific evidences linking the antioxidant properties of such carotenoids (Bartley and Scolnik 1995; Kiokias and Gordon 2003) with reduction of the reactive oxygen species/nitric oxide induced by diabetes (Rossoni-Júnior et al. 2012) and the risk of cancer and other chronic conditions such as coronary heart disease (Cunningham and Gantt 1998). In addition, carotenoids have important functions in relation to human health and nutrition, since all species that contain a β-ring can be converted into retinol and, therefore, are precursors of vitamin A. The identification of the carotenoid biosynthesis genes in plants and other organisms has offered the foundations to the biotechnological overproduction of carotenoids of interest in crops (Fraser and Bramley 2004). These above mentioned economic and scientific importances have attracted the attention of researchers and in the past 15 years, several basic and applied relevant information on this species have been generated.

Annatto is a cross-pollinated species and thus highly heterozygous. The conventional propagation is mainly through seeds as plant material available to growers. Vegetative propagation via cuttings has limitations because of the intense leaching of a gummy substance and phenolics from the cutting ends, which difficult rooting (D'Souza and Sharon 2001). Therefore, the application of a reliable in vitro clonal propagation system would unquestionably aid in the multiplication of elite types, in especial those with higher carotenoid contents. The main purpose of applying in vitro culture is to establish and optimize a new method of vegetative propagation and the potential advantages of such system would be to reduce the heterogeneity, to increase the production of the pigment bixin, and to provide a foundation for the subsequent genetic manipulation and control of the biosynthesis of specific secondary metabolites like bixin.

In this particular, plant cell and tissue culture techniques have long attracted interest to characterize better and understand the plasticity of somatic plant cells and related morphogenic events resulting from in vitro cell reprogramming toward the acquisition of regeneration competence (Verdeil et al. 2007; Sugimoto 2015; Sugiyama 2015). Notwithstanding, the foundations for the regulation of the in vitro morphogenetic somatic organogenesis and embryogenesis processes at the cellular,

molecular, and physiological levels have been increasing (Kurczyńska et al. 2012; Elhiti et al. 2013; Sugiyama 2015).

A prerequisite for any approach relying on tissue culture is the existence of a reliable regeneration system, based either on organogenesis or embryogenesis. However, there have been relatively few studies involving tissue culture of annatto. As a woody perennial species, the genetic improvement aiming to increase the seed pigment contents is very slow and limited by its long life cycle. Thereby, genetic transformation of annatto to overexpress key enzymes of biosynthetic pathways of metabolites like bixin could facilitate the generation of plants with increased pigment accumulation on seed coats (Paiva Neto et al. 2003a).

Annatto is a species in which pluripotency of cells and tissues has been proven from various types of explants derived from seedlings, such as shoot and nodal segments (D'Souza and Sharon 2001), hypocotyl segments (Paiva Neto et al. 2003b; Parimalan et al. 2007), inverted rooted hypocotyls (Paiva Neto et al. 2003/4), cotyledonary nodes (Carvalho et al. 2005), cotyledons (Parimalan et al. 2007), root segments (Cruz et al. 2014), petiole segments (Mohammed et al. 2015), and from nodal and internodal adult stem segments (Siril and Joseph 2013; Cruz et al. 2015). Additionally, several plant growth regulators were tested on these explants, such as auxins, i.e., indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), and indole-3-butyric acid (IBA) alone or, most commonly, in combination with cytokinins like 6-γ,γ-dimetylalylamino-purine (2-iP), zeatin (ZEA), kinetin (Kin), 6-benzyladenine (BA), and thidiazuron (TDZ)

The acquisition of competence for somatic embryogenesis (Paiva Neto et al. 2003/4; Parimalan et al. 2011) and the expression of totipotency have been rarely reported. Moreover, there are no systematic works covering the different aspects of somatic embryogenesis and gene expression. So, here we will describe some achievements involving the somatic embryogenesis of *B. orellana* in the past years.

13.2 Annatto as a Key Species to Understand Metabolic Pathways of Carotenoid Biosynthesis

The aryls of annatto seeds are a rich source of orange–red pigments that are widely used in the food, textile, and cosmetic industries. These pigments are commercially known as annatto (E160b), a mixture of bixin ($C_{25}H_{30}O_4$) as the main color component and traces of norbixin, bixin dimethyl ester, and other minor apocarotenoids. The ability to synthesize bixin and, thereby, its biosynthetic pathway has been the focus of intense interest in some plants like annatto (Mercadante et al. 1997; Mercadante and Pfander 1998; Narváez et al. 2001; Jako et al. 2002; Bouvier et al. 2003; Rodríguez-Ávila et al. 2011a, b; Soares et al. 2011; Rivera-Madrid et al. 2013; Cárdenas-Conejo et al. 2015), *Costus pictus* (Annadurai et al. 2012), *Crocus sativus,* and *Vitis vinifera* (Ramamoorthy et al. 2010). Bixin is a dicarboxylic

monomethyl ester apocarotenoid derived from the oxidative cleavage of carotenoids. Based on a heterologous in vitro expression system, it has been proposed that the pathway for bixin biosynthesis in annatto involves the actions of a lycopene cleavage dioxygenase (BoLCD), a bixin aldehyde dehydrogenase (BoBADH), and a norbixin methyltransferase (BonBMT), which catalyze the synthesis of bixin in a series of reactions proceeding sequentially from the C_{40} carotenoid precursor lycopene (Bouvier et al. 2003). However, the expression and significance of these gene products for the biosynthesis of bixin in annatto have not been confirmed in in vivo studies, so that the elucidation of the later biosynthetic steps and associated genes involved in the biosynthesis of bixin in annatto awaits further investigations. A preliminary analysis of mRNA expression of the genes involved in the early steps of carotenoid biosynthesis, namely *DXS* (1-deoxy-D-xylulose 5-phosphate synthase), *PSY* (phytoene synthase), *PDS* (phytoene desaturase), *ε-LCY* (lycopene epsilon-cyclase), and *β-LCY* (lycopene *β*-cyclase), was investigated in two *B. orellana* cultivars of contrasting carotenoid and bixin accumulation by reverse transcription-polymerase chain reaction (RT-PCR) analysis, and the expression of three of them (*PSY*, *PDS,* and *β-LCY*) could be associated with pigment accumulation (Rodríguez-Ávila et al. 2011b). Two carotenoid cleavage dioxygenases (CCDs) genes, one encoding a CCD subclass 1 (BoCCD1) (Rodríguez-Ávila et al. 2011a) and another CCD subclass 4 (BoCCD4) (Soares et al. 2011), have been also implicated in the biosynthesis of bixin based on their expression profiles in different tissues of *B. orellana*, including developing seeds. Recently, a *de novo* transcriptome sequencing in *Bixa orellana* has been published (Cárdenas-Conejo et al. 2015), showing that bixin production involves a coordinate expression of genes related to methylerythritol phosphate, carotenoid and bixin biosynthesis in immature seed.

The advent of next-generation high-throughput DNA/RNA sequencing (NGS) and CRISPR/Cas9 genome editing technologies has created unprecedented opportunities toward the elucidation of the metabolic pathway of carotenoid and bixin biosynthesis and their regulation in annatto. The NGS technologies provide high-throughput reads at a relatively low cost as compared to the Sanger method (Mardis 2008), generating highly reproducible and informative data and accurately quantifying transcripts (Marioni et al. 2008; Wang et al. 2009). On the other hand, the CRISPR/Cas9 genome editing technology is an easy and affordable tool that enables the precise manipulation of specific genomic sequences, allowing the generation of targeted knockout mutants for functional characterization of plant genes in a single generation (Belhaj et al. 2015). This simple system consists of a prokaryotic 'clustered regularly interspaced short palindromic repeat' (CRISPR)-associated protein 9 (Cas9) endonuclease and a small RNA molecule, the 'single guide' RNA (sgRNA), which instructs the nuclease to recognize and cleave a specific DNA target site. The increasing list of plant species that have been successfully used for targeted genome modification by the CRISPR/Cas9 system (see review by Belhaj et al. 2015) and the possibility of assembling multiple sgRNAs and the Cas9 encoded gene into a single delivery vector emphasize both the ease of employing this genome editing tool and the powerful of applying this technology for the rapid elucidation of metabolic pathways and their regulatory mechanisms in

plants. And in this sense, somatic embryogenesis may play a key role in allowing adequate and reproducible systems for genetic transformation aiming at unraveling metabolic pathways of carotenoid biosynthesis and functional gene expression studies linked to that.

13.3 Somatic Embryogenesis as a Key Morphogenic Pathway for Genetic Transformation in Annatto

Due to the great commercial appeal of bixin, the establishment of efficient transformation protocols for *Bixa orellana* can assist to obtain transgenic lines and potential commercial varieties with high bixin yields (Kumar et al. 2007). To date, surprisingly, genetic transformation of annatto has not been systematically and comprehensively investigated yet. Few studies involving a genetic transformation in annatto have been performed, including direct and indirect somatic embryogenesis-based transformation system (Parimalan et al. 2011) and transient transformation from hypocotyls (Zaldívar-Cruz et al. 2003), all mediated by *Agrobacterium tumefaciens*. However, there is a need for the establishment of more efficient transformation protocols, where transformed explants could show a higher regenerative rate, which is essential for the success of genetic transformation (Anami et al. 2013).

Somatic embryogenesis is a unique system that has also become an appropriate method for studying the morphophysiological and molecular aspects of cell differentiation. On top of that, there is a growing body of literature that reports the usefulness of the embryogenic pathway in genetic transformation protocols (Jin et al. 2005; Bull et al. 2009; Ribas et al. 2011; Yang et al. 2014; Nyaboga et al. 2015). This topic is extensively reviewed by Ochoa-Alejo in Chap. 23 (this book).

Somatic embryo, a bipolar structure with shoots and root domains, is an efficient system to enable regeneration of transformed plants. The bipolarity linked to the single cell origin of the embryos may reduce the development of chimeras or mixoploidy in the regenerants (Pathi et al. 2013). Somatic embryogenesis may occur directly or in an indirect way. The former occurs without a callus stage, whereas the latter undergoes a callus phase. The direct embryogenesis provides a more efficient transformation because it preserves a higher genetic stability of the plant material (Wang and Wang 2012; Kreis et al. 2015).

In annatto, direct and indirect somatic embryogenesis have been achieved from immature zygotic embryos, hypocotyl, and root segments (Paiva Neto et al. 2003a, b, c; Parimalan et al. 2011). Annatto explants have high plasticity and may exhibit in vitro embryogenic and/or organogenic responses, depending on the growth regulators used, as demonstrated in root segments (Cruz et al. 2014), expanding the possibilities for genetic transformation.

Somatic embryos of annatto may be used for nuclear transformation but also to potentially generate transplastomic plants, as reported for cotton (Kumar et al. 2004a), carrot (Kumar et al. 2004b) and soybean (Dufourmantel et al. 2004). Plastids are important organelles in plant cells, where several biosynthetic pathways

occur, such as the production of bixin (Nisar et al. 2015; Louro and Santiago 2016). The plastid genome transformation has become an alternative for the nuclear transformation, because of some advantages such as gene containment —since there is no plastid genome transmission through pollen, precise transgene integration—exclusively by homologous recombination, expression of multiple genes—through the construction of clusters, production of high protein levels, absence of epigenetic effects, and gene silencing (Bock 2015).

Although annatto is an excellent model of study, and highly efficient regeneration protocols have been already established, there is still a lack of studies to develop optimized transformation methods. It is necessary to establish methods ensuring the improvement and enhancement of transformation efficiencies, enabling the generation of plants with phenotypes of interest unattainable by conventional breeding.

13.4 Somatic Embryogenesis in Annato: Origin and Developmental Stages of Somatic Embryos

Somatic embryogenesis (SE) is a morphogenic route where embryos (bipolar structures) are generated from single cells or from multicellular clusters, which may be formed directly from the original explant tissue or induced after a preceding callus stage. This is an alternative technique with potential applications in the clonal propagation of plants, besides of being excellent tool for basic studies and analysis of molecular and biochemical events that occur during plant embryogenesis (Santos et al. 2005; Cangahuala-Inocente et al. 2009; Kurczyńska et al. 2012; Steiner et al. 2012; Rocha and Dornelas 2013; Steiner et al. 2016). This morphogenic pathway has been widely applied for a variety of species, from basic models to industrial and agronomical crops, as well-documented in this book (Chaps. 11–22) in a variety of applications as outlined in Chaps. 23–27.

In annatto the SE was first reported by Paiva Neto et al. (2003a) from immature zygotic embryos (IZE), where it was possible to observe somatic embryos (SE) in various stages of development few days after the beginning of induction. In addition, Parimalan et al. (2011) obtained somatic embryos from calluses with the intention of establishing a viable system of genetic transformation in annatto using *Agrobacterium*.

Knowledge of all stages of this process can facilitate the in vitro propagation of the species and the application of genetic breeding techniques. Associated with different techniques, anatomical analyzes are essential to characterize the cellular alterations that occur in the somatic embryogenesis process. They can help us to understand better the factors that lead somatic cells to resume meristematic characteristics and acquire competence to form somatic embryos; furthermore, histodifferentiation in SE can provide the information necessary to understand the embryogenesis process in plants (Corredoira et al. 2006; Moura et al. 2008; von Aderkas et al. 2015). Histochemical analyzes have allowed a better understanding

of the mobilization of reserves and energetic demands during the somatic embryos development (Cangahuala-Inocente et al. 2004, 2009; Moura et al. 2010; Rocha et al. 2012; Jariteh et al. 2015). This subject is well-approached in Chap. 23 (this book) by Rocha and co-workers.

13.4.1 Structural Changes Involved in the Somatic Embryogenesis Program in Annatto

The formation of somatic embryos in annatto is related with plant growth regulators (PGRs) added to the culture medium, the age of the zygotic embryo, besides being dependent on the genotype (Paiva Neto et al. 2003a). Only under optimal conditions embryogenic cells, genetically determined, will develop and form embryos in response to specific signals and also in cells in which the physiological conditions are appropriate (Fehér 2008).

Considering the morphological and anatomical aspects and profiles of gene regulation, it is noticed that there is a need for further studies on somatic embryogenesis. The IZE of annatto consists of two cotyledons and a cylindrical embryonic axis (Fig. 13.1a, b). These embryos show green color and smooth surface which starts to get wrinkled in the initial days of in vitro culture. After the initial days of the induction, in the medium occurs several external and internal changes in IZE leading to the formation of SE (Fig. 13.1c). After around 30 days of culture, somatic embryos at the globular stage are observed on the surface of the IZE (Fig. 13.1c, d). These structures have a spherical shape, smooth surface, and are greenish (Fig. 13.1d). After 52 days of induction, somatic embryos at different stages of development can be viewed throughout the explant, denoting the lack of synchrony of the embryogenic process in annatto (Fig. 13.1f).

At the beginning of induction, IZEs have uniseriate smooth epidermis with isodiametric cells, uniform and undifferentiated mesophyll and procambium cords distributed in the parenchyma (Fig. 13.1b). Changes continue, and pro-embryogenic zones are evident in certain regions of both sides of the cotyledons standing out initially as protuberances. These zones are formed from the epidermis and the underlying parenchyma (Fig. 13.1c). Epidermal cells divide in an anticlinal way, while in the mesophyll parenchyma cells immediately below the epidermis are divided into different levels, leading to the formation of pro-embryogenic zones (Fig. 13.1c). This sequence of changes leads to SE and, depending on the exposure time, some cells acquire meristematic activity and undergo numerous mitosis (Fehér 2003, 2005, 2008; Paim Pinto et al. 2011; Steiner et al. 2016). In annatto, these changes promote deformations, which are caused by cell walls in different section planes and finish with the formation of the somatic embryos. Rocha et al. (2012) reported the formation of protuberances on the adaxial surface of the cotyledon in passion fruit (*Passiflora cincinnata*) throughout SE induction. However, these protuberances originated during differentiation of tissue explant did not produce embryogenic callus nor somatic

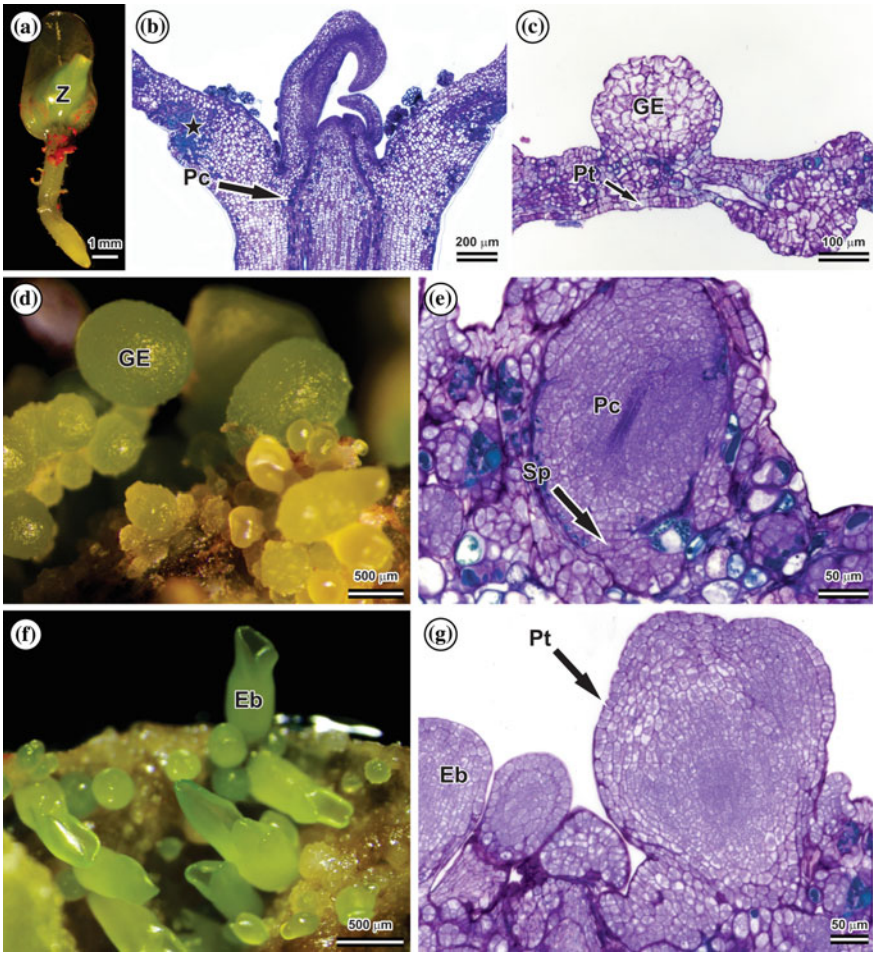

Fig. 13.1 Somatic embryogenesis in *Bixa orellana* L. **a** Immature zygotic embryo (*Z*) showing *greenish* coloring and the presence of bixin at the initiation of culture. **b** Longitudinal section of *Z* showing the presence of the procambium in the embryonic axis and phenolic compounds in the cotyledons. **c** Cross section in *Z* cotyledon is confirming different origins of a globular somatic embryo (*GE*). The presence of early *GE* on the adaxial surface of the *Z* protoderm. **d** *GE* visualized after 30 days of culture. **e** Cross section of *Z* with completely individualized somatic embryos (*Eb*). The presence of procambium and suspensor on *Eb* and phenolic compounds on *Z*. **f** Eb in several stages of development after 52 days cultivation, highlighting the lack of synchronization on somatic embryogenesis system of annatto. **g** Cross section of the *SE*. The presence of uniseriate protoderm and individualized *SE*. *GE* Globular Embryo. *Abbreviations Pc* Procambium. *Pt* Protoderm. *Sp* Suspensor. *Z* Immature zygotic embryo. * Phenolic compounds. *Eb* Somatic embryos

embryo. It occurs in a different way in annatto by the fact that these protuberances observed in this species originate somatic embryos in both the adaxial and abaxial surface of cotyledon.

The SE also has an uniseriate protoderm, ground meristem, and some are connected to the tissue explant through embryo suspensor-like structures (Fig. 13.1e). The observation of the process after 52 days cultivation allows noting the full development of SE in late developmental stages (torpedo and cotyledonary embryo) in addition to being individualized (Fig. 13.1f, g). In these stages, the presence of differentiated procambium can be observed (Fig. 13.1e). It is noticed that the SEs originated in this system have both unicellular and multicellular origin (Fig. 13.1c).

All these events are marked by the presence of phenolic compounds which were stained by toluidine blue (Fig. 13.1b). Phenolic compounds are associated with the antioxidant activity by eliminating free radicals or lessening their effects (Alemanno et al. 2003; Peixoto et al. 2007; Almeida et al. 2012). We note that these compounds are shown in IZE of annatto as the induction time increases. The presence of phenolics in this organ may be associated with the protection of tissues from free radicals since these compounds are present in cells bordering the somatic embryos.

The dedifferentiation of epidermal cells of *B. orellana* and, therefore, the formation of somatic embryos may also be explained by a possible effect of stress condition on the cultured explants. Whereas the presence of PGRs in the culture medium can be a stress factor, the amount of 2,4-D can be toxic to certain tissues or plants, and the changes that occur in these cells can be justified by stress (Zavattieri et al. 2010). This stressful condition causes changes in the normal endogenous hormonal balance of the cells and leads them to leave a fully differentiated state and to resume meristematic activity, showing the plasticity to hormones in plants (Fehér et al. 2003; Zavattieri et al. 2010; Pandey et al. 2012; Rahman 2013).

In addition to the factors listed before, a new perspective has been considered mutually active in the process of activation/silencing of genes involved in somatic embryogenesis: DNA methylation, histone modifications, and chromatin remodeling (Fehér 2008; Pandey et al. 2012). The subject has been reviewed elsewhere (De-la-Peña et al. 2015; Mahdavi-Darvari et al. 2015) and approached (Chap. 6, this book) by De-la-Peña and colleagues. Activation/silencing of genes is observed in somatic cells acquiring embryogenic competence during the induction period (Rocha and Dornelas 2013). Normally, these cells perceive external signals, often in neighboring cells, which can be cell wall components, or nutrients present in the culture medium, as boron (Pandey et al. 2012). Boron is an essential nutrient in the synthesis and organization of the cell wall and plasma membrane structures (Brown and Hu 1997; Pandey et al. 2012), and it is involved in the induction of somatic embryogenesis by stress signaling pathways.

13.5 Involvement of *SERK* Genes Expression During Somatic Embryogenesis of Annatto

The onset of somatic embryogenesis is dependent on a complex network of interactions that will modulate the expression of several genes. Among the genes expressed in somatic embryogenesis, the Somatic Embryogenesis Receptor-Like Kinases family

has been the most studied. Its expression pattern in several embryogenic systems has been linked to the acquisition of embryogenic competence from differentiated cells. It has a wide and remarkable involvement in several developmental signal transduction pathways. Despite extensive studies since its discovery, there are several scientific gaps regarding the particular interactions in the complex network of the somatic embryogenesis that still limits to unravel a more comprehensive and definitive role for this gene family. The latest achievements report that it is activated or upregulated in response to endogenous or exogenous signals, particularly by auxin exposition and stress-related responses. It has been hypothesized that *SERK* gene expression may be associated to cellular reprogramming that triggers a new developmental program (Nolan et al. 2003; Fehér 2008; Nolan et al. 2009; Fehér 2015). Additionally, studies showed that the constitutive overexpression of *SERK* increased the embryogenic responses (Hecht et al. 2001).

The first homolog of *SERK* was isolated from *Daucus carota* (*DcSERK*) in a subpopulation of competent embryogenic cells, derived from the proliferation of provascular elements of hypocotyls in the presence of 2,4-D (Schmidt et al. 1997). In the Arabidopsis genome, five distinct members were identified (*SERK1* to *SERK5*) (Hecht et al. 2001). Along two decades it has been demonstrated that these genes are present in the genome of all higher plant groups, monocots and eudicots, gymnosperms, and lower plants as well (Sasaki et al. 2007; Steiner et al. 2012; Aan den Toorn et al. 2015).

Genes of this family encode a transmembrane protein belonging to the LRR-RLK II group of the superfamily of receptor-like kinases and contain a highly conserved structural organization. The extracellular domain is constituted by a signal peptide followed by a leucine zipper, 4.5-5 LRR, and a proline-rich region that contains the SPP motif. In the intracellular domain are the catalytic serine/threonine or tyrosine kinase domain and the c-terminal region (Hecht et al. 2001; Shiu and Bleecker 2001a, b; Albrecht et al. 2008; Aan den Toorn et al. 2015).

We have isolated two putative members of the SERK family expressed during somatic embryogenesis in annatto by amplifying cDNA from embryogenic callus using degenerated primers (Baudino et al. 2001) followed by Rapid Amplification of cDNA Ends (RACE). Results of BLAST search with the deduced amino acids sequence in the National Center for Biotechnology Information (NCBI) and UNIPROT (http://www.uniprot.org/) databases revealed high similarity of both sequences with homologs of SERK proteins of other species, such as *Theobroma cacao*, *Coffea canephora*, *Ricinus comunis*, *Carica papaya*, *Citrus sinensis*, *Gossipium hirsutum*, among others. The contig1 exhibited a partial coding sequence of 340 amino acids that showed identity between 96 and 98 % with annotated sequences as SERK1 and SERK2 in the databases. The contig2, in turn, had a complete coding sequence of 589 amino acids that showed identity between 75 and 84 % with annotated homologs as SERK3/BAK1 of various eudicots. In relation to Arabidopsis thaliana it was found through BLAST search from TAIR database (www.arabidopsis.org) that contig1 showed 74–76 % identity with AtSERK1 and AtSERK2, whereas the contig2 showed an identity of 72 % with AtSERK3/BAK1. Due to the identity of sequences, the contigs 1 and 2 were named, respectively, as BoSERK-Like1 and BoSERK-Like3.

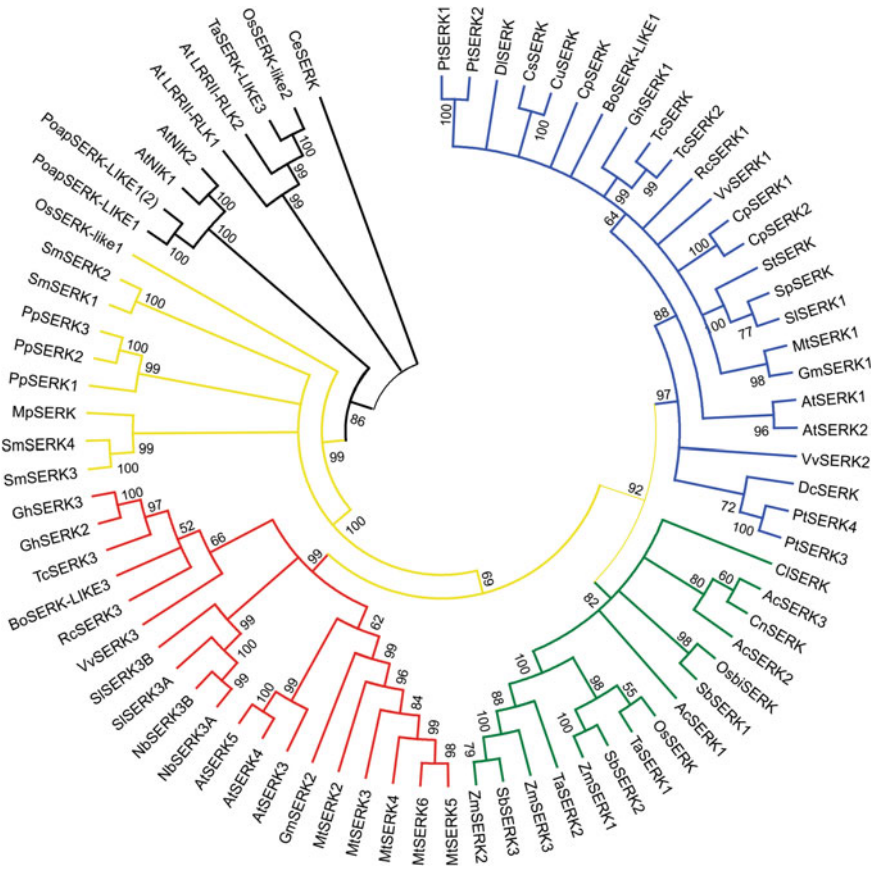

Fig. 13.2 Evolutionary history of *SERK* genes inferred using the Neighbor-Joining method (Saitou and Nei 1987). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The branches with a percentage below 50 % were cut. The evolutionary distances were computed using the Dayhoff matrix-based method (Schwarz and Dayhoff 1979) and are in the units of the number of amino acid substitutions per site. The analysis involved 77 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 782 positions in the final dataset. Four distinct groups of SERK proteins are inferred, according to Aan den Toorn et al. (2015): SERK dicots 1/2 that grouped with AtSERK1 and AtSERK2 (*blue*); SERK monocots (*green*); SERK dicots 3/4 that grouped together with AtSERK3, AtSERK4 and AtSERK5 and SERK Non-vascular plants (*yellow*). The *black branches* refer to a clade of LRR proteins that grouped together with the *A. thaliana* non-SERKs. BoSERK-Like1 was grouped together to SERK dicots 1/2 while BoSERK-Like3 was positioned into the SERK dicots, 3/4 group. Evolutionary analyzes were carried out in MEGA6 (Tamura et al. 2013)

The identity of conserved intra and extracellular domains was verified using the Pfam program and by alignment of sequences with related species (data not shown). The partial sequence of BoSERK-LIKE1 encodes part of the leucine zipper, the five LRR and SPP motifs, the transmembrane domain and part of the kinase domain.

BoSERK-Like3 contains all structural domains characteristic of this gene family (data not shown).

A phylogenetic relationship was investigated (Fig. 13.2) based on plant lineages (Aan den Toorn et al. 2015). According to the authors, evolutionary changes in the extracellular and intracellular domains were important to specify different members of the SERK family. The analysis of our data led to the separation of the SERK proteins in four major clusters: non-vascular SERK proteins, monocot SERK proteins, and two clusters of eudicot SERK proteins formed by SERK dicots S1/S2 and S3/S4 (Fig. 13.2). Here, we aligned the BoSERK sequences with the same 67 sequences of SERK proteins and six further proteins that showed homology with BoSERK-Like1 and BoSERK-Like2 in the BLAST results. Two other LRR-RLK groups (NIK proteins and two of unknown function) were included as outliers. For eudicots, there was a bifurcation forming a cluster for SERK1/SERK2 and other for SERK3/SERK4. For monocots, a single group was formed and another cluster of the non-vascular SERK proteins. The contig BoSERK-Like1 grouped together to the cluster corresponding to SERK1/SERK2 of the Eudicotyledons, closely to sequences of *Carica papaya*, *Theobroma cacao*, *Gossypium hirsutum,* and *Ricinus communis*, whereas BoSERK-Like3 was grouped into the cluster corresponding to SERK3/SERK4, being more closely related to SERK3-BAK1 proteins. These data suggest that the contigs named BoSERK-Like are indeed the possible orthologs of SERK1/SERK2 and SERK3 in *B. orellana*.

13.5.1 Localization of BoSERK-Like Transcripts by In Situ Hybridization

In situ hybridization assay has been an important tool of functional genomics applied to studies of the development of plants. This technique associated with cytological and structural studies has been largely used in attempts to understand in vitro morphogenesis, providing information on the localization of key transcripts and changes in the transcriptional state of the tissues subjected to different inducing conditions.

Here, in situ hybridization analysis was performed in order to locate the patterns of temporal and spatial expression of *BoSERK-Like* during the embryogenic callus and somatic embryos differentiation from immature zygotic embryos in *B. orellana*. For this, sense and antisense digoxigenin labeling RNA probe were synthetized from a cloned fragment characterized as BoSERK-LIKE1. Transcripts of *BoSERK-Like* were observed spread throughout the entire immature zygotic embryo, revealing the occurrence of a basal expression of genes from this family previously to embryogenesis induction and the meristem nature of the young tissues in the development of the embryo. However, a strong signal was detected in the protoderm cells, procambium region and initial cells of the shoot apical meristem during the induction time in the presence of 2,4-D and kinetin. The expression of

SERK was maintained in the cells of epidermis and mesophyll in the division. These cells have given origin to pro-embryogenic mass and subsequently to somatic embryos asynchronously. After 30 days of culture, proembryos, and globular somatic embryos showed a strong signal of hybridization. Finally, after 52 days of culture, somatic transcripts of SERK were detected in all phases of development of the somatic embryos. Somatic embryos in advanced stages showed weak signal in

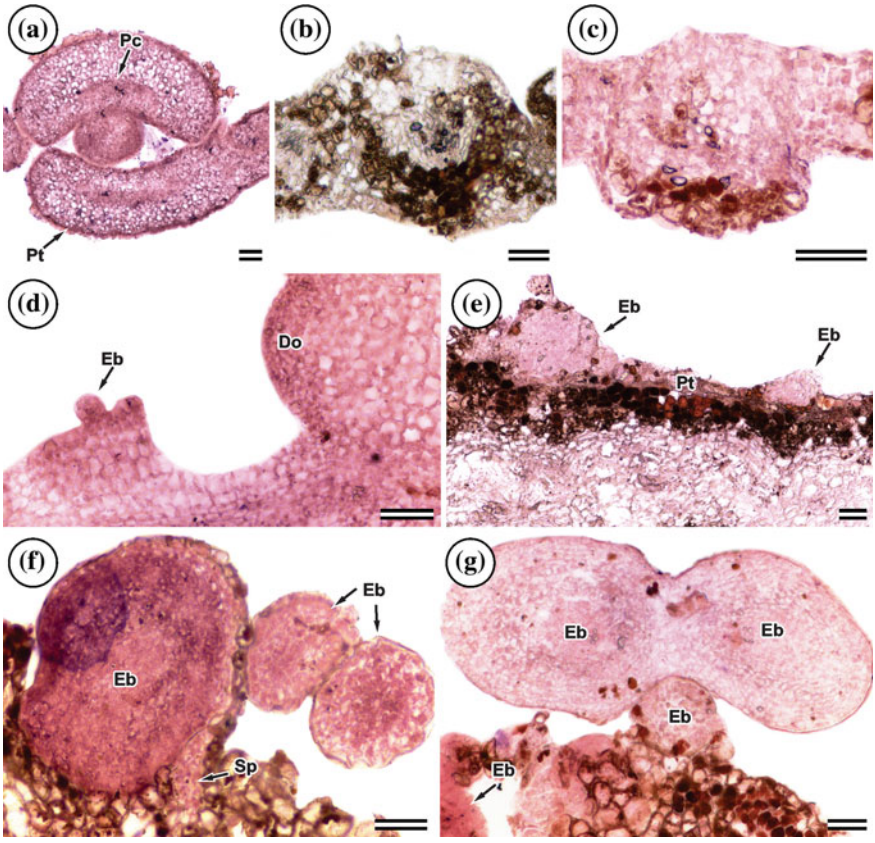

Fig. 13.3 Localization of *BoSERK-Like* transcripts during somatic embryogenesis in *Bixa orellana* by means of in situ hybridization. **a** Cross section of an immature zygotic embryo showing transcripts spread throughout all the cotyledons and shoot apical meristem before induction. A strong signal was detected in the protodermal cells and procambial region (*arrow*). **b** Cross section of the cotyledons after 20 days of induction did not hybridize with sense probe (negative control). **c** Cross section of cotyledons after 20 days with signal expression in all the explant. **d** Somatic embryo being formed from epidermal cells showing strong hybridization signal (*arrow*). *BoSERK-Like* expression was also observed in cells of the apical dome of the zygotic embryo (*arrow*). **e**, **f**, and **g** Somatic embryos at different developmental stages after 52 days of induction are shown. Hybridization signal was visualized by the formation of a *pink* or *purple* precipitate. *Abbreviations Eb* Somatic embryo; *Do* Apical dome; *Sp* Suspensor; *Pc* Procambium; *Pt* Protodermal. Bars = 50 μm

comparison to early stages, reflecting a pattern of basal expression similar to that observed in zygotic embryos before induction. No signal of expression was observed in tissues hybridized with the sense probe (Fig. 13.3).

13.6 Concluding Remarks and Future Road Map

The comprehension of the developmental events during the induction phase as well as the development of somatic embryos is essential to regulate each stage of the somatic embryogenesis developmental program. Additionally, the development of efficient protocols of somatic embryogenesis in annatto may be useful for applications in genetic transformation systems with the final aim to obtain annatto plants with increased carotenoids synthesis and accumulation. The advent of NGS and CRISPR/Cas9 genome editing technologies has created unprecedented opportunities towards the elucidation of the metabolic pathway of carotenoid and bixin biosynthesis and their regulation in annatto. We are willing to expand the number of genes involved in somatic embryogenesis by means of a transcriptome-based dataset. Also, proteomics- and metabolomics-associated aspects will be instrumental approaches to be looked, and to explore further possibilities on the arabinogalactan and pectin epitopes and the competence acquisition for somatic embryogenesis in this species.

Acknowledgments This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Brazil), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) (Belo Horizonte, MG, Brazil) and Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) (Salvador, BA, Brazil). The company Chr. Hansen Brasil (Valinhos, SP, Brazil) is acknowledged for kindly supplying annatto seeds to our laboratory.

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Chapter 14
Somatic Embryogenesis in *Capsicum* spp.

Neftalí Ochoa-Alejo

Abstract *Capsicum* species represent a horticultural crop of great importance worldwide, which is frequently exposed to different adverse environmental abiotic or biotic factors that affect field yields. Biotechnology merges as an alternative to improve agriculturally important characteristics in *Capsicum* through modern approaches. Tissue culture together with molecular biology techniques is the foundation of plant biotechnology. Efficient in vitro plant regeneration protocols need to be developed and established to provide appropriate systems for micropropagation and manipulation through genetic engineering for modern crop improvement programs. Organogenesis and somatic embryogenesis are the two ways of generating entire plants in vitro. Somatic embryogenesis, the asexual process of producing bipolar structures that resemble the sexually generated zygotic embryos, has been described for a number of plant species; however, in the case of *Capsicum* species the in vitro embryogenesis systems have faced recalcitrancy problems. In this chapter, the information on somatic embryogenesis in *Capsicum* is presented, and the recalcitrancy problems are discussed.

14.1 Introduction

Chili pepper pods are important items in many countries around the world because they are used as ingredients for different typical dishes or salads, and also chili pepper fruits are of importance for the food and pharmaceutical industries since they synthesize and accumulate pigments (anthocyanins and carotenoids), vitamins (A, B and C) and capsaicinoids, the pungent compounds (Ochoa-Alejo and Ramírez-Malagón 2001; Kothari et al. 2010). Like any other horticultural crop, chili

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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_14

pepper production is frequently threatened and affected by different pathogens, pests, or environmental factors (for example, water and salt stress, and low temperatures). Any crop improvement program using traditional techniques is high time consuming and very common takes a long time (5–10 years or more). If the goal for crop improvement involves a very specific trait, for example, a change in capsaicinoid, carotenoid, anthocyanin or vitamin content in chili pepper fruits, then the time to achieve it may be even longer. We have been interested in applying genetic engineering techniques for crop improvement and metabolic engineering of *Capsicum* species (Ochoa-Alejo and Ramírez-Malagón 2001), and a prerequisite for this is to develop efficient in vitro plant regeneration and transformation systems (Kothari et al. 2010). Different authors have reported protocols for in vitro plant regeneration through organogenesis in *Capsicum* species, but low-efficiency values are the rule due to recalcitrance problems involving developmental failures during bud/shoot formation and difficulties for bud/shoot elongation (Kothari et al. 2010). Somatic embryogenesis in chili pepper has also been documented, but regeneration of entire plants has been a recalcitrant process of low efficiency (Kothari et al. 2010).

14.2 Somatic Embryogenesis Systems

Somatic embryogenesis has been exclusively reported in *Capsicum annuum* and to a lesser extent in *C. chinense*. Harini and Lakshmi Sita (1993) were the first to report the direct regeneration of somatic embryos from immature zygotic embryos of *C. annuum* L. var. California Wonder cultured on MS (Murashige and Skoog 1962) medium with 2,4-dichlorophenoxyacetic acid (2,4-D; 1–2 mg/L), 10 % coconut water, and sucrose (8–10 %). Somatic embryos were formed mainly on the embryonal axis and cotyledons of the immature zygotic embryos (5–6 mm in length), and the optimum concentration of 2,4-D and sucrose in the culture medium was 2 mg/L and 10 %, respectively. Induction and maturation of somatic embryos were achieved on the same medium, whereas germination occurred in the presence of 1 mg/L gibberellic acid (GA_3). Finally, the regenerated plants were cultured in liquid medium without growth regulators to promote further growth before transplanting to soil. Binzel et al. (1996) described the direct somatic embryogenesis in *C. annuum* New Mexico-6 and Rajur Hirapur cultivars using immature zygotic embryos (7–8 mm long) as explants cultured on MS medium supplemented with 9 μM 2,4-D + 10 μM thidiazuron (TDS), 10 % coconut water, and 8–10 % sucrose. Somatic embryos were produced in the embryo apex, embryo axis, and cotyledons, and the induction and maturation occurred on the same culture medium, whereas germination of somatic embryos was observed in the presence of GA_3 (2.8 μM) or TDZ (0.05 μM). Jo et al. (1996) tested the embryogenic capacity of immature zygotic embryos of *C. annuum* cv. Nokkwang at different stages of development cultured on MS medium varying the 2,4-D and sucrose

concentrations, and they found a dependence of somatic embryogenesis for the size of the zygotic embryos as well as the level of 2,4-D in the culture medium. Direct somatic embryos were induced at 90 % frequency from zygotic embryos 1–2 mm long cultured on MS medium with 2 mg/L 2,4-D incubated in the dark, while indirect embryogenesis was attained at 10 % on callus generated from zygotic embryos 3–4 mm long grown on MS medium with 5–10 mg/L 2,4-D under continuous illumination. Somatic embryo maturation occurred at 25 % on MS medium with 0.5 mg/L 2,4-D, 0.5 % activated charcoal, 3 % sucrose and in the presence of 2 μM abscisic acid (ABA) or 20 μM $AgNO_3$ (an inhibitor of ethylene action). The somatic embryogenic process was followed by an electron microscopy analysis. Direct or indirect regenerated somatic embryos were converted into entire morphologically normal plants. Using mature zygotic embryos of *C. annuum* var. Ace as explants, Buyukalaca and Mavituna (1996) reported indirect somatic embryogenesis on MS medium supplemented with 9.05 μM 2,4-D and 3 % sucrose, where embryogenic callus was generated and then subcultured in MS liquid medium containing 4.52 μM 2,4-D and 3 % sucrose to increase the embryogenic callus mass as a suspension culture. This cell suspension was further pretreated with potassium citrate (6 g/L) and then cultured in the initiation medium consisting of MS with 6 g/L proline and a reduction in ammonium concentration from 20 to 10 mM. Maturation of somatic embryos and conversion into plants was achieved at 97 % efficiency on paper bridges in a half-strength MS medium with 1.89 μM ABA. Somatic embryogenesis in chili pepper was also reported using young leaves of *C. annuum* cv. Colombo (Kintzios et al. 2000); in this case, the explants were cultured on semisolid MS medium with 9 μM 2,4-D, 12.9 μM benzyladenine (BA) and 8 % sucrose for embryogenic callus induction (induction medium) or they were exposed to liquid medium with 129 μM BA + 9 μM 2,4-D + 3 % sucrose for 24 h and then cultured on semisolid MS induction medium. Embryogenesis was observed more frequently in explants derived from the two upper leaves of the donor plants. Pretreatment of leaf explants with 129 μM BA affected negatively the somatic embryos proliferation, but not the percentage of globular embryos, which were capable of converting into torpedo-shaped embryos and lately germinating. The effect of light and darkness was also investigated, and a positive effect on heart- and torpedo-shaped embryo formation was observed after incubation of callus tissue in darkness for 3 weeks and then exposed to light. These authors extended their studies to the effect of vitamins and micronutrients on the somatic embryogenesis in callus cultures derived from leaf tissue of chili pepper and they concluded that nicotinic acid (0.1 mg/L) and a tenfold increase in copper in the induction medium promoted 9.2 % globular embryos formation over the control without affecting embryo maturation and germination (Kintzios et al. 2001). Bodhipadma and Leung (2002) analyzed the responses of Sweet Banana, California Wonder, Yolo Wonder, and Ace pimiento cultivars (*C. annuum* L.) with the protocols reported by Harini and Lakshmi Sita (1993), Binzel et al. (1996), and Buyukalaca and Mavituna (1996). In general, somatic embryogenesis was recorded in immature embryos cultured under the conditions described by Harini and Lakshmi Sita (1993), except that the initiation medium lacked the 10 %

coconut water, and those of Binzel et al. (1996), whereas only callus formation occurred in mature embryos cultured as described by Buyukalaca and Mavituna (1996). Conversion of somatic embryos into plants decreased approximately 50 % when the explants were cultured for prolonged time (from 2 to 4 weeks) on the induction medium. Furthermore, these authors tested the effect of addition of 10 % glucose, fructose, maltose, or sucrose to the induction medium on the somatic embryogenesis using immature embryos of the Yolo Wonder cultivar and they observed no somatic embryo formation in the presence of glucose and fructose or in the absence of sucrose, and maltose could replace sucrose. Steinitz et al. (2003) tested the effect of indoleacetic acid (IAA), naphthaleneacetic acid (NAA), phenylacetic acid (PAA), 3,6-dichloro-2-methoxybenzoic acid (dicamba), 4-amino-3,5,6-trichloropicolinic acid (picloram), 2,4-D, centrophenoxine [(4-dichlorophenoxy) acetic acid 2-(dimethylamino) ethyl ester], and quinclorac (quinolinecarboxylic acid) on the direct somatic embryogenesis of zygotic embryo explants from twelve genotypes of pimiento peppers (*C. annuum*). Five out of twelve genotypes exhibited 82–100 % explants with somatic embryos, and a mean of 4–8 somatic embryos per explant when they were cultured on an induction medium based on the salts of the MS medium and the vitamins of B5 medium (Gamborg et al. 1968) with 1.2 mm centrophenoxine, 5 g/L activated charcoal and 200 mM sucrose (6.85 %). Germination of somatic embryos was achieved on a modified induction medium containing 90 mM (3.08 %) sucrose. However, the regenerated somatic embryos exhibited morphological anomalies (absence of cotyledons, a single deformed cotyledon, a cup-shaped cotyledon or the absence of a shoot). Khan et al. (2006) described the direct somatic embryo formation on stem and shoot tip explants of *C. annuum* cv. Pusa Jwala cultured on MS medium supplemented with 0.5 μM TDZ. Approximately 22 and 16 somatic embryos were produced per stem and shoot tip explant, respectively. Maturation and germination of somatic embryos were observed on the same culture medium while rooting of shoots developed from somatic embryos was induced on MS medium with 1 μM indolebutyric acid (IBA). The regenerated plants were adapted to the soil (85 % survival), and they exhibited normal morphology. The effect of cytokinins on somatic embryogenesis in *C. annuum* Blue Star, Bendigo, and Bell Boy chili pepper cultivars was analyzed by Kaparakis and Alderson (2008) using immature embryos at the liquid endosperm stage (5–6 mm long) cultured on MS medium with 2 mg/L 2,4-D and 10 % sucrose (Harini and Lakshmi Sita 1993) combined with BA, isopentenyladenine (2iP), kinetin (Kin), or zeatin (Z) at 0.01 or 0.1 mg/L, or 10 % coconut water. Embryogenic responses and the production of somatic embryos per responding explant were dependent on the cultivar and culture medium; for example, cultivar Blue Star exhibited the highest embryogenic response followed by Bell Boy and Bendigo, while the number of somatic embryos in responsive explants was higher in Bendigo and to a lesser significant extent in Blue Star and Bell Boy; cultivar Bell Boy showed a higher somatic embryogenesis formation on the culture medium without coconut water than in its presence. In general, explants cultured on medium without coconut water, 2iP (0.01 and 0.1 mg/L) and Kin (0.01 mg/L) displayed the highest embryo responses, and to a

lesser extent those on medium with BA and Z. Germination of somatic embryos occurred equally in the absence or in 1 mg/L GA_3. An interesting approach to try to overcome the plant regeneration recalcitrant problem in chili pepper was the regeneration of transgenic plants from Fiesta, Ferrari, and Spirit pimiento hybrids (*C. annuum*) using cotyledon explants infected with *A. tumefaciens* bearing the *Brassica napus* BABY BOOM AP2/ERF transcription factor gene (Heidmann et al. 2011). Cotyledon or leaf explants from transformed seedlings cultured on MS medium with 10 μM dexamethasone (DEX), 1 mg/L TDZ or 10 μM DEX + 1 mg/L TDZ exhibited abundant somatic embryos, which were subsequently converted into plants on a rooting induction medium.

Regarding somatic embryogenesis in *Capsicum chinense* Jacquin, López-Puc et al. (2006) were the first to describe the direct regeneration of somatic embryos in cotyledon, hypocotyl, cotyledonary leaf, zygotic embryo, and germinated zygotic embryo explants of cv. BVII-03 cultured on the induction medium consisting of the MS medium with 9.05 μM 2,4-D, 3 % sucrose, and 0.8 % Gelrite. The best response was achieved in hypocotyl explants (175 ± 20 somatic embryos per explant). Somatic embryos treated with 1.89 μM ABA and then cultured on the germination medium (MS medium with 1.1 μM GA_3) exhibited radicle emergence and expansion of cotyledonary leaves at a 60 %, but frequent abnormalities were detected, and no conversion into entire plants was achieved. An ontogenetic study of direct somatic embryogenesis in *C. chinense* cv. BVII-03 by histological analysis was further described (Santana-Buzzy et al. 2009). Indirect somatic embryogenesis in *C. chinense* cv. Rux-02 was also reported in cell suspensions derived from callus tissue produced in hypocotyl explants (from axenic seedlings) cultured on the induction medium described earlier (López-Puc et al. 2006) and established as suspensions in the liquid induction medium with 4.5 μM 2,4-D and subsequently exposed to a liquid medium with 3.4 μM TDZ (Zapata-Castillo et al. 2007). This indirect somatic embryogenesis process was complemented by a morphohistological and ultrastructural study (Avilés-Viñas et al. 2013). Solís-Ramos et al. (2010) established a protocol for the induction of indirect somatic embryogenesis in *C. chinense* starting with mature zygotic embryos segments as explants, which were cultured on MS medium supplemented with 8.9 μM NAA, 11.4 μM IAA, and 8.6 μM BA to produce embryogenic callus; only 8 % of calli produced somatic embryos and when torpedo-stage somatic embryos were detached from callus and cultured on MS medium without growth regulators a 75 % conversion into plantlets was attained; these plants were finally transplanted to soil and adapted to greenhouse conditions. Very recently, Valle-Gough et al. (2015) analyzed the influence of the polyamines putrescine, spermidine, and spermine at 0, 0.01, 0.1, and 1.0 mM on the efficiency of somatic embryogenesis and the morphology of the regenerated somatic embryos in *C. chinense* var. Mayan Ba'alche applying the protocol described by Avilés-Viñas et al. (2013). In general, no positive effect of the polyamines on the number of somatic embryos was recorded, but an improvement in the somatic embryo development and morphology was observed because they

exhibited harmonic apex-radicle morphology, a greenish coloration, and the formation of two tiny cotyledonary leaves. When the expression of *WUS*, *WOX1,* and *WOX3* genes involved in the determination of apical meristem integrity or shoot maintenance was comparatively analyzed by q-RT-PCR in somatic embryos treated or non-treated with spermine and zygotic embryos, the transcript levels differed between each other; for example, zygotic embryos showed higher transcript levels of *WUS*, *WOX1,* and *WOX3*, whereas somatic embryos treated with spermine presented higher transcript levels of all these genes in comparison with the non-treated ones, and, in particular, the expression pattern of *WOX1* was similar to that of zygotic embryos suggesting that *WUS* and *WOX* genes are involved in developmental processes related to meristem maintenance.

14.3 In Vitro Plant Regeneration Recalcitrancy

In vitro plant regeneration in chili pepper has been found to be recalcitrant and the reasons for this are still unknown. However, in vitro morphogenetic responses in plant species are dependent on the explant source (genotype, type of plant, physiological conditions of the donor plant, type of organ or tissue) and also on the prevailing culture conditions (composition of the culture medium, light, photoperiod, temperature, and pH). Undoubtedly the genotype plays the main role in the somatic embryogenesis competency, but although up to now all the tested cultivars or varieties of *Capsicum* species have shown differences in embryogenic capacity (Binzel et al. 1996; Bodhipadma and Leung 2002; Steinitz et al. 2003; Kaparakis and Alderson 2008), the number of investigated *Capsicum* species and cultivars is scarce and the embryogenic efficiency is still low compared with that showed by other members of the Solanaceae family such as tobacco, tomato, and potato. In order to improve the embryogenesis efficiency in *Capsicum* species, the inhibitor of ethylene action $AgNO_3$ has been added to the culture media (Jo et al. 1996), and no other factors have been extensively investigated to overcome embryogenesis recalcitrancy; for example, approaches to increase calcium levels in cytosol have been shown to be beneficial to somatic embryogenesis (Malabadi and Staden 2006), and also, ammonium and nitrate ratios of the culture medium can improve somatic embryogenesis in different plant species (Grimes and Hodges 1990; Greer et al. 2009), also organic acids, vitamins B12 and E promoted embryogenic initiation in recalcitrant pinus (Pullman et al. 2006), sugars such as D-xylose and D-*chiro*-inositol have been found to increase embryogenic tissue initiation for several *Pinus* species (Pullman et al. 2009), oxidation-reduction agents such as sodium thiosulfate and sodium dithionite increases embryogenic initiation in loblolly pine (*Pinus taeda*) and Douglas fir (Mirb) Franco (*Pseudotsuga menziensii*), whereas glutathione disulfide stimulated somatic embryo germination (Pullman et al. 2015); glutathione also improves early somatic embryogenesis in *Araucaria angustifolia* (Bert.) O. Kuntze (Vieira et al. 2012).

14.4 Future Work

Somatic embryogenesis in *Capsicum* has been reported only for *C. annuum* and *C. chinense* and for a limited number of genotypes, cultivars, or varieties with relatively low plant regeneration efficiencies due to recalcitrancy problems. It is imperative to devote more efforts to overcome this problem by increasing the research on the embryogenic capacity of a number of *Capsicum* species, cultivars, and genotypes and also on the environmental factors that could influence the embryogenic responses.

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Chapter 15
Somatic Embryogenesis in *Coffea* spp.

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Abstract Coffee is a very important commodity in the world. It is a perennial plant and as a consequence its genetic improvement had been difficult. The biotechnology, in particular somatic embryogenesis (SE) has helped to the propagation of commercial coffee cultivars. Along almost 50 years, different research groups around the world have developed uncountable SE processes to produce an efficient, reproducible, and reliable SE process. Many of the factors that affect the embryogenic response have been studied. It has been demonstrated that the genetic modification of coffee is possible. This transformation can lead to the improvement of varieties with very attractive agronomic traits, such as low caffeine content, resistant to diseases and pests.

15.1 Introduction

Coffee is one of the two major commodities in the world. The cultivation of coffee gives employment to more than 80 million people, and every year, more than 11 million hectares are cultivated around the world.

All coffee species are trees or perennial woody bushes. The coffee plants differ among themselves in different aspects such as morphology, size, and ecological adaptation. Although there are more than 124 species of the *Coffea* genus, only two are used to produce coffee commercially, *Coffea arabica* and *C. canephora*, which accounts for 60 and 40 % of world coffee production, respectively. *C. canephora* (2n = 2x = 22) is an outcrossing diploid, allogamous, consisting of polymorphic populations of strongly heterozygous individuals and with high caffeine content. *C. arabica* is an allotetraploid (2n = 4x = 44) derived from a spontaneous

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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_15

hybridization between *C. canephora* and *C. eugenioides*, autogamous and with low caffeine content. *C. arabica* is very susceptible to pathogens whereas *C. canephora* has some degree of resistance to diseases.

The growing world population, less land, and water available to cultivate crops and an increasing deforestation are only some of the major challenges for modern agriculture. The searches for new varieties resistant to pests and/or pathogens, the extreme conditions of the environment, as well as an increase in the productivity, are the challenges for the agronomist today.

The generation of a new variety of coffee with stable, improved traits, using conventional breeding, takes around 30 years. This is the time that requires six–seven self-pollination cycles from seed to seed through conventional breeding. The most viable alternative is biotechnology. Biotechnology is a more reliable technique for perennial crops. Among all the biotechnological tools, somatic embryogenesis (SE) is the most promise alternative.

15.2 Early Years

The first reports of SE of *Coffea* spp. appeared at the beginning of the 70s of the last century. Staritsky (1970), Söndahl and Sharp (1977) laboratories published the first papers on SE of *C. canephora* and *C. arabica*, respectively. Since then, numerous papers have been published on the subject (Table 15.1). The source of explants can be almost every part of the plant. Some of them possess a higher embryogenic potential.

The source of explants that has been used are perisperm (Sreenath et al. 1995), orthotropic and plagiotropic shoots (Nassuth et al. 1980; Raghuramulu et al. 1987; Staritsky 1970), foliar tissue (Cid et al. 2004; Dublin 1981; Etienne et al. 2013; Quiroz-Figueroa et al. 2001, 2002, 2006; Ramakrishna et al. 2012; Söndahl and Sharp 1977), and ovules outer covering (tegument) (Lanaud 1981). So far, leaves are the source of explant more widely used. They are abundant and all year accessible.

Staritsky (1970), in his pioneer work, used cultured internodal segments of orthotropic and plagiotropic shoots from *Coffea arabica*, *C. canephora* (Robusta) and *C. liberica*. Explants were cultured on two different media culture (Heller 1953; Linsmaier and Skoog 1965), supplemented with kinetin (Kin, 0.46 μM), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.45 μM) or α-naphthaleneacetic acid (NAA, 5.37 μM). *C. canephora* was the only responsive species to the embryoid formation.

Herman and Haas (1975) reported the formation of structures like embryos from *C. arabica* leaf-derived calli grown on modified LS medium supplemented with 0.46 μM Kin and 0.45 μM 2,4-D. The embryo-like structures developed roots in Gresshoff and Doy medium (Gresshoff and Doy 1972) containing 0.53 μM NAA (Herman and Haas 1975).

An improvement of the *C. arabica* embryogenic response came from Söndahl laboratory. They used two different media to produce SE from leaf explants of

Table 15.1 Summary of protocols for the induction of somatic embryogenesis in *Coffea* spp.

Species	Explant	Medium	Growth regulators	Comments	References
Coffea arabica	Le	LS	2,4-D (0.452 μM) KIN (0.464 μM)	Large numbers of coffee organoids were produced from callus culture	Herman and Haas (1975)
	EC	MS (S)	KIN (2.3 μM) NAA (0.27 μM)	50–100 embryos per culture bottle (30 ml)	Söndahl and Sharp (1977)
	Le	MS (S)	KIN (20 μM) 2,4-D (5 μM)	100–200 somatic embryos develop from clusters of embryogenic tissue	Söndahl et al. (1985)
	EC	MS (S)	BA (5 μM) NAA (0.05 μM)	186 ± 90 somatic embryos per callus mass	Yasuda et al. (1985)
	Le	Söndal and Sharp Medium	2,4-D (18.8 μM) KIN (13.92 μM)	6–8 weeks of culture give many clusters of 50–60 somatic embryos	Guimaraes et al. (1987)
	Le	MS (L)	NAA (0.53 μM) KIN (2.32 μM)	–	Santana et al. (1988)
	EC	MS (S)	BA (4.4 μM)	Maximum embryogenesis rate (100 %) was achieved in 2 weeks	Bertrand-Desbrunais et al. (1988)
	Le	YS	BA (4.4 μM)	50–80 %	Michaux-Ferrière et al. (1989)
	P	Protoplast culture medium (L)	2,4-D (2.26 μM) BA (2.26 μM) NAA (2.26 μM)	Yields of protoplast ranged from 1×10^5 to 6×10^5 g^{-1} fresh weight	Acuña and de Pena (1991)
	P	Liquid medium	Pre-induction: MS + B_5 + 3 % Sucrose	The regenerated embryos contain the inserted foreign DNA	Barton et al. (1991)
		Liquid medium	Full induction: 0.5 x MS + 38 mM KNO_3 + B-5 + 2 % Sucrose		
	Le	MS	NAA (0.5 μM) KIN (5.0 μM)	–	Nakamura et al. (1992)

(continued)

Table 15.1 (continued)

Species	Explant	Medium	Growth regulators	Comments	References
	Le	MS	2,4-D (4.52 μM) KIN (18.56 μM)	94.5 %	Neuenschwander and Baumann (1992)
	Le	MS Modified (S)	BA (4.43 μM)		Bieysse et al. (1993)
	P	MS (S)	BA (5 μM)	–	Tahara et al. (1994)
	Le	MS/2 + Organic constituents of B5 (S)	2iP (5 mM) *Group 1* 0, 50, 100 μM putrescine and spermidine *Group 2* 0 and 20 μM spermidine and spermine		Calheiros et al. (1994)
	EC	MS (S)	2,4-D (10 μM)	–	Tahara et al. (1995)
	Le	MS(S)	BA (5 μM)	185 ± 93 per explant	Yasuda et al. (1995)
	Le	MS	KIN (0.92 μM)		Mycock et al. (1995)
	Le	MS	2iP (5 μM)	86 embryos/callus	Nishibata et al. (1995)
	CS	MS/2(L)	BA (4.4 μM)	12,300 somatic embryos gram inoculum^{-1}	Van Boxtel and Berthouly (1996)
	CS	MS (L)	2,4-D (4.5 μM)	Production of more than 9,000 embryos and plants per container after 6 months	Etienne et al. (1997b)
	Le	MS (S)	2,4-D (4.5 μM) BA (35.5 μM)	After 8 months in culture, 65 % of the coffee calli became highly embryogenic producing on the average 87 somatic embryos per explant	Menéndez-Yuffá and de Garcia (1997)
	Le	MS	2,4-D (4.5 μM)	Effective plant conversion in soil (78 %)	Etienne-Barry et al. (1999)
	ZE	MS	NAA (5.37 μM) BA (32.78 μM)	–	Pasqual et al. (2000)
	Le	MS/2	NAA(0.5 μM) KIN (2.5 μM)	–	Rani et al. (2000)
	CS	MS	2,4-D (4.52 μM) KIN (4.65 μM)	–	Etienne and Bertrand (2001)

(continued)

Table 15.1 (continued)

Species	Explant	Medium	Growth regulators	Comments	References
	Le	MS/2	BA (13.3 μM)	12–72 %	Molina et al. (2002)
	EC	MS/2 (S)	2,4-D (4.52 μM) BA (17.76 μM)	Approximately 800 embryos L^{-1} per bioreactor. Small and medium cotyledons had the highest frequencies of conversion into plantlets after direct sowing (43 and 63 %, respectively)	Barry-Etienne et al. (2002)
	CS	MS (L)	BA (22.15 μM)		De Feria et al. (2003)
	EC	MS/2 (S)	BA (48 μM)	Approximately 44–90 embryos per 5 explants	Cid et al. (2004)
	Le	MS/2 (L)	TRIA (4.55 and 11.38 μM) BA (1.1 μM) IAA (2.28 μM)	260 ± 31.8 and 59.2 ± 12.8 somatic embryos per culture	Giridhar et al. (2004c)
	Le	MS	TDZ (2.27–11.35 μ)	Direct somatic embryogenesis wherein 70 % of in vitro leaf explants (161.6 ± 2.5 embryos) and 50 % of in vitro stalk explants of regenerated plants produced embryos (39.9 ± 1.25 embryos)	Giridhar et al. (2004b)
	EC	MS/2 (S)	2,4-D (4.5 μM) BA (17.7 μM)	3,081 embryos L^{-1} per bioreactor. 75 % plant conversion	Albarrán et al. (2005)
	CS	MS/2	2,4-D (4.5 μM) BA (17.7 μM)	At the end of the developmental phase, each bioreactor contains around 8,000 torpedo-shaped somatic embryos	Etienne (2005)
	Le	MS/2 (S)	2,4-D (2.25 μM) 2-iP (4.93 μM)	35–306 per 100 mg embryogenic calli	Samson et al. (2006)
	Le	YS (MS/4)	–	Explants produced somatic embryos (2.7 ± 0.5) after 12 weeks of culture	Gatica et al. (2007)
	CS	MS(L)	BA (22.5 μM)	23.7 somatic embryos in torpedo stage $L^{-1} \times 10^3$	Barbón et al. (2008)
	Le	YS (SS)	BA (1.1 μM) IAA (2.85 μM) TRIA (4.55 μM)	The highest number of embryoids explants was obtained using Yasuda semisolid medium (3.2–6.0)	Gatica-Arias et al. (2008)
	CS	MS	2,4-D (4.52 μM) KIN (18.56 μM)	255.8 ± 142.1 mg of somatic embryos	Gatica et al. (2008)

(continued)

Table 15.1 (continued)

Species	Explant	Medium	Growth regulators	Comments	References
	Le	MS/2	BA (50 μM	–	Papanastasiou et al. (2008)
	CS	MS/2 (L)	2,4-D (4.52 μM) BA (17.76 μM)	After 4 months each bioreactor produced 8,000 torpedo-shaped somatic embryos	Menéndez-Yuffá et al. (2010)
	Le	MS	BA (4.4 μM) 2,4-D (13.6 μM)	80,000 embryos L^{-1}	Mukul-López et al. (2012)
	CS	MS/2 (L)	2,4-D (4.52 μM) KIN (4.65 μM)	Germination, characterized by radicle emergence, was high (91 ± 4.0 % for SE). Plant conversion frequency was 55 % on average after 22 weeks	(Etienne et al. (2013)
	EC	MS/2 (S)	2,4-D (4.5 μM) BA (17.7 μM)	10,000 embryos	Bobadilla Landey et al. (2013)
Coffea canephora	S	LS (S)	KIN (0.46 μM) 2,4-D (0.45 μM) NAA (5.3 μM)		Staritsky (1970)
	IN	MS	AIB (24.6 μM) BA (4.4 μM)	–	Nassuth et al. (1980)
	Le	MS/2	2iP (4.92 μM) IBA (24.6 μM)	–	Pierson et al. (1983)
	P	B-5 (L)	without growth regulators	–	Schöpke et al. (1987)
	P	B-5 (S)	KIN (2.32 μM) 2,4-D (2.26 μM) NAA (26.85 μM)	–	Schöpke et al. (1987)
	CS	MS (L)	BA (5 μM)	450,000 somatic embryos L^{-1}	Zamarripa et al. (1991a)
	Le	MS/2 (L)	2-iP (5 μM) KIN (5 μM) BA (5 μM)	Under optimal conditions, each explant formed more than 100 embryoids with little callus and few adventitious roots	Hatanaka et al. (1991)

(continued)

Table 15.1 (continued)

Species	Explant	Medium	Growth regulators	Comments	References
	Le	MS/2 + Organic constituents of B5 + 87 mM Sucrose (S)	2iP (5 mM) *Group 1* 0, 50, 100 μM putrescine and spermidine *Group 2* 0 and 20 μM spermidine and spermine	Exogenous application of polyamines in coffee can be detrimental to somatic embryo formation, nevertheless the amount of embryos produced varied among genotypes	Calheiros et al. (1994)
	P	MS (L)	BA (5 μM)	–	Yasuda et al. (1995)
	Le	MS (S)	2-iP (5 μM) ZE (5 μM) KIN (5 μM) BA (5 μM)	Number of embryos/explant 2-iP: 68.1 ± 4.5 ZE: 13.9 ± 1.8 KI: 9.4 ± 1.2 BA: 11.3 ± 2.4 After 2 months of culture	Hatanaka et al. (1995a)
	Le	MS (S)	2-iP (5 μM)	Number of embryos/explant was from 41 to 69 after 2 months of culture	Hatanaka et al. (1995b)
	EC	MS (L)	NAA (5.37 μM) BA (22.19 μM)	–	Sreenath et al. (1996)
	CS	MS/2 (L)	BA (4.4 μM)	120,000 somatic embryos per gram inoculum	Van Boxtel and Berthouly (1996)
	EC	Basal medium (S)	2,4-D (4.4 μM) BA (17.8 μM)	80,000 ± 15,000 somatic embryos within 3–4 months	Berthouly and Michaux-Ferrière (1996)
	EC	YS/4 + MS/2 (L)	BA (4.98 μM)	One g from a 3-month-old cell line produces 56,000 plantlets	Ducos et al. (1999)
	Le	MS (L)	2-iP (5 μM)	Percentage of embryo-forming explants was 100 % for genotypes. Proliferation of embryogenic tissue was observed in 80 % of the explants	Fuentes et al. (2000)

(continued)

Table 15.1 (continued)

Species	Explant	Medium	Growth regulators	Comments	References
	CS	MS (L)	BA (25 μM)	5,067 trees from 5 to 7 month-old embryogenic suspension cultures	Ducos et al. (2003)
	Le	MS/2 (L)	TRIA (4.55 and 11.38 μM) BA (1.1 μM) IAA (2.28 μM)	59.2 ± 12.8 somatic embryos per culture	Giridhar et al. (2004a, c)
	Le	MS	TDZ (2.27–11.35 μ)	In *C. canephora* 274 variety 60 % of in vitro leaf explants and 72 % in vitro stalk of regenerated plants produced direct somatic embryogenesis from the margins and surface, respectively, (180.1 ± 3.0 and 102.8 ± 6.8 embryos) on medium	Giridhar et al. (2004c)
	Le	YS	BA (5 μM)	–	Quiroz-Figueroa et al. (2006)
	Le	MS/2 (S)	2,4-D (2.25 μM) 2-iP (4.93 μM)	16,127 per 100 mg embryogenic calli.	Samson et al. (2006)
	Le	MS/2	KIN (23.2 μM)	–	Priyono et al. (2010)
	Le	MS	BA (4.4 μM) 2,4-D (13.6 μM)	80,000 embryos/L	Mukul-López et al. (2012)
	Le	MS + B5 (vitamins)	Serotonin (100 μM) KIN (0.93 μM) IAA(11.4 μM)	85 embryo/callus	Ramakrishna et al. (2012)
Coffea dewevrei	H, Le, S	MS/2 (L)	IAA (2.85 μM) ABA (8.87 μM)	4.9–59.35 [explant^{-1}]	Sridevi and Giridhar (2014)
Coffea heterocalyx	Le	MS/2 (S)	2,4-D (2.25 μM) 2-iP (4.93 μM)	18–285 per 100 mg embryogenic calli	Samson et al. (2006)

(continued)

Table 15.1 (continued)

Species	Explant	Medium	Growth regulators	Comments	References
Coffea arabusta	H	MS (S)	KIN (0.46 μM) IAA (2.85 μM)	More than 100 embryos per 1 g of callus	Dublin (1980a)
	Le	MS/2 (S)	BA (44.3 μM) IBA (0.492 μM)	Somatic embryos were developed within 9–12 weeks of culture	Afreen et al. (2002)
C. congensis x *C. canephora*	EC	MS (S)	ABA (3.78 μM)	–	Sreenath et al. (1995)

C. arabica (Söndahl and Sharp 1977; Söndahl et al. 1979). One media was for "conditioning" the explants and the other for the "induction". As in most of the embryogenic systems, so far reported, the concentration of growth regulators added to the culture medium plays a central role in the induction of SE.

15.3 Plant Growth Regulators

Several members of the plant growth regulators (PGRs) family play a key role in the induction of SE. The concentration of auxin, before, during, and after the induction of SE is a limiting factor for an embryogenic response (Halperin and Wetherell 1965; Söndahl and Sharp 1977). In coffee, the presence of 2,4-D in combination with Kin markedly increased the high frequency somatic embryogenesis (HFSE; >60 %) in the conditioning medium. Other auxins, such as indole-3-butyric acid (IBA) and NAA combined with Kin, were not effective for HFSE induction (10–20 %), but they increased low frequency somatic embryogenesis (LFSE) induction up to 60 %, especially when NAA was used. Depletion or reducing the concentration of auxins, and in some cases Kin in the induction medium, appeared to be essential for somatic embryo development in *Coffea* spp. We still do not know why 2,4-D is so effective in promoting HFSE. One possible explanation could be the lower degradation of 2,4-D in relation with that of other auxins.

Coffee SE shows two different developmental patterns. One is indirect SE, where embryos are derived from an embryogenic dedifferentiated tissue (callus) and is associated with dedifferentiated cells that require dedifferentiation, proliferation, and the induction of determined embryogenic cells (Söndahl et al. 1985). The other pattern is direct SE, where embryos are directly formed from the explant (Quiroz-Figueroa et al. 2002) and is associated from the beginning with determined, proembryogenic cells (DPEC).

Calli induced by NAA and 2,4-D can be phenotypically similar. However, the biochemical and physiological status of the two types of callus may be different. The use of IAA during SE induction in explants of Arabusta (F1 hybrid from *C. arabica* X *C. canephora*, 4X) (1980b) was indispensable for the development of somatic embryos. On the other hand, Hatanaka et al. (1991) reported that all auxins tested (NAA, IBA, IAA, and 2,4-D) were inhibitory to somatic embryo formation of *C. canephora*, but was promoted by cytokinins such as 2iP, BA, and kinetin at 5 μM. Under these conditions, over 100 embryos per explant were formed.

Ayil-Gutiérrez et al. (2013) found that a preincubation of the source of the explants with a combination of NAA and Kin for 2 weeks, produced sharp increases in the content of IAA. The transference of the explants onto the SE induction medium caused a very fast decrease in the content of endogenous auxins. It is possible that different auxins induce a different pattern of endogenous indole-3-acetic (IAA) and, in consequence, different calli phenotypes.

Addition of 1-triacontanol, an enhancer of the growth in plants, to leaf explants of *C. arabica,* and *C. canephora,* cultured on a medium containing IAA 2.28 μM and BA 1.1 μM BA in half strength MS basal medium, induced SE (Giridhar et al., 2004a).

Direct SE can also be observed on a cytokinin rich medium in the absence of auxins. Yasuda et al. (1985) and Ayil-gutiérrez et al. (2013) reported a simple method for SE induction in leaf explants from *C. arabica* var. Typica and *C. canephora*, respectively, using 5 μM BA as the sole growth regulator.

A combination of auxins and cytokinins is the most common used PGRs to produce SE. de García and Menéndez (1987) utilized a combination of 35.5 μM BA and 4.52 μM 2,4-D to produce proembryogenic calli. When these cultures were transferred to a medium supplemented with 10 % coconut milk 75 embryos per explant were observed. A different combination of PGRs (26.6 μM IBA, and 4.92 μM 2iP), supplemented with 10 mg L^{-1} hydrolyzed casein, produced HFSE. The regenerative capacity of calli could be maintained for more than 2 years. Santana et al. (1988) obtained similar results with *C. arabica* var. Caturra and 5 Catimor lines, using different combinations of BA and 2,4-D. Neuenschwander and Baumann (1992) used a combination of 2,4-D (4.5 μM) and kinetin (18.4 μM) to induce SE in calli of *C. arabica*. The transfer to a liquid medium with a different combination of PGRs (NAA 0.23 μM and Kin 2.7 μM) for 18–24 weeks synchronized the SE process. In some cases, the combination of two different auxins and a cytokinin is used. This combination was tested in leaf explants of *C. canephora* using 2,4-D, IBA and 2iP; highly embryogenic callus was obtained (Berthouly and Michaux-Ferrière 1996). This embryogenic mass was able to produce embryos with a high conversion capacity into plants.

Other less conventional PGRs also play a role in the induction of SE in *Coffea* spp. Ethylene and salicylic acid are two PGRs that modified both the induction of SE and the development of somatic embryos. The use of a pharmacological approach has been very important to elucidate the role of ethylene (1995b). Addition of 30–60 μM $AgNO_3$ increased the production of somatic embryos in 5 genotypes of *C. canephora* (Fuentes et al. 2000). However, higher $AgNO_3$ concentrations were inhibitory. The treatment was effective in *C. arabica* and *C. canephora*, as reported by Giridhar et al. (2004b) using hypocotyls explants to induce direct somatic embryogenesis in the presence of silver nitrate. These authors used MS medium supplemented with 1.1 μM de BA, 2.85 μM IAA and 40 μM of silver nitrate to produce a maximum of 144 and 69 embryos per explant of *C. canephora* and *C. arabica*, respectively.

Salicylic acid (SA) affects the growth of embryogenic suspension cultures in a concentration-dependent way. Concentrations of 10^{-12} and 10^{-10} M of SA increased cellular growth and somatic embryogenesis by twofold in *C. arabica* cv. Caturra Rojo (Quiroz-Figueroa et al. 2001). It is possible that the increase in the number of somatic embryos is the result of the better growth induced by SA.

15.4 Effect of Genotype in the SE

During the induction of SE in different species, it has been evident that genotype plays a major role in the induction of SE. The genus *Coffea* is not the exception. *C. arabica* has both embryogenic and non-embryogenic cultivars (Michaux-Ferrière et al. 1989). In an embryogenic line, Michaux-Ferrière et al. (1989) found that two kinds of calli were formed. One of them was produced during the first 20 days of culture; this callus lost its embryogenic capacity very soon. Later, the second kind of callus appeared after 60 days of culture. This callus produced proembryos and later somatic embryos. In then non-embryogenic cultivar the callus also appeared after 60 days of cultures; however, this callus was not embryogenic. Interestingly both kind of calluses possessed similar cytological characteristics.

The embryogenic capacity character is fixed in early generations (F3 or F4) (Molina et al. 2002). In consequence, there is a relationship between the embryogenic response of the progenies and their progenitors. The embryogenic capacity varied greatly among genotypes of *Coffea* spp.; Molina et al. (2002) found variations ranging between 4.8 and 72.7 %.

Testing different media for the induction of SE in recalcitrant genotypes can yield good results. After using different media and gelling agents, Bieysse et al. (1993) found that a combination of Yasuda medium (Yasuda et al. 1985) and gelrite-gelled medium was able to induce somatic embryos and plantlets from two recalcitrant genotypes of *C. arabica*.

Aponte (1993) also was successful in producing proembryogenic tissues from five different coffee genotypes using two different media. One of the medium was half strength Murashige and Skoog (MS; 1962) basal salts, organic constituents from B_5 medium (Gamborg et al. 1968), 30 g L^{-1} sucrose, and 13.31 μM BA, while the other was one quarter-strength MS basal salts, organic constituents from B_5 medium, 30 g L^{-1} of sucrose, and 4.92 μM 2iP. Similar results were also reported by Hatanaka et al. (1991).

In some cases, the use of the same protocol produces somatic embryos in different *Coffea* spp. A liquid medium containing MS salts with 4.5 μM 2,4-D, and an inoculum density of 10 g L^{-1} and subcultured every 7 days was able to induce the grown of callus from leaf explants of *C. canephora*, *C. arabica*, and the hybrids Congusta (*C. congensis* X *C. canephora*), and Arabusta (*C. arabica* X *C. canephora*) (Van Boxtel and Berthouly 1996). The elimination of 2,4-D and reducing the inoculum density to 1 g L^{-1} produced embryogenic calli.

15.5 Other Factors

PGRs are not the only factor affecting the SE response. Other factors are the origin of the explant, the physiological status of the explant, the culture medium, among others. There was an important difference in the embryogenic response when the

explants were collected from plants in the vegetative state or plants in the reproductive stage (Santana-Buzzy et al. 2004).

There was an increase in the callus growth by 60–70 % after reducing the concentrations of nitrogen and increasing the concentrations of boron and magnesium (Marques 1987). The increase in the osmotic potential and the presence of casein hydrolysate, as well as the incubation of cultures in the dark, favors the embryo proliferation. In our laboratory, we also observed similar results after decreasing the nitrogen concentration in the medium and changing the relationship between oxidized and reduced nitrogen (Fuentes-Cerda et al. 2001). The use of amino acids or reduced organic nitrogenous compounds also promoted SE of coffee (Fuentes-Cerda et al. 2001), e.g., asparagine was shown to be effective in SE induction (Nishibata et al. 1995). SE in coffee could also be promoted by exogenous application of other nitrogenous compounds, such as polyamines (Calheiros et al. 1994).

SE is also affected by the carbon source in the medium. The change of sucrose by fructose during the induction of SE in *C. canephora* genotypes N91 and N128 increased the number of somatic embryos. For the genotype N75, the most effective carbon source was maltose. The change of glucose by sucrose in genotype N91 produced a more synchronous embryo development (Fuentes et al. 2000).

Dissolved oxygen (DO) has been shown to affect somatic embryo development of *C. arabica* cv. Catimor 9722. The numbers of somatic embryos increased in the presence of 80 % DO. However, most of the embryos were at the globular and heart-shaped stages and only 6.6 % were torpedo shaped (De Feria et al. 2003). A decrease to 50 % DO increased the number of torpedo-shaped embryos to 20 %. These results suggested that a high respiration rate was required during the early phases of development of the somatic embryos.

The position of the leaves used as explants also affects the response to SE induction. The poorest response was for the first two pairs of leaves from the apex. In a leaf, explants that originated from the distal end were less responsive than those from the basal end. Leaf explants from nodes 2, 3, and/or 4 of *C. canephora* var. Robusta and *C. arabica* vars. Caturra and Catimor 9722 were most effective in the formation of embryogenic callus. In addition, leaves from orthotropic branches were more responsive than leaves from plagiotropic branches (Santana 1993).

The age and cellular structure of embryogenic callus, as well as inoculum density in *C. canephora* var. Robusta is other two important factor to take into consideration during the induction of SE (Montes et al. 1995). The use of 2–3 month-old callus with an inoculum density of 2–3 g L^{-1} produced the best embryogenic suspension cultures.

Embryo formation is also affected by the inoculum density in *C. arabica* (Zamarripa et al. 1991a, b). When the inoculum density was high, the SE was inhibited. This inhibition could be partially suppressed by periodically renewing the medium. In our laboratory, we found that the explants of *C. arabica* secreted a set of phenolic compounds, mainly caffeine and chlorogenic acid, and both of them inhibited SE (Nic-Can et al. 2015). The methylation of DNA decreased 50 % in the *C. arabica* explants.

15.6 Scale-Up

The SE process of *Coffea* spp. has been scaled-up. Zamarripa et al. (1991a, b) cultivated embryogenic tissues of *C. canephora* in Erlenmeyer flasks or in a bioreactor and yielded up to 200,000 somatic embryos. Ducos et al. (1993) reported the production of 600,000 embryos L^{-1} of medium in a bioreactor.

The use of the method known as automated temporary immersion system (recipient à immersion temporaire automatique (RITA)) has been shown to be beneficial for the production and germination of somatic embryos of coffee (Etienne and Berthouly 2002; Etienne-Barry et al. 1999). This method yielded synchronous production and germination of somatic embryos of *C. arabica* F1 hybrids (Berthouly et al. 1995; Etienne et al. 1997a) without the need of selection before acclimatization another advantage of the uses of RITA (Etienne-Barry et al. 1999). The yield of normal torpedo stage embryos using RITA was usually >90 % and with high capacity to convert into plants (75 % conversion) (Albarrán et al. 2005). The yield of somatic embryos ranged from 15,000 to 50,000 per gram of embryogenic suspension cell mass depending on the genotypes.

The hyperhydricity problem associated with the use of liquid medium can be overcome using short immersion times (Albarrán et al. 2005; Etienne and Berthouly 2002).

The direct sowing of germinated somatic embryos into the RITA produced a high frequency conversion of embryos into plants. The addition of sucrose at a higher concentration (234 mM) 2 weeks before sowing promoted plant conversion in soil (78 %) and vigorous vegetative growth of the plants (Etienne-Barry et al. 1999).

The absence of variation among the propagated plants has been tested in large-scale field trials. Ducos et al. (2003) made a trial in the field with more than 5,000 plants. The study of morphological characteristics and yield did not produce any significant differences between the plants derived from embryogenic tissues and microcuttings. The most productive lines showed a cumulative yield of green coffee of more than 3,000 kg ha^{-1}.

Somaclonal variation at the DNA level or the occurrence of phenotypic variants that could appear later during the development of the plants cannot be excluded (Ducos et al. 2003). In our laboratory, we used amplified fragment length polymorphism (AFLP) to evaluate the variability in regenerated plantlets of *C. arabica* obtained by direct or indirect SE. Our results suggested that the SE process induced rearrangements at the DNA level (Sánchez-Teyer et al. 2003).

15.6.1 Coffea *Genetic Transformation*

The genetic transformation of *Coffea* spp. is very attractive. There are several traits that are susceptible to be modified by genetic engineering. Plants with resistance to

difference diseases and/or pests, as well as with lowest caffeine content are among the most important transformation goals to carry out.

The first transformation of coffee was reported by Barton et al. (1991), Ocampo and Manzanara (1991). Both papers were presented during the 14è Colloque Scientifique Internationale sur le Café in San Francisco in 1991.

Barton et al. (1991) used the method of protoplast electroporation of *C. arabica*, followed by regeneration through SE. The gene encoding the chloramphenicol acetyltransferase was cloned into a CAT gene construct. The expression of this gene confers kanamycin resistance to the transformed cells. After 3–4 months of kanamycin selection, the selected cultures regenerated transgenic plants. However, the roots of the plantlets were very weak and the plants did not produce seeds.

Ocampo and Manzanara (1991) transformed hypocotyls of *C. arabica* cv. Caturra with different *Agrobacterium tumefaciens* strains. The tumors produced by the infection grew in a rich medium in the absence of PGRs. Most of the tumors grew until 6 months, and thereafter they stopped to growth and died.

Transformation of coffee embryogenic cultures is a viable and powerful tool both for its genetic improvement and for the study of important genes.

15.6.2 Agrobacterium *spp.*

15.6.2.1 Agrobacterium tumefaciens

A. tumefaciens has been the most used vector for the transformation of coffee. Both commercial species of coffee have been transformed with *Agrobacterium* spp. There are differences in the transformation efficiency of coffee among the different strains of *A. tumefaciens*. *C. arabica* was more efficiently transformed with nopaline strains than with octopine strains (Freire et al. 1994). Recently Ribas et al. (2011a) transformed *C. arabica* with the strain LBA1119 of *A. tumefaciens* harboring pBin 35S GFP or harboring the plasmids pBIN19 and pMDC32 with the marker gene *GFP5* and the *hygromycin phosphotransferase* selectable marker gene (Déchamp et al. 2015). After establishing the adequate parameters such as density of the bacteria culture, the maintenance conditions for the embryogenic cultures, the age of the culture, and the phenotype of the embryogenic cultures, the efficiency of transformation reached more than 90 %.

C. canephora has also been transformed. *A. tumefaciens* LBA4404 (Leroy et al. 1997, 1999, 2000), strain EHA101 harboring pIG121-Hm, containing β-glucuronidase (GUS), hygromycin phosphotransferase (HPT), and neomycin phosphotransferase II genes (Hatanaka et al. 1999), strain C58C1 harboring the binary vector pER10 W-35SRed without (Canché-Moor et al. 2006) or with the gene *WUSCHEL* under an estradiol-inducible transcriptional control (Arroyo-Herrera et al. 2008), and strain GV 3101 harboring pCAMBIA 1305.2 binary vector (Sridevi et al. 2010) are among the different strains used for its transformation.

In some cases, the infection is carried out directly on wounded somatic embryos (Leroy et al. 1997), intact somatic embryos (Arroyo-Herrera et al. 2008), or incubating the embryogenic mass with the bacteria (Hatanaka et al. 1999). In other cases, the initial explant, such as hypocotyls (Sridevi et al. 2010) or leaf explants (Canché-Moor et al. 2006) are transformed prior to the induction of the SE process.

The efficiency of transformation can vary widely; for example, variations between 30 and 80 % of the calli used for transformation (Leroy et al. 1997), 2–5 % when the transformation was carried on the explants used for the induction of the SE (Sridevi et al. 2010), or 33 % of the transformed embryos (Canché-Moor et al. 2006) have been recorded.

In several studies genetic transformation has been demonstrated in coffee plantlets regenerated from somatic embryos. Hatanaka et al. (1999) reported a strong GUS activity in all organs of the regenerated plants. They also found that β-glucuronidase and *HPT* genes were stably integrated into the genome of the regenerated coffee plants.

There are two aspects of coffee transformation that are the most studied. The cloning of genes involved in the SE process, with the goal of increasing the efficiency of the process (Arroyo-Herrera et al. 2008), and the production of plants with resistance to different kind of stresses (Leroy et al. 1999, 2000; Perthuis et al. 2005). During the last years our knowledge on coffee pests and disease resistance genes (Lashermes et al. 2010; Noir et al. 2003; Ribas et al. 2011b), drought resistance genes (Marraccini et al. 2011), and specific promoters (Marraccini et al. 1999; Petitot et al. 2013) has made possible to target specific genes to improve *Coffea* spp.

Bacillus thuringiensis (Bt) has been in use since many years ago. Was until 1996 that the first transgenic crops expressing Bt were commercially available (Soberón et al. 2015). The bacterium is pathogenic to insects because produces a crystal toxin (Cry). There are two proposed models for its mechanism of action. First, an osmotic imbalance produced by the pores in the cell membrane or second, an opening of ion channels producing the cell death (Melo et al. 2016).

Coffee berry borer (CBB, *Hypothenemus hampei*) is one of the most important causes of losses in the coffee industry. Most of the *B. thuringiensis* have little insecticidal activity again CBB. However, some of them are toxic to CBB (Méndez-López et al. 2003). Another important pest for coffee is the leaf miner (*Leucoptera coffeella*).

The *crylAc* gene of *B. thuringiensis* was introduced into *C. canephora* and *C. arabica* to try to confer resistance to leaf miner. The *A. tumefaciens* strain LBA4404 harboring the modified *crylAc* gene, the reporter *uidA* gene coding for β-glucuronidase and the *esr1-1* gene conferring resistance to the herbicide chlorsulfuron was used for genetic transformation. Some of the transgenic plants showed an important resistance to the pest (Leroy et al. 1999, 2000). In a field experiment of 4 years carried out with transgenic *C. canephora* harboring the cry1Ac insecticidal protein or a synthetic *cry1Ac* gene, the transformed plants showed a stable resistance to the leaf miner (Perthuis et al. 2005, 2014).

In our laboratory, we have transformed *C. canephora* leaf explants with *A. tumefaciens* strain C58CI harboring the binary vector pER10 W-35SRed (Canché-Moor et al. 2006) or harboring the gene *WUSCHEL* under an estradiol-inducible transcriptional control (Arroyo-Herrera et al. 2008). The expression of *WUSCHEL* induced a 400 % increase in somatic embryo production.

15.6.2.2 Agrobacterium rhizogenes

There is evidence showing that sometimes it is easier to regenerate plants from hairy roots that from any other tissue. This approach was used to transform somatic embryos of *C. canephora* with *A. rhizogenes* harboring the gene for β-glucuronidase. After the infection, the embryos developed calli, and later on formed secondary somatic embryos. These embryos exhibited positive β-glucuronidase activity (Spiral et al. 1993). Somatic embryos of *C. canephora* at the torpedo stage were wounded with a scalpel and infected with *A. rhizogenes* strain A4. The histochemical GUS test was positive in the embryogenic callus, as well as in the roots and leaves of young seedlings from the regenerated plantlets (Leroy et al. 1997).

Transformation of cotyledons, hypocotyl, and leaf explants from three *C. arabica* cultivars, with cucumopine and mannopine strains of *A. rhizogenes*, yielded calli and hairy roots (Freire et al. 1994). Infection with *A. rhizogenes* strain IFO 14554 harboring a mikimopin type Ri plasmid produced calli and adventitious roots after 2 months. Adventitious embryos were produced directly from hairy roots after 6 months of subculture. The adventitious embryos regenerated plantlets with short internodes and many roots (Sugiyama et al. 1995). Déchamp et al. (2015) used *A. rhizogenes* strain A4RS harboring the plasmid pBin19 having the *uidA* bacterial gene and produced hairy roots.

15.6.3 RNA Interference

The RNAi technique has been used to inhibit the caffeine biosynthesis. Ogita et al. (2003) used the 3′-untranslated region of the 7-*N*-methylxanthine methyltransferase (CaMXMT1; theobromine synthase) messenger RNA. The construct was introduced into *A. tumefaciens* strain EHA101. This transformed *A. tumefaciens* were used to transform *C. canephora*. Some of the regenerated cells were hygromycin-resistant. The phenotype of both the transgenic seedlings and the nontransformed plants look the same. However, the content of purine alkaloids in the transgenic lines, expressing RNAi, showed a decrease of 30–80 % theobromine and a 50–70 % reduction in caffeine content compared with the controls (Ogita et al. 2003). In the second paper of the series, the same authors showed that the RNAi transgenic lines of embryogenic mass from *C. arabica* and transgenic plantlets of *C. canephora* showed a clear reduction in (*CaMXMT1*) transcripts in relation to the controls. Other transcripts, such as the xanthosine methyltransferase (*CaXMT1*) and 3,7-dimethylxanthine

methyltransferase (*CaDXMT1*; caffeine synthase) genes were also reduced (Ogita et al. 2004). These results strongly suggest that it is possible to obtain coffee plants with less caffeine content.

To achieve decaffeination of coffee, Mohanan et al. (2013) used a set of different posttranscriptional gene silencing techniques. Since the genes codifying for the *N*-methyltransferases have a high degree of homology, it was possible, using a 339 bp fragment, to develop sense, antisense, and invert repeat constructs to inhibit caffeine biosynthesis. RNAi technology was more effective than cosuppression and antisense technologies for gene silencing. The use of RNAi decreased by more than 90 % the content of caffeine.

15.6.4 Other Transformation Methods

In addition to transformation with *Agrobacterium* spp., there are other transformation methods that have been used to generate transgenic coffee. Among these other methods are vacuum infiltration (Canché-Moor et al. 2006), electroporation (Fernandez-Da Silva and Menéndez-Yuffá 2003) and biolistic or particle bombardment (Albuquerque et al. 2009; Gatica-Arias et al. 2008; Ribas et al. 2005; Rosillo et al. 2003; Van Boxtel et al. 1995).

The electroporation method was used to transform different tissues of *C. arabica* cv. Catimor with the plasmid pCAMBIA3201, containing *GUS* and *BAR* genes. After the electroporation of torpedo shape embryos, these were cultivated in liquid media with 35.44 μM BA, which led to the production of secondary transgenic somatic embryos. The secondary embryos were positive for *GUS* expression (Fernandez-Da Silva and Menéndez-Yuffá 2003).

The biobalistic method has been used to transform leaves, somatic embryos, and suspension cultures of *C. arabica*, *C. canephora,* and Arabusta, using four different promoter sequences. The transformation produced transient expression of the leaves of microcuttings with the *EFlct-A1* gene promoter of *Arabidopsis thaliana* been the most effective (Van Boxtel et al. 1995). When the tissue target is suspension cultures of *C. arabica*, the osmotic preconditioning of the cells is an important parameter to consider. The use of two coffee promoters: *α-tubulin* and *arabicin* genes reduced the expression of the gene *uidA*. However, when leaf tissues were bombarded, only with the CaMV35S and *arabicin* gene promoters were observed histochemical activity in coffee endosperms (Rosillo et al. 2003).

The particle bombardment of leaf explants bearing somatic embryos and embryogenic tissue, with tungsten particles carrying the plasmid pCAMBIA3301 harboring the *uidA* and *BAR* genes produced stable transformation. The regenerated plantlets were GUS positive, and the transgenic plants growing in the greenhouse were resistant to the herbicide Finale™ (Ribas et al. 2005). The same technique used on *C. arabica* cv Cataui suspension cultures with particles carrying the plasmids pCAMBIA 1301, pCAMBIA 1305.2 and pCAMBIA 1301-BAR produced the stable GUS visualization (Gatica-Arias et al. 2008).

The bombardment of embryogenic calli of *C. arabica* cv. Catuaí Vermelho with the pBI426 vector containing *uidA* and *nptII* genes produced embryos resistant to kanamycin and positive for β-glucuronidase activity. The plants generated from transgenic embryos showed normal development and produced fruits. In these plants, GUS expression was localized in the flowers and fruit organs. The T1 progeny of these transgenic plants showed a 3:1 Mendelian segregation of the *uidA* gene (Albuquerque et al. 2009).

15.7 Concluding Remarks

Coffee is one of the most important commodities crop in the world. As a perennial plant, its genetic improvement is difficult. As a consequence, there are few improved varieties of *Coffea* spp. However, biotechnology can help to its genetic improvement. In particular, SE is a powerful tool to reach this goal, as well to study the mechanistic of the development of the embryo. There are many factors that affect the embryogenic response. Nevertheless, the SE of coffee is used to propagate some selected cultivars of economic importance.

The genetic modification of coffee can lead to improved varieties with very attractive agronomic traits. Among these trails, low caffeine content, resistance to diseases and pests is under study in distinct laboratories around the world. The recent publication of the complete sequence of the genome of *C. canephora* (Denoeud et al. 2014) opens new windows of opportunities to develop new coffee varieties.

Acknowledgments The work from VMLV laboratory was supported by a grant received from the National Council for Science and Technology (CONACyT, 157014).

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Chapter 16
Somatic Embryogenesis in *Agave* spp.

Benjamín Rodríguez-Garay

Abstract The genus *Agave* is a monocotyledonous group of species that belong to the Asparagaceae family. Because of its CAM metabolism and other botanical features, the genus *Agave* is gaining importance throughout the world to address the challenges that climate change is imposing with regard to food, medicine, and bioenergy. On the other hand, it is important to point that in order to develop protocols and methods for somatic embryogenesis in species of this genus, the knowledge of its counterpart, the natural zygotic embryogenesis is crucial. Methodologies for the production of somatic embryos in this genus have been reported for *A. victoria-reginae, A. sisalana, A. salmiana, A. tequilana, A. angustifolia, A. vera-cruz, A. fourcroydes,* and *A. sisalana*; and the uni- and multicellular origin of the somatic embryos is a key characteristic that should be taken into account for special purposes and uses. The importance of culture medium, plant growth regulators, genotype, and special conditions for culture incubation will be discussed.

16.1 The Genus *Agave*

This genus conforms a group of plant species of the Asparagaceae family (formerly Agavaceae) that belongs to the monocot class of angiosperms (APG III 2009). Nowadays, the genus *Agave* is distributed in the tropical and subtropical areas of the world and represents a large group of succulent plants, and its center of origin is probably limited to México (Gentry 1982). The genus *Agave* has about 200 species of which approximately 150 are endemic to México (García-Mendoza 2002), and it is divided into two subgenera, *Littaea* and *Agave,* based on the architecture of the inflorescence; subgenus *Littaea* has a spicate or racemose inflorescence while plants

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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_16

of the subgenus *Agave* bear a paniculate inflorescence with flowers in umbellate clusters on lateral branches (Gentry 1972).

Recent studies have found that the genus *Agave* is a young genus, which is between 7.8 and 10.1 million years old (Good-Avila et al. 2006). The subgenus *Agave* and particularly the sections Rigidae and Sisalanae are cultivated because of their commercial importance for diverse purposes: (a) alcoholic beverages such as tequila and mezcal; (b) natural long and hard fibers; and (c) sapogenins as natural precursors of steroidal compounds and medicinal principles as those species of the Amolae group (Blunden et al. 1980; Gentry 1982; Cedeño 1995); and unarmed species lacking spines are frequently used as ornamental plants among many other uses. *Agave tequilana* Weber var. Azul, which is the raw material for the production of tequila is the most extended species in plantations with about 100,000 ha in the region of the appellation of origin "tequila" in México (CRT 2015). Today, the cultivation of this species involves a high degree of mechanization and the use of modern agronomical inputs with a high degree of success in the production (Valenzuela 2010). However, the cultivation of other *Agave* species used in México for the elaboration of diverse products such as mezcal, in a majority of cases still being produced under ancient practices.

Besides their economic importance for the production of alcoholic beverages and fibers, agaves are becoming key plant species for the pharmaceutical industry and to tackle climate change in the near future for the production of biofuels because of their rusticity and because they do not compete with food crops.

In general, wild and cultivated species of *Agave* perform well in areas where rainfall is not sufficient for many cultivated C_3 and C_4 plants; this is because their crassulacean acid metabolism (CAM) allows them to tolerate dry and hot environments by opening the stomata at night for CO_2 uptake, thus avoiding loss of water. This CAM photosynthetic pathway allows that most agave species may have higher productivity in areas of prolonged droughts and with water restrictions than many other plant species (Kant 2010). Escamilla-Treviño (2012) made a detailed analysis of biomass productivity with regard to drought tolerance according to approximate rainfall requirements and based on the reports of several authors. He found that *Agave* species such as *A. salmiana, A. mapisaga, A. deserti, A. fourcroydes,* and *A. tequilana* have a higher degree of tolerance to drought as compared to *Panicum virgatum* var. Alamo, *Zea mays* (grain and stover), *Populus* spp. *Miscanthus giganteus, Saccharum officinarum,* and *Sorghum bicolor.*

Because of the above characteristics, agaves have emerged as a potential solution for the production of biofuels for the reduction of greenhouse emissions. The countries that have ratified the Kyoto Protocol are committed to fulfilling the commandments of the Clean Development Mechanism, whose distinctive element of the Kyoto Protocol is its demand that countries must reduce their greenhouse gas emissions (UNFCCC 1998). Furthermore, some *Agave* species have proven to be low recalcitrant lignocellulosic feedstock for biofuels when compared to non-agave plants (Li et al. 2014). Lignin together with hemicellulose and cellulose are the principal elements of plant cell walls. For the purpose of biofuel production, lignin hinders the hydrolysis of the polysaccharides to convert the lignocellulosic mass to

biofuel making some plant species highly recalcitrant (Escamilla-Treviño 2012). Recently, it has been reported that *A. americana* leaves, *A. salmiana* leaves, *A. tequilana* leaves, and *A. americana* stem have 8.2, 9.8, 11.9, and 7.3 g/100 g biomass based on oven-dried material, respectively, while Poplar and Switchgrass have a lignin content of 23.4 and 18.8 g /100 g biomass, respectively (Li et al. 2014). Thus, some estimated theoretical maximum ethanol yields for *A. americana*, Poplar, and Switchgrass are from 963 to 3,273, 1,273, and 1,403 gallons /ha year, respectively (Li et al. 2012).

It is important to note that there exist several agave landraces belonging to the *A. angustifolia* ssp. *tequilana* complex with domestication syndrome for sugars that may be useful for biofuel production in the near future (Valenzuela 2010).

On the other hand, recently the use of fructans, especially those from several agave species are gaining importance as healthy food ingredients as soluble dietary fiber and also because of their prebiotic characteristics benefiting the gastrointestinal flora of humans and some animals (López and Urías-Silvas 2007; Espinoza-Andrews and Urías-Silvas 2012). Agave fructans possess a particular core structure for which they have been called "agavins." This particular structure escapes from the action of digestive enzymes, thus serving as substrates (prebiotics) for the microflora living in the colon (López and Urías-Silvas 2007; Velázquez-Martínez et al. 2014).

16.2 Zygotic Embryogenesis in *Agave tequilana*

The somatic embryogenesis process cannot be understood without extensive knowledge of the zygotic embryogenesis in the plant. The formation of the embryo sac and subsequent double fertilization and the early development of the embryo and endosperm have recently been studied in *Agave tequilana* Weber var. Azul. This study was carried on clarified mature and immature ovules without cutting the tissues with a microtome in order to maintain the cells in their original site inside the embryo sac. In short, the female gametophyte originates from a single haploid cell originated by the meiotic division of a megaspore mother cell. This, in turn, undergoes three mitotic divisions that occur in a synchronized way at both extremes of the embryo sac giving rise to an eight-nucleated embryo sac. In this study, it was corroborated that the mature embryo sac is of the monosporic Polygonum-type and at this stage is already cellularized and consists of seven cells: three antipodal cells located at the chalazal pole, the central cell formed by two polar nuclei located just below the antipodals, and the egg apparatus located at the micropylar pole and composed of one egg cell and two synergids (González-Gutiérrez et al. 2014). The keynote is that all structures and cells studied were highly polarized and aligned to the micropylar-chalazal axis. In this manner, the development of the embryo sac, the egg cell, the zygote, and the early embryo were polarized as in most of the angiosperms (Huang and Sheridan 1994; Dodeman et al. 1997; Sundaresan and Alandete-Saez 2010). The polarity of the egg cell and the zygote is evident from the position of the nucleus located toward the

cytoplasm-rich chalazal extreme while the micropylar pole is highly vacuolated (see Figs. 4d, 5a and additional file 2 Fig. S5 in González-Gutiérrez et al. 2014). At 6 days after pollination (DAP), the zygote elongates about 50 % its original size. Finally, at nine DAP the zygote suffers a first asymmetric cell division giving rise to a two-celled proembryo consisting of cells with different developmental fates; the basal cell that will form the suspensor and the apical cell which is the first cell of the embryo proper and that through a series of coordinated cell divisions will form the embryo. This observed process was similar to what is described for the majority of angiosperms (Lau et al. 2012; González-Gutiérrez et al. 2014; Leljak-Levanić et al. 2015). Furthermore, the same pattern has been observed in another genus member of the Asparagaceae family (formerly Agavaceae): *Polianthes tuberosa* (González-Gutiérrez, to be published elsewhere).

16.3 Zygotic Embryogenesis Versus Somatic Embryogenesis in *Agave tequilana*

Somatic embryogenesis in plants is intrinsically linked to zygotic embryogenesis. Early somatic embryogenesis stages resemble those of zygotic embryogenesis, and many phenotypic and molecular features are shared between both types of embryogenesis (Jin et al. 2014). In *A. tequilana,* the rare occurrence of dicotyledonar zygotic embryos was recently reported (Ayala-González et al. 2014). *A. tequilana* is a plant species of the Asparagaceae family that belongs to the monocot class of angiosperms. Therefore, it should contain only one cotyledon. From a total of 1,164 analyzed embryos, 4 % showed two cotyledons (or dicotyledonar embryos), 44 % showed two fused cotyledons, and 52 % showed only one cotyledon. This means that about 50 % of the analyzed embryos were of a kind of dicotyledonous nature. It is possible that PIN proteins and adjacent genetic elements are being expressed as in dicots such as *Arabidopsis thaliana* (Jenik et al. 2007).

It is considered that monocots must have evolved from a primitive dicot. If a monocotyledon is derived from a dicotyledon, it must have happened through the process known as syndactyly (Bancroft 1914). Syndactyly is a concept used for the description of the fusion of two cotyledons to form one member (Sargant 1903; Bancroft 1914; Socoloff et al. 2014). Furthermore, recently dicotyledonar somatic embryos have appeared in some genetic lines of *A. tequilana* (unpublished results). Histological sections of embryogenic cell cultures showed the early formation of somatic dicotyledonar embryos (Fig. 16.1). Most of the embryos showed two fused cotyledons and after germination, they reached the form of a normal seedling. It seems that this phenotypic trait is the expression of a genetic nature and not due to particular environmental conditions of the in vitro culture, thus being this an example of shared common characteristics between zygotic embryogenesis and somatic embryogenesis. Other cytological characters resembling those of the zygotic embryos of this species will be discussed below in the corresponding section.

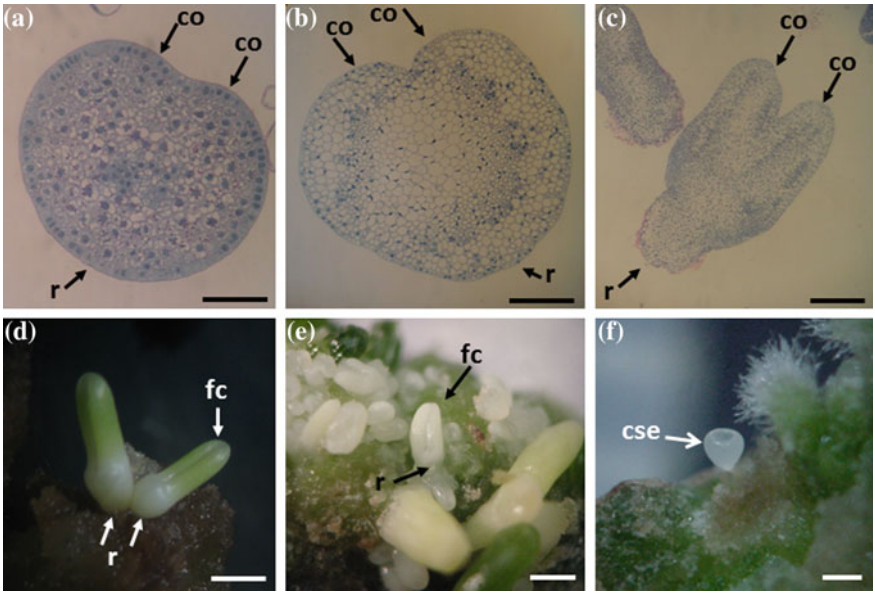

Fig. 16.1 Dicotyledonar somatic embryos of *Agave tequilana* Weber var. Azul. **a**–**b** Histology of early somatic embryos at the heart stage. Bar = 75 µm. **c** Mature somatic embryo with two cotyledons. Bar = 350 µm. **d**–**e** Somatic embryos with fused cotyledons. Bar = 1 mm. **f** *Cup-shaped* somatic embryo. Bar = 0.5 mm. *co* cotyledon; *r* radicle; *fc* fused cotyledon; *cse* cup-shaped somatic embryo

16.4 Somatic Embryogenesis in *Agave* spp.

For ease of time and space, in this revision, only basal media and growth regulators of the revised protocols will be mentioned, and some particular procedures and materials will be discussed where applicable. In this context, Table 16.1 summarizes general aspects of explant and medium composition for the somatic embryogenesis of several agave species.

16.4.1 Agave victoria-reginae

The first report on the somatic embryogenesis in the genus *Agave* was on the ornamental species *A. victoria-reginae* (Rodríguez-Garay et al. 1996). Direct somatic embryos were produced from young leaf blades harvested from in vitro propagated plantlets. The induction medium consisted of MS medium (Murashige and Skoog 1962) supplemented with 0.3 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Embryo germination was achieved by transferring globular embryos to growth regulator-free half strength MS medium; however, the germinated embryos

Table 16.1 Plant growth regulators used for induction of somatic embryogenesis in *Agave* spp.

Agave species	Explant	Response	Medium + PGR mgL^{-1}	References
victoria-reginae	In vitro leaf blades	Direct SE	MS + 0.3 2,4-D	Rodríguez-Garay et al. (1996)
sisalana	In vitro stems	Indirect SE	MS + 0.25 2,4-D + 1.0 BAP	Nikam et al. (2003)
victoria-reginae	Seedling stem segments	Indirect SE	MS + 0.52 2,4-D	Martínez-Palacios et al. (2003)
salmiana	In vitro leaf blades	Indirect SE	MS + 0.5 NAA + 1.1 BAP	Flores-Benítez et al. (2007)
tequilana	In vitro leaf blades	Indirect SE	MS + 2.0 2,4-D + 0.3 BAP	Portillo et al. (2007)
vera-cruz	In vitro leaf blades	Indirect SE	MS + 1.0 NAA + 0.2 ZEA	Tejavathi et al. (2007)
angustifolia	Zygotic embryo	Indirect SE	MS + 3.0 2,4-D + 1.0 BAP	Arzate-Fernández and Mejía-Franco (2011)
tequilana	In vitro leaf blades	Indirect SE	MS + 3.0 2,4-D + 0.3 BAP SH + 3.0 2,4-D + 0.3 BAP	Rodríguez-Sahagún et al. (2011)
fourcroydes	In vitro stems	Direct SE	MS + 0.5 DIC MS + 0.5 PIC	Monja-Mio and Robert (2013)
sisalana	Bulbils	Indirect SE	MS + 3.0 2,4-D + 20.0 BAP	Carneiro et al. (2014)

PGR Plant Growth Regulators; *SE* Somatic embryogenesis; *2,4-D* 2,4-dichlorophenoxyacetic acid; *BAP* 6-benzylaminopurine; *NAA* naphthaleneacetic acid; *ZEA* zeatin; *DIC* dicamba; *PIC* picloram; *MS* Murashige and Skoog (1962); *SH* Schenk and Hildebrandt (1972)

became hyperhydric. Hyperhydricity was completely eliminated by the use of vented Petri dishes, where the vents were covered with filter paper to facilitate gas exchange and MS medium with the concentration of NH_4NO_3 reduced to 5 mM. Plantlets from somatic embryos resulted habituated for growth regulators and at present are still propagated by shoot proliferation in completely hormone-free MS medium. Finally, the adaptation of several hundreds of plants to their natural habitat was successful. Moreover, Martínez-Palacios et al. (2003) successfully produced indirect somatic embryos from seedling stem segments in the same species by the addition of 0.5 mgL^{-1} 2,4-D to MS medium. These authors claimed a multicellular origin of the somatic embryos.

16.4.2 Agave sisalana

Agave sisalana (also known as Sisal) is a cultivated pentaploid species ($2n = 5x = 150$) (Castorena-Sánchez et al. 1991), which is used in many countries for the extraction of fibers from leaves and also for the secondary metabolites of pharmacological importance (Nikam et al. 2003; Debnath et al. 2010; Carneiro et al. 2014).

The first report of somatic embryos in this species is that of Nikam et al. (2003). They found that under a prolonged culture of 5–7 weeks in MS medium supplemented with 1–2 mg L^{-1} kinetin (KIN) or 0.25–0.5 mg L^{-1} naphthaleneacetic acid (NAA) + 1–1.5 mg L^{-1} KIN or 6-benzyladenine (BA), new embryos developed from embryogenic callus. However, the most effective medium for the induction of somatic embryos was supplemented with 0.25 mg L^{-1} 2,4-D, in which the embryogenic potential was maintained for about 48 months. In this protocol, MS medium + 0.1 or 0.2 mg L^{-1} KIN were used for embryo expression and germination which was achieved in 5 weeks. Histological analyzes of somatic embryogenesis showed that both unicellular and multicellular processes were the origin of somatic embryos.

In a more recent study on the somatic embryogenesis of *A. sisalana* conducted by Carneiro (2014), the best culture medium was half the concentration of MS salts supplemented with 3.0 mg L^{-1} 2,4-D + 20.0 mg L^{-1} BA. The cytological and histological analyzes of embryogenic cultures showed a clear unicellular origin as it has been found in other studies conducted in *A. tequilana* (Gutiérrez-Mora et al. 2004; Portillo et al. 2007; Santacruz-Ruvalcaba and Portillo 2009).

16.4.3 Agave salmiana

This species is cultivated in several regions of México and is used for the alcoholic beverages pulque and mezcal. Also, it is widely used for ethnomedical purposes and fodder in desert lands (Colunga-GarcíaMarín et al. 2007; Flores-Benítez et al. 2007). The somatic embryogenesis was achieved on a study about the genetic transformation of the species by using leaf blades from in vitro-produced plantlets. Somatic embryos were produced on MS medium with the addition of 0.5 mg L^{-1} NAA and 1.1 mg L^{-1} BA, and supplemented with a mixture of vitamins and amino acids reported by Mere-Villanueva and Vázquez-Alejandro (2003) that consisted of 306.38 μM glycine, 804.84 μM myoinositol, 12.18 μM nicotinic acid, 7.30 μM pyridoxine HCl, 8.90 μM thiamine HCl, 66.62 μM L-asparagine, 4.10 μM biotin, 57.40 μM L-arginine, 56.35 μM L-aspartic acid, 410.67 μM glutamine, 51.0 μM glutamic acid, 2.26 μM folic acid, 0.26 μM riboflavine and 749.25 μM urea (Flores-Benítez et al. 2007). Finally, in this work, *A. salmiana* transformed plants were regenerated from embryogenic callus co-cultivated with *Agrobacterium tumefaciens.*

16.4.4 Agave tequilana

As stated above, *A. tequilana* is the most widely cultivated species of agave in México with about 100,000 ha. Since the 1990s, this species has been severely attacked by diverse diseases caused by bacteria and fungi, and exposed to a multitude of natural abiotic stressors, which reduce both quality and yield of fermentable juices. During the past few years, the bacterium *Erwinia carotovora* and the fungus *Fusarium oxysporum* have been causing severe damage to *A. tequilana* plantations in México, including the states of Guanajuato, Jalisco, Michoacán, Nayarit, and Tamaulipas (Jiménez-Hidalgo et al. 2004; Ávila-Miranda et al. 2010). Moreover, constant high temperatures imposed by climate change have been of strong impact on agaves. While *A. tequilana* is commercially reproduced by rhizomatous suckers for new plantations, blooming plants have shown severe abnormalities in their flowers, mainly in the female reproductive apparatus. This means that the whole plant is under stress, diminishing the possibilities of a good productivity for the tequila industry (Rodríguez-Garay et al. 2014). The previously mentioned problems have pushed researchers to find biotechnological alternatives for the micropropagation and the genetic improvement of this important species.

The first protocol for the somatic embryogenesis in *A. tequilana* was reported by Portillo et al. (2007). Somatic embryos were produced from leaf blades collected from six in vitro micropropagated genotypes and cultured on MS medium supplemented with L2 vitamins (Phillips and Collins 1979) with the addition of several growth regulators. In this study, it was found that for the induction of somatic embryogenesis some genotypes gave good embryo production under high cytokinin concentration and low auxin concentration while other genotypes showed a good response to relatively high auxin concentration and low cytokinin concentration. In this manner, the genotype named S3 produced somatic embryos with 10.0 and 15.0 mg L^{-1} BA and 1.0 mg L^{-1} 2,4-D. On the other hand, genotype S7 produced somatic embryos with 2.0 mg L^{-1} 2,4-D and 0.3 mg L^{-1} BA. These highly contrasting responses can only be attributed to the genotype of the mother plant. In all cases, the expression and maturation of embryos were achieved on MS medium without growth regulators and supplemented with 500 mg L^{-1} L-glutamine and 250 mg L^{-1} casein hydrolysate. Moreover, in this work the unicellular origin of the somatic embryos was demonstrated (Fig. 16.2). On the other hand, an elegant demonstration of the unicellular origin of the *A. tequilana* somatic embryos was reported by Rodríguez-Domínguez (2000). In this study, gamma rays were used for the bombardment of highly embryogenic cells causing a mutation of the apical cell that resulted from a first cell division giving rise to an albino plantlet with green radicle.

In general terms, the initial embryonic cell is immersed in a proembryogenic cell mass and emulates the zygote which is its zygotic equivalent; its polarity is evident and contains large amounts of starch granules (Fig. 16.2a, b). The first and second divisions are highly polarized and start to show the first suspensor cells (Fig. 16.2c, d). After some rounds of cell divisions, the somatic embryo shows initials of a

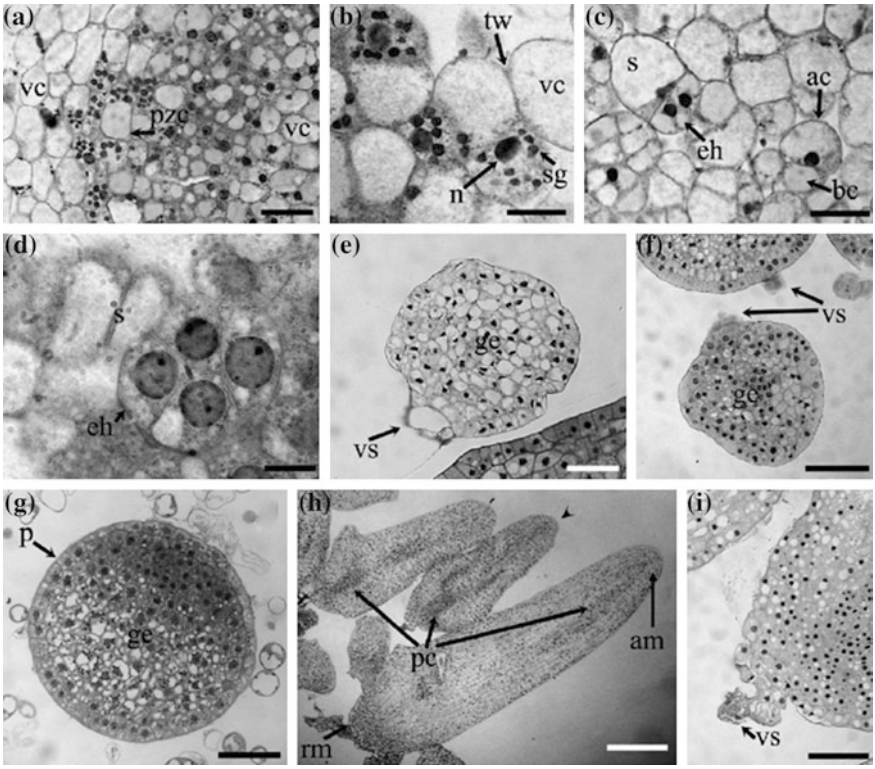

Fig. 16.2 Early stages of somatic embryogenesis in *A. tequilana* Weber var Azul. **a** Callus formed by highly vacuolated noncompetent cells and embryogenic cells containing large amounts of starch granules. Bar = 40 μm. **b** Polarized embryogenic cells containing large amounts of starch granules. Bar = 20 μm. **c** Polarized embryogenic structures resulting from first and second division of embryogenic cells. Bar = 30 μm. **d** Four-celled proembryo with a suspensor-like structure. Bar = 10 μm. **e** and **f** Globular embryos with vestigial suspensor. Bars = 50 and 100 μm. **g** Globular somatic embryo without suspensor. Bar = 75 μm. **h** Torpedo stage embryos showing procambial initials. Bar = 350 μm. **i** Close-up of the vestigial suspensor of one of the embryos in (*h* arrow head). Bar = 20 μm. *Pzc* polarized cell; *vc* vacuolated cell; *sg* starch grain; *n* nucleus; *tw* thick wall; *eh* embryo head; *s* suspensor; *ac* apical cell; *bc* basal cell; *ge* globular embryo; *vs* vestigial suspensor; *p* protoderm; *pc* procambial strands; *rm* root meristem; *am* apical meristem. From Portillo et al. (2007) Somatic embryogenesis in *Agave tequilana* Weber cultivar Azul. In Vitro Cell Dev-Pl 43:569-575. Copyright 2007 by the Society for In Vitro Biology, formerly the Tissue Culture Association. Reproduced with permission of the copyright owner

remaining suspensor and reaches its globular stage with an evident and well-formed protoderm (Fig. 16.2e, f, g). The embryos in the torpedo stage show the procambial initials and are ready for germination (Fig. 16.2h). At this point, it is important to mention that this cytological and morphological characteristic (suspensor) in the somatic embryo, is initiated from the basal cell of the very first division of the embryogenic cell; in the somatic embryo this is a key point for the formation of the

radicle and the final polarity of the new plant (Gutiérrez-Mora et al. 2012). On the other hand, it has been demonstrated that the medium basal composition plays an important role in the success of somatic embryogenesis in *A. tequilana*. When SH medium (Schenk and Hildebrandt 1972) was used instead of the MS medium, the production of somatic embryos was highly reduced. It is possible that the higher concentration of some ions in the MS medium is responsible for this effectivity. Furthermore, in this work, it was found that light quality exerts an important effect on the induction, maturation, and germination of the agave somatic embryos. Blue light produced a high number of embryos (an average of 20 per explant). However, the production of embryos increased when the white or red light was used for the induction period and then wide-spectrum light for the expression and maturation phase (Rodríguez-Sahagún et al. 2011).

Moreover, it is known that arabinogalactan proteins (AGPs) exert an important control on zygotic and somatic embryogenesis by stimulating both processes (Samaj et al. 2006). Recently, a study was conducted to investigate the distribution of AGPs and pectin in *A. tequilana* by using immunolabeling with anti-AGP monoclonal antibodies JIM4, JIM8 and JIM13 and anti-methyl-esterified pectin-antibody JIM7. Besides the presence of starch granules, it was found that AGPs and pectin are directly related to the embryogenic capability of somatic cells. These findings may be useful for selecting embryogenic genotypes, providing a new tool for the optimization of the somatic embryogenesis process (Portillo et al. 2012).

In regard to genetic improvement, somatic embryogenesis protocols have been used to produce trisomic, triploid and haploid plants. This goal was achieved with induction of trisomy by exposing embryogenic cells to 8 mg L^{-1} *para*-fluorophenylalanine (PFP) added to the induction medium. Obtained plants were trisomic with $2n = 2x = 61$; and there were differences in chromosome arm ratio (long arm/short arm) in eight chromosome pairs and more than 13 homologous chromosome pairs exhibited structural changes; all these aberrations in the chromosome complement of trisomic plants were putatively caused by inversions, deletions, and/or duplications produced by high concentrations of PFP; and the presence of a single extra chromosome could have been induced by the effect of PFP on the mitotic spindle by inducing nondisjunction of sister chromatids, resulting in cells with $2n + x$ and $2n - x$ chromosomes. In vitro-produced plants were transferred to soil and have continued to grow under ex vitro conditions. Trisomic plants showed remarkable morphological characteristics, such as longer terminal spines and wider leaves, as compared as to wild-type or normal plants (Ruvalcaba-Ruíz et al. 2012). Moreover, triploid plants have been regenerated from somatic embryos produced from the immature triploid endosperm of *A. tequilana*. Age (45 days after pollination), the genotype of the parents, growth regulators and light quality played important roles in the production of triploid embryos. Two embryogenic calluses were obtained by culture on *N* medium (Nitsch and Nitsch 1969) and MS medium with the addition of 2 mg L^{-1} 2,4-D, and 0.3 mgL^{-1} BA. After the induction period, embryogenic calluses were transferred to LOG medium (Castro-Concha et al. 1990) without growth regulators and supplemented with 4 mg

L^{-1} BA and exposed to red light (λ = *630 nm) for 15 days. It was claimed that red light was a key element for the regeneration of triploid plants from the endosperm. These two calluses regenerated two plants that had triploid cells with 90 chromosomes (Ruvalcaba-Ruíz 2003). On the other hand, haploid plants are important individuals in plant breeding programs for a vast number of genetic methodologies, such as the production of completely homozygous plants and the selection of recessive traits among many other uses. Ruvalcaba-Ruíz (2003) produced a haploid plant by culturing unpollinated ovaries of* A. tequilana *on NPB medium supplemented with 90 gL^{-1} maltose, 300 mgL^{-1} casein hydrolysate, 2.0 mgL^{-1} 2,4-D and 0.3 mgL^{-1} BAP for the induction of somatic embryogenesis. Embryogenic callus was transferred to MS medium with the addition of 4.0 mg L^{-1} BA and incubated for 15 days under red light (λ = 630 nm). A plant was obtained from a regenerated somatic embryo which was found to be haploid with 30 chromosomes.*

Current work is focused on the application of several strategies with the development of cell and tissue culture methodologies as well as in casa hybridization techniques, which include embryo rescue (to be published elsewhere) in order to be used in the genetic improvement of this important industrial agave species.

16.4.5 Agave angustifolia

This species is widespread all over México and cultivated in many countries. In México, one of the most important uses is for mezcal production among other industrial and medicinal purposes. The somatic embryogenesis in *Agave angustifolia* was recently achieved by the use of zygotic embryos as explants. These embryos were cultured for the induction process on 25 % MS medium supplemented with 3.0 mg L^{-1} 2,4-D, 1.0 mg L^{-1} BA, and 60 g L^{-1} sucrose and incubated under dark conditions. The expression and germination medium consisted of half strength MS medium without growth regulators. Regenerated plants were obtained 140 days after the beginning of the in vitro culture (Arzate-Fernández and Mejía-Franco 2011). It is well known that diverse kinds of stresses and plant growth regulators play an important role in somatic embryogenesis. The utilization of 25 % MS medium and the addition of 60 g L^{-1} sucrose in the induction process could have acted positively as starvation and osmotic stresses, respectively, as it has been demonstrated in other plant species (Jin et al. 2014).

16.4.6 Agave vera-cruz

This species is an unknown plant in México. *A. vera-cruz* is cultivated in some regions of South India for fiber, food, and medicine and it is known as "Grey Aloe of India" (Tejavathi et al. 2007); and it has been studied and well characterized

since the early 1950s in India as a source of carbohydrates (Srinivasan and Bratia 1953; Cairns 1993).

Shoot apices, cotyledons, and leaf segments from 3 months old seedlings were used as explants. Diverse vitamin compositions were tested, finally for all somatic embryogenesis experiments L2 vitamins (Phillips and Collins 1979) were chosen because of its ease of rapid production of callus.

Embryogenic callus was induced on MS medium supplemented with 1.0 mg L^{-1} NAA and 0.2 mg L^{-1} zeatin (ZEA) with the addition of 40 g L^{-1} sucrose. Expression and germination of somatic embryos were achieved in the same MS medium.

Rooted plantlets were transferred to soil with a survival of 96–98 % without any hardening procedure. The authors claimed that the origin of somatic embryos was of a multicellular kind (Tejavathi et al. 2007).

16.4.7 Agave fourcroydes

This *Agave* species known as "henequén" is well adapted to the arid areas of México and Central America including Caribbean countries such as Cuba. *A. fourcroydes* is a pentaploid, long-lived plant, asexually propagated and grown mainly for the manufacture of ropes, woven sacks of high quality, and for the extraction of medicinal precursors (Gentry 1982). Recently, Monja-Mio and Robert (2013) reported the direct somatic embryogenesis in this species through thin cell layer culture (tTCLs), which will be a useful biotechnological technique for in vitro germplasm conservation, genetic improvement and for micropropagation as it has been reported for many plant species. For this purpose, thin tissue segments (tTCLs) of 0.5–1.0 mm from stems taken from in vitro propagated plantlets were used as explants. Induction of somatic embryogenesis was achieved by culturing the explants on MS medium supplemented with L2 vitamins (Phillips and Collins 1979), 0.5 mg L^{-1} dicamba (DIC) or 0.5 mg L^{-1} picloram (PIC), 30 g L^{-1} sucrose, solidified with 3 g L^{-1} agar and 3 g L^{-1} Phytagel and incubated under dark conditions. The embryogenic response was improved when the explant donor plantlets were maintained for one month on a culture medium containing 10 mg L^{-1} BA. Again in this agave species, the embryogenic response was strongly dependent on the genotype. Somatic embryos did not show any vascular connection with the original explant tissue and seemed to be generated through uni- and multicellular events. These embryos germinated when they were transferred to half strength MS medium and regenerated plantlets were transferred to soil and maintained under greenhouse conditions with a survival rate of 85 %.

16.5 Concluding Remarks

Somatic embryogenesis has been achieved in several species of the genus *Agave*. Scientific reports have indicated that the production of somatic embryos is feasible in species from both subgenera, *Littaea* and *Agave* such as *A. victoria-reginae* and *A. tequilana,* respectively. At this point, it is important to remark that more knowledge in depth is necessary about zygotic embryogenesis in order to understand the cytological, biochemical, and molecular mechanisms for developing protocols for somatic embryogenesis; being this one of the most important biotechnological tools for conservation, micropropagation, and the genetic improvement of *Agave* species of ecological and economic importance.

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Chapter 17
Somatic Embryogenesis in *Agave*: An Overview

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Abstract This chapter describes the details of somatic embryogenesis of some species of the genus *Agave*. Various factors that influence the induction of the embryogenic process, such as explant type, growth regulator type, growth regulator concentration and genotype, are discussed. Furthermore, embryo development and conversion, as well as the first works using temporary immersion bioreactors, are also discussed.

17.1 Introduction

The genus *Agave* belongs to the Agavaceae family and is the largest member of this family with 166 species, of which 125 inhabit Mexico (Rocha et al. 2006). This genus comprises perennial plants that are polyploid and can be wild or cultivated.

Some agaves are used to obtain a wide range of products such as alcohol, fibers, cellulose, and inulin, which have great demand as industrial feedstock (Robert et al. 2006; Narváez-Zapata and Sánchez-Teyer 2009). Despite this fact, its cultivation has been based on ancient practices that have been improved very little; therefore, crops are affected by health problems and low productivity. The lack of quality plants is a common problem, especially in the case of the species of agro-industrial interest (Robert et al. 2004).

Micropropagation is the best method to obtain a large number of selected cloned plants and their consequent establishment in plantations (Das 1992). Therefore, efficient and reproducible protocols are important for the establishment of morphogenetic pathways (organogenesis or somatic embryogenesis).

The objective of most of the reported works on plant tissue culture in the genus *Agave* is propagation for conservation purposes (germplasm bank) and the mass micropropagation of commercial species. Agave plantlets produced in vitro have

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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_17

been obtained through organogenesis and somatic embryogenesis (SE), both directly and indirectly.

SE is a process of highly multiplicative potential that can greatly increase the micropropagation of this species at a low cost. Furthermore, the use of indirect embryogenesis enables the generation of genetic variability that can be utilized in breeding programs, in addition to being a basis for epigenetic studies. Although SE has been reported in some species of the genus *Agave*, further studies are necessary for this process to be used for commercial propagation.

This chapter describes various factors affecting the induction of the embryogenic process, such as explant type, genotype, and growth regulator.

17.2 Induction of SE

SE is a process by which a cell (Haccius 1978) or a group of cells (Williams and Maheswaran 1986) from somatic tissue form an embryo. In *Agave*, few reports exist on SE in economically important species (Table 17.1). The establishment of the culture conditions for each phase of the process is necessary to achieve reproducible and highly efficient protocols.

17.2.1 Type of Explant

Different tissues, such as the leaves, stems, and roots, have been used to induce ES in different species of agaves. These tissues have been obtained from in vitro plant seeds, bulbils, or axillary bud propagation. Other explants such as zygotic embryonic axes and bulbils have also been used.

Tissues obtained from a leaf base have been reported for *A. victoriae-reginae* (Rodríguez-Garay et al. 1996), *A. vera-cruz* (Tejavathi et al. 2007), and *A. tequilana* (Portillo et al. 2007; Rodríguez-Sahagún et al. 2011).

Stem tissues have been used for *A. victoriae-reginae* (Martínez-Palacios et al. 2003) and *A. fourcroydes* (Monja-Mio and Robert 2013). In the first case, plantlets from seeds were used, and in the second case, plantlets from selected mothers were used. Furthermore, using the technique of "thin cell layer" in this last work allowed a greater number of layers of stem cells, which were mostly advantageous tissues.

The use of roots as a source of explant has been reported in *A. tequilana* (Portillo and Santacruz-Ruvalcaba 2006b). The authors mentioned some qualitative advantages of using roots as explants rather than using leaf explants, such as low oxidized phenols and absence of hyperhydricity in the callus from the root (Portillo and Santacruz-Ruvalcaba 2006b).

The use of bulbils as explants has been reported in *A. fourcroydes* (González Oramas et al. 2002) and *A. sisalana* (Dos Santos Carneiro et al. 2014). In the latter species, Nikam et al. (2003) reported that callus obtained from the stems of bulbils

Table 17.1 References for the induction of SE of *Agave*

Species of *Agave*	Type of SE	Type of explant	Culture medium	Growth regulator	Production	Time (days)	References
Agave victoriae-reginae Moore	Direct	Leaf blade	MS medium supplemented with L2 vitamins	1.4 μM 2,4-D	Dates not reported	42	Rodríguez-Garay et al. (1996)
Agave fourcroydes Lem.	Indirect	Apices of bulbils	MS with the nitrogen sources modified	1.12 μM 2,4-D	17.14 embryogenic aggregates/treatments	60	González Oramas et al. (2002)
Agave victoriaereginae	Indirect	Seedling stems	MS	2.26 μM 2,4-D	50 % of explants with embryogenic masses	70	Martínez-Palacios et al. (2003)
Agave sisalana Perr. ex. Engelm	Indirect	Young shoots raised in vitro from the stem portion of the bulbil	MS	1:1.07 μM NAA and 6.98 μM KIN or 2:1.13 μM 2,4-D and 4.44 μM BA	1:9.1 embryos/responding callus 2:9.5 embryos/responding callus	35	Nikam et al. (2003)
Agave tequilana Weber var. Azul	Indirect	Roots of 1 cm length	MS medium supplemented with L2 vitamins	13.6 μM 2,4-D and 1.3 μM BA	Obtained embryogenic callus	30	Portillo and Santacruz-Ruvalcaba (2006 b)
Agave vera-cruz Mill.	Indirect	Leaf of seedlings	MS medium supplemented with L2 vitamins	4.52 μM 2,4-D or 5.37 μM NAA	Formed a creamy green-colored embryogenic nodular callus	–	Tejavathi et al. (2007)
Agave tequilana Weber var. Azul	Indirect	Leaf segments of in vitro-maintained plantlets	MS medium supplemented with L2 vitamins	9.0/1.3, 13.6/4.0, 4.52/ 66.6 μM, 2,4-D and BA	Embryogenic callus (friable and creamy yellowish in color)	40	Portillo et al. (2007)

(continued)

Table 17.1 (continued)

Species of *Agave*	Type of SE	Type of explant	Culture medium	Growth regulator	Production	Time (days)	References
Agave tequilana Weber var. Azul	Indirect	Leaf segments of in vitro propagated plants	MS	13.6 μM 2,4-D, 1.3 μM BA	Embryogenic callus	40	Rodríguez-Sahagún et al. (2011)
Agave angustifolia Haw	Indirect	Zygotic seed embryonic axes	MS medium (25 % of its original concentration) supplemented with L2 vitamins and 60 g L^{-1} sucrose	13.6 μM 2,4-D and 4.44 μM BA	Embryogenic callus (creamy white, soft and friable consistency)	30	Arzate-Fernández and Mejía-Franco (2011)
Agave fourcroydes Lem.	Direct	tTCLs stem in vitro propagated plants	MS medium supplemented with L2 vitamins	2.26 μM dicamba or 2.07 μM picloram	92 embryos/stem (dicamba) and 81.72 (picloram) in genotype K9	30	Monja-Mio and Robert (2013)
Agave sisalana Perrine	Indirect	Bulbils	Half-strength MS medium	13.6 μM de 2,4-D and 88.8 μM BA	Embryogenic callus	30	Dos Santos Carneiro et al. (2014)

did not lead to the induction of embryos, whereas callus obtained from the base of the shoots (from bulblets) that were placed on induction media generated embryos.

The use of zygotic embryonic axis explants has been reported in *A. angustifolia,* demonstrating their ability to form an embryogenic callus (Arzate-Fernández and Mejía-Franco 2011).

17.2.2 Culture Media, Vitamins, Carbon Sources, and Gelling

The most commonly used culture medium in the induction of embryogenesis in agaves is MS medium (Murashige and Skoog 1962), but SH medium has also been used (Schenk and Hildebrandt 1972). The importance of culture media composition in the production of agaves embryos was reported in *A. tequilana*, and explants produced 95 and 75 % of callus when cultured on MS and SH, respectively (Rodríguez-Sahagún et al. 2011). Both media differed in their total ionic strength, nitrogen level, ammonium concentration, and the ammonium-to-nitrate ratio (Rodríguez-Sahagún et al. 2011).

In most reports, MS medium has been used in its original concentration (Table 17.1); however, there were also reports of nitrogen sources being modified, as in the case of *A. fourcroydes* (González Oramas et al. 2002), which used the formulation of MS with the nitrogen sources modified by Robert et al. (1992). In other cases, the embryogenic induction was obtained favorably with reduction of the concentration of MS medium. Half the concentration of salts in the MS medium was used in the embryogenic induction of *A. sisalana* (Dos Santos Carneiro et al. 2014). In *A. angustifolia*, the embryogenic capacity of the callus induced on MS medium with 25 % of its original concentration was almost six times higher (85 %) than that obtained with MS media at its original concentration (15 %) (Arzate-Fernández and Mejía-Franco 2011) (Table 17.1).

The effect of vitamins in the induction medium of agaves has not been well studied; most studies have reported the use of the vitamin L2 (Phillips and Collins 1979). Tejavathi et al. (2007) mentioned that prior to the induction of *A. vera-cruz,* they tested various combinations of vitamins plus MS medium salts, with the most favorable being L2 vitamins in terms of the time required for the initiation and growth of the culture. The difference in the composition of vitamin L2 (Phillips and Collins 1979) in relation to vitamins of the MS medium (Murashige and Skoog 1962) is that it has a greater amount of myo-inositol (2.5 times) and thiamine (20 times; Table 17.2).

Sucrose has been the carbon source used in the induction of SE in agaves, and its concentration has mostly been 30 g L^{-1}. However, embryo formation in *A. angustifolia* increased by 2.7 times in the embryogenic callus obtained with 60 g L^{-1} of sucrose with respect to the medium with 30 g L^{-1} (Arzate-Fernández and Mejía-Franco 2011). Apparently, the sucrose concentration may affect the

Table 17.2 Components of MS vitamins (Murashige and Skoog 1962) and L2 (Phillips and Collins 1979)

Components (mg L^{-1})	MS vitamins (Murashige and Skoog 1962)	L2 vitamins (Phillips and Collins 1979)
Myo-inositol	100	250
Thiamine-HCl	0.1	2
Pyridoxin-HCl	0.5	0.5
Nicotinic acid	0.5	–

morphogenic induction as a nutrient source and as a regulator of the osmotic potential of the culture medium (Körbes and Droste 2005).

The concentration of gelling agent in the induction medium varied from 6 g L^{-1} in *A. fourcroydes* (Portillo and Santacruz-Ruvalcaba 2006b; Monja-Mio and Robert 2013); 7 g L^{-1} in *A. sisalana* (Dos Santos Carneiro et al. 2014); 8 g L^{-1} in *A. angustifolia* (Arzate-Fernández and Mejía-Franco 2011) *A. tequilana* (Portillo et al. 2007; Rodríguez-Sahagún et al. 2011), *A. sisalana* (Nikam et al. 2003) and *A. vera-cruz* (Tejavathi et al. 2007); and 10 g L^{-1} in *A. fourcroydes* (González Oramas et al. 2002).

17.2.3 Growth Regulators

Auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is the most widely used growth regulator in the induction of SE (Gaj 2004; Jiménez 2005) and agaves are no an exception. This auxin has been used alone or in combination with cytokinins, but other auxins have also been used, such as α-naphthalene acetic acid (NAA), 3,6-dichloro-2-methoxybenzoic acid (dicamba), and 4-amino-3, 5, 6-trichloro-2-pyridinecarboxylic acid (picloram). Among the cytokinins, 6-benzyladenine (BA) and kinetin (KIN) were used.

The use of 2,4-D alone has been reported in *A. victoriae-reginae* (Rodríguez-Garay et al. 1996; Martínez-Palacios et al. 2003), *A. fourcroydes* (González Oramas et al. 2002), and *A. vera-cruz* (Tejavathi et al. 2007).

The combination of 2,4-D and BA was used in the SE of *A. sisalana* (Nikam et al. 2003; Dos Santos Carneiro et al. 2014), *A. tequilana* (Portillo and Santacruz-Ruvalcaba 2006b; Portillo et al. 2007; Rodríguez-Sahagún et al. 2011), and *A. angustifolia* (Arzate-Fernández and Mejía-Franco 2011). Portillo et al. (2007) mentioned that induction with 2,4-D in *A. tequilana* alone generated only callus without embryos while the addition of BA allowed embryogenic callus induction.

In *A. fourcroydes*, Monja-Mio and Robert (2013) found that 2,4-D was less effective than other auxins, such as dicamba and picloram, which induced the highest embryogenic response. Picloram has been used in the embryogenic

induction of other monocots such as *Bactris gasipaes* (Steinmacher et al. 2007) and *Elaeis guineensis* Jacq. (Scherwinski-Pereira et al. 2010), and dicamba was also used in wheat (Filippov et al. 2006).

Regarding the concentrations used, the auxins ranged from 1.07 to 13.6 μM, while cytokinins ranged from 1.3 to 88.8 μM (Table 17.1). High concentrations of 2,4-D with the addition of cytokinins favored the induction of embryogenic callus in *A. tequilana* (Portillo and Santacruz-Ruvalcaba 2006b; Portillo et al. 2007; Rodríguez-Sahagún et al. 2011), in *A. angustifolia* (Arzate-Fernández and Mejía-Franco 2011), and in *A. sisalana* (Dos Santos Carneiro et al. 2014). Portillo et al. (2007) observed that high concentrations of cytokinins can induce a high number of embryos of *A. tequilana*, and the embryos obtained were green in contrast to those obtained on the medium with low concentrations of cytokinins.

17.2.4 Genotype

Genotype is one of the most important factors that determines the ability of SE (Fehér et al. 2003) and is one of the main reasons for the lack of reproducibility of the many protocols. The difference in the embryogenic response may be due to variations in levels of endogenous growth regulators (Jiménez 2005), qualitative and quantitative genetic differences (Fehér 2008) or epigenetic differences, including chromatin condensation (Fehér 2006).

In *A. tequilana*, Portillo et al. (2007) observed that although all genotypes responded to induction, the number of embryos was higher in genotypes obtained from germinated seeds than of genotypes obtained from axillary buds.

In *A. fourcroydes*, Monja-Mio and Robert (2013) found significant differences in the production of embryos from different clonal lines. Therefore, the selection of embryogenic genotypes is one of the key steps in the establishment of somatic embryogenesis protocols in agaves.

17.2.5 Type of SE

Most studies on SE of the *Agave* species have reported indirect SE (González Oramas et al. 2002; Martínez-Palacios et al. 2003; Nikam et al. 2003; Portillo and Santacruz-Ruvalcaba 2006a, b; Portillo et al. 2007; Tejavathi et al. 2007; Arzate-Fernández and Mejía-Franco 2011; Rodríguez-Sahagún et al. 2011; Dos Santos Carneiro et al. 2014) (Table 17.1). Direct SE has been reported only in *A. victoriae-reginae* (Rodríguez-Garay et al. 1996) and *A. fourcroydes* (Monja-Mio and Robert 2013).

In indirect embryogenesis, the types of callus reported varied according to species. Friable calluses were reported in *A. tequilana* (Portillo et al. 2007) and *A. angustifolia* (Arzate-Fernández and Mejía-Franco 2011). Compact and nodular calluses were reported in *A. victoriae-reginae* (Martínez-Palacios et al. 2003), *A. vera-cruz* (Tejavathi et al. 2007), and *A. sisalana* (Nikam et al. 2003; Dos Santos Carneiro et al. 2014).

The origin of somatic embryos can be unicellular (Haccius 1978) or multicellular (Williams and Maheswaran 1986). In agaves, a unicellular origin was observed in *A. tequilana* (Portillo et al. 2007), while a multicellular origin was observed in *A. victoriae-reginae* (Martínez-Palacios et al. 2003) and *A. vera-cruz* (Tejavathi et al. 2007). Both origins were observed in *A. sisalana* (Nikam et al. 2003) and *A. fourcroydes* (Monja-Mio and Robert 2013). In *A. sisalana*, it was observed that embryos were generated from epidermal and subepidermal cells inside the callus (Nikam et al. 2003).

17.3 Development and Maturation of Somatic Embryos

For developing embryos in agaves, different methods have been used, such as the increase of the carbon source, the removal of auxin, the addition of cytokinin, the addition of nitrogen compounds, or the reduction of the medium culture concentration.

It has been reported that concentrations of carbohydrates stimulate histodifferentiation (Ramsay et al. 2003). In *A. vera-cruz* (Tejavathi et al. 2007) and in *A. victoriae-reginae* (Martínez-Palacios et al. 2003), an increased concentration of sucrose (40 g L^{-1}) was required to achieve the development of embryos.

The addition of cytokinins was used in the development of *A. fourcroydes* (González Oramas et al. 2002), *A. sisalana* (Nikam et al. 2003), *A. vera-cruz* (Tejavathi et al. 2007), and *A. sisalana* (Dos Santos Carneiro et al. 2014).

The addition of nitrogen compounds, such as hydrolyzed casein, was used in the expression phase of *A. tequilana* (Portillo and Santacruz-Ruvalcaba 2006b; Portillo et al. 2007; Rodríguez-Sahagún et al. 2011).

Reducing the concentration of the culture medium to half its ionic strength was used in developing embryos of *A. victoriae-reginae* (Rodríguez-Garay et al. 1996) and *A. angustifolia* (Arzate-Fernández and Mejía-Franco 2011). In the expression phase of somatic embryos of *A. tequilana*, the nitrate content was reduced to 5 mM (Portillo et al. 2007; Rodríguez-Sahagún et al. 2011).

The use of MS medium at half its ionic strength for embryo germination was reported in *A. fourcroydes* (Monja-Mio and Robert 2013) and *A. angustifolia* (Arzate-Fernández and Mejía-Franco 2011) (Table 17.3).

Table 17.3 Maturation and germination of somatic embryos of *Agave*

Species of *Agave*	Culture medium	Growth regulator	Time (days)	Maturation or germination	References
Agave victoriae-reginae Moore	Half-strength MS medium	Without growth regulators	56	Dates not reported	Rodríguez-Garay et al. (1996)
	Half-strength SH medium	Without growth regulators	28	Dates not reported	
Agave fourcroydes Lem.	MS with the nitrogen sources modified	0.11 μM 2,4-D and 22.2 μM BA	60	75 % embryos germinated	González Oramas et al. (2002)
Agave victoriae-reginae	MS medium	Without growth regulators	56	Dates not reported	Martínez-Palacios et al. (2003)
Agave sisalana Perr. ex. Engelm	MS medium	0.1 mg L^{-1} KIN	35	75 % embryos germinated	Nikam et al. 2003
Agave tequilana Weber var. Azul	MS medium supplemented with 500 mg L^{-1} l-glutamine, 250 mg L^{-1} casein hydrolysate		Not reported	101.73 embryoid by repetition (four root pieces of 1 cm length)	Portillo and Santacruz-Ruvalcaba (2006 b)
Agave vera-cruz Mill.	MS medium supplemented with L2 vitamins	5.37 μM NAA plus 0.91 μM zeatin	56	46 embryos/explant	Tejavathi et al. (2007)
Agave tequilana Weber var. Azul	MS expression medium (NH_4NO_3 reduced to 5 mM), supplemented with L2 vitamins, 500 mg L^{-1} l-glutamine, 250 mg L^{-1} casein hydrolysate	Without growth regulators	90	9.0 μM, 2,4-D plus 1.3 μM BA: 557 embryos per dish 13.6 μM, 2,4-D plus 4 μM BA: 465 embryos per dish Each dish contained 4 segments of leaf	Portillo et al. (2007)

(continued)

Table 17.3 (continued)

Species of *Agave*	Culture medium	Growth regulator	Time (days)	Maturation or germination	References
Agave tequilana Weber var. Azul	MS expression medium (NH_4NO_3 reduced to 5 mM), supplemented with L2 vitamins, 500 mg L^{-1} l-glutamine, 250 mg L^{-1} casein hydrolysate	Without growth regulators	60	18 per explant germinated embryos	Rodríguez-Sahagún et al. (2011)
Agave angustifolia Haw	For maturation: Half-strength MS medium For germination: Half-strength MS medium plus 60 g L^{-1} sucrose	0.1 mg L^{-1} de 2,4-D for maturation medium Without growth regulators for germination medium	70 40	182 embryos/embryonic axes 100 % germination of cotyledonary embryos	Arzate-Fernández and Mejía-Franco (2011)
Agave fourcroydes Lem.	Half-strength MS medium	Without growth regulators	60	Dates not reported	Monja-Mio and Robert (2013)
Agave sisalana Perrine	Half-strength MS medium	39, 96 μM BA and 30 g L^{-1} sucrose 13, 32 μM BA and 40 g L^{-1} sucrose	90	26.16 embryos/250 mg embryogenic callus 23.33 embryos/250 mg embryogenic callus	Dos Santos Carneiro et al. (2014)

17.4 SE of Agaves in Liquid Media and Temporary Immersion Bioreactors (TIB)

In *A. tequilana*, the feasibility of using a temporary immersion bioreactor (Orbitabión®) was assessed, and production was possible because the number of embryoids was statistically equal in this system compared to on solid medium; however, in all TIBs, a high percentage of hyperhydric embryos was observed (Portillo and Santacruz-Ruvalcaba 2006a). In the same species, a hybrid technique

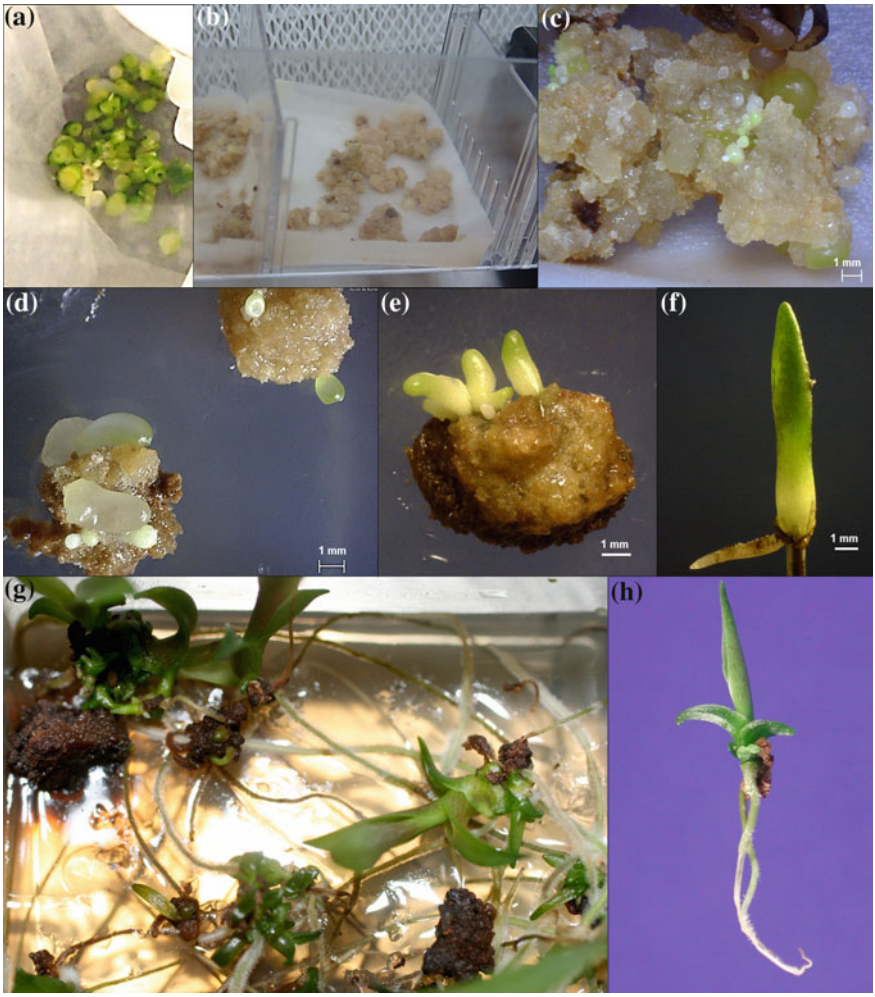

Fig. 17.1 SE in *A. tequilana* using TCL and the BioMINT™ bioreactor. **a** tTCLs on filter paper. **b** Embryogenic calluses obtained after 30 days. **c** Globular structures after 60 days of culture. **d** Somatic embryos at different stages. **e** Embryos at mature stages. **f** Germinated embryo with apical and radicular tips. **g** Asynchrony in embryo germination. **h** Plantlet obtained through SE

called “thin cell layer suspension” was used, in which a liquid suspension of embryogenic calluses was inoculated on a semisolid medium for the expression and development of somatic embryos, and under these conditions, the absence of hyperhydricity was observed in the generated embryos (Santacruz-Ruvalcaba and Portillo 2009).

In *A. fourcroydes*, the induction phase was successfully performed using thin layer stem cells (tTCLs) in temporary immersion bioreactors (RITA and BioMINT) (Monja-Mio et al. in press) (Fig. 17.1). Directly obtaining somatic embryos of *A. fourcroydes* in TIB could be an advantage for use as explants, especially during the early stages of development due to high embryogenic capacity and loss of the control group organized cells of the embryo (Raemakers et al. 1995).

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Chapter 18
Somatic Embryogenesis in *Cocos nucifera* L.

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Abstract In our coconut laboratory micropropagation has been the subject of research for nearly three decades, as this plant species is highly recalcitrant for in vitro regeneration and so far only achieved through somatic embryogenesis as the sole path for coconut regeneration. Of all the explants tested, plumules have proved to be the most responsive and the process efficiency has been improved by indirect embryogenesis and thereafter secondary embryogenesis and callus multiplication, this strategy is currently applied in floral explants. Two different approaches have been used to find ways to have a more efficient protocol. The first one, a direct and practical method, included plant hormones and activated charcoal. On the other hand, the indirect approach consisted in basic studies on: morphohistological development, biochemical and physiological aspects such as uptake of exogenous auxin, levels of endogenous auxin; shoot apical meristem formation and maintenance (*KNOX* gene family); the occurrence and expression of genes related to the cell cycle control (Cyclin-Dependent Kinase), and somatic embryogenesis (*Somatic Embryogenesis-Related Kinase*); and the establishment of a transformation protocol. A better understanding of the somatic embryogenesis of coconut was achieved by these approaches. This way, in the short term there is no doubt that we will have mass propagation options based not only in plumule explants but also on rachillae, unfertilized ovary, and leaf explants.

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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_18

18.1 Introduction

Coconut (*Cocus nucifera* L.) is an important perennial crop, widely distributed in humid tropical climates. It is cultivated on an estimated twelve million hectares, and it is an important participant to achieve food security, improved nutrition, employment, and income generation. It is a monospecific palm species which includes various ecotypes, varieties, and hybrids with a variety of desirable agronomical characteristics and a diverse number of uses. Coconut is often referred to as "the tree of life" given the many applications that have been developed for it. Ethnic diversity is an important factor in the ways that coconut products are manufactured and used (Foale 2005); varieties and hybrids with diverse agronomic characteristics of interest for different packed water, virgin oil, coconut milk, coconut sugar, fiber-derived products for the automobile industry, and oil derivatives such as surfactants and biodiesel. In the Philippines, an industrial plant was launched in 2006 for the production of 75 million liters/year of coco-diesel where it is being used as a fuel additive (Lao 2009). A mixture of diesel with 2 % coconut oil has shown to limit harmful exhaust emissions (opacity, K value) by 63 % (Lao 2008).

Most coconut plantations need to be replanted due to loss either by palm senescence or by diseases as lethal yellowing in the American continent (Harrison and Oropeza 2008), by the lethal disease in Africa (Eden-Green 1997), and cadang-cadang in Asia (Hanold and Randles 1991). Unfortunately, selected disease-resistant planting materials are scarce, and seed propagation does not yield sufficient material to satisfy the rapidly growing demands. For this reason, new methods for the propagation of selected planting material need to be developed, and the potential of massive propagation of in vitro propagation or micropropagation via somatic embryogenesis seems to provide an encouraging alternative.

18.2 Early Findings on Somatic Embryogenesis with Different Explants

During the 1970s and early 1980s various laboratories reported coconut somatic embryogenesis starting from different explant sources: shoot apical meristem (Apavatjrut and Blake 1977), endosperm (Fisher and Tsai 1978; Kumar et al. 1985), leaves (Buffard-Morel et al. 1988, 1992; Karunaratne et al. 1991), roots (Fulford et al. 1981), zygotic embryos (Bhalla-Sarin et al. 1986; Karunaratne and Periyapperuma 1989).

18.2.1 Rachilla Explants

Most of the early progress were achieved using inflorescence explants obtained slicing rachilla tissues (Verdeil et al. 1994; Blake and Hornung 1995). When cultured,

these explants developed a partly dedifferentiated callus that was referred to as "calloid" (Brackpool et al. 1986). Afterward, the somatic embryos were formed and germination followed to form clonal *plantlets* (Blake 1990; Verdeil et al. 1994; Blake and Hornung 1995). Initially, protocols using rachilla explants were inefficient and lacked reproducibility, but further studies allowed for a reproducible regeneration protocol to be generated, although with an undefined efficiency concerning the formation of somatic embryos and plantlet conversion defined (Verdeil et al. 1994).

18.2.2 Plumule Explants

In the middle of the 1990s CICY (Mexico) collaborating with Wye College (UK) tried testing different parts of the zygotic embryo, including the plumule (shoot meristem surrounded by leaf primordia) that was the most responsive part regarding the formation of embryogenic callus, somatic embryos, and the subsequent conversion to plantlets larger than those reported previously with rachilla explants, nearly twofold for calli formation (60 %) and tenfold for the formation of calli with somatic embryos (12–24 %) (Chan et al. 1998). Ex vitro acclimatization was successful, and several plots have already been established in field conditions (CICY, unpublished data). Research at CICY continued, and an improved micropropagation protocol was developed based on embryogenic callus multiplication (see below).

18.2.3 Zygotic Embryo Slices

Immature and mature zygotic embryo slices were also tested as a source of explant, where different conditions and compounds were tested (Adkins et al. 1999; Samosir et al. 1999). The number of immature zygotic embryos producing embryogenic callus was significantly greater than that of the mature ones. Using a combination of 60 μM of 2,4-D and 1.25 g L^{-1} AC, 50 % of the immature embryos produced embryogenic callus while only 3 % of the mature ones did. Slices taken from the center of the embryos were the best for producing embryogenic callus (58 %). This response significantly decreased as the slice source moved further away from the center of the embryogenic axis (Adkins et al. 1999).

18.2.4 Anther and Unfertilized Ovary Explants

Studies testing floral tissue explants other than rachillae have also been reported. Formation of callus and embryos was reported from anther coconut explants (Perera et al. 2008). Depending on conditions embryos could give rise to new embryos. One hundred and twenty-five embryos or calli per 100 anthers were obtained, and

27 % of those were germinated, and 20 plantlets were obtained (Perera et al. 2008). They did ploidy analysis showing that one-half of the tested plantlets was haploid and the other half was diploid. In order to determine the origin of the diploid plantlets, polymorphic fragments of the parental palm and its segregation patterns of anther-derived plantlets were used. All tested diploid plantlets were established to have a microspore-derived origin (i.e., were homozygous) and were therefore considered as candidates for coconut breeding programs (Perera et al. 2008).

Perera et al. (2007) also tried unfertilized ovaries isolated from immature female flowers of coconut, and 41 % produced callus cultured in a medium containing 100 μM 2,4-D, and 0.1 % activated charcoal. Somatic embryos formed when calli were subcultured into a medium containing 5 μM abscisic acid. Many of the somatic embryos were complete with shoot and root poles, and when cultured in a medium containing 5 μM 6-benzyladenine (BA), they could germinate and form normal shoots, but no quantitative data on the efficiency was reported (Perera et al. 2007). Further studies with unfertilized ovaries showed consistent callogenesis when cultured in CRI 72 medium containing 100 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 % activated charcoal. Callusing was improved by the application of 9 μM thidiazuron (TDZ). Somatic embryos formed on embryogenic calli when subcultured onto a medium containing 66 μM 2,4-D (Perera et al. 2009). Maturation of somatic embryos was obtained in Y3 medium without growth regulators. Conversion of somatic embryos was induced by adding gibberellic acid (GA_3) to conversion medium containing 5 μM BA and with 2-isopentyl adenine (2iP) the frequency of plant regeneration was increased. A total of 83 plantlets were produced from 32 cultured ovaries (Perera et al. 2009).

As mentioned above several explants were tested with diverse results, but being the most responsive rachillae from immature inflorescences, anthers, unfertilized ovaries, and particularly plumules. For this reason, they have been more extensively used to study and improve the different developmental changes within the process: callogenesis, embryo formation, germination, and conversion.

18.3 Optimizing Coconut Micropropagation via Somatic Embryogenesis

18.3.1 Somatic Embryogenesis Using Plumule Explants

In order to increase the efficiency of somatic embryogenesis using coconut plumules, two separate strategies were evaluated, multiplication of embryogenic callus and secondary somatic embryogenesis. Plumule explants were used to produce a primary somatic embryo, which in turn formed both secondary somatic embryos and embryogenic callus. After three multiplication cycles, they produced embryogenic calluses that were capable of producing somatic embryos. The system's efficiency was evaluated in three different stages, beginning with the first step of

primary somatic embryogenesis induction followed by three stages of secondary embryogenesis induction, another three stages of secondary somatic embryogenesis induction, and the final production of somatic embryos from callus (Pérez-Nuñez et al. 2006). The total yield from one plumule was 98,000 somatic embryos. Comparing this to the yield obtained from primary somatic embryogenesis results in about a 50,000-fold increase (Pérez-Nuñez et al. 2006).

Our protocol represents an important step forward to the practical application by showing a way to improve the efficiency of the production of coconut somatic embryos (Fig. 18.1). It still has, however, some limiting factors, such as the relatively low yield in embryogenic calli formation (40–60 %), of calli with somatic embryos (12–24 %) and the limited number of somatic formed per callus (2–10). Different plant growth regulators and compounds have been tested in order to avoid many steps of multiplication by optimizing this process and increasing the aforementioned yields.

18.3.2 Brassinosteroids

The effects of 22(S), 23(S)-homobrassinolide, a brassinosteroid, were tested on the formation of somatic embryo, embryogenic callus, and initial callus in coconut plumule explants. Explants were treated with increasing concentrations (0.01–4 μM) of the brassinosteroid during a 3- or 7-day preculture. An improved capacity of initial callus, embryogenic callus, and somatic embryo formation was observed showing a favorable response of the explants to the brassinosteroid. In this regard, the highest yield in somatic embryos (10.8 somatic embryos/explant) was observed by a 3-day exposure of the explants to the brassinosteroid at 0.01 or 0.1 μM contrasting with 3.8 somatic embryos/explant obtained from untreated explants, therefore increasing 2.8 times the yield of somatic embryos (Azpeitia et al. 2003).

18.3.3 Gibberellic Acid

The addition of GA_3 to the medium for the culture of calli with somatic embryos has been used to promote their germination as reported by Perera et al. (2009), but no numerical data was presented. On the other hand, if added earlier (0.5 μM), when embryogenic calli derived from plumule was subcultured into a medium for inducing embryo formation, its effect was found to be more extensive. The number of embryogenic calli forming somatic embryos increased from 20 to 60 %, and somatic embryos per callus increased initially 3.2-fold for globular embryos decreasing afterward to twofold for coleptilar embryos (Montero-Cortés et al. 2010b). Furthermore, the evaluated effect of GA_3 on the germination of somatic embryos showed positive results. An increase in the proportion of calli with germinating embryos from nearly 20 to 40 % was observed and the number of

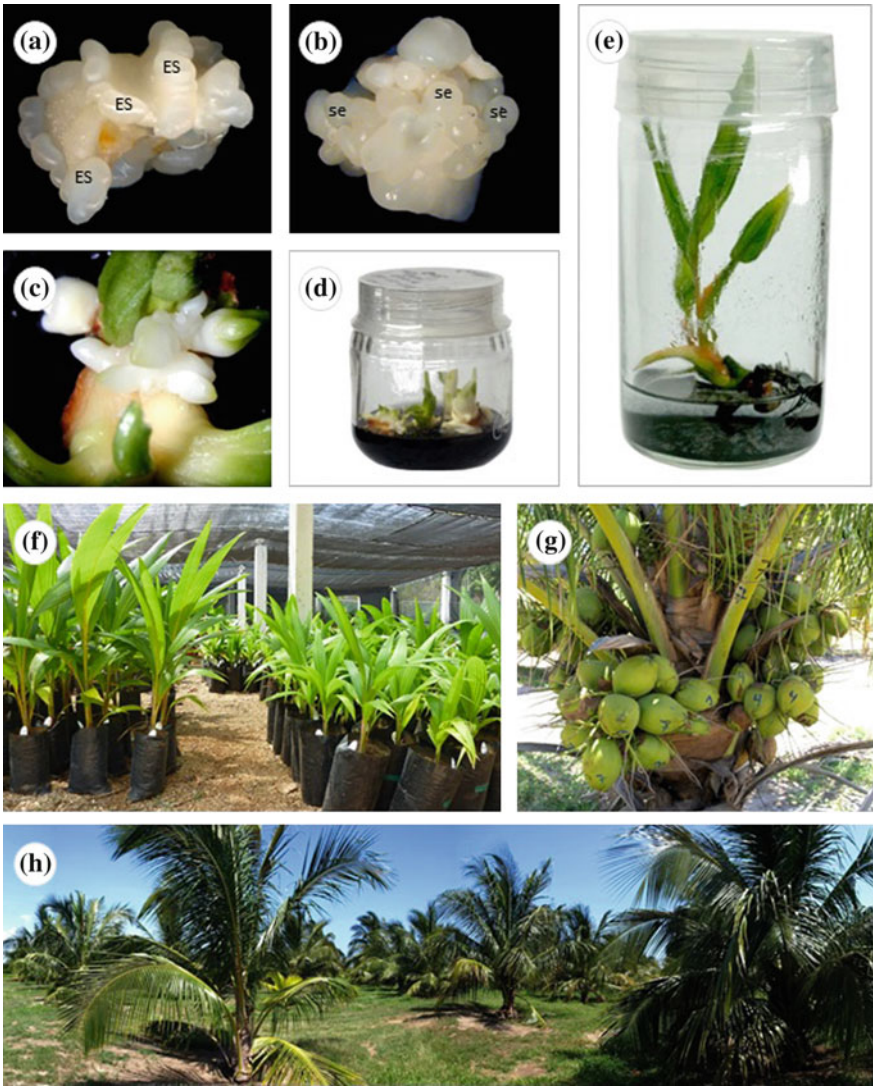

Fig. 18.1 Clonal propagation of coconut palm via somatic embryogenesis. Embryogenic callus (**a**), callus with somatic embryos (**b**), callus with germinating embryos (**c**), shoots from germinating embryos (**d**), in vitro plantlets (**e**), plantlets in nursery (**f**), clonal coconut palm with fruits (**g**), clonal coconut palms in the field (**h**). *ES* Embryogenic structures. *se* somatic embryos

germinating somatic embryos per callus increased from 2.5 to more than 5. Therefore, combining these results, a fourfold overall increase in the number of germinating embryos was obtained with GA_3 treatment (Montero-Cortés et al. 2010b). Then all together, the use of GA_3 was positive both for the formation of somatic embryos and on their germination.

18.3.4 Activated Charcoal

Activated charcoal (AC) has been included in the formulation of in vitro culture protocols for several plant species (Pan and van Staden 1998), either to reduce or eliminate undesirable compounds and thus improving explants morphogenic responses. However, culture medium components such as copper and zinc (Van Winkle and Pullman 2003), plant growth regulators (PGR) (Mohamed-Yaseen 2001) and vitamins (Weatherhead et al. 1978) can also be bound to CA as gibberellins (Mohamed-Yaseen 2001). For this reason, an important effort has been made to comprehend the relationship between AC and some of the culture media components. For instance, significant absorption of 2,4-D (auxin) and 6-BAP (cytokinin) has been found while studying the effects of AC on medium Y3, an usual medium for coconut explant culturing. In fact, AC can bind up to 99 % of the original amounts of PGRs in the medium (Ebert and Taylor 1990; Ebert et al. 1993) and therefore, the addition of AC into a well-defined culture medium can, therefore, transform it into an undefined medium.

This phenomenon was also studied by Sáenz et al. (2010a). Eight types of AC were evaluated for their effects on the free 2,4-D level, pH, conductivity, the osmolarity of the culture medium, and on the frequency of embryogenic callus induction. There were important differences in the 2,4-D adsorption capacity among the different ACs tested. This is particularly relevant since the morphogenetic response of in vitro-cultured coconut explants in this system depends on the presence of this auxin and its concentration (Chan et al. 1998; Pérez-Nuñez et al. 2006), and as we know this is affected by AC (Ebert and Taylor 1990). Concurrently, the type of AC used also influenced embryogenic callus induction, with the percentage of explants responding varying from below 20 to 60 % (Sáenz et al. 2010a). The best responses were obtained with media containing AC from SIGMA (acid washed for plant cell and tissue culture), DARCO, and United States Pharmacopeia (USP), in all cases promoting the formation of embryogenic callus in 60 % of the explants, but with different optimal 2,4-D concentrations (Sáenz et al. 2010a). Small particle fraction (<38 μm) was abundant in all charcoal type, even though profiles varied among all. Higher frequencies of embryogenic callus (70 %) were obtained with small particle fractions in regard to whole charcoal or large charcoal fractions (40 %) (Sáenz et al. 2010a).

18.3.5 Polyamines

A recent study (Rajesh et al. 2014) reported the induction of somatic embryogenesis and plant regeneration as the effect of treatments with polyamines putrescine and spermidine using plumules from Malayan Yellow Dwarf (MYD) and Chowghat

Green Dwarf (CGD) as explants. The results showed good yields of embryogenic callus formation varying from 37 to 50 % for MYD explants and from 55 to 63 % for CGD explants, also from this somatic embryogenesis was obtained varying from 20 to 43 % for MYD and from 26 to 43 for CGD. Formation of shoots and plantlets and their acclimatization are also reported.

18.3.6 Abscisic Acid and Osmotic Agents

As mentioned above, slices of immature zygotic embryos were successfully used as explants, since good yields of embryogenic callus formation were obtained (Adkins et al. 1999). In this system, osmotic compounds such as mannitol, sorbitol, and polyethylene glycol (PEG) were tested to improve the formation, maturation of somatic embryos, and shoot formation with good results particularly with the combination of abscisic acid and PEG (Samosir et al. 1999).

18.3.7 Rachilla Explants for Massive Propagation

The use of plumule explants combined with embryogenic callus multiplication and secondary somatic embryogenesis (Pérez-Nuñez et al. 2006) allows for massive propagation of coconuts with good performance provided that we can produce seed by controlled pollination of the selected parents, either of the same variety or of different varieties (hybrid seed). However, if we are interested in cloning palms with known agronomic traits, we cannot do so from plumule explants, and we need to start from vegetative explants from adult palms such as unfertilized ovaries as has been tested by Perera et al. (2007) with promising results or rachillae from immature inflorescences. In order to further explore this alternative, CICY (unpublished results 2015) tested rachilla explants from immature inflorescences culturing them in different media with different combinations of 2,4-D and BAP concentrations. With some of these combinations, there was the formation of calli bearing some pearly white embryogenic structures, resembling those from plumule-derived embryogenic calli (Pérez-Nuñez et al. 2006). The rest of the body of these calli consisted of spongy or necrotic tissue. Excising the pearly white embryogenic structures and subculturing produced with each time that this operation was repeated, better calli and eventually obtaining full embryogenic calli, able to be multiplied as in the case of the plumule system (Pérez-Nuñez et al. 2006). From these calli, somatic embryos could be obtained and germinated leading to plantlet formation. With these results, the basis for a protocol for mass propagation of adult palms with known agronomic traits has been established. The development of such a protocol is currently in progress (CICY, unpublished results 2015).

18.4 True-to-Typeness Studies

The first study of true to type was carried out with regenerated palms from Malayan Green Dwarf plumule explants 5 years after planted that showed the development of reproductive organs. No differences in the number of percentage of germination, number of pollen grains and their viability, the number of female flowers and rachillae per inflorescence, and in the number of inflorescences were observed when compared with sexually propagated palms. Also, the ovary anatomy of seed palms and of micropropagated palms was very similar. It is worth to mention that this is the first report on coconut micropropagated palms reaching sexual maturity on the field (Chuc-Armendariz et al. 2006). A molecular approach was carried out at the University of Queensland, Australia by B. Carroll et al. using the technique of amplified methylation polymorphism in different sets of palms, each consisting of four or five palms cloned from a single plumule explant. The results showed no differences between the clonal plantlets within each set. These results showed the potential of coconut micropropagation to produce true-to-type palms.

18.5 Understanding Coconut Somatic Embryogenesis

18.5.1 Histological and Ultrastructural Changes During Somatic Embryogenesis

Microscopy techniques have been extremely useful in the understanding of embryogenesis in different plant species (Yeung 1995; Ferreira et al. 2010; Steinmacher et al. 2011). In the case of coconut, there is a study that showed the ultrastructural variations in calli that are associated with the acquisition of embryogenic competence in explants obtained from the inflorescence (Verdeil et al. 2001). Special features of embryogenic cells are described, particularly related to the nucleus, cytoplasm, and cell wall: deep invaginations of the nuclear envelope, the proliferation of dictyosomes, with the emission of Golgi vesicles, with a direct relation to an increase in the thickness of cell wall. Using gold-conjugated probes, modifications to the cell wall structure were studied; special attention was paid to the cytolocalization of callose and pectin epitopes and of 1,4-glucans. Early changes (after 2,4-D increase) were related to the breaking of symplastic continuity, closure of plasmodesmata, and callose deposition. Acquisition of embryogenic competence was related to the formation of an outer layer of fibrillar material that contained pectin epitope (mainly un-methyl-esterified), which completely coated the embryogenic cells (21 days after the induction treatment). Some of the observed ultrastructural changes during somatic cell reprogramation toward embryogenesis are comparable to those observed during the maturation of female gamete cells in other plant species. In the case of plumule explants Fernando et al. (2003) reported histological studies of embryogenic callus development from Sri Lanka Tall

plumules contained active meristematic domes that give rise to leaf initials. The histological observations of tissues fixed 1 week after culturing in callusing medium showed new meristematic cells forming by the division of provascular cells of preliminary leaves. Peripheral meristematic masses appeared after meristematic cells were formed by the continuous division of provascular cells and apparently embryogenic nodules developed from these meristematic masses. Active cell division in the discontinuous zone led to the formation of protuberances composed of meristematic cells followed by the formation of the epidermis. These developments resulted in the formation of embryogenic calli on which somatic embryo formation was induced by ABA treatment. The evidence presented and supported a multicellular origin of somatic embryos.

Sáenz et al. (2006) also studying somatic embryogenesis from plumule explants excised from Malayan Green Dwarf coconut seed, reported a detailed histological study of the development of embryogenic callus and regeneration from it (Fig. 18.2). No apparent growth was found on the explants after day 15 of culture, however on a transverse section, noticeable growth of the plumular leaves was

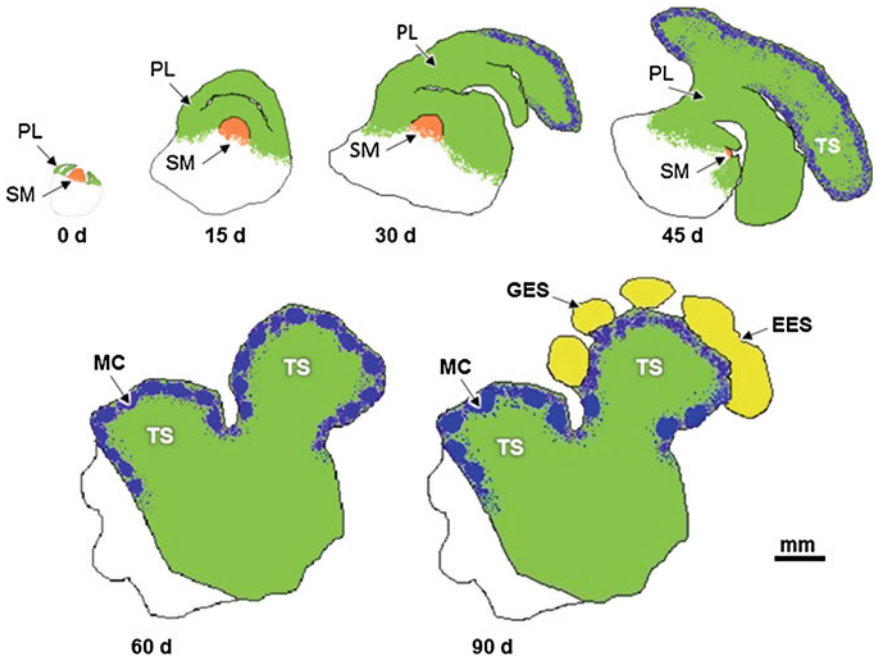

Fig. 18.2 Schematic representation of the changes that occur during the formation of initial callus and embryogenic callus from plumule explants cultured in vitro. At day 15, the coalescence of the external plumular leaves (PL) is observed. At day 30, the PL have grown and formed an initial callus. At day 45, the formation of a meristematic zone (MZ) near the periphery of this callus is observed. The callus forms translucent structures (TS). At day 60, the formation of meristematic centers (MC) in the TS is observed. After 75–90 days, the formation of globular (GES) and elongated (EES) embryogenic structures took place. *White areas* correspond to other tissues

observed forming a ring around the inner leaves and the shoot meristem which did not show any growth. They found that after day 30, plumular leaves growth continued, and the shoot meristem growth started. By day 45, explants were still compact and of with color, however, partial dedifferentiation and meristematic cell proliferation preceding the development of translucid and ear-like-shaped callus structures could be observed. After day 60 these meristematic cells gave rise to meristematic masses as reported by Fernando et al. (2003) and evolved into nodular structures by day 75. Initially, they were pearly globular structures on the surface of the translucent structures. These globular structures became more abundant giving rise to elongated structures, probably by fusing themselves with each other. Both the globular and the elongated structures were referred to as embryogenic structures, as from them somatic embryos that exhibited well-developed root and caulinar meristems. Several of them could germinate and form plantlets that were successfully transferred into ex vitro conditions. The pattern of development of embryogenic callus described above is highly reproducible, each time showing the same sequence of events, associated with the corresponding subcultures in specific media as described by Sáenz et al. (2006) and Pérez-Nuñez et al. (2006).

18.5.2 Uptake of Nutrimental Media Components by Explants

Several approaches have been taken into account to study the nutritional requirements of in vitro cultured inflorescence explants. A detailed study on two lines of in vitro-cultured inflorescence explants was carried out by Dussert et al. (1995). The line L1, of unicellular origin, was obtained decreasing the concentration of 2,4-D in the culture medium while the other line, L7 also of unicellular origin, was obtained by increasing the level of 2,4-D in the culture medium. Studies were performed during multiplication and somatic embryogenesis induction conditions. Somatic embryogenesis was linked to the specific uptake of NH_4^+, Mg^{2+}, sucrose and Ca^{2+} in both strains. No difference in the uptake of other nutrients (SO_4^{2+}, Cl^-, H_2PO^{4-}, K^+ and NO_3^-) per g of dry matter was observed when comparing calluses cultured on embryogenesis initiation medium and cultured on the multiplication medium. For the initiation of somatic embryogenesis, the uptake of the reduced form of inorganic nutrients was preferred. This preference toward NH_4^+ can be credited the increase in protein synthesis during the beginning of somatic embryogenesis. Magnaval et al. (1995) determined the amino acid composition of coconut calli on a similar model. In this study, six amino acids were detected: threonine, valine, leucine, proline, alanine, and serine. A significant difference was observed between calli on the multiplication medium and calli on the induction medium; this difference was characterized by a drop in the contents of serine, valine, and proline. The tendency toward embryogenesis was characterized by an increase in leucine, proline, and valine while alanine levels remained unchanged; histological analysis showed the

appearance of storage proteins concomitantly with the aforementioned increases in amino acid contents.

18.5.3 Uptake of Media 2,4-D by Explants

In order to understand what happens to the auxin 2,4-D when rachilla explants from immature coconut inflorescence are cultured in a medium containing the auxin, Oropeza and Taylor (1994) carry out uptake studies using ^{14}C-2,4-D. They found that the auxin was rapidly taken up by the explants against a 2,4-D concentration gradient. Most of the radioactivity disappeared from the medium within the first 24 h of culture. This proportion increased slowly afterward to about 90 % by day 7. The pH of the medium was also found to decrease rapidly to become acidic. In different pH media, ^{14}C-2,4-dichlorophenoxyacetic acid uptake was greater and the pH of the medium was lower. The fall in pH and the extent of the ^{14}C-2,4-D uptake by the explants were much reduced when sucrose was omitted from the culture medium (Oropeza and Taylor 1994). The synthetic auxin was metabolized during culture to polar conjugates. The metabolite fraction increased to 24 and 32 % after 3 and 7 days, respectively (CICY, unpublished results 2015). The authors proposed that ^{14}C-2,4-D is taken up by coconut explants through facilitated diffusion driven by a pH gradient that requires energy for its formation and maintenance. Conjugation could also contribute to ^{14}C-2,4-D uptake but in a proportion smaller than facilitated diffusion.

This phenomenon was also studied with coconut plumule explants cultured for 120 days to follow the induction and development of embryogenic callus in a medium containing ^{14}C-2,4-D (Sáenz et al. 2005). The first week of culture showed a high rate of auxin uptake, slowing down afterward until day 90 where it reached a plateau. The rate of the auxin uptake within the first 20 days of culture, prior to the appearance of any morphogenic response, ^{14}C-2,4-D reached a maximum concentration value in the explants. In this system and using different techniques, evidence was found for the occurrence of tyrosine phosphorylated proteins and tyrosine kinase activity, which is important for signal transduction and that activity, was highest during initial callus formation and decreased afterward when embryogenic callus developed (Islas-Flores et al. 2000). In addition, sequence analysis showed the occurrence of a SERK ortholog referred to as *CnSERK* in coconut (Pérez-Nuñez et al. 2009, see below) and the expression analysis showed that it was being expressed in embryogenic tissues even before any observable embryo development. Alternatively, its expression was reduced or undetectable in nonembryogenic tissues, suggesting an association between somatic embryogenesis induction and the expression of *CnSERK* in coconut tissues cultured in vitro (Pérez-Nuñez et al. 2009). The timing of the increased activity of tyrosine kinase activity and of this CnSERK expression suggests that there might be a consequence of the buildup of 2,4-D concentration that does occur immediately after the explants are placed in the culture medium containing this auxin and starts taking it up.

18.5.4 Endogenous Cytokinin Contents

Cytokinins are phytohormones derived from purine. They influence several plant processes such as leaf expansion, lateral buds growth, and leaf senescence (see Davies 1995). In combination with auxins, cytokinins can as well influence cell division and morphogenesis of in vitro cultures (see Krikorian 1995). For example, low levels of exogenous cytokinin inhibit embryogenesis of embryogenic genotypes of *Dactylis glomerata* (Wenck et al. 1988). Additionally, supplementation of anti cytokinins stimulated embryo formation in reduced embryonic genotypes (Somleva et al. 1995). Analysis of the endogenous cytokinin contents of different *D. glomerata* genotypes revealed the inverse relation of the cytokinin levels and the somatic embryogenic potential of the explants (Wenck et al. 1988). When studying endogenous cytokinins of *Corylus avellana*, Centeno et al. (1997) found a direct relation to the embryogenic potential to the Z type and an inverse relation to the iP type. Conversely, exogenous cytokinins have been found to favor embryogenesis in different plant species (see Gaj 2004).

Endogenous cytokinins were analyzed during three stages of in vitro plumule culture: embryogenic callus, nonembryogenic callus, and initial callus. Cytokinins with aliphatic and aromatic side chains were found through these analyses, namely four aromatic and fourteen aliphatic cytokinins were discovered in each type in varying proportions. The predominant cytokinins found in the samples were dihydrozeatin riboside, dihydrozeatin, isopentenyladenine riboside, zeatin riboside, zeatin-9-glucoside, and isopentenyladenine-9-glucoside in increasing order. Total active cytokinin content was compared between the three types of calli, and it was found to be lower in embryogenic calli (9.66 ± 1.17 pmol g^{-1} FW) compared to nonembryogenic calli (26.59 ± 7.22 pmol g^{-1} FW) or initial calli (32.83 ± 2.09 pmol g^{-1} FW) The same pattern was observed for major individual cytokinins (Sáenz et al. 2010b).

18.5.5 Molecular Studies

18.5.5.1 Control of Cell Cycle

Plant morphogenesis involves close control and coordination of proliferative activity through regulation of the cell cycle in meristematic tissues (Planchais et al. 2000). A prerequisite for the generation and establishment of dedifferentiated meristematic cells is the artificial initiation and maintenance of cell division. Cells under division can follow different developmental pathways in cell cultures, for instance, as somatic embryo formation, root and shoot initiation, and as unorganized callus growth. In the case of somatic embryo formation, a cellular state similar to that of the zygote established after egg cell fertilization is generated by the division of somatic or dedifferentiated cells (Fehér et al. 2003).

Sandoval et al. (2003) published a study reporting on the regulation of regeneration during the cell cycle of in vitro cultured coconut palm tissues. Different types of in vitro tissue cultures were compared: fast-growing calli (FGC), slow-growing calli (SGC), immature leaf explants and shoot meristems excised from embryos and culture in vitro. With the only exception of FGC, all the studied tissues showed a high percentage of G0/G1 phase cells (~90 %). The high accumulation of cells in G0/G1 phase was connected to the culture conditions through a kinetic study done before and after leaf explants. The mentioned experimental data need to be kept in context to the slowness of the morphogenesis process which is characteristic of in vitro regeneration of the coconut palm.

The *CDKA* (*Cyclin-Dependent Kinases A*) gene is linked to cell proliferation and maintenance of cell division competence in differentiated tissues during plant development (Martinez et al. 1992; Hemerly et al. 1993). *CDKA* was isolated from *Cocos nucifera* L. and a comprehensive expression analysis was performed during somatic embryogenesis. The most important conserved residues were identified by the analysis of the deduced amino acid sequence and *Picea abies* showed the highest homology (96 %). A steady increase of the putative *CnCDKA* gene expression can be observed during the embryogenic callus formation phase after embryogenic competence is reached. The transcripts were localized, by in situ hybridization, in mainly a few cell layers inside the meristematic centers in 90-day old embryogenic calli cultures. A decrease in the expression of *CnCDKA* was detected when analyzing different stages of somatic embryo formation; the lowest level of expression being in germinated somatic embryos (Montero-Cortés et al. 2010a).

18.5.5.2 Formation and Maintenance of Shoot Apical Meristem (KNOX Family Genes)

Expression of the class I KNOX (KNOTTED-like homeobox) genes appears to have an important role during somatic embryogenesis. Overexpression of *HBK3*, a class I *KNOX* homeobox gene, has shown to improve the development of somatic embryos. On the other hand, lines in which *HBK3* was downregulated showed a limited ability to generate immature somatic embryos and were unable to fulfill the maturation process (Belmonte et al. 2007). Two complete sequences of *KNOX*-like genes were obtained: *CnKNOX1* and *CnKNOX2*. Highly conserved domains, which are characteristic of the *KNOX* gene family, were detected by the deduced amino acid sequence. A high homology was found between KNOX I class proteins and CnKNOX1. With the exception of the globular stage, the expression of *CnKNOX1* could be detected in all stages of the embryogenesis process, with the maximum being observed at the coleoptilar stage. On the other hand, in calli with aberrant embryos no detectable expression of *CnKNOX1* could be observed. When gibberellic acid has added the expression of *CnKNOX1* was stimulated earlier, its expression was higher in all subsequent stages. Oppositely, gibberellic acid treatment reduced the expression of *CnKNOX2*, even though it was expressed at all

stages in normal culture conditions (with a maximum expression at the globular stage) (Montero-Cortés et al. 2010b).

18.5.5.3 Somatic Embryogenesis (Somatic Embryogenesis-Related Kinase)

Several events of differential gene expression and several signal transduction pathways, activating or repressing various gene sets, are involved in the somatic embryogenesis (Chugh and Khurana 2002). *Somatic Embryogenesis Receptor-Like Kinase* (DcSERK) is one of the several stage-specific genes involved in somatic embryogenesis. *DcSERK* was first isolated from embryogenic cells of *Daucus carota* suspension cultures (Schmidt et al. 1997). Expression of *DcSERK* was not observed in nonembryogenic cultures. *SERK* expression occurs in the early globular stage, during *D. carota* embryogenesis. Conversely, no expression of this gene could be found in any other tissue, but the introduction of a *SERK* promoter-luciferase reporter gene into other cells allowed them to form somatic embryos (Schmidt et al. 1997). Studies in other dicots have shown similar results. The expression of the *AtSERK1* gene, in *A. thaliana*, was observed during early embryogenesis, the development of the embryogenic cells in culture and in planta forming ovules, particularly in the embryo sac cells up to fertilization, and in the cells following fertilization of the growing embryo up to the heart stage (Hecht et al. 2001). Seedlings of *A. thaliana* overexpressing *AtSERK1* showed an increase in efficiency for somatic embryogenesis initiation three to four times higher; consequently, the increase in the expression of *AtSERK1* granted embryogenic competence in culture (Hecht et al. 2001).

SERK-like gene in *C. nucifera* was sequenced and referred to as *CnSERK*. After analyzing CnSERK through a predicted sequence analysis it was established that it encodes a SERK protein with the reported domains typical for SERK proteins described in other plant species. These domains are a Serine-Proline-Proline domain which is a characteristic domain in the SERK proteins, a leucine zipper domain, a signal peptide, five LRR, a single transmembrane domain, the kinase domain containing 11 subdomains, and the C-terminal region. Expression analysis demonstrated that its expression could be identified sooner than embryo formation could be observed. Oppositely, low or no expression of the gene was detected in nonembryogenic tissues, indicating the association of *CnSERK* expression and the somatic embryogenesis, on this basis *CnSERK* could potentially be an appropriate marker for competent cells in the formation of somatic embryos from coconut tissues cultured in vitro (Pérez-Nuñez et al. 2009).

18.5.5.4 Transcriptomics of Coconut Tissue Culture

A transcriptome analysis (RNA-Seq) of coconut embryogenic calli, derived from plumular explants of West Coast Tall cultivar, has been undertaken on an Illumina

HiSeq 2000 platform by Rajesh et al. (2015). After de novo transcriptome assembly and functional annotation, it has been obtained 40,907 transcripts. Fourteen somatic embryogenesis-related genes were identified: *Somatic Embryogenesis Receptor Kinase* (*SERK*); *Glutathione S-transferase* (*GST*); *WUSHEL* (*WUS*); *Embryogenic cell protein*; *Germin-like protein* (*GLP*); *PICKLE* (*PKL*); *WRKY* transcription factor; *CLAVATA1* (*CLV*); *Mitogen-activated protein kinase*; *AP2/ERF* domain-containing transcription factor; *SAUR* family protein; *Arabinogalactan protein*; *Late embryogenesis-abundant protein*; *Aintegumenta*. Quantitative real-time PCR (qRT-PCR) analyses of seven of these genes (*SERK*, *GST*, *GLP*, *CLV*, *WUS*, *PKL*, *WRKY*) were carried in six developmental stages. The result showed that CLV gene was upregulated in the initial stage of callogenesis. *GLP*, *GST*, *PKL*, *WUS,* and *WRKY* were expressed more in the somatic embryo stage. Expression of *SERK* was higher in the embryogenic callus compared to initial and somatic embryo stage. This study has provided until now the most comprehensive analysis of the gene expression patterns during somatic embryogenesis in coconut.

18.5.5.5 Genetic Transformation Protocol

A transformation protocol of embryogenic calli has been developed in our laboratory. The essays for Agrobacterium-mediated transformation were developed using green and red fluorescent protein genes, which are both suitable as reporter genes in coconut transformation. The established protocol for coconut genetic transformation combined biobalistics that generates micro-wounds on the explants followed by vacuum infiltration and coculture with *Agrobacterium tumefaciens* (C58C1 + pER10W-35SRed containing the embryogenesis-related gene WUSCHEL) (Andrade-Torres et al. 2011). Calli treated with the combined protocol showed red fluorescence with greater intensity and greater area than calli treated with either biobalistics or infiltration, followed by bacteria coculture. PCR amplification of the extracted DNA from the transformed embryogenic callus, using WUSCHEL primers, showed a band with the expected size (862 bp). Oppositely, no bands were observed when using VirE2 primers (Andrade-Torres et al. 2011).

18.6 Conclusions and Perspectives

In the last 10 years, the coconut water industry has exploded into one of the fastest growing beverage categories in the US and the UK, and similar trends are occurring for other coconut products such as virgin coconut oil, coconut water, coconut milk, etc. This market growth has to be coupled with sustaining or increasing coconut production. Unfortunately, this is not occurring but the opposite. Plants are dying due to diseases or senescence, and there are not enough plants being produced for replanting, so it would impossible to think of replanting with selected elite palms unless we are able to mass propagate, and the only way is through micropropagation.

So far, from what we have learnt with research, this should be through somatic embryogenesis. If we have this capacity, we will certainly be able to think not only of replanting programs all over the world but to doing it with elite palms, selected for resistance to diseases and high productivity.

From what is presented in this review, we can see that a sustained and huge effort has been made throughout decades, since the 1970s by several researchers working in institutions located in countries in all the continents, and most of the time in collaboration. Unfortunately, progress has not been easy because coconut happened to be one of the most recalcitrant species for inducing morphogenic responses in in vitro culture conditions. Nevertheless, relevant progress has been achieved, particularly in the past 15 years.

The coconut research community has generated knowledge on what kind of explants can be used to obtain morphogenic responses leading to the production of plantlets and that in all cases the path was through somatic embryogenesis. We have learnt different ways to optimize this process and that plantlets obtained have been true to type and can be successfully acclimatized to ex vitro conditions and be productive in the field. Basic research is being carried exploring each time in more depth the understanding of somatic embryogenesis in coconut, learning about physiology, biochemistry, and molecular events involved in the induction embryogenic calli, somatic embryos, and their conversion into plantlets.

Most important, perhaps is that we have now the potential for mass propagation of coconuts. The progress achieved using plumules development for a process through embryogenic callus multiplication and secondary somatic embryogenesis is highly efficient. It cannot propagate adult palms of known traits that might distinguish them from other individuals of the same variety, hybrid, but it can be used to propagate the progeny of elite palms by controlled pollination and used the resulting seed. Furthermore, progress is being achieved using explants such as rachillae from immature inflorescences for the successful production of embryogenic callus and their multiplication and therefore with the potential to develop a process for mass propagation as in the case of the current plumule-based process that is currently in the process to be transferred to a facility for scaling up in Mexico and start producing plants for pilot planting programs.

We have still a lot to do, starting from the basic research that has to continue so we can gain knowledge that will allow us to develop better tools that facilitate the progress in protocol development or improvement. At the same time, we have to improve what we have with the current tools. We need to make callus production more efficient as close to hundred percent as possible, that the amount of somatic embryos derived from and embryogenic callus is higher, or test new approaches such as cell suspension cultures, we need to improve conversion of embryos into plantlets. Also, it will be very important to continue working on the use of explants such as those from floral tissues or leaf tissues. As mentioned before a protocol for mass propagation based on rachillae is being developed, but using the same procedure the use of unfertilized ovaries can be further explored. This way, in the short term there is no doubt that we will have mass propagation options based not only in plumule explants but also on rachillae, unfertilized ovary, and leaf explants.

Studies to understand somatic embryogenesis in coconut should continue. In this sense, the study of the genetic control is essential and it is therefore very important to better understand the role of the already isolated genes, in addition to other components of the genetic control of somatic embryogenesis. Even more, the transcriptome profile and the epigenetic changes that occur during the different phases of the somatic embryogenesis could help, not only to better understand the phenomenon, but also to open new opportunities for the advancement to an improved efficiency and quality of clonal propagation of coconuts.

In order to take full advantage of the progress that has been done, there is still a lot of research ahead. Specially to be able to master the in vitro clonal mass propagation of elite palms, our best selected disease and pest resistance/tolerance, adverse environmental conditions and high productivity for the different markets, and the growing coconut industry to improve the livelihoods of all the members of the coconut production chain, particularly those with the lowest incomes.

Acknowledgments The authors would like to thank V. Hocher and J-L. Verdeil IRD/CIRAD Montpellier, France, respectively, where the isolation of *CnCDKA* and *CnKNOX* were carried out and M. Strnad for the analysis of cytokinin content (Institute Experimental Botany, Czech Republic). Partial funding of the research reported here was from CONACyT, México (Grant no. 43834-Z) and a scholarship was awarded to T. Pérez-Nuñez (No. 162930), M. Montero-Cortés (No. 183253), A. Azpeitia (No. 119335), A. Andrade-Torres (No. 204774). G. Rivera-Solís (No. 242979). G. Sandoval-Cancino (No. 300614).

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Chapter 19
Somatic Embryogenesis for More Effective Breeding and Deployment of Improved Varieties in *Pinus* spp.: Bottlenecks and Recent Advances

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Abstract Global transition towards a bioeconomy sets new demands for wood supply (bioenergy, biomaterials, biochemicals, etc.), and the forestry sector is also expected to help mitigate climate change by increasing carbon fixation. For increased biomass production, the use of improved, genetically superior materials becomes a necessity, and vegetative propagation of elite genotypes provides a potential delivery mechanism for this. Vegetative propagation through somatic embryogenesis alone or in combination with rooted cuttings obtained from somatic young trees can facilitate both tree breeding (greater selection accuracy and gains,

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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_19

breeding archives of donor material for making crosses after selection) and the implementation of deployment strategies for improved reforestation materials. To achieve these goals, progress in the efficiency of pine somatic embryogenesis biotechnology has been made for a few commercial pine species, and a better understanding has been gained of the molecular mechanisms underpinning somatic and zygotic embryo development.

19.1 Introduction

Pines (*Pinus* spp.) are native to most of the Northern Hemisphere and are also extensively planted in many parts of the Southern Hemisphere, with 119 species in the genus (Tang and Newton 2008). *Pinus* spp. make up 46 % of the estimated 53.4 million ha of planted production forest worldwide, with *Eucalyptus* spp. being the next largest at 26 % (FSC Strategic Review on the Future of Forest Plantations 2012). The most planted conifer in the world, originally from California (USA), is Monterey pine (*Pinus radiata*) covering over 10 million acres (4.05 million ha) of plantations. Other species include loblolly pine (*Pinus taeda*), a dominant commercially planted species in Southeastern USA, where it covers 25 million acres (10.1 million ha); Scots pine (*P. sylvestris*), a European species, is also planted in North America as well as in New Zealand (Tang and Newton 2008), and maritime pine (*P. pinaster*), native to the Mediterranean basin (ca. 4 million ha), is the most important timber species cultivated in France, Spain and Portugal, where it is the dominant species in over 2.3 million ha. Breeding programmes have been established for these commercially important pine species and protocols for somatic embryogenesis (SE) are being refined as a means of vegetative propagation (Klimaszewska et al. 2007, 2016). Conifer SE is important for tree improvement owing to its potential for making possible the selection and mass propagation of elite genotypes from a broad genetic base. In particular, it offers significant opportunities to improve management of breeding populations and to accelerate delivery of improved material to plantations through clonal forestry (Park et al. 1998; Cyr and Klimaszewska 2002; also see below *Implementation of SE in tree breeding and forest regeneration*).

A typical explant for initiation of SE in pines (and other conifers) is an immature zygotic embryo (*ze*), most frequently at the early to late cleavage polyembryony through early to late dominance stages (von Aderkas et al. 1991; Cairney and Pullman 2007), with a few species best responding at the cotyledonary stage of development. Since the first reports on SE induction in *P. lambertiana* and *P. taeda* (Gupta and Durzan 1986, 1987), a large number of publications reporting the production of somatic trees through SE have been published, the majority of which concerns the most economically important timber species such as *P. taeda* (Pullman and Bucalo 2014 and references therein), *P. radiata* (Hargreaves et al. 2011), *P. pinaster* (Lelu-Walter et al. 2006; Trontin et al. 2016a) and *P. sylvestris* (Lelu-Walter et al. 2008; Aronen 2016). Because SE in pines and other conifers is a

multistage process, each stage requires adjustments of in vitro treatments to maximize its efficiency. After SE is initiated and proliferating embryonal masses (EM) reach several hundreds of mg in fresh mass, they have to be bulked up through serial subcultures, most frequently on media of the same composition, until the required amount of EM is obtained. At this time, a suitable amount of EM is cryopreserved and stored in liquid nitrogen to ensure ample supply of EM lines (genotypes) for future tree production, when the progenies (regenerated trees from EM lines) have been field proven. After storage, the next crucial stage in conifer SE is the development and maturation of EM into mature somatic embryos (*se*) on a medium of different composition. Once these are mature, which is usually judged by visual inspection, they may be germinated on a different medium until plantlets are obtained. The next step is to transfer somatic seedlings (*ss*) to a soil-less substrate for acclimatization in a greenhouse before their transfer to a nursery for further growth and quality assessment before plantation in the field. Notwithstanding the recent progress made with respect to the optimization of laboratory protocols for SE of pine species, there are still some problems that need to be solved. Depending on the species, these problems include either all or some of the following: low SE initiation frequency from the seed explants, reduction or cessation of *se* regeneration capacity concomitant with the increased chronological age of EM; low genotype capture at the maturation step and low number of mature *se*; poor *se* quality at the end of the maturation stage; and inferior initial growth of *ss* compared with seedlings in the field (Fig. 19.1). In this review, we highlight the progress made but also the bottlenecks in pine SE, the ongoing research aimed at understanding the underlying causes, and we recommend potential solutions.

19.2 Induction of Somatic Embryogenesis in *Pinus* spp.

19.2.1 Initiation from Seed Embryos

In *Pinus* spp., SE is initiated most efficiently from immature *ze* enclosed within the whole megagametophyte. However, in some species such as *P. radiata*, SE induction has also been induced successfully using excised *ze* (Table 19.1). The two most commonly used media for pine SE are DCR (Gupta and Durzan 1985) and LV (Litvay et al. 1985) as modified by Klimaszewska et al. (2000) (mLV).

mLV has been initially used for SE in *P. strobus* with a high degree of success with multiple seed families (Klimaszewska et al. 2001). Later, it has been utilized for SE of *P. strobus* x *P. wallichiana* F2 hybrid seeds with initiation frequencies of over 50 % in control-pollinated seed families (Daoust et al. 2009). Cultures on mLV consistently produce high initiation rates in maritime pine (Table 19.1) with both French (Lelu-Walter et al. 2006; Trontin et al. 2016a) and Spanish (Humánez et al. 2012) seed families. In maritime pine, comparison of DCR (Gupta and Durzan 1985) and modified DCR (Breton et al. 2005) (mDCR) in a parallel experiment revealed that SE initiation rates were reduced by approximately 50 % compared

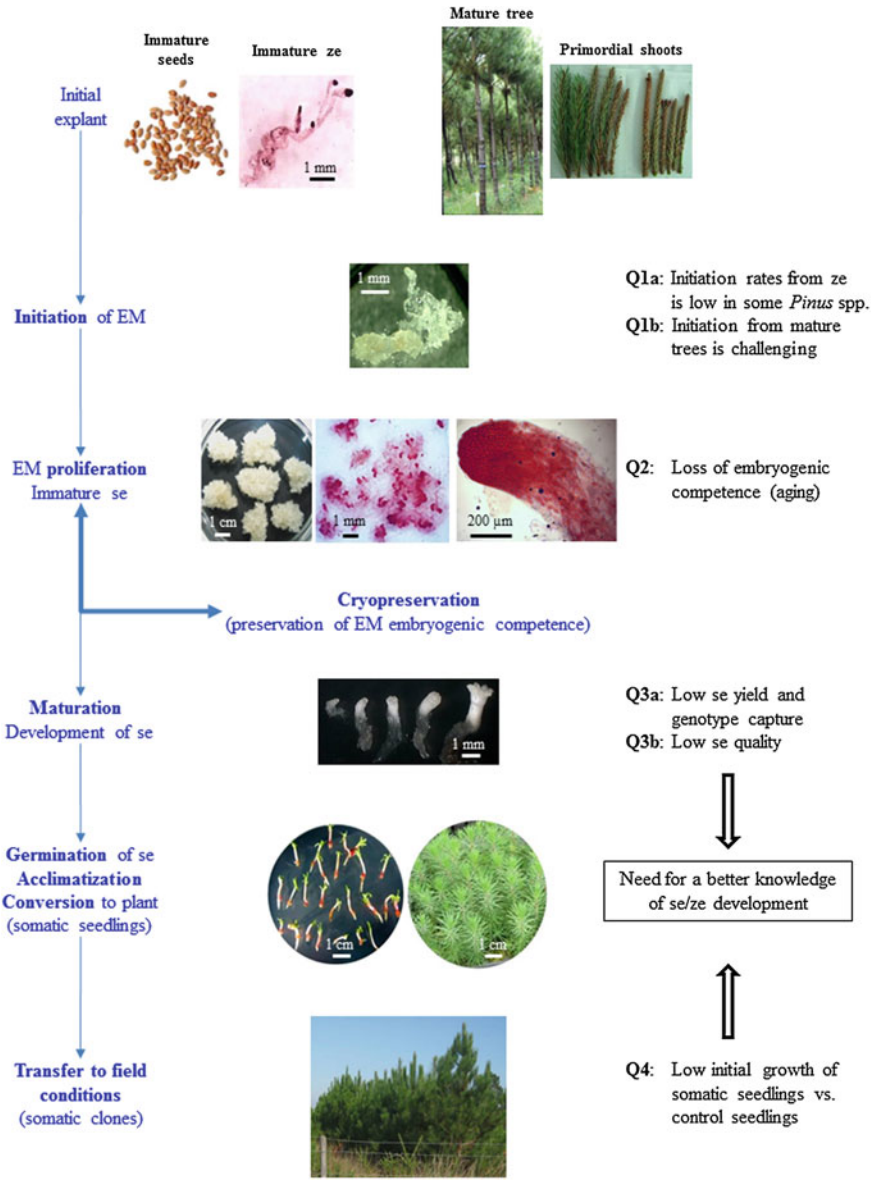

Fig. 19.1 Stages of somatic embryogenesis in pines and critical questions (Q). Illustrations are from maritime pine (FCBA/INRA). *EM* embryonal mass; *se* somatic embryo; *ze* zygotic embryo

with mLV. The data, based on a large selection of full- or half-sib seed families, showed that the mean initiation rate was 67.5 % on mLV and only 23.3 % on mDCR (Trontin et al. 2016a). Plant growth regulators, usually an auxin (2,4-D/2,4-dichlorophenoxyacetic acid) and a cytokinin (BA/benzyladenine), are

Table 19.1 Initiation rate of somatic embryogenesis in *Pinus* spp.: some recent reports

Pinus species	*Ze* dev. stage	Seed origin	Initiation response (% range)	Reference
radiata	*Dissected ze*			
	Pre-coty.	19 open PT	47–97	Hargreaves et al. (2009)
	Pre-coty.	20 control PT	44–93	Hargreaves et al. (2011)
	Undissected ze			
densiflora	n/a	12 open PT	n/a	Kim and Moon (2014)
halepensis	Pre-coty.	7 open PT	1–7	Montalbán et al. (2013)
luchuensis	Coty.	1 open PT	1	Hosoi and Maruyama (2012)
nigra	n/a	12 open PT	1–10	Salaj et al. (2014)
oocarpa	Early stage	2 open PT	2–9	Lara-Chavez et al. (2011)
pinaster	Dominant	5 open PT	0–82	Humánez et al. (2012)
	Dominant	16 control, 4 open PT	65–96	Trontin et al. (2016a)
pinea	Pre-coty.	5 open PT	<1	Carneros et al. (2009)
radiata	Pre-coty.	20 control PT	0–73	Hargreaves et al. (2011)
		2 open PT	24–60	Montalbán et al. (2015)
sylvestris	Early stage	6 open PT	0–30	Aronen et al. (2009)
strobus x *wallichiana*, F2	Dominant	12 control PT	52	Daoust et al. (2009)
taeda	Early to Pre-coty.	11 control, 1 open PT	6–43	Pullman et al. (2015)

Coty. cotyledonary; *dev. stage* development stage; *ze* zygotic embryo; *PT* pollinated trees; *n/a* not available

required for SE initiation at a high rate on mLV (Lelu-Walter et al. 2006; Humánez et al. 2012), but initiation can also be achieved at a relatively high frequency without PGR in *P. pinaster* (Lelu et al. 1999). Alternatively, in mLV (but not mDCR), 2,4-D and BA could be substituted by a potent cytokinin (the phenylurea CPPU), which increased SE induction efficiency to 77 % compared with 34 % in its absence (Park et al. 2006). Results from multiyear experiments conducted with maritime pine at FCBA established the optimal CPPU concentration at 1 µM (Trontin et al. 2016a).

As it is a potent cytokinin with a putative role in ageing and vigour in pine (Valdés et al. 2003; Klimaszewska et al. 2009), CPPU supplementation during the

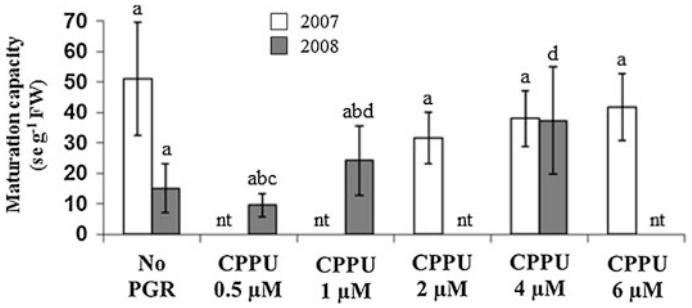

Fig. 19.2 Mean maturation capacity of maritime pine embryogenic lines initiated at the FCBA in 2007 or 2008 on mLV medium containing various concentrations of phenylurea CPPU (0–6 µM). 2007: 19–20 lines (from four families) matured per condition after 16–29 weeks of subculture (on average, post-initiation) on multiplication medium (nine maturation experiments with 0.5 g FW/line). 2008: 14–19 lines (from four families) matured per condition after 12–22 weeks of subculture (on average, post-initiation) on multiplication medium (nine maturation experiments with 1 g FW/line). mLV medium (modified LV medium from Litvay et al. 1985) was used for both multiplication and maturation of embryogenic lines. *FW* fresh weight; nt: not tested; *se*: somatic embryo. *Bars* represent 95 % confidence limits. For each year, significant variations between means (t-tests, $\alpha = 0.05$) are indicated by different letters

induction phase could have an adverse and delayed effect on the maturation capacity of initiated embryogenic lines. Hence, experiments with maritime pine were carried out in 2007 and in 2008 with 14–20 EM lines (from four seed families) to compare *se* maturation yields after 12–29 weeks of subculture following induction (Fig. 19.2). Mean maturation capacity of lines initiated on media with CPPU at 0.5–6 µM was compared with lines initiated without CPPU (no PGR). In 2007, no CPPU effect (2–6 µM) could be detected. In 2008, CPPU (0.5–4 µM) was confirmed as a good substitute for 2,4-D/BA in initiation medium as maturation capacity was found to be either similar (0.5–1 µM CPPU) or even significantly higher (4 µM CPPU) compared with control lines initiated without PGR. Interestingly, a general trend for increased maturation capacity as a function of CPPU concentration was observed during these experiments with some significant differences in 2008, i.e. higher *se* yield for lines initiated with 1–4 µM compared with 0.5 µM CPPU (Fig. 19.2). These results suggest that the type and concentration of PGR used in the SE induction medium may affect other SE steps up to maturation step (*se* development).

In these multiyear experiments, the seed family effect remained significant, but the genotype capture was in the range of 65–96 % and no adverse effect of CPPU on the development of *se* during maturation was observed. Hence, SE induction frequency in maritime pine fulfils the requirements for implementation of this biotechnology in the French breeding programme (Trontin et al. 2016a).

In *P. radiata*, another modified LV medium (GLITZ) was also found to be very effective for SE initiation with special benefits for immature excised *ze* (Hargreaves et al. 2009, 2011). Results with 19 open- and 20 control-pollinated seed families have shown initiation rates in excess of 50 % irrespective of the collection time of

immature cones; at the optimum *ze* developmental stage for each cross, an average of 70 % of the explants produced established cell lines. Similar to *P. pinaster*, these are the two *Pinus* spp. that can no longer be considered recalcitrant to SE in reference to the initiation stage (Table 19.1).

19.2.2 Initiation from Mature Trees

SE initiation from vegetative tissues of individual trees at adult vegetative or reproductive growth phases, when the tree's characteristics and growth performance are demonstrated, is highly desirable for implementation of efficient multi-varietal forestry. This has never been more important than today with the increasing pressures of a changing climate and new disease incursions into our global forestry environment. The direct propagation of selected trees would significantly reduce the costs and efforts of delivering elite varieties in commercial plantation forestry. In maritime pine, SE from mature trees could produce a new variety ready for deployment in less than 5 years (Fig. 19.3). In contrast, SE from seed embryos of unknown performance requires that somatic clonal trees be field tested until they reach the reproductive growth phase (10–15 years). Then, the selected tree varieties may be mass propagated from the cryopreserved juvenile stock established at the

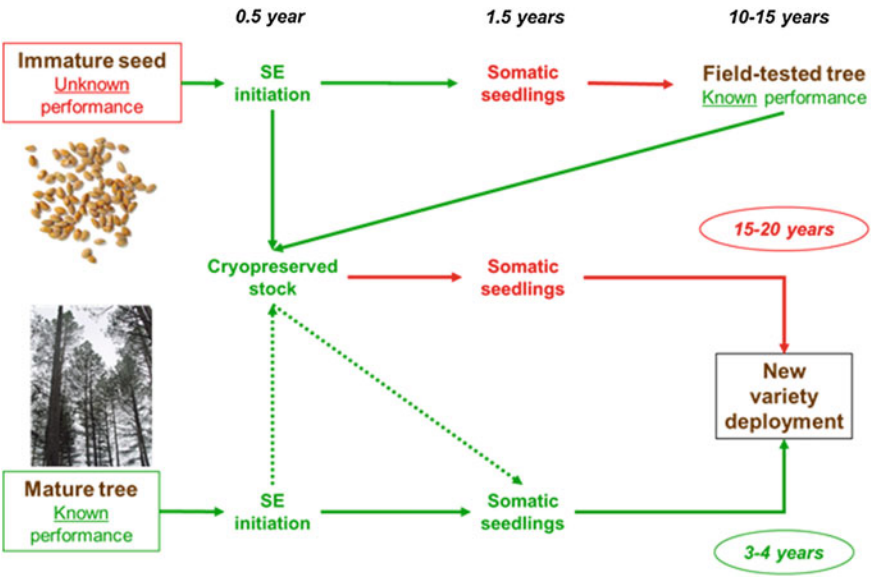

Fig. 19.3 Somatic embryogenesis from immature seed or mature tree in maritime pine as a means to implement multi-varietal forestry through the deployment of tested/selected elite varieties. *Dashed lines* indicated optional steps

time of SE initiation. Therefore, the whole process for new variety development takes over 15–20 years to be completed (Fig. 19.3).

Unfortunately, SE initiation from mature tree tissues is still as challenging in pines as in other conifers. In the recent international, multiyear project conducted with six pine species (*P. contorta*, *P. patula*, *P. pinaster*, *P. radiata*, *P. strobus* and *P. sylvestris*) based on published and original protocols (reviewed in Trontin et al. 2016c), strong evidence for SE initiation from primordial shoot explants was reported for only one species (*P. sylvestris*, Aronen and Ryynänen, see Trontin et al. 2016c). A few abnormal or cotyledonary *se* were obtained in two embryogenic lines expressing embryogenesis-related genes (*VP1*, *WOX2*), but these *se* were developmentally arrested and did not germinate. Moreover, embryogenic lines were apparently genetically unstable as various mutations were detected at several microsatellite loci. In other species, a few initiated cell aggregates were shown to have embryogenic-like micromorphology (*P. pinaster*, Trontin et al. 2016c) or to express putative embryogenesis-related genes (reviewed in Trontin et al. 2016b; Miguel et al. 2016), such as *LEC1* in *P. radiata* (Garcia-Mendiguren et al. 2015) or *WOX2* in *P. contorta* (Park et al. 2010). However, those cell aggregates could not be sustainably propagated. Overall, these results suggest that some initial stages of SE induction did occur in primordial shoot tissues of mature pine trees, but the process did not progress. In conifers, demonstrated evidence for SE initiation from mature trees, i.e. with plant regeneration, has only been reported from somatic trees in *Picea abies* (Harvengt et al. 2001) and *Picea glauca* (reviewed in Klimaszewska and Rutledge 2016). Apparently, *se*-derived trees may have higher embryogenic potential than seed-derived trees (Klimaszewska et al. 2011; Trontin et al. unpublished).

19.3 Multiplication of EM and Progressive Reduction in Somatic Embryo Production Capacity as a Result of the Culture's Increased Chronological Age

Rapid EM multiplication is easily achieved, especially when using the culture method over a filter paper disk as first developed for *P. strobus* (Klimaszewska and Smith 1997). EM culture subdivision during proliferation at both spatial (Petri dish) and temporal levels (subline) did not affect the *se* production capacity of *P. pinaster* EM lines (Breton et al. 2006), indicating that environmental conditions were homogeneous and standardized. In contrast, EM growth was strongly impacted by cell density on the filter paper (optimal in the range of 50–100 mg fresh mass for most embryogenic lines) and medium formulation. mLV promoted a higher proliferation rate (up to 1500 % relative increase in fresh mass within 2 weeks) compared with mDCR (Trontin et al. 2016a).

Cryopreservation of embryogenic lines shortly after initiation (usually within 2–4 months post-initiation) is necessary because *se* production capacity decreases

rapidly during proliferation (between 6–10 months of subculture; Breton et al. 2006; Trontin et al. 2011). Considering the ratio of fully developed cotyledonary *se* to precotyledonary and abnormal *se* at various sampling times during proliferation, Breton et al. (2006) showed that the maturation of *se* was qualitatively unchanged, but that it quantitatively and progressively reduced as the number of consecutive subcultures and, hence, the chronological age of the cultures increased.

Ageing of EM cultures during proliferation not only impacts yield; it also impacts cotyledonary *se* quality. Breton et al. (2006) observed a significant reduction in *se* total mean length from ca. 3 mm in young sublines (subcultured for 10–12 weeks) to less than 2 mm in older sublines (subcultured for >30 weeks). In particular, an overall size reduction of about 33 % in the hypocotyl region occurred after 15 weeks of subculturing (Fig. 19.4). As expected, the smaller size of the cotyledonary *se* resulted in a lower embryo quality that showed lower germination rates compared with se matured from younger cultures. In an experiment with six embryogenic lines subcultured for 5–22 weeks, a clear trend towards decreased germination was observed, particularly after 12 weeks at which time se germination dropped below 50 % (Fig. 19.5). However, sporadic lines (genotypes) produced *se* that germinated at a relatively high frequency even after more than 20 subcultures.

The ageing mechanisms responsible for the progressive loss of *se* productivity in embryogenic lines during prolonged EM proliferation are still not well understood in pines as in other conifer genera. Variation of both genetic and/or episomaclonal origin can be involved in the instability of in vitro propagated embryogenic lines (reviewed by Miguel et al. 2016; also, see below *Molecular aspects of SE and ZE in pines*).

Interestingly, in maritime pine, the ageing process could be delayed (but not suppressed) by more frequent subcultures on fresh proliferation medium with

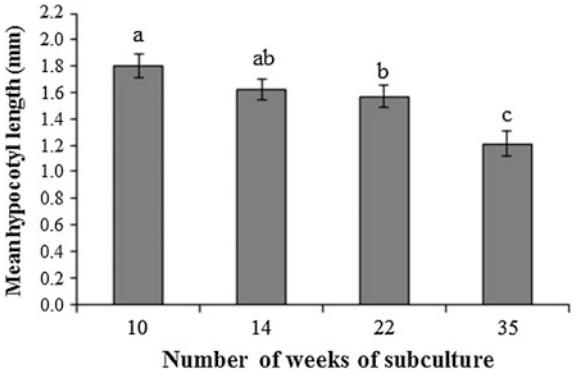

Fig. 19.4 Mean hypocotyl length of cotyledonary somatic embryos (*se*) in maritime pine (PN519 embryogenic line) as a function of the number of weeks of proliferation since EM reactivation from the cryopreserved stock. PN519 was proliferated and matured at the FCBA on mLV medium (modified LV from Litvay et al. 1985). Cotyledonary *se* were harvested after 12 weeks of maturation. *Bars* represent 95 % confidence limits. For each sampling date (N = 36–90 cotyledonary *se*), significant variation between means (t-tests, $\alpha = 0.05$) is indicated by different letters

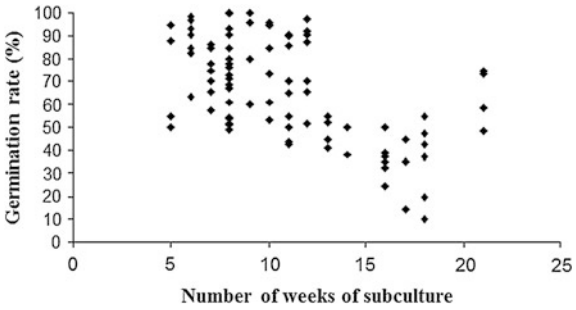

Fig. 19.5 Germination capacity of cotyledonary somatic embryos (*se*) in maritime pine as a function of the number of weeks of subculture on proliferation medium since EM reactivation from the cryopreserved stock. Data have been collected at the FCBA from six lines originating from five families (PN519, AB774, AAF04005, AAY06006, NL04045, NL04048). Lines were proliferated and matured on mLV medium (modified LV from Litvay et al. 1985). Cotyledonary *se* were harvested after 12 weeks of maturation. ANOVA of this dataset (three SE classes: 5–8, 9–12 or >12 weeks of subculture) revealed a significant impact of subculture duration ($F = 21.01$, $p < 0.01$) and genotype ($F = 13.46$, $p < 0.01$) on germination rates and also a significant interaction between genotype and subculture duration ($F = 2.12$, $p < 0.05$)

maltose instead of sucrose and no PGR (Breton et al. 2005). For the time being, cryopreservation is still necessary to retain young clones of initiated lines until alternative culture conditions are discovered to overcome the ageing problem. Alternatively, initiation of secondary SE from cotyledonary *se* can be used to "rejuvenate" aged embryogenic lines. It has been shown that secondary SE lines initiated from three out of four tested genotypes of maritime pine had similar or improved *se* maturation capacity compared with young lines initiated from *ze* (Klimaszewska et al. 2009). Various genes may be involved in the "rejuvenation" process. For example, Uddenberg et al. (2011) reported that initiation rate of secondary embryogenesis from young germinants in Scots pine was significantly improved by treatment with the histone deacetylase inhibitor trichostatin A that apparently activates embryogenesis-related genes such as *LEC1*/*HAP3A* and *ABI3*/*VP1*. Comparison of epigenomic markers in young versus aged cultures could lead to a better understanding of the changes associated with ageing and possibly to the discovery of factors that could be used to modify gene expression and make the cultures productive again (see below *Molecular aspects of somatic and zygotic embryogenesis in pines*).

19.4 Somatic Embryo Development and Maturation

As for other conifers, pine *se* development and maturation were improved on media containing between 40–250 μM of abscisic acid (ABA) depending on the species (Table 19.2). Another critical factor was reduced water availability, which could be imposed by a high gellan gum concentration in the maturation medium to promote

Table 19.2 Somatic embryo yield obtained in *Pinus* spp. using different formulations of maturation medium

Pinus species	Tested lines		ABA (µM)	Sugar (M)/PEG (%)[b]	Gellan gum (g)	*se* yield g^{-1} FW Max.	Reference
	Nb	Maturing[a]					
densiflora	15	11	250	S (0.2)	12	798	Kim and Moon (2014)
halepensis	13	13	75	M (0.16)	9	10–270	Montalbán et al. (2013)
luchuensis	1	1	100	M (0.08)/15	6	282	Hosoi and Maruyama (2012)
nigra	6	5	95	M (0.16)	10	235	Salaj et al. (2014)
oocarpa	2	2	40	M (0.16)/12	6	21	Lara-Chavez et al. (2011)
pinaster	26	15	80	S (0.2)	10	0–274	Humánez et al. (2012)
	39	32	80	S (0.2)	9	0–192	Trontin et al. (2011)
	346	323	80	S (0.2)	9	0–652	Trontin et al. (2016a)
pinea	7	4	121	S (0.17)	10	n/a (low)	Carneros et al. (2009)
radiata	24	24	60	S (0.16)	9	10–1550	Montalbán et al. (2010)
	4	3	212	S (0.16)	3	2–42	Find et al. (2014)
sylvestris	22	20	80	M (0.18)	9	127	Aronen et al. (2009)
	81	57	80	S (0.2)	10	977	Latutrie and Aronen (2013)
strobus x *wallichiana*, F2	261	138	80	M (0.18)	10	350	Daoust et al. (2009)
taeda	5	5	20	M (0.06)/13	2.5	150[c]	Pullman and Johnson (2009)

ABA abscisic acid; *FW* fresh weight; *n/a* not available; *PEG* polyethylene glycol; *se* somatic embryo

[a]Giving rise to cotyledonary *se*

[b]*S* sucrose; *M* maltose

[c]Yield expressed in cotyledonary *se* per ml (proliferation in liquid suspension cultures)

cotyledonary *se* development. This was first demonstrated for *P. strobus* by Klimaszewska and Smith (1997) and then successfully applied to a large number of *Pinus* species (Table 19.2).

19.4.1 Maturation: Improved Yield on mLV but Still Low Genotype Capture

In maritime pine, the best maturation conditions involve EM culture for 12–14 weeks on mLV medium supplemented with 0.2 M sucrose, 80 µM ABA and 9–10 g L^{-1} gellan gum (Lelu-Walter et al. 2006; Humánez et al. 2012; Morel et al. 2014a, b). The switch from EM proliferation to *se* development and maturation has recently been investigated by both transcriptome and proteome profiling (Morel et al. 2014a; Plomion et al. 2016; also, see below *Molecular aspects of somatic and zygotic embryogenesis in pines*).

For initiation and multiplication of embryogenic lines, mLV was confirmed to be a superior formulation compared with mDCR, with a mean maturation yield of ca. 50 cotyledonary *se* g^{-1} FW in a multiyear experiment conducted with 346 embryogenic lines (Trontin et al. 2016a). A majority of the lines (93 %) demonstrated some *se* regeneration capacity (Table 19.2), and a significant subset (61 %) of lines produced mean cotyledonary *se* yield of at least 10 *se* g^{-1} FW. A maximum yield of up to 652 cotyledonary *se* g^{-1} was obtained with some individual lines. However, based on micromorphological observations, only a tiny fraction of early *se* developed to the cotyledonary stage. It has been estimated, on both mDCR (Breton et al. 2006) and mLV (Trontin et al. unpublished results), that only ca. 10 % of early *se* differentiates into cotyledonary *se*. The development of the majority of early *se* is apparently arrested, asynchronous and/or abnormal.

The majority of the embryogenic lines cryopreserved at FCBA from 2000 to 2005 were initiated and propagated on mDCR medium formulations and revealed only poor maturation capacity (mean of ca. 3 cotyledonary *se* g^{-1} FW; Trontin et al. 2016a). One critical question was thus to investigate the maturation capacity of cryopreserved mDCR lines on the more favourable mLV maturation medium. Maturation data were compiled over 15 years (2000–2015) for a model line (PN519; Breton et al. 2006) initiated on mDCR in July 1999 and involved in various maturation experiments with mDCR (2000–2005) and mLV medium (since 2005). The maturation capacity of PN519 drastically and consistently increased on mLV (Fig. 19.6). Mean maturation capacity of PN519 was 12.7 *se* g^{-1} FW on mDCR and 94.0 *se* g^{-1} FW on mLV. Line age since its initiation affected maturation yield on both mDCR and mLV, as previously discussed. In conclusion, PN519 maturation capacity was increased ca. sevenfold on mLV. Interestingly, these data indicate that the maturation capacity of an embryogenic line initiated in suboptimal conditions (mDCR) is not corrupted but may be only revealed on the optimal maturation medium. It seems that the maturation capacity of an initiated line is far from being determined only by the genotype, but also by the culture conditions or other unidentified environmental factors.

Because of the progressive decrease in *se* maturation capacity concomitant with line ageing, genotype capture at the maturation step is particularly difficult to estimate in pines. In maritime pine, maturation yield has been shown to be non-optimal during a period of up to 12 weeks following line thawing from the cryopreserved stock (Breton et al. 2006). Therefore, genotype capture has been

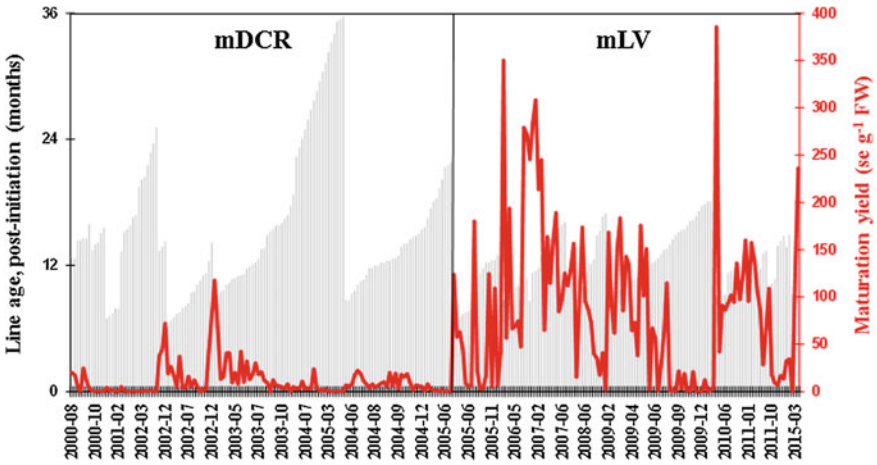

Fig. 19.6 Maturation capacity of a maritime pine embryogenic line (PN519) tested at the FCBA over 15 years (2000–2015) on mDCR or mLV medium (0.2 M sucrose, 80 µM ABA, 9 g L^{-1} gellan gum). The original PN519 line was initiated on July 29, 1999, proliferated, and initially cryopreserved on 8 December 1999 (mDCR media). The line was later re-cryopreserved several times to maintain the cryopreserved stock. Maturation yield (cotyledonary *se* g^{-1} fresh weight/FW) is shown for the experiment involving the original non-cryopreserved line (2000) and then 19 sublines thawed from the cryopreserved stock (2001–2015). The original line and thawed sublines were proliferated and matured on either mDCR (2000–2005) or mLV (2005–2015). mLV medium started being routinely used in 2005 following the results of Park et al. (2006). *Solid bars* (in *grey*) show the chronological line age since initiation (in months) at the time of each maturation experiment (N = 250 data), i.e. the cumulative proliferation time from (i) initiation to cryopreservation (4.2 months) and (ii) line regrowth from the cryopreserved stock to the maturation experiment. *mDCR* modified DCR medium from Gupta and Durzan (1985); *mLV* modified LV medium from Litvay et al. (1985)

redefined as the number of embryogenic lines producing at least 50 cotyledonary *se* g^{-1} FW on mLV medium (Trontin et al. 2016a) after ca. 4 months of proliferation (18 weeks). According to this definition, genotype capture at the maturation step (39 lines tested) was estimated to be 43.6 % (Trontin et al. 2011). Considering only lines yielding at least 100 cotyledonary *se* g^{-1} FW, genotype capture is reduced to 23.1 %. In a considerably larger sample of embryogenic lines matured several times after 2–6 months proliferation on mLV at FCBA (346 lines; Table 19.2), genotype capture was only 28 % (>50 *se* g^{-1} FW) or 16 % (>100 *se* g^{-1} FW).

19.4.2 Conversion of Cotyledonary Somatic Embryo to High-Quality Somatic Seedlings

In maritime pine, cotyledonary *se* are arbitrary harvested after a 12 week culture on a maturation medium (Lelu et al. 1999; Ramarosandratana et al. 2001; Breton et al.

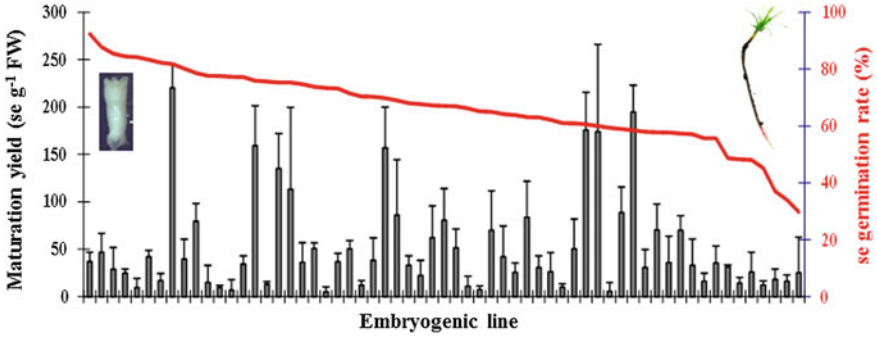

Fig. 19.7 Ranked germination rates (%) of cotyledonary somatic embryos (*se*) and maturation capacity of the corresponding embryogenic lines in maritime pine. *Solid bars*: maturation capacity (*left axis*); red line: germination rate (*right axis*). Data are presented for 61 embryogenic lines (from 23 seed families) initiated at the FCBA and proliferated for 7–12 weeks post-thawing from the cryopreserved stock. EM proliferation and maturation as well as cotyledonary *se* germination were performed on mLV (modified LV medium from Litvay et al. 1985). Cotyledonary *se* were harvested after 12 weeks of maturation. *FW*: fresh weight. *Bars* represent 95 % confidence limits for the maturation yield

2006; Klimaszewska et al. 2009; Humánez et al. 2012). Biological parameters (dry weight, water content) and biochemical analyses (total protein and carbohydrate contents) showed that cotyledonary *se* harvested after 10 or 14 weeks did not differ markedly and thus confirmed that harvesting cotyledonary *se* after 12 weeks is appropriate (Morel et al. 2014b). Cotyledonary *se* germinated at a high frequency (>70 %, reviewed in Trontin et al. 2016a), although differences were observed among lines. In a sample of 61 productive lines initiated from 23 seed families, mean germination rate of cotyledonary *se* on mLV medium was 66.5 %, with a 30–92 % variation range (Fig. 19.7). Interestingly, germination rate was not correlated with maturation ability (Fig. 19.7) suggesting that *se* development (as judged by cotyledonary *se* yield) and *se* quality (as judged by the germination rate) are not necessarily linked.

Cotyledonary *se* capacity to germinate into plantlets that can be acclimatized in the greenhouse and readily converted into growing somatic seedlings (*ss*) in the nursery was studied at FCBA with six embryogenic lines (Trontin et al. 2016a). The frequency of cotyledonary *se* conversion to *ss* was significantly improved using mDCR germination medium (48.6 %) as compared with mLV (only 34.5 %). The negative effect of mLV on *se* germination rate and conversion to plants could be already determined during the maturation phase (Trontin et al. 2016a), suggesting that the quality of harvested *se* is suboptimal.

Zygotic embryos and/or seedlings were introduced as the reference standard for estimating initial growth and vigour of cotyledonary *se* harvested after 12 weeks of maturation on the best maturation conditions for maritime pine (mLV, 0.2 M sucrose, 80 µM ABA and 9 g L^{-1} gellan gum). As a very important limitation to SE implementation in breeding programmes, *se* growth post-acclimatization is far

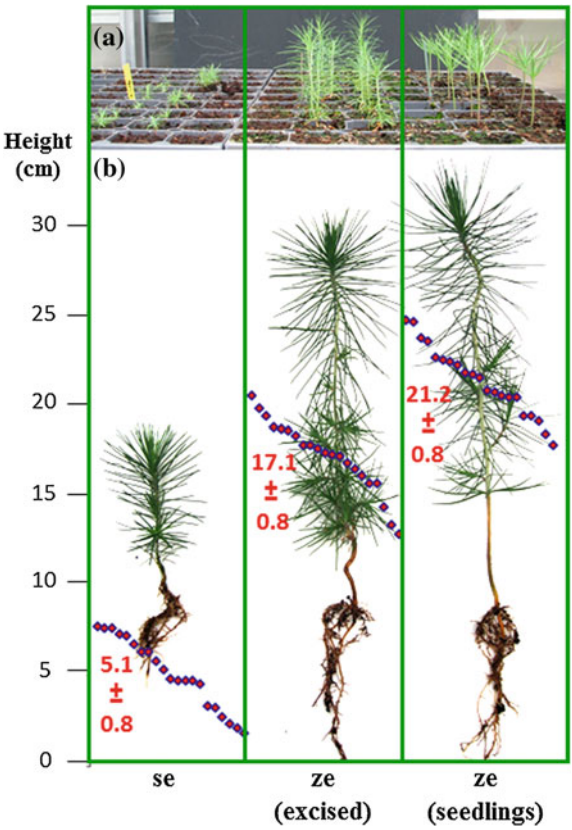

Fig. 19.8 Growth behaviour (shoot height) of somatic seedlings from a maritime pine embryogenic line (NL04045) and excised *ze* from mature seeds or seedlings from the corresponding NL full-sib family after 6 (A) or 22 weeks (B) of development (*se*, excised *ze*), or post-germination (seedlings). Cotyledonary *se* and excised *ze* were germinated in vitro in the same conditions for 10 weeks before acclimatization (mLV medium). Corresponding seedlings were sown at the time of *se*/excised *ze* acclimatization after stratification for 5 weeks at 4 °C. Mean heights ±95 % confidence limits (in *red*) and individual height (*red dots*, N = 21 plants) are both presented in panel B. Observed differences between means are significant (t-tests, $\alpha = 0.05$)

from matching that of seedlings and is significantly lower than dissected *ze* germinated under the same in vitro conditions as cotyledonary *se*. As an example, Fig. 19.8 shows the shoot height of *ss* from one embryogenic line after 22 months of growth in the greenhouse and the corresponding height of dissected *ze* from mature seeds and seedlings from the same family. Both mean height and individual height of *ss* are below that of control family. The striking difference observed with dissected *ze* shows that the intrinsic quality of cotyledonary *se* is not appropriate to obtain high initial vigour that is similar to that of seedlings. Several field trials established with *ss* and control seedlings confirmed the initial low growth rate of somatic clones in maritime pine (Trontin et al. 2016a; also, see below *Field performance of the se-derived pines*). These results have huge practical implications for refining the maturation conditions in maritime pine towards the production of high-quality somatic seedlings.

For the production of *ss* of eastern white pine hybrids the most critical factor was to synchronize the acclimatization in a greenhouse with the beginning of the natural vegetative season and the application of suitable fertilization regime (Fig. 19.9, Klimaszewska et al. unpublished).

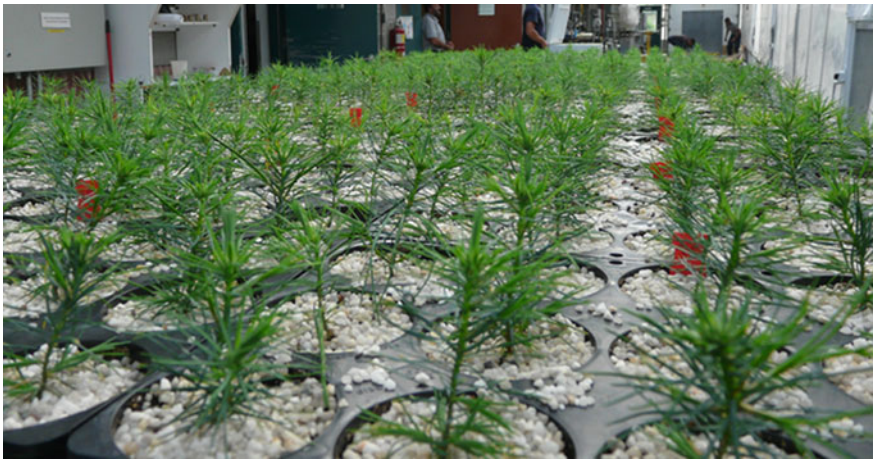

Fig. 19.9 Somatic seedlings of *Pinus strobus* x *P. wallichiana* F2 hybrids produced for the selection of white pine blister rust tolerance at the Laurentian Forestry Centre (CFS-NRCan) Quebec, Canada

19.5 Molecular Aspects of Somatic and Zygotic Embryogenesis in Pines

Although impressive advances have been made in the establishment of SE in *Pinus* spp. (see above *Induction of somatic embryogenesis in Pinus* spp.), further progress is expected concomitant with a better understanding of the molecular mechanisms of embryo development and pattern. This fundamental knowledge is important to achieve and/or further optimize all stages of SE, from induction to *se* maturation and *ss* regeneration (Miguel et al. 2016). With these goals in mind, several approaches and methodologies targeting different layers of molecular information have been applied in conifers (Trontin et al. 2016b). Genes, transcripts, proteins, metabolites and epigenetic mechanisms have been analyzed. Comparative analyses between SE and zygotic embryogenesis (ZE), regarded as the model for SE, have highlighted important processes during embryo development. Moreover, molecular profiling studies conducted either with *se* or *ze* (or comparative studies of both) have generated basic knowledge on embryo developmental pathways that could be used to optimize SE. Transfer of knowledge from more efficient conifer SE systems, e.g. *Picea abies*, has also contributed to the recent progress (von Arnold et al. 2016).

However, these efforts have been hindered by the intrinsic characteristics of these long-lived species, which include huge genome sizes and, consequently, the lack (until recently) of large and comprehensive molecular resources compared with model angiosperm species. Specifically, since reverse genetics approaches are mostly based on SE for producing mutant lines and plants, their success largely depends on the availability of an effective SE system for the species under study.

Despite these obstacles, progress has been reported from targeted and genomewide studies (transcriptomics, proteomics and metabolomics) and much more is expected in the coming years, as the genome sequences of several pine species (e.g. *P. taeda*, *P. radiata* and *P. pinaster*) are being decoded and released (Plomion et al. 2016).

19.5.1 Molecular Markers to Evaluate Genetic Stability

The first molecular studies of SE in conifers, including pines, were performed with the aim of evaluating genetic stability of the cultures (Fourré 2000). The occurrence of genetic alterations, which may include changes in chromosome number and/or structure and in DNA sequence, is one of the most often suggested causes for loss of *se* maturation capacity in aged conifer EM and for deviation from normal embryo development. Such alterations may affect the function or regulation of genes involved in embryogenesis, potentially leading to variable success at different SE stages or to abnormalities in the *se* phenotypes, and have been suggested to be an adaptation response to the stress imposed either by in vitro culture or by environmental conditions in general, thus reflecting plant developmental plasticity. Therefore, investigation of genetic instability and its putative association with embryo or EM developmental characteristics has been performed in several *Pinus* species using DNA markers, karyological and flow cytometric analyses (Burg et al. 2007; Marum et al. 2009a, b; Miguel et al. 2016).

Due to their ubiquity in genomes and high mutability, simple sequence repeats (SSRs, or microsatellites) are considered sensitive markers for monitoring putative mutation events in cultured cells. The analysis of four nuclear microsatellite loci during the establishment, proliferation and maturation of embryogenic cultures of *P. sylvestris* made it possible to conclude that genetic instability within family correlated positively with embryogenecity (the ability to establish embryogenic cell lines), but negatively with the frequency of cotyledonary embryo formation (Burg et al. 2007). Although the variability was higher during SE in four out of ten families, *ze* also showed some variation, suggesting that instability in the analyzed SSRs might reflect the plasticity of the family during adaptation to environmental conditions. As pointed out by the authors, it would be important to further investigate whether instability reflects alteration in functional genes that are somehow involved in embryogenecity or in the embryo developmental process.

Putative correlations between genetic instability and abnormal embryos or *ss* morphology were also investigated in *P. pinaster* (Marum et al. 2009a, b). While no major changes were detected in ploidy level as analyzed by flow cytometry, even in *se* with abnormal phenotype (Marum et al. 2009a), variation in SSR loci was found in both proliferating EM lines and *ss*. However, genetic instability in the analyzed loci could not be correlated with abnormal *ss* phenotype (Marum et al. 2009b). Due to the lack of clear conclusions from these and other studies in conifers (reviewed in Miguel et al. 2016), the effectiveness of such approaches to monitor SE is still a matter of debate.

19.5.2 SE Induction and EM Multiplication

The molecular studies focusing on SE induction and EM propagation that have been conducted in pines were based on either targeted or genomewide expression analyses and were mostly aimed at the identification of markers with embryogenic potential (Table 19.3). *LEC*, *SERK* and *ABI3/VP1* well-known genes putatively coding for transcription factors such as the *WUSCHEL* (*WUS*)-related homeobox (*WOX*) family and associated with plant embryogenesis, particularly with SE induction (Elhiti et al. 2013; Mahdavi-Darvari et al. 2015), have been investigated. Transcript expression profiling in *P. radiata* shoot-derived calli and ze-derived EMs showed correlation of *WOX2* and *ABI3* expression with embryogenic potential whereas *LEC1* was expressed in both EM and non-embryogenic calli (Garcia-Mendiguren et al. 2015). This is consistent with previous studies conducted in other conifer species (Palovaara and Hakman 2008; Klimaszewska et al. 2011), including *P. contorta* where *PcWOX2* was expressed in EM from immature ze and EM-like tissues derived from mature trees but not in non-embryogenic calli derived from a seedling needle (Park et al. 2010). Also, *ABI3/VP1* appeared to be expressed at low levels during EM proliferation in several conifer species including *P. sylvestris* (Uddenberg et al. 2011). On the other hand, a *LEC1*-like transcript (*PcHAP3A*) in *P. contorta* appeared to be mainly associated with non-embryogenic calli, but it was also expressed at a high rate in EM (Park et al. 2010), while in *P. sylvestris*, *LEC1/PsHAP3* was highly expressed during early SE or ZE (Uddenberg et al. 2011). Expression of a *SERK1* gene was similarly detected in proliferating EMs from *P. massoniana* and also at later embryo developmental stages (Yan et al. 2010). Given their different expression patterns in different conifer species as reported in functional studies (Klimaszewska et al. 2010, 2011; reviewed in Trontin et al. 2016b; Miguel et al. 2016), the role of these genes in embryogenesis remains unclear. Other targeted gene and transcriptomic studies in *P. radiata* have also highlighted some additional genes that, according to their differential expression in embryogenic versus non-embryogenic tissues or during SE, might play important roles in specific stages of the process (Bishop-Hurley et al. 2003; Aquea and Arce-Johnson 2008; Aquea et al. 2008) such as a group of five gene families (Table 19.3) that includes four putative extracellular proteins (Bishop-Hurley et al. 2003).

19.5.3 Somatic Embryo Development and Maturation

The switch from proliferation of EM to embryo maturation represents one of the most challenging and critical steps of SE in pines, being dependent on the genotype, culture conditions and the age of the cultures. It has become evident that molecular studies can prove valuable in understanding the effect of these factors and optimizing SE accordingly.

Table 19.3 Recent studies focusing on the molecular aspects of somatic and zygotic embryo development in *Pinus* spp.

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
P. contorta	EM, EM-like, NET	qRT-PCR	*PcWOX2*, *LEC1* (*PcHAP3a)*	*WOX2* specifically expressed in EM and EM-like obtained from primordial shoot explants of mature tree	Park et al. (2010)
P. massoniana	Early-late *ze*	2-DE DIGE, MS/MS	Proteome	Proteins associated with *ze* stages up to the coty. stage	Zhen et al. (2012)
	Early-late *se*	qRT-PCR	*PmSERK1*	*PmSERK1* is expressed in proliferating EMs and coty. *se*	Yan et al. (2010)
P. nigra	EM	HPCE HPLC	Genomic DNA methylation Free polyamines level	Low % 5-mdC and low free polyamines associated with high embryogenic capacity of EMs	Noceda et al. (2009)
P. oocarpa	Early-late *se*	qRT-PCR	*Legumin-like*, *vicilin-like*, *LEA*, *HD-Zip I*, *26S proteasome subunit S2* (*RPN1*), *clavata-like*.	*P. oocarpa* versus *P. taeda se*: variation of *legumin*-like, *vicilin*-like, *LEA and HD-Zip I* expression occurred at late coty. stages; d*issimilar RPN1* and *Clavata-like* expression; lower *se* quality in *P. oocarpa*	Lara-Chavez et al. (2012)
P. pinaster	Early-late *se*, *ze*, seedlings	*In situ* hybridization, northern blot	*Glutamine synthase (GS1a*, *GS1b)*, *Arginase*	*GS1b* associated with vascular pattern formation *Arginase* expressed in coty. *se* but not in *ze*	Pérez Rodríguez et al. (2006)
	Early-late *ze*, seedlings	qRT-PCR, *in situ* RT-PCR	Rab-related small GTP-binding protein (*PpRab1*)	*PpRab1* upregulated at the early *ze* stages	Gonçalves et al. (2007)
	Young versus aged EMs	HPCE HPLC MSAP	Genomic DNA methylation Hormones/polyamines level Methylation pattern	5-mdC level not associated with EM ageing Inconsistent hormones/polyamines levels EM ageing associated with net (de) methylation	Klimaszewska et al. (2009)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
	Mature *se*, *ze*	HPCE	Genomic DNA methylation	Lower 5-mdC level in abnormal *se*	Marum (2009)
	Early-late *ze*	qRT-PCR	Alpha-Amylase Inhibitors Lipid Transfer Seed Storage protein (*PpAAI-LTSS1*)	*PpAAI-LTSS1* expressed in pre- and early coty. *ze*	Simões et al. (2011)
	Early-late *se*, seedlings	qRT-PCR, genetic transformation	*Clavata1-like*	*Clavata1-like* may play a role in caulogenesis Promoter drives expression from early to late *se* development	Alvarez et al. (2013)
	Early-late *ze*	cDNA microarray	25848 cDNA clones	Most changes at transitions from early to pre-coty. and from coty. to mature *ze*; epigenetic (transposable elements, histone modification, small/microRNA) and auxin-mediated regulation (transcription factors) of *ze* development.	de Vega-Bartol et al. (2013)
	Early *se*	RNA-seq (Illumina) 2-DE, MS/MS	Transcriptome Proteome	Unfavourable maturation conditions (4 g L^{-1} gellan gum) enhance glycolytic pathways, resulting in cell proliferation Favourable conditions (9 g L^{-1}) activate protective pathways and ABA-mediated responses, promoting *se* development	Morel et al. (2014a)
	Late *se*, *ze*	HPLC, Bradford test 1/2-DE, MS/MS	Carbohydrates/proteins level Proteome	Coty. *se* matured for 10-14 weeks are similar Coty. *se* are similar to fresh, undesiccated coty. *ze* 23 protein markers of fresh coty. *se*/*ze* stage	Morel et al. (2014b)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
P. pinea	Seedlings	qRT-PCR	*Clavata1-like*	*Clavata1-like* may play a role in caulogenesis	Alvarez et al. (2013)
P. radiata	Early-late *se*, NET	cDNA library, RT-PCR, northern blot	28 embryogenesis-related cDNA clones	*Germin*, *α-expansins*, *21 kDa precursor*, *cytochrome P450* and an unknown gene (*PRE87*) expressed from early to late *se* development.	Bishop-Hurley et al. (2003)
	Early *se*, NET (seedlings)	cDNA-AFLP, qRT-PCR	Otubain-like cystein protease (*PrOTUBAIN*)	*PrOTUBAIN* highly and preferentially expressed in embryogenic tissue	Aquea et al. (2008)
	Early *se*, NET (needles)	cDNA-AFLP, RT-PCR	4000 transcript-derived fragments (TDFs)	50 TDFs upregulated (cellular metabolism, stress response) and 32 TDFs downregulated (proteolysis, cell wall modification, signalling pathways) in early *se*	Aquea and Arce-Johnson (2008)
	Early-late *se*	qRT-PCR	GRAS family (*PrSHR*, *PrSCL1* and 13 other GRAS genes)	GRAS gene expression is high in coty. *se* Increased expression of *PrSHR*, *PrSCL1* and five other GRAS genes at the beginning of *se* differentiation	Hernández et al. (2011)
	EM, NEC	qRT-PCR	*LEC1*, *WOX2*, *ABI3*, *Histone 4*, *PCNA*, *SKN1-4*, *WOX4*	Putative embryogenesis-related *LEC1* is expressed in NEC from axillary primordial shoot explants of mature tree *WOX2* and *ABI3* are associated with embryogenecity	Garcia-Mendiguren et al. (2015)
P. strobus	Late *se*, *ze*, seedlings	1-DE, MS/MS	Storage proteins	Most abundant proteins are 11S-globulin and 7S vicilin-like Less storage proteins accumulated in *se* versus *ze* (11S-globulin)	Klimaszewska et al. (2004)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
sylvestris	Early-late *se*, *ze*, seedlings	*In situ* hybridization, northern blot	*GS1a*, b (*glutamine synthase*), *rbcs (rubisco small subunit), psbO (33 kDa protein of PSII)*	*GS1b* associated with vascular pattern formation *GS1a, rbcs* and *psbO* expressed in coty. *se* but not *ze*	Pérez Rodríguez et al. (2006)
	Early-late *ze*	qRT-PCR, *in situ* hybridization, HPLC	Polyamines levels, *Arginine decarboxylase* (*ADC*)	Polyamines content increases in early *ze* and decreases in late *ze* (except free putrescine fraction); *ADC* expression increases from early to late *ze*	Vuosku et al. (2006)
	Early-late *se*, NEC	RT-PCR, gas chromatography	*1-aminocyclopropane-1-carboxylate synthase* (*PsACS1*, *PsACS2*),	*PsACS2* expressed only at the maturation step and correlated with both ethylene production and higher *se* maturation yield	Lu et al. (2011)
	Early-late *se*, *ze*	qRT-PCR	*LEC1/PsHAP3A/PsHAP3B*, *ABI3/PsVP1*	*PsHAP3A* expression is high in EMs and early *ze* *PsVP1* expression increases with *se*/*ze* development	Uddenberg et al. (2011)
	Early-late *ze*, FG	qRT-PCR, *in situ* hybridization	*Autophagy related ATG5*, *retinoblastoma related* (*RBR*), *catalase* (*CAT*),	*ATG5*, *CAT* and *RBR* are involved in embryo development and cell death processes	Vuosku et al. (2015)
taeda	Early-late *se*, *ze*	cDNA array, differential display	326 cDNA clones	Evidence for difference in gene expression between early and late *se* and between *se* and *ze*	Cairney et al. (2000)
	Early-late *se*, *ze*, seedlings	Northern blot, RT-PCR	*Aquaglyceroporin* (*PtNIP1;1*)	*PtNIP1;1* preferentially expressed in suspensor tissue	Ciavatta et al. (2001, 2002)
	Early-late *se*, *ze*	cDNA array	326 cDNA clones	Gene expression in coty. *se* is most similar to early coty. *ze*	Pullman et al. (2003)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
	Early-late *se*, *ze*	Biochemistry, 1-DE	Triacylglycerol and storage protein contents	Triacylglycerol increases during se maturation but is lower than in *ze*; higher storage (soluble) proteins content in *se*	Brownfield et al. (2007)
	Late *se*, *ze*	cDNA array	326 cDNA clones	Increased ABA during maturation makes the *se* mRNA profile more like *ze* for *starch synthase*, *small HSP*, *HSP70*, *LEA* (x2), *XETG-like*, *40S ribosomal protein*, *cyclic phosphodiesterase* and four unknown genes	Vales et al. (2007)
	Early *se*, early-late *ze*, FG, seedlings	Northern blot, qRT-PCR	AGO genes (miRNA metabolism, *PtAGO1,9L*), five micro RNAs and putative target: *miR159/MYB33*, *miR166/class III HD-ZIP*, *miR167/ARF8*, *miR171/scarecrow* and *miR172/apetala 2*	*miR166* detected in early *se* but not *miR167* Stage-specific modulation of five miRNA in FG and *ze* *PtAGO1L* and *PtAGO9L* are stage-specific and inversely regulated in *ze* and FG The peak levels of *miR166* in FG and *ptAGO9L* in coty. *ze* occurred at a critical transition point where *se* maturation often stops	Oh et al. (2008)
	Early-late *se*, *ze*	Semi quantitative RT-PCR	*Serine palmitoyltransferase* (*PtSPT1, PtSPT2*), *ceramide kinase* (*PtCERKL*, *PtCERKS*)	*PtSPT1* expression is lower in *se* than in *ze*, and either constant (*se*) or increasing (*ze*) from early to late embryogenesis Only *PtCERKL* expressed in *se* and in a gradually increasing pattern, whereas expression is constant from early to late *ze*	Zhu (2008)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
	Early *se*	GC/MS	Metabolome	The regenerative capacity of an embryogenic line can be accurately predicted using a subset of 47 metabolites related to nutrient uptake/allocation at the transition to maturation and response to stress during proliferation Primary metabolites with the strongest influence in the model are sucrose, glutamine, fructose-6-phosphate, dehydroascorbate, malic acid, an unidentified purine, threonine, asparagine, glycine, serine and proline	Robinson et al. (2009)
	Mid-late *ze*	qRT-PCR	ABA-responsive genes (*ABI3,4,5*) and six genes involved in root development: *woodenleg* (*PtWOL*), *short root (PtSHR)*, *hobbit (PtHBT)*, *bodenlos (PtBDL)*, *scarecrow(PtSCR)* and *monopteros* (*PtMP*)	*ABI3,4,5* exhibited a three-phase pattern of expression in coty. *ze* that may relate to an oscillating pattern of sugar accumulation *PtSCR* and *PtSHR* are overexpressed at the time of cell differentiation around RAM in coty. *ze* *PtBDL*, *PtMP*, *PtHBT* and *PtWOL* specifically expressed at the time of establishment of germination competence	Jones (2011)

(continued)

Table 19.3 (continued)

Pinus spp.	Material[a]	Method[b]	Target	Main result[c]	Reference
	Early-late *se*, *ze*	qRT-PCR	*Legumin-like*, *vicilin-like*, *LEA*, *HD-Zip I*, *26S proteasome subunit S2* (*RPN1*) and *clavata-like*	Gene expression similarities between *se* and *ze* Higher gene expression in *se* (except *LEA*) may be attributed to continual exposure to ABA *P. taeda se* are of higher quality than those of *P. oocarpa*	Lara-Chavez et al. (2012)

[a]*EM* embryonal mass; *FG* female gametophyte; *NET* non-embryogenic callus (NEC) or vegetative tissue; *se* somatic embryo; *ze* zygotic embryo
[b]*AFLP* amplified fragment length polymorphism; *cDNA* complementary DNA; *1/2-DE* one/two-dimensional gel electrophoresis; *DIGE* difference gel electrophoresis; *GC/MS* gas chromatography coupled with mass spectrometry; *HPCE* high-performance capillary electrophoresis; *HPLC* high-performance liquid chromatography; *MSAP* methylation-sensitive amplified polymorphism; *MS/MS* tandem mass spectrometry; *PSII* photosystem II; *qRT-PCR* reverse transcription quantitative polymerase chain reaction; *RNA-seq* RNA sequencing
[c]*Coty.* cotyledonary; *5-mdC* 5-methyldeoxycytidine

Recent studies in pines and other conifers using different molecular approaches, predominantly at the transcript level, pointed to major pathways that are essential to normal *se*/*ze* development (Trontin et al. 2016b). These include developmentally regulated programmed cell death (PCD), megagametophyte function and/or signalling, cell wall modifications, auxin response machinery and other important regulators of embryo patterns including epigenetic regulation, ABA-mediated developmental switch, changes in carbohydrates, proteins and energy metabolism, stress-related responses and the maintenance of redox homeostasis (Table 19.3).

A striking example of the importance of molecular approaches for understanding the effect of culture conditions on SE is the analysis of water availability during embryo maturation. Embryo maturation in pines is drastically affected by the gel strength of the culture medium, which determines water availability. In *P. strobus*, *P. sylvestris* and *P. pinaster*, reduced water availability (9–10 g L^{-1} gellan gum) results in the formation of cotyledonary *se* within 12 weeks, which are able to germinate (Klimaszewska and Smith 1997; Klimaszewska et al. 2000; Ramarosandratana et al. 2001; Lelu-Walter et al. 2006, 2008; Aronen et al. 2009), while increased water availability (4 g L^{-1} gellan gum) leads to the excessive proliferation of EM, thereby interfering with embryo maturation (Morel et al. 2014a). In *P. pinaster*, significant differences between both conditions were detected at the molecular level after only 1 week of culture on the maturation medium (Morel et al. 2014a). Under increased water availability, the hexose/sucrose ratio was high in the proliferating EM, suggesting stimulation of the glycolytic pathway, corroborated by both transcriptomic and proteomic analyses. Also, proteins involved in cell division and DNA replication, such as DNA replication licensing factor MCM3 homolog 3 and cell division protein FtsQ, were overexpressed, in accordance with the transcriptomic studies and fresh weight increases. One of the most significantly overexpressed proteins was related to a 26S proteasome regulatory subunit involved in the selective breakdown of proteins and could be an important player in the regulation of cell senescence, in agreement with the necrotic cells observed as early as 2–3 weeks after the beginning of maturation. On the other hand, under reduced water availability, gene expression was apparently reoriented through genomewide mechanisms such as chromatin modification (high expression of various ubiquitin protein ligase genes involved in the activation of the small ubiquitin-related modifier) and de novo protein biosynthesis from controlled proteolysis (expression of proteasome subunit beta 1 proteins, ubiquitin/26S proteasome pathway). Concomitantly, a major increase in endogenous ABA occurred after 4 weeks of maturation and is thought to be an ontogenetic signal for ABA-mediated molecular and physiological responses promoting embryo development. After only 1 week of maturation, an upregulation of transcripts related to ABA-mediated molecular response (high expression of protein phosphatase 2C and serine/threonine kinase genes; see also Bishop-Hurley et al. (2003) for *P. radiata*) occurred, as well as an activation of protective pathways such as synthesis of secondary metabolites (flavanone 3-hydroxylase, flavonol metabolism; Morel et al. 2014a) or other defence genes (see Aquea and Arce-Johnson (2008) for *P. radiata*).

At the proteomic level, many overexpressed proteins showed control of the proteasome-dependent proteolysis pathway of PCD (disulfide isomerase, chitinase), of the regulation of oxidative stress and maintenance of cell redox homeostasis (superoxide dismutase proteins, germin-like proteins and genes; see also de Vega-Bartol et al. (2013) for *P. pinaster*, Bishop-Hurley et al. (2003) and Aquea and Arce-Johnson (2008) for *P. radiata*, and Pullman et al. (2015) for *P. taeda*), of the cell division oriented towards cell remodelling (expansins, tubulin beta-2, GTP-binding nuclear protein Ran-1), and of ABA-mediated molecular responses. Overexpression of various proteins (glucose-1-phosphate adenylyltransferase AGPP, 4-alpha-glucano-transferase DPE2, disproportioning enzyme) also suggested an activation of the biosynthesis of starch granules (Morel et al. 2014a). Starch accumulation has been associated with early embryo development in maritime pine (see Tereso et al. 2007). A chart representing early physiological, cellular and molecular events during SE in maritime pine, in the presence of either low or high gellan gum concentration (Morel et al. 2014a), can be found in Plomion et al. (2016).

A proteomics study of early ZE in *P. massoniana* (Zhen et al. 2012) also revealed that some of these large functional molecular classes were successively overexpressed during seed development, including in the cleavage polyembryony, dominant embryo, columnar embryo and early cotyledonary embryo stages. Proteins involved in carbon and energy metabolism were overrepresented at the cleavage polyembryony and columnar embryo stages, which is consistent with a higher energy and carbohydrate requirement, and were also possibly involved in starch synthesis. A significant amount of proteins required for embryo morphogenesis seem to be then synthesized and assembled under the control of chaperones and heat shock proteins. This intense activity during embryo morphogenesis is accompanied by an essential regulation of the redox cell system (Zhen et al. 2012).

A well-known limitation of *Pinus* SE is the variation in the capacity of EM to produce cotyledonary *se*, i.e. embryo maturation capacity as a function of the genotype or other factors such as ageing. Ageing of EM during the subculture process has been pointed out as one of the main factors leading to the systematic loss of maturation capacity (Breton et al. 2006; Klimaszewska et al. 2009). In maritime pine, Marum et al. (2009b) detected variations at 3 SSR loci after proliferation for 6, 14 and 22 months in two out of 17 embryogenic lines. However, logistic regression did not reveal any significant effect of the number of subcultures on mutation rate. The apparent lack of association between accumulation of genetic mutations and maturation ability is consistent with the results obtained at FCBA for this species. Strikingly, none of the ca. 2,000 embryogenic lines (genotypes) tested in the past 15 years were stable over time during proliferation (i.e. the loss of maturation ability is general) in contrast to what is observed in some other conifers (e.g. *Picea*, *Larix*). Epigenetic mechanisms are probably involved since it is rather unlikely that mutations should systematically affect the same genes involved in maintenance of *se* regeneration capacity in different genotypes. Moreover, in maritime pine, it was demonstrated that spatial separation (in different Petri dishes) of EM sublines (from the same genotype) with different maturation capacity had erratic consequences during subsequent subcultures on the maturation capacity

which changed from low to high yield or vice versa (Breton et al. 2006). Random, reversible changes in *se* maturation yield are strongly indicative of environmentally induced modifications of gene expression. Epigenetic variations can be induced by culture conditions through various mechanisms involving DNA methylation, histone modifications and chromatin remodelling as well as expression of small RNAs (Miguel and Marum 2011). Such processes are interconnected and are the part of the epigenetic complex of regulation of gene expression, in particularly that of embryogenesis-related genes involved in maintenance of the embryogenic state and regeneration capacity (reviewed in Trontin et al. 2016b; Miguel et al. 2016).

In an attempt to identify the underlying causes of this phenomenon, several studies have been conducted based on biochemical analyses and DNA methylation (discussed in the section below). The levels of polyamines or hormones were analyzed in lines with varying capacity for embryo maturation in *P. pinaster* (Klimaszewska et al. 2009) and *P. nigra* (Noceda et al. 2009). While inconsistent polyamine and hormone profiles were displayed by EM cultures of different genotypes with similar maturation capacity in *P. pinaster*, an inverse relationship between total contents of free polyamines and maturation capacity was reported in *P. nigra*. Polyamine and hormone profiles were also compared between young (productive) and aged EM (non-productive) of the same genotype. No difference could be detected for one genotype, whereas a second genotype showed a higher level of indole-3 aspartate and lower levels of zeatin riboside, free spermidine and spermine in young EM cultures (Klimaszewska et al. 2009). Interestingly, in *P. taeda*, a subset of 47 metabolites identified by GC/MS-based metabolomics of propagated embryogenic lines correlated with maturation capacity and can be used as a predictive screening tool because the corresponding model is largely independent of the genotype (Robinson et al. 2009).

19.5.4 Epigenetic Analyses Throughout se Development

Epigenetic mechanisms have emerged as being critical in the control of both SE and ZE in different plant species (Nodine and Bartel 2010; Miguel and Marum 2011; De-la-Peña et al. 2015; Trontin et al. 2016b) by ultimately determining gene expression patterns through modulation of access to DNA and definition of distinct chromatin states. Chromatin remodelling may occur during proliferation and early embryo development in pine as observed in *P. sylvestris* (Uddenberg et al. 2011) and *P. pinaster* (de Vega-Bartol et al. 2013; Morel et al. 2014a).

A special attention has been given to DNA methylation analysis, as a number of tools for detecting this type of variation are readily available (Miguel and Marum 2011). Noceda et al. (2009) found that the global methylation levels analyzed in *P. nigra* embryogenic cell lines gradually decreased with the increased maturation capacity of the line, varying from 18 to 30 % 5-methyldeoxycytidine (5-mdC) in the lines showing the highest (80 mature *se* g^{-1} EM FW) and null maturation capacity, respectively. This type of study suggests that demethylating agents used to

manipulate the DNA methylation status might also be used to improve the regeneration capacity of pine embryogenic cultures. However, when testing this hypothesis in *P. pinaster* EM, Klimaszewska et al. (2009) found inconsistent changes in DNA methylation in the cultured EM. Treatment of aged EM with the hypomethylating drug 5-azacytidine led only to a slight increase in maturation capacity. The analysis of both global DNA methylation and methylation patterns as detected by MSAP (methylation-sensitive amplification polymorphism) in EM cultures of different ages (i.e. different regeneration capacity) revealed an association of ageing with net DNA demethylation or methylation at specific target sequences, but not with global DNA methylation levels, thus suggesting discreet instead of large epigenetic changes. In another study, global DNA methylation was reported to vary among genotypes but it could not be related with *se* maturation capacity (Trontin et al. 2016a). Also in *P. pinaster*, Marum (2009) observed that the relative percentages of 5-mdC quantified by HPCE both in mature *se* and *ze* as well as in derived plants were very similar (23–24 % 5-mdC for embryos and 17 % for plants). However, mature *se* with abnormal phenotype presented 3.5 % less 5-mdC when compared with *se* of normal phenotype.

The relevance of several genes involved in the maintenance of chromatin silencing, regulation of histone acetylation or methylation and regulation of DNA methylation has also been highlighted by functional category analysis of differentially expressed genes in *P. pinaster* ZE (de Vega-Bartol et al. 2013). During early embryogenesis, gene silencing mechanisms seem more active, probably to control transposable elements, while from mid to late embryogenesis stages, upregulation of several putative chromatin-remodelling ATPases (CHC1, RAD5 and BSH) was detected. Additionally, differential regulation of several transcripts with homology to known regulators of small RNA biogenesis, processing, and function was identified across all stages of pine embryo development. Genes involved in micro RNA (miRNA) biogenesis and various miRNAs were specifically modulated at different development stages of *ze* and in female gametophyte in *P. taeda* (Oh et al. 2008). Some miRNAs were also regulated during early SE, particularly *miR166* targeting a *class III HD-Zip* transcription factor gene apparently involved in the critical transition point during late ZE where *se* maturation often stops in *P. taeda*.

19.5.5 Comparative Molecular Analyses for Evaluation of Embryo "Quality"

In order to obtain high-quality embryos, leading to vigorous *ss*, it is essential to optimize the maturation conditions, including the duration of the treatment before the beginning of germination. Embryos are usually harvested based on morphological criteria that do not necessarily reflect their maturation status or "quality".

When characterizing the relative expression of six developmentally regulated genes during ZE in *P. taeda* and *se* development/maturation in *P. taeda* and *P. oocarpa*, Lara-Chavez et al. (2012) showed that expression levels usually tended

to be higher in *P. taeda se* compared with similar *ze* stages. Such difference may be attributed to continuous exposure to ABA during SE. Additionally, differences in the expression profile of several genes involved in late maturation (e.g. coding for storage proteins) were consistent with a higher similarity of late cotyledonary *se* to mature *ze*, confirming the empirical choice of that stage for germination (Lara-Chavez et al. 2012). Furthermore, they suggested that the differences in transcript levels observed at late stages of cotyledonary *se* between *P. oocarpa* and *P. taeda* could explain the low germination success and overall lower quality of *P. oocarpa se*.

Expression of genes normally induced in a shoot pole during germination (*arginase*) or in photosynthetic tissue (*glutamine synthase/GS1a*, *rubisco small subunit/rbcs*, *33 kDa protein of photosystem II/psbO*) was detected in cotyledonary *se* but not in *ze* of *P. pinaster* or *P. sylvestris*, suggesting precocious germination and therefore inferior quality of *se* (Pérez Rodríguez et al. 2006). These results suggest that cotyledonary *se* did not undergo the desiccation-induced dormancy that normally occurs in a seed and which separates the embryo maturation stage from germination and post-germination growth (see Pérez Rodríguez et al. 2006 and references therein).

The biochemical composition of *ze* during late maturation has also been investigated in an attempt to define the optimal timing for transferring cotyledonary *se* to a germination medium (Brownfield et al. 2007; Vuosku 2011; Morel et al. 2014b; Pullman and Bucalo 2014). The type and contents of proteins, sugars, ethylene and polyamines were suggested to be critical for *se* post-maturation stages affecting *ss* quality.

During embryogenesis there is a typical trend towards an increase in the levels of spermine and spermidine during early developmental stages and a decrease of these levels during late embryo development. Putrescine remains relatively stable throughout embryo development. Therefore, the spermidine/putrescine ratio has been proposed to follow maturation in *P. sylvestris* (Vuosku et al. 2006).

Considering protein analyses, the same storage proteins or their coding transcripts have been identified in the *se* of *P. strobus* (Klimaszewska et al. 2004), *P. pinaster* (Morel et al. 2014b), *P. taeda* and *P. oocarpa* (Lara-Chavez et al. 2012), and have been shown to accumulate in different conifer species from precotyledonary to (mainly) cotyledonary stages (Lippert et al. 2005; Lara-Chavez et al. 2012). In *P. sylvestris*, it has also been reported that storage proteins were present in equivalent amounts between mature *se* and *ze* (Lelu-Walter et al. 2008).

In *P. pinaster*, no differences were observed in cotyledonary *se* after 10–14 weeks of maturation with respect to contents of total proteins, various mono- or polysaccharide sugars, water and dry matter (Morel et al. 2014b). When 12-week-old *se* were compared with *ze* at different stages of maturity (fresh cotyledonary to desiccated cotyledonary stages) using the same molecular and other biological and physiological parameters, it was clear that they corresponded the most to the fresh, maturing cotyledonary *ze* found in green cones that are present in France in late July/early August. Genomewide proteomic profiling of both *se* and *ze* revealed 94.5 % similarity of detected proteins. At this stage, *ze* just completed

their morphological development (histodifferentiation, embryo patterning). As stated above, this work further confirmed that cotyledonary *se* successfully completed morphogenesis under the current "optimized" maturation conditions, but that they only partially executed the further desiccation process that should result in fully mature *se*. A similar result was reported in *P. taeda* following cDNA macroarray profiling of cotyledonary *se* and *ze* (Pullman et al. 2003). Differences between late *se* and *ze* were also apparent after profiling of several genes involved in sphingolipids (Zhu 2008) or storage protein synthesis (Lara-Chavez et al. 2012) in *P. taeda*, as well as triacylglycerol and/or storage protein contents in *P. taeda* (Brownfield et al. 2007) and *P. strobus* (Klimaszewska et al. 2004). ABA present at increased concentrations during *se* maturation in loblolly pine affected their gene expression profile, which was more similar to that of mature *ze* (Vales et al. 2007).

Cotyledonary *ze* undergo many changes during the late maturation and post-maturation stages. Water loss is accompanied by a sudden increase in protein content (over 50 % per mg DW), the synthesis of storage proteins (vicilin- like proteins, legumin-like proteins, albumin-3 and pine globulin-1) and a decrease in sugar content (by 40 % per mg DW) (Morel 2014). Additionally, a change in the type of sugars is observed, namely the disappearance of hexoses and an increase in the raffinose family of oligosaccharides (RFO) (Pullman and Buchanan 2008; Morel et al. 2014b), which are known to be involved in the acquisition of desiccation tolerance (Lipavská and Konrádová 2004). It may be important to stimulate those biochemical changes as well as post-maturation treatment (Maruyama and Hosoi 2012) in *se* through the use of adequate tissue culture conditions to ensure high germination capacity and conversion rates into plants.

19.6 Implementation of SE for Tree Breeding and Forest Regeneration

According to a survey done in 2007, pines are among the forest tree species for which the use of vegetative propagation (VP) through rooted cuttings is wide spread, with approximately 164 million plants produced annually worldwide (Lelu-Walter et al. 2013). For the production of these materials, SE, either alone or in combination with organogenesis and rooted cutting propagation, are used. Likewise, VP including SE can facilitate both tree breeding (greater selection accuracy and gains, breeding archives of donor material for making crosses after the selection) and implementation of deployment strategies for improved reforestation materials.

19.6.1 Integration into Breeding Programmes

The overall scheme of implementation of SE for tree breeding and for the production of improved forest regeneration material is shown in Fig. 19.10, and has

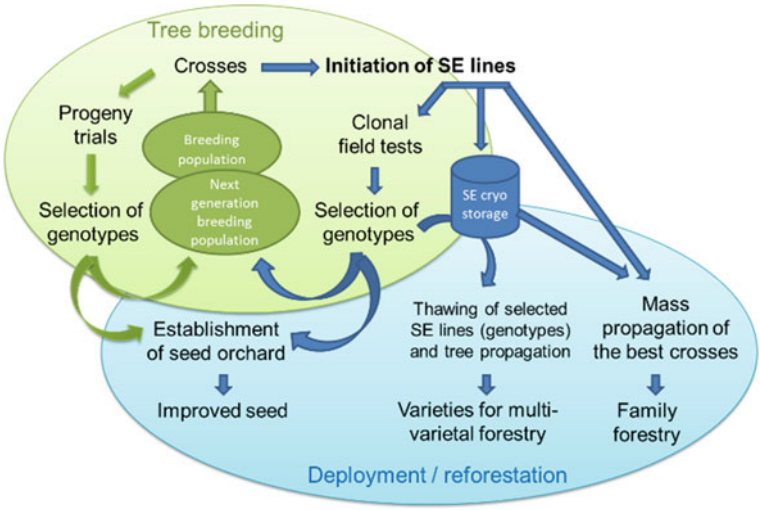

Fig. 19.10 Diagram of the implementation of somatic embryogenesis (SE) in *Pinus* tree breeding and in the production of improved forest regeneration material

recently been reviewed by Krakau et al. (2013) and Lelu-Walter et al. (2013). A description of SE applications in pine improvement and deployment strategies in plantation forestry can also be found in Klimaszewska et al. (2007). In short, VP (e.g. by means of SE) provides several advantages for breeding through clonally replicated genetic tests (clonal testing for forward selection): when multiple copies of the candidates to be tested can be placed in various environments, microenvironmental and genotype by environment interactions as well as phenotypic plasticity can be estimated more accurately than by progeny testing. As a result, both genetic parameter estimation and selection accuracy are improved following clonal testing. Shortening of breeding cycles is another remarkable benefit achieved by clonal testing when compared with progeny testing in which extra time is needed to allow candidates to flower prior to testing their progenies. In *P. sylvestris*, the cycle length was estimated to be 33–36 years when using different breeding strategies based on a separate progeny testing step, and only 23 years when selection is performed in field tests with cloned replicates rather than seedlings (Rosvall and Mullin 2013). VP is also useful at the time of genetic testing for varietal selection prior to deployment. In such varietal tests, a number of selected families, varieties per families (genotype/progeny) and ramets per variety are field tested for accurate selection of tree varieties that will be used in plantation forestry (Weng et al. 2011). In practice, varietal tests can be advantageously set up to maximize genetic gains at a genetic diversity level above a fixed threshold of acceptability.

SE is also well suited for early selection schemes, in which molecular markers for specific traits (maker-assisted selection/MAS) or genomewide information

(genomic selection/GS) is used as the basis for selection. For example, in *P. taeda,* genomic selection is expected to lead to 53–112 % higher selection efficiency per time/unit, i.e. a reduction of 50 % in the breeding cycle (Resende et al. 2012). Integration of both MAS or GS selection and SE into conventional breeding programmes is likely to result in high synergies over the next decades for the implementation of multi-varietal forestry (El-Kassaby and Klápště 2015; Plomion et al. 2016). Defined as the use of tested tree varieties in plantation forestry, multi-varietal forestry is an efficient way of preserving productivity and adaptability of intensively managed conifer plantations using all available genetic variance, i.e. both additive and non-additive genetic variances (Park 2002; Weng et al. 2011).

However, it is well known that there is variation among families in the success of SE, i.e. it can be difficult to obtain SE lines from all the elite crosses, and the potential impacts of low or variable genotype capture on the outcomes of the breeding have raised some concerns. However, a simulation study by Lstibůrek et al. (2006) showed that variation in the success of clonal propagation does not lead to reduced genetic gains in the selected clonal mixtures since most of the variation in the breeding population is within-family variation, and superior clones may thus originate from other crosses of the very best parents. It should be noted that significant progress has been made in improving genotype capture at the initiation step from crosses of interest in *P. pinaster* and *P. radiata* in recent years (see above *Induction of somatic embryogenesis* in *Pinus* spp.). As a result, the prospects for incorporation of SE technology into broader forestry programmes are good (Lelu-Walter et al. 2006; Hargreaves et al. 2009, 2011; Trontin et al. 2016a).

In practice, clonal testing is seen as complementary to progeny testing, and tree breeding programmes combine the two to achieve the best selection accuracy (Baltunis et al. 2009). For example, a new breeding strategy adopted by the Radiata Pine Breeding Company for New Zealand and New South Wales, Australia, includes an elite population tested both as progeny and as clones (Dungey et al. 2009). The idea is that clonal populations will capture the greatest gains in traits having low heritability, and the progeny will make it possible to test more families and individuals for traits with higher heritability. The presence of clones will also allow destructive testing for selection criteria such as wood quality characteristics. The elite population is managed intensively following a "rolling front" approach, i.e. new material is being created and testing is conducted continuously. At the same time, pre-deployment selections from the best parents are done for the development of commercial clonal varieties. In *P. sylvestris*, Rosvall and Mullin (2013) showed through computer simulations of alternative breeding strategies that the genetic gain per time unit depends on the selection intensity and accuracy and also on the time required to complete the breeding cycle. A breeding strategy based on clonal testing will have a moderate selection intensity (because of the high resources needed to produce clonal ramets for each tested tree), but a high selection accuracy, a short cycle time and a high potential for genetic gains. Conversely, strategies based on progeny testing can offer higher selection intensity, but the breeding cycle time is longer and the potential for genetic gains per year is therefore reduced.

Total cost per breeding population and breeding cycle for a strategy based on clonal replicates (short cycle time) has been estimated at € 233 k in *P. sylvestris* (Rosvall and Mullin 2013). This is slightly higher than the costs estimated for other strategies based on progeny testing and with long cycle time (€ 204–218 k) and can mainly be explained by the need for the production of propagules. SE may help to reduce the cost of producing clonal propagules since it is potentially a very efficient multiplication technology.

19.6.2 Options for Deployment

Traditionally, in pines, improved forest regeneration materials are produced in seed orchards in which the selected trees are clonally propagated, often by grafting, and mate to produce seeds. If the planting stock for reforestation can be vegetatively propagated, e.g. through SE, several benefits are achieved (Klimaszewska et al. 2007; Dungey et al. 2009; Lelu-Walter et al. 2013). Improved material will be available for production forestry faster since there is no delay due to the time needed for seed orchards to grow and start flowering. Likewise, irregular flowering or pests and pathogens affecting seed yields do not cause uncertainty in the supply of improved material. In VP, the genetic gains achieved by breeding are transferred as a whole (both additive and non-additive variation) to production forestry. It is also possible to maximize genetic gains by implementing multi-varietal forestry, i.e. by focusing on the multiplication of the very best individuals. This is currently being demonstrated with *P. radiata*. In parallel with optimizing varietal production methods, Forest Genetics (www.forest-genetics.com) has been actively developing an advanced generation of improved varieties. This was done by creating new varieties from crosses among the best clones already in production (Carson et al. 2015).

Deployment through VP provides an opportunity to select clones for specific end uses or specific site conditions. The material to be propagated can also consist of hybrids. SE is compatible with the propagation of pine hybrids. A good example of this is the ongoing work conducted with *P. attenuata* x *P. radiata* hybrid SE lines in New Zealand, aiming to improve performance under dry and cold growth conditions that are not well suited for either of the parent species (Anonymous 2015). SE methodologies may be of special benefit when using hybrid pines to facilitate the capture of EM before any incompatibility issues with the crossing of different species may arise that could result in empty seeds at maturity.

With VP, the growing stock will be more uniform, which could reduce harvesting and processing costs; also, genetic diversity may be reduced. This may increase the risk for disease or pest damage if susceptible clones are widely used, and some concern has also been raised on clonal stability over various environments (Bettinger et al. 2009). However, recent results, (e.g. from *P. radiata*)

suggest that clones performing well and showing stability over large areas can be found (Baltunis and Brawner 2010). Moreover, genetic diversity can be maintained by establishing polyclonal plantations as well as mosaics of species and genotypes at the landscape level, and by the continuous release of new and better clones (Bettinger et al. 2009; Carson et al. 2015). Multi-varietal forestry can be advantageously used to guarantee that the genetic diversity of a varietal mixture is fixed to a given acceptable level and that genetic gain is maximized under this constraint (Weng et al. 2011 and reference therein).

Options for the deployment of SE (or other types of VP) are presented in Fig. 19.10: SE can be utilized for the mass production of tested and selected clones that are marketed either as single varieties or as varietal mixtures. Alternatively, the best seed families can be multiplied without clonal testing, resulting in family forestry (Dungey et al. 2009; Lindgren 2009; Krakau et al. 2013; Lelu-Walter et al. 2013). Yet another option is to convert clonal trials into varietal tests and, ultimately, into seed orchards after testing for and removing of inferior clones (Dungey et al. 2009), followed by traditional seed production. In such a scenario, testing efforts should be carefully estimated using computer simulations to optimize the costs of establishment of clonal trials, varietal tests, and/or seed orchards (Weng et al. 2011).

When SE is used for mass propagation, *ss* can be either directly used as planting stock, or they can be grown as cutting donors in nursery stool beds or used as stored embryogenic cultures. In *P. taeda* (Soresson 2006; Bettinger et al. 2009), in which rooting of shoot cuttings is difficult, direct planting of germinated *se* cannot be used. In *P. radiata*, production of rooted cuttings from plants lined out in the nursery bed works well, which results in cheaper planting stock than that obtained with the direct use of *ss* (Carson et al. 2015). Several further improvements have been made to integrate *P. radiata* SE technology into less intensive propagation methods. Germinated *se* can be used to provide shoots for the initiation of organogenic cultures, which is done by trimming the root and hypocotyl from *se* and placing them on standard multiplication medium (details on transfer strategies and media are provided in Hargreaves et al. 2005; Hargreaves and Menzies 2007). Plantlets produced from organic cultures derived from *se* are shown in Fig. 19.11. Other approaches include using the first plants that arise from *se* as stool plants, that can be intensively managed in pots and propagation houses (where small needle fascicle cuttings can be taken multiple times throughout the growing season) or lined out in nursery beds as described earlier. Some attempts have been made to use *se* tissue (cotyledons and epicotyl material) to regenerate shoots via adventitious meristem formation (Montalbán et al. 2011). However, research by Hargreaves et al. (2005) indicates that the process of adventitious shoot formation may confer a legacy of increased maturation, which results in slower in vitro and ex vitro growth and poorer root initiation. Phenotypic differences in bud development were also observed after 18 months in the nursery (Hargreaves et al. 2005).

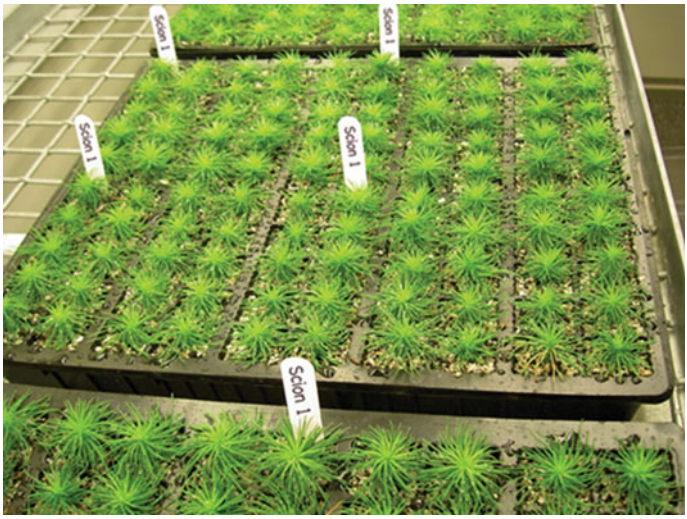

Fig. 19.11 Organogenic shoots of *Pinus radiata* generated at Scion, New Zealand, from germinated somatic embryos (root initiation phase)

19.6.3 Field Performance of SE-Derived Pines

The predicted gains associated with multi-varietal forestry, i.e. the use of vegetatively propagated planting stock instead of seedlings, are high: productivity of the selected elite clones is expected to increase by up to 50 %, with potential simultaneous improvements in stem and wood quality and tree resistance (Soresson 2006). Since the application of SE to pine species is still a relatively novel approach, the data on actual field performance of pine *ss* is still scarce, although it is accumulating all the time.

A recent study by Antony et al. (2014) in the Southern US compared the growth and wood properties of *P. taeda ss* with full-sib and half-sib seedlings after four growing seasons in the field. They found that overall growth properties were better in full-sib seedlings than in *ss*, but *ss* had higher wood density. Furthermore, it was possible to find clones showing both better growth and higher wood density, two traits that are generally negatively correlated. Wood et al. (2015) examined an 8-year-old field trial of *P. taeda* established with *ss* and concluded that selecting clones based on total height and stem diameter will result in varieties with the largest volume growth, even if stem taper is not taken into account.

In *P. radiata*, assessment of the stem diameter of 5-year-old *ss* and 4- to 13-year-old seedlings showed that the deployment of the top 5 % of clones would improve more than 100 % of the family forestry (seedlings from controlled crosses of the selected parents) at the same selection intensity (Baltunis et al. 2009). As in *P. taeda*, "correlation breakers", i.e. clones having both improved growth and wood quality can be found in *P. radiata* (Cown and Sorensson 2008). The recent

assessment of 9-year-old *P. radiata* trials has revealed *se*-derived clones having 11–21 % better height growth and 5–14 % larger stem diameter and, at the same time, remarkably improved wood quality (+8–17 % wood density, +38 % core-wood stiffness) when compared with seedlings of either seed orchard or controlled crosses origin. An important part of field evaluation of *se*-derived *P. radiata* clones in New Zealand included selection for resistance against the fungal disease *Dothistroma* needle blight, which can have a significant impact on early tree growth (Carson et al. 2015).

In France, eight field trials have been established with *P. pinaster ss* and control seedlings in a nursery (since 1999) or in the forest (since 2004) (Trontin et al. 2016a). After 15 years of growth, the oldest test demonstrated that *ss* had completed all development phases, from juvenile growth to the adult vegetative and reproductive phases (flowering and production of cones). In both nursery and forest plots, the mean initial height of somatic tree clones was lower than that of control seedlings of the same or improved families, and a similar conclusion was obtained for most clones at age 6–7 years. Such a poor initial growth performance of *ss* in *P. pinaster* was recently associated with inappropriate biological, physiological and molecular characteristics of cotyledonary *se* harvested after 12 weeks of maturation as compared with the quality standard of seedlings (Morel et al. 2014b, see *Molecular aspects of SE and ZE in pine* in this chapter). However, growth rates of somatic clones, computed as the mean relative increase in height 6–7 years after plantation, were reported to be either similar or better than those of control seedlings (Trontin et al. 2016a). It was concluded that somatic clones could recover from initial low vigour after a few years under field conditions and subsequently perform as seedlings. Selecting the top ranking clones within each family may therefore already provide opportunities for improved traits. However, further refinement of maturation conditions to improve the quality of cotyledonary *se* is necessary to achieve full genetic gains with varietal mixtures of somatic clones.

Behaviour of *P. sylvestris ss* under the field conditions in comparison with seedlings of the same genetic background was recently studied in Finland by Aronen (2016). At planting time, *ss* were smaller than the seedlings, but they grew normally. During the first six growing seasons, their annual height growth was comparable or, in the case of some clones, inferior to that of the seedlings. However, the number of clones studied was small, so no definite conclusion could be made. There are currently no large-scale efforts to use SE in breeding or reforestation of *P. sylvestris*, but the *ss*-derived clones having variable contents of phenolics are being tested for their fungal resistance both in the field and under controlled conditions.

The propagation methods used for *ss*-derived plants of *P. radiata* in New Zealand have gone a long way towards reducing earlier differences in field performance observed in other *Pinus* species described in this section. A significant proportion of the planting stock in New Zealand is derived from seedling cuttings of control-pollinated crosses (Hargreaves et al. 2011). The use of either *se* or shoots derived from organogenic cultures to subsequently form stool beds means that the production methods are identical; cuttings are sturdy with good fibrous root systems.

19.6.4 Regulations and Other Factors Affecting Implementation

Implementation of SE into practical forestry is advancing in pine, but it also faces to same challenges as any breeding programme, including biological (target species, ecological conditions), economic (value of target traits, return on investment, operating funds), institutional (cooperation between academic, private and government organizations) and sociopolitical (social expectations, legal requirements) factors (Rosvall and Mullin 2013). Globally, there are no specific rules governing the use of vegetatively propagated reforestation materials. In the European Union, however, many member states have rather strict regulations concerning testing requirements, the maximum number of plants per clone, the minimum clone number, the maximum percentage of area where clonal materials are allowed in forestry and so on. Another obstacle is that none of the current forest certification standards approve vegetatively propagated materials (Bettinger et al. 2009; Lelu-Walter et al. 2013).

Also, the lack of public acceptance and lack of interest on the part of forest owners to pay more for improved planting stock may hinder the application of VP in forestry. However, as with regulations, these issues vary a lot depending on the region and the species. For SE, development of cost-efficient mass propagation technology is a key issue, especially for the species for which production costs cannot be lowered by further propagation via rooted cuttings. The price for *P. taeda ss* is about six times the price of a seedling, and even if the expected economical outcome at the final harvest is substantially better with clonal material than when using seedlings (Bettinger et al. 2009), establishment costs may still be too high for some of the landowners. The SE costs can be reduced by lowering planting densities and by mixing clonal plants with seedlings (Bettinger et al. 2009; Lelu-Walter et al. 2013), but eventually, the development of automated technology for *ss* production will be needed. This work is currently ongoing and either published (Aidun and Egertsdotter 2012; Anonymous 2014) or patented, e.g. in the case of *P. taeda* by the Weyerhaeuser Company with various published patents, in particular in the field of manufactured seed construction (see Gupta et al. 2014 for patent numbers).

19.7 Conclusions and Future Research

Somatic embryogenesis coupled with long-term storage of EM genotypes at low temperature is a modern and powerful tool for scaling up the production of genetically improved conifer varieties and storage of genetic resources. Protocols for both SE and cryopreservation of embryogenic tissues are being developed for an increasing number of conifer species including pines (Lelu-Walter et al. 2013; Klimaszewska et al. 2016). In contrast, achieving SE from explants of mature trees is still challenging (Klimaszewska and Rutledge 2016; Trontin et al. 2016c) but

remains to be a highly strategic research field because direct propagation of elite trees with field-proven performance would result in a significantly shorter breeding cycle and overall reduced costs to deliver new varieties in plantation forestry. In New Zealand, two companies are commercially producing planting stock derived from the SE process (Forest Genetics and Arborgen). Plants are produced via a combination of propagation techniques using *se* directly as stock plants for fascicle cuttings or incorporating an in vitro organogenesis step to provide plants that are used as planting stock or for stool bed production (then cuttings). The laboratory methodologies are still relatively expensive and SE-derived planting stocks cost more to produce; any progress towards more efficient or possibly automated technologies is essential.

In addition to cell culture improvements, molecular studies have been undertaken in several economically important pine species (mainly *P. pinaster*, *P. radiata*, *P. sylvestris* and *P. taeda*). These results have increased our knowledge of genes and molecular pathways involved in (i) embryogenecity (determined by SE initiation rate), (ii) the notorious loss of embryogenic competence (based on maturation capacity) in ageing cultures and (iii) inferior embryo quality (as estimated by comparison with *ze*: lower *se* germination capacity and lower initial growth rate of *ss*). A number of molecular tools are already available for use to discover and study the effect of genetic instability on SE initiation frequency, *se* maturation yield and embryo quality. However, the biological significance of observed variations in pine and other conifers is still questionable and would require more functional studies (Miguel et al. 2016). New knowledge about the expression of gene cohorts involved in normal embryo patterning, from early embryo proliferating in EM to late embryo cotyledonary stages, has been gathered from targeted gene and transcriptomic or proteomic profiling in pines and other conifers (Trontin et al. 2016b). In pines, there is already strong evidence in pine of stage-specific modulation of embryogenesis-related genes by a number of transcription factors and epigenetic mechanisms (Oh et al. 2008; Klimaszewska et al. 2009; de Vega-Bartol et al. 2013; Morel et al. 2014a).

A number of genes and associated pathways have emerged as excellent candidates to provide molecular tools of practical interest to refine SE at the maturation step but they require confirmation studies in different pine species (Miguel et al. 2016). Comparative biochemical, transcriptomic and proteomic analysis of SE and ZE can be used to gain a better understanding of *se* quality. Careful examination of the molecular data from these comparative studies may prove essential for refining maturation or post-maturation protocols in pines.

Acknowledgements The "Genetics and Biotechnology" team of the FCBA is gratefully acknowledged for its contribution to the technical and scientific developments presented throughout this chapter for maritime pine somatic embryogenesis. We especially thank Isabelle Reymond, Francis Canlet, Sandrine Debille, Karine Durandeau, Pierre Alazard and Luc Harvengt. We also thank Alain Bouvet for statistical support.

In France, the maritime pine multiyear project was supported by grants from the "Conseil Régional de la Région Centre" (EMBRYOME project, contract 33639; IMTEMPERIES, contract 2014-00094511), the "Conseil Régional de la Région Aquitaine" (Embryo2011, contract

09012579-045), the French Ministry of Foreign Affairs and the French Ministry of Higher Education and Research through the France/Czech Republic Science Cooperation BARRANDE Program. Data analysis and experiments were made possible through the involvement of INRA's GenoToul bioinformatics platform in Toulouse (France) and the XYLOFOREST platform (ANR-10-EQPX-16), especially the XYLOBIOTECH technical facility located at INRA Orléans and FCBA Pierroton (France). In Portugal, the preparation of this chapter was supported through projects funded by (1) the European Community's Seventh Framework Programme (FP7/2007-2013, Grant Agreement N°289841-PROCOGEN), and (2) Fundação para a Ciência e Tecnologia (FCT), through grants GREEN-it (UID/Multi/04551/2013) and IF/01168/2013. In Canada, KK was supported by Natural Resources Canada, Canadian Forest Service. Ms Isabelle Lamarra (NRCan-CFS) is thanked for English editing.

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Chapter 20
Direct and Indirect Somatic Embryogenesis in Mango Ginger (*Curcuma amada* Roxb.)

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Abstract Somatic embryogenesis is a developmental restructuring of somatic cells in the embryogenic pathway and forms a basis for the concept of totipotency in plant cells. Understanding the process of transition from vegetative to embryogenic cells and factors involved in the somatic embryogenesis and subsequent plant development represents a challenge for plant tissue culture studies in any selected species. Although several reviews have been published dealing somatic embryogenesis in several plant species, there is no recent information on somatic embryogenesis in mango ginger (*Curcuma amada*). Two different types of somatic embryogenetic pathways are available in mango ginger. They are indirect somatic embryogenesis (IDSE) and direct somatic embryogenesis (DSE). Indirect somatic embryogenesis occurs in friable embryogenic callus from leaf sheath explants that undergo an extreme proliferation before the development of somatic embryos, whereas in the direct somatic embryogenesis, two-step system of culture was followed. Initially, leaf sheath explants were subjected to a pretreatment under dark condition before they were transferred to the subculture medium for embryo development. Histological and scanning microscopic studies showed that the formation of translucent globular embryogenic cell suspension is an important visible stage during early somatic embryogenesis through indirect method. In the direct pathway, upon pretreatment, epidermal and subepidermal cells of the leaf sheath explants formed globular and elongated structures. The presence of clear protoderm in the globular embryos and procambial structures are crucial visible stages.

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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_20

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
NAA	Naphthaleneacetic acid
BA	6-Benzyladenine
GA_3	Gibberellic Acid
Kn	Kinetin
TDZ	Thidiazuron
MS	Murashige and Skoog medium
SEM	Scanning electron microscopy
SE	Somatic embryo
DSE	Direct Somatic Embryogenesis
IDSE	Indirect Somatic Embryogenesis

20.1 Introduction

Curcuma amada is an aromatic spice crop of the family Zingiberaceae, a group of important tropical monocots, which includes turmeric, ginger, and cardamom. It is commonly known as mango ginger and possesses a raw mango-like flavor blended with that of ginger (Banerjee et al. 2012). The aromatic smell raised from *C. amada* is mainly attributed to the presence of car-3-ene and *cis*-ocimene compounds (Gholap and Bandyopadhyay 1984; Rao et al. 1989; Choudhury et al. 1996). Its rhizomes contain fibers, starch, and more than 68 volatile, aromatic essential oils which are used in food, beverages, cosmetics, and medicines (Srivastava et al. 2001; Mustafa et al. 2005; Policegoudra and Aradhya 2008).

20.1.1 Uses of C. amada

C. amada possesses several medicinal properties, such as stomachic, carminative, aphrodisiac, antipyretic, and laxative properties, and is a potential source of compounds with cholesterol-lowering activities (Kirtikar and Basu 1984; Warrier et al. 1994; Srinivasan et al. 2008). The major bioactive compounds of *C. amada* include curcuminoids, curcumin, demethoxycurcumin, and bis-demethoxy curcumin. These compounds have been used for their antioxidant, anti-inflammatory, antidepressant, and platelet aggregation inhibition activities (Policegoudra et al. 2011). It also contains a labdane-type diterpenoid (labda-8(17), 12-diene-15 and 16-dial), which exhibits activity against tuberculosis (Singh et al. 2010).

20.1.2 Propagation

C. amada is mainly propagated by division of rhizomes which are relatively slow to multiply. Genetic improvement by conventional breeding is also difficult in *C. amada* because of a lack of sexual reproduction (Balachandran et al. 1990; Prakash et al. 2004). Furthermore, genetic engineering and molecular studies of its resistance to rhizome rot (caused by *Pythium* sp.) and bacterial wilt (caused by *Ralstonia solanacearum*) requires a standardized, simple morphogenic protocol (Prasath et al. 2011) that can be suitable for large-scale propagation of this rhizomatous spice.

Somatic embryogenesis represents a promising tool for mass propagation as well as for genetic transformation (Nhut et al. 2000; Manrique-Trujillo et al. 2013). Somatic embryos are preferred as a tissue for production of alginate-encapsulated synthetic seeds (Ganapathi et al. 2001; Remakanthan et al. 2013). Tissue culture studies in *C. amada* have shown mostly adventitious plantlet formation from rhizome and leaf sheath explants (Prakash et al. 2004; Das et al. 2010; Banerjee et al. 2012). This attempt aims to elaborate on somatic embryogenesis in *C. amada* as studied in our laboratory (Soundar Raju et al. 2013, 2014).

20.2 Indirect Somatic Embryogenesis

20.2.1 Medium for Callus Induction

Murashige and Skoog medium was found to be the most effective for callus induction and subsequent indirect regeneration in several other species of *Curcuma* including *C. longa* (Salvi et al. 2001), *C. aromatica* (Mohanty et al. 2008), *C. kwangsiensis* (Zhang et al. 2011), and *C. attenuata* (Kou et al. 2012). 2,4-D has been proven to be the most potent and effective growth regulator for callus induction in many plant species (Woo et al. 2000; Okamura et al. 2001; You et al. 2011). Prakash et al. (2004) obtained semi-friable organogenic callus from leaf sheath explants of *C. amada* on MS medium containing 2,4-D alone. Soundar Raju et al. (2013) suggested that the MS medium supplemented with 2,4-D in combination with BA is a prerequisite for white friable embryogenic callus formation in *C. amada*. 2.0 mg/L 2,4-D and 0.5 mg/L BA was found to be the most efficient (26.43 %) for the induction of embryogenic calli from leaf sheath explants (Fig. 20.1a, b).

20.2.2 Induction of Somatic Embryogenesis

In several plant species, indirect somatic embryogenesis is usually induced in the presence of auxin alone or in combination with cytokinins (Gaj 2004; Paul et al.

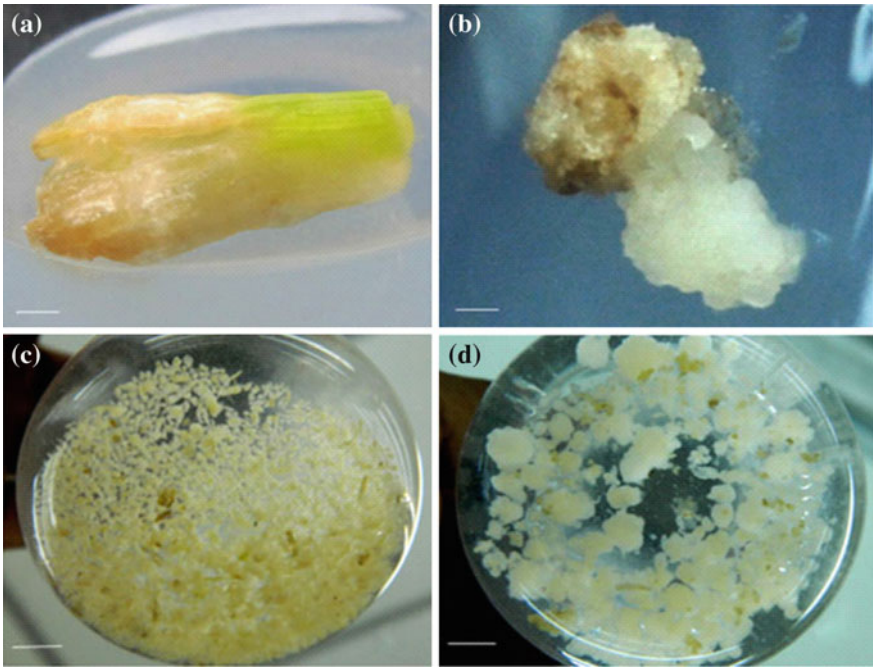

Fig. 20.1 **a** Callus initiation from leaf sheath explant. **b** White and friable embryogenic callus. **c** Induction of somatic embryos from embryogenic callus derived suspension. **d** Development of secondary somatic embryos. Scale bars a=1.5 cm; b, c=400 μm; d=4mm

2011; Pinto et al. 2011; Bao et al. 2012) and in a few systems cytokinins alone were found to be effective in somatic embryogenesis (Nanda and Rout 2003; Carra et al. 2006). Soundar Raju et al. (2013) found that the embryogenic callus subcultured in medium containing BA gave best results on somatic embryogenesis in *C. amada*.

The concentration of BA significantly influenced the somatic embryo induction. Among the series (0.1–0.6 mg/L) of BA concentration, the frequency of somatic embryo induction was higher (62.93 %) in the medium supplemented with 0.3 mg/L BA (Fig. 20.1c). The occurrence of somatic embryos with 0.1–0.2 mg/L BA was 26.20–46.67 %, while the frequency was further reduced to 5.33–23.95 % on 2,4-D (0.1–0.6 mg/L) containing media (Table 20.1). The presence of higher concentrations (0.4–0.6 mg/L) of BA induced secondary somatic embryogenesis with fused margins of embryos (Fig. 20.1d).

This finding is concordant with the results of a similar study in *Zingiber officinale* (Guo and Zhang 2005). The concentration of BA also significantly affected the somatic embryogenesis. Similar phenomenon of secondary somatic embryogenesis has been previously reported in the monocot species of *Lepidosperma drummondii* and *Baloskion tetraphyllum* (Panaia et al. 2011).

Table 20.1 Effects of MS liquid medium supplemented with 2,4-D and BA on the rate of indirect somatic embryogenesis

Plant growth regulators (mg/L)	Frequency of somatic embryogenesis (%)	Number of somatic embryos per culture (%)
2,4-D		
0	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
0.1	16.52 ± 0.24^{b}	13.04 ± 0.16^{a}
0.2	23.95 ± 0.27^{a}	15.80 ± 0.41^{a}
0.3	14.57 ± 0.32^{b}	11.80 ± 0.14^{a}
0.4	7.14 ± 0.28^{c}	5.90 ± 0.16^{b}
0.5	5.33 ± 0.17^{c}	0.00 ± 0.00^{c}
0.6	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
BA		
0.1	26.20 ± 0.34^{f}	32.33 ± 0.29^{d}
0.2	46.67 ± 0.45^{c}	45.66 ± 0.20^{c}
0.3	62.93 ± 0.34^{a}	54.52 ± 0.21^{a}
0.4	53.07 ± 0.18^{b}	47.71 ± 0.20^{b}
0.5	41.73 ± 0.27^{d}	34.61 ± 0.29^{d}
0.6	36.26 ± 0.20^{e}	23.42 ± 0.16^{e}

Data represented mean ± SE of three replicates, each with 15 cultures. Means having the same letter in a column were not significantly different by Duncan's multiple range test ($p = 0.05$)

20.2.3 Role of Osmoticum

Theoretically, the osmotic potential of culture medium should be directly affected by its strength. A change in strength directly influenced the formation and maturation of somatic embryos (Komatsuda et al. 1992). In *C. amada,* somatic embryogenesis was better on half-strength MS medium (Soundar Raju et al. 2013). This is probably because, prevalence of optimal concentration of inorganic nutrients in the culture medium results in a favorable optimal osmotic potential for dedifferentiation of embryogenic callus. The inhibitory effects of high MS strength on SE development have been reported in other plant species (Choi and Jeong 2002; Chen et al. 2010). Sugar not only provides the required carbon sources for plant growth, but in its original form it also influences the osmotic potential in the culture medium. Our results showed that 3.0 % sucrose concentration was optimal for increasing the number of SEs, and above this concentration, SE development has been gradually decreased in numbers.

20.2.4 Histological Analysis

Light and scanning electron microscopic studies revealed that the white friable embryogenic callus consisted of heterogenous cells. The embryogenic cells were

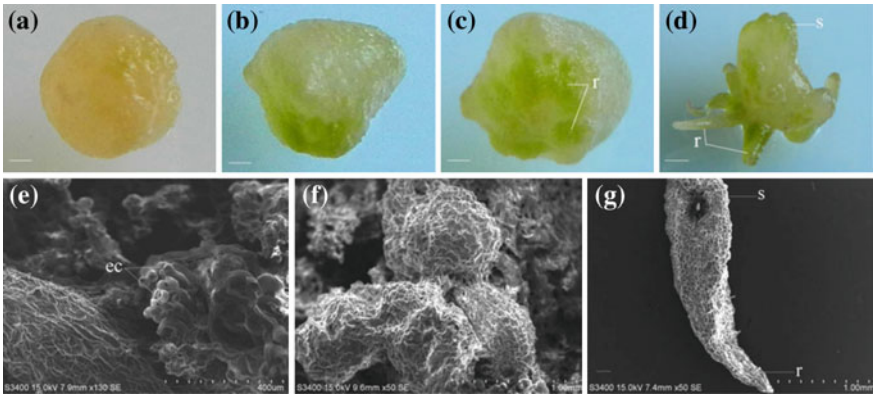

Fig. 20.2 Stereo microscopy and SEM images showing development stages of somatic embryos. **a** Translucent globular pro embryo. **b** and **c** Slightly torpedo shaped embryos. **d** Germinating somatic embryo. **e** Development of embryogenic cells. **f** Secondary somatic embryogenesis from primary somatic embryos. **g** Germinating somatic embryos. Scale bars a=1 mm; b-d=2mm

smaller and more spherical in shape. After transfer of embryogenic cells to suspension medium, globular and torpedo shaped embryos were developed (Fig. 20.2a–g).

The initial features of somatic embryos obtained in *C. amada* were translucent globular in appearance, which were similar to somatic embryos of banana (Escalant et al. 1994; Cote et al. 1996; Grapin et al. 1996, 2000; Navarro et al. 1997; Becker et al. 2000; Ganapathi et al. 2001; Khalil et al. 2002). Translucent globular shape was an important visible stage during early somatic embryo development.

20.2.5 Hardening

Mature embryos were germinated to produce complete plantlets under dark condition. This result was in agreement with Jalil et al. (2008) who observed that the dark condition induced germination of somatic embryos in banana. Well-grown plantlets (5–7 cm shoots and 3–5 cm roots) were transferred to light to facilitate chlorophyll formation and further development of root and shoot.

20.3 Direct Somatic Embryogenesis

Direct somatic embryogenesis is a more desirable approach to obtain regenerated plants, similar to the parent plants, since callus formation may cause somaclonal variation (Mizukami et al. 2008). Direct embryogenesis has been also reported to be

useful for the regeneration of transgenic plants (Manoharan et al. 1998; Tokuji and Fukuda 1999). In our laboratory, a high percentage of somatic embryo induction directly from leaf sheath explants was obtained. In addition, this method is easier and requires less time than the previously described method of indirect regeneration. Therefore, DSE from leaf sheath explants could prove to be another effective regeneration system for more rapid propagation of mango ginger.

20.3.1 Pretreatment of Explants

In *C. amada* primary treatment of explants on MS medium containing a low concentration of 2,4-D and BA for 2 wk is essential to trigger DSE. This pretreatment triggers the morphogenetic competency of the explant, leading to the reception of the signals for embryo development as reported in other species (Franklin et al. 2006; Dam et al. 2010).

After treatment, the leaf sheath explants became swollen and soft (Fig. 20.3a). The explants eventually became crimped, and white clusters of small translucent

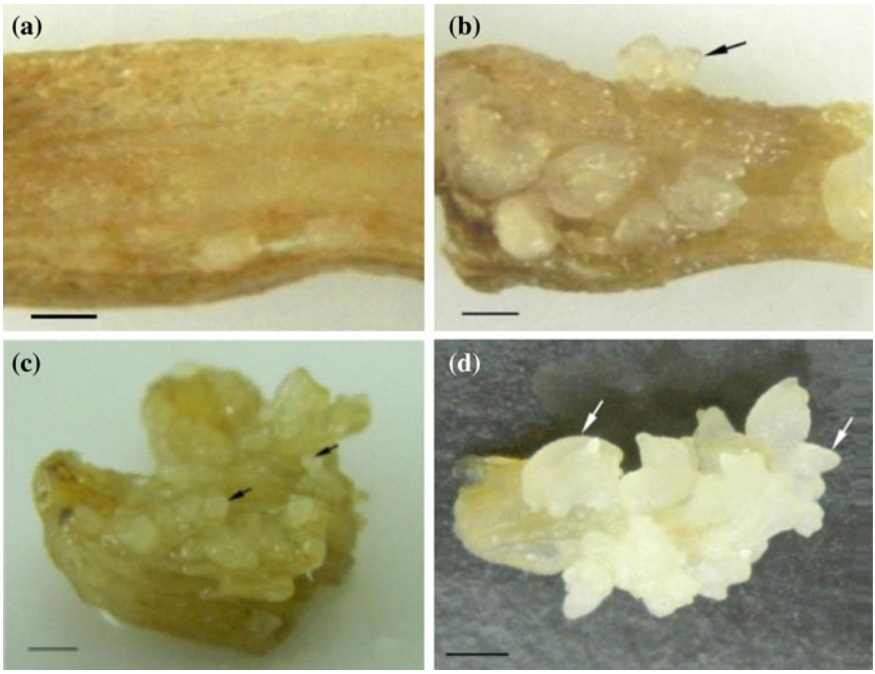

Fig. 20.3 Morphological and development stages of direct somatic embryogenesis from leaf sheath explants. **a** General view of leaf sheath explants. **b** Development of translucent spheres (*arrow*). **c** Early stages of globular embryo development(*arrow*). **d** Elongated stages of somatic embryos (*arrow*). Scale bars a=100 µm; b, c=500 µm; d=700 µm

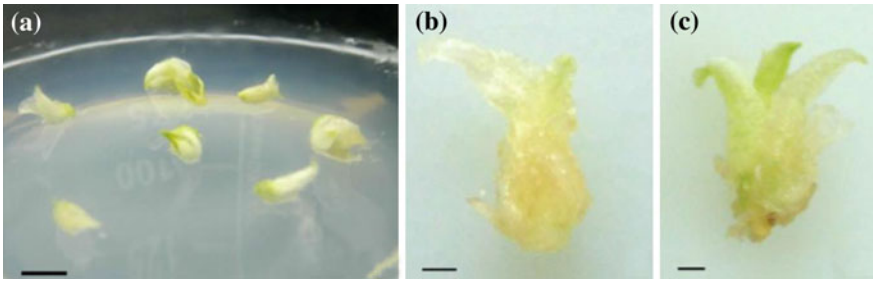

Fig. 20.4 **a** Germination of somatic embryos. **b** and **c** Germinated somatic embryo. Scale bars a=1 mm; b, c=1.5mm

spherical structures were formed on SIM 2 medium containing cytokinin (BA, Kn, TDZ) alone or in combination with NAA (Fig. 20.3b). Later, these structures differentiated into globular and elongated stages (Fig. 20.3c, d).

20.3.2 Germination of Somatic Embryos

In somatic embryos of certain species, particularly of plants that undergo dormancy in natural seeds, the germination and growth of embryos into plants can be stimulated by the application of GA_3 to the culture medium (Gaj 2004; Manrique-Trujillo et al. 2013). The germination of somatic embryo of some monocots was enhanced by dark conditions (Nhut et al. 2000). In *C. amada*, somatic embryos cultured on half-strength MS medium containing GA_3 under dark condition showed enhanced germination (Fig. 20.4a–c).

The highest rate of germination (86.7 %) was obtained from somatic embryos cultured in the dark on medium containing 1.44 μM GA_3 (Fig. 20.5).

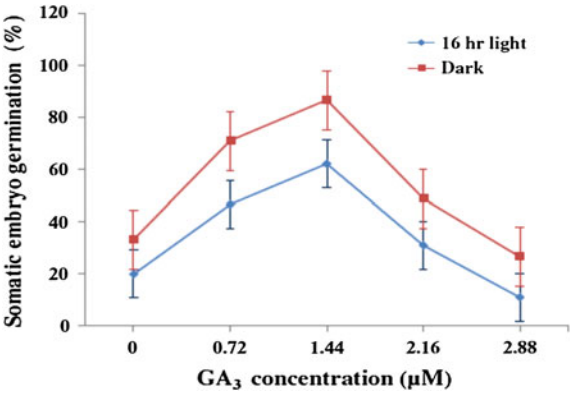

Fig. 20.5 Effects of GA_3 and light condition on *C. amada* somatic embryo germination

20.3.3 Histological Analysis

The histological study on DSE in *C. amada* showed that epidermal and subepidermal cells were the source of somatic embryos in *C. amada*. The initial cell divisions occurred in the epidermal and subepidermal cells of the explants (Fig. 20.6a–c). This regeneration pathway is common in several monocots including river lily (Slabbert et al. 1995) and açaí palm (Scherwinski-Pereira et al. 2012). Further, the formation of embryogenic cells with prominent nuclei, small vacuoles, and dense cytoplasm was common for several plant species (Paul et al. 2011). Globular and elongated embryo stages were regarded as key stages in the identification of somatic embryos (Fig. 20.6d, e). The presence of protoderm and procambial strands in the developing embryos is the additional key features, which could be used as indicators for somatic embryo formation(Fig. 20.6 f–h).

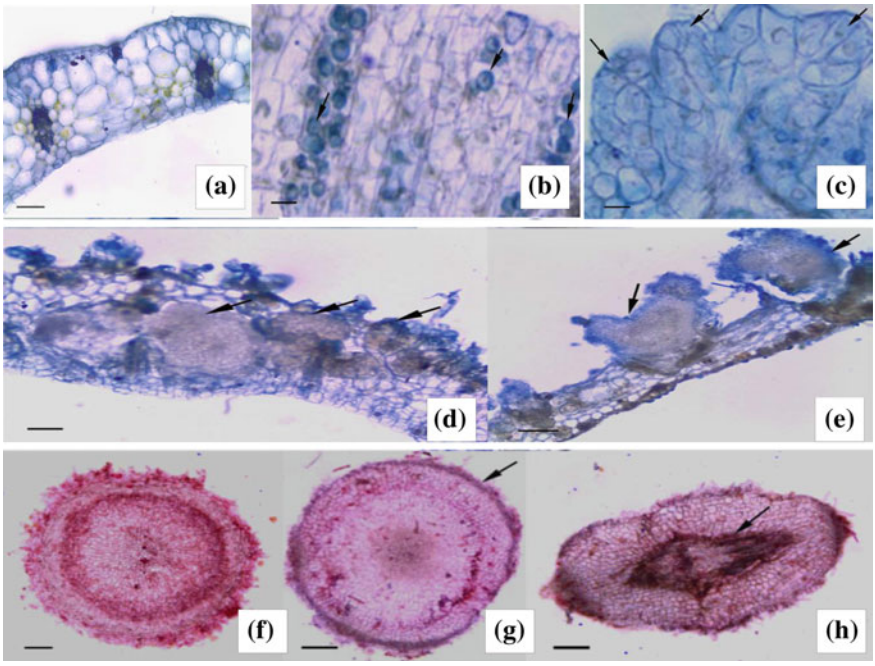

Fig. 20.6 **a** Transverse section of leaf sheath explants. **b** Presence of embryogenic cells with prominent nucleus, small vacuole, and dense cytoplasm(*arrow*). **c** Active cell division and formation of embryo(*arrow*). **d** and **e** Development of globular somatic embryos directly from epidermal and subepidermal regions of the leaf sheath explant(*arrow*). **f** and **h** Initial stages of somatic embryos showing protoderm and procambial strands (arrow). Scale bars a, b=100 µm; c, d=300 µm; e-g=500 µm; h=700 µm

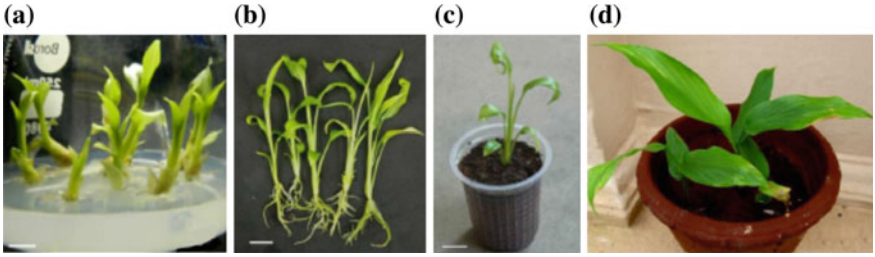

Fig. 20.7 **a** Plantlets regenerated under light condition. **b** Plantlets showing well developed shoot and roots. **c** and **d** Secondary hardening. Scale bars a, b=1 cm; c, d=1.2 cm

20.3.4 Hardening

Plantlets germinated from somatic embryos of both indirect and direct pathways were further transplanted to plastic pots containing the potting mixture and successfully transferred to field after hardening (Fig. 20.7a–d).

20.4 Conclusion

Components of the culture medium and physiological conditions are the important determinants for the occurrence of both indirect and DSE from leaf sheath explants of *C. amada*. It was found that the friable fast growing embryogenic calli obtained from leaf sheath explants are the suitable starting material for IDSE. Initial features of somatic embryogenesis obtained through indirect pathway of *C. amada* are similar to the indirect somatic embryogenesis pathway of banana. Pretreatment of leaf sheath explants on medium containing 2,4-D and BA under dark condition was critical for DSE, and initial cell division for somatic embryos occurred in epidermal and subepidermal cells of the explants. The somatic embryogenesis protocol described here is useful for the multiplication of valuable germplasm on a large scale at a much faster rate, and is easier, compared to the previous methods of organogenesis in mango ginger. Furthermore, these methods should also be useful for the introduction of genes conferring resistance to pathogens using genetic engineering.

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Chapter 21
Somatic Embryogenesis in Banana, *Musa* ssp.

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Abstract In *Musa* (Musaceae family), as for other angiosperms, somatic embryo formation from somatic cells exemplify a distinctive phenomenon of plant cell developmental plasticity. Somatic embryogenesis (SE) through embryogenic cell suspension (ECS) cultures is an important milestone method for accelerating bananas' mass-propagation due to its high regeneration potential, and serves as powerful cellular tool for its non-conventional improvement. Protocols for SE have been standardized for several genotypes of wild *Musa* species (having AA and/or BB genomes), dessert (AA, AB and AAA), cooking (ABB), and plantain (AAB) bananas using different types of explants; however, in some cases, the protocols are limited by the low embryo germination and plant conversion rates. Therefore, efforts are needed to understand the physiological, biochemical, and genetic processes underpinning banana embryo development (zygotic and somatic), in order to inaugurate robust SE protocols with high rates of embryo germination and plant conversion. Here, we present an overview of the general progress in banana plant regeneration through somatic embryogenesis.

21.1 Background Information

Bananas and plantains, collectively known as bananas, belong to the genus *Musa* (Family: Musaceae); these monocotyledonous giant herbs are among the most important horticultural crops widely distributed throughout the humid tropical and

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sub-tropical regions of the world where they are cultivated. Both dessert and cooking bananas are important perennial fruit crops on which millions of people depend for their livelihood and as their main staple foods. The present-day cultivated bananas are diploid and triploid hybrids largely originated form natural inter-(sub) specific- and inter-specific crosses between diploid ($2n = 2x = 22$ chromosomes) wild subspecies, mainly of *Musa acuminata* Colla (A genome) and *Musa balbisiana* Colla (B genome) bananas (D'Hont et al. 2000; Husin et al. 2014), both belonging to section *Musa* (Häkkinen 2013).

Traditional breeding in bananas is limited due to polyploidy and vegetative parthenocarpy. Vegetative propagation through the conventional procedure of using banana lateral shoots that develop from the rhizome, so-called suckers, cannot always match the demand of elite genotypes for cultivation. Consequently, propagation through biotechnological approaches, viz., organogenesis and SE, has made a great contribution to large-scale production of demanded plants. In particular, ECS cultures are powerful tools for genetic improvement and clonal propagation of bananas, given the constrain production in many countries due to pests, diseases, and abiotic stresses and the troublesome of its conventional breeding. These procedures are of use for the conservation of the genetic diversity of the wild bananas ancestors of current edible plants (Asif et al. 2001), they also support breeding strategies by embryo rescue of seeds from open-pollinated seedy bananas and controlled new hybrids (Afele and de Langhe 1991; Bakry 2008; Uma et al. 2012). Propagation of edible clones for synthetic seed technology (Ganapathi et al. 2001b), and the use of somatic embryogenic cultures for in vitro selection of somaclonal variants (Ghag et al. 2014b) or genetic transformation for example edible vaccine against hepatitis B (Kumar et al. 2005) and resistance to biotic stress (Ghag et al. 2014a) are examples of the active topics of research in *Musa*.

21.2 Somatic Embryogenesis

Somatic embryogenesis is a process that illustrates the expression of plant developmental plasticity and of cellular totipotency, with tremendous potential for fundamental research and commercial application. This is a biological process in which under inductive conditions somatic cells reach embryogenic competence and after a series of biochemical and morphological changes precisely organized embryos are produced (Quiroz-Figueroa et al. 2006). The somatic embryos have a bipolar structure with apical and radical meristems and showed morphological characteristics resembling those found in zygotic embryos. Plant regeneration in banana through SE is important because of the recognized unicellular origin of embryos (Strosse et al. 2004; Roux et al. 2004; Escobedo-GraciaMedrano et al. 2014) and the risk of generating plants with chimeric tissues after genetic transformation is minimized. SE can be initiated directly from the explant of donor plant without callus formation (direct SE), or indirectly by means of a callus stage (indirect SE) (Quiroz-Figueroa et al. 2006). In the latter case, SE usually follows a similar path,

such as: (i) initiation of calli followed by selection of the embryogenic callus, (ii) proliferation of the embryogenic cells of the callus (undifferentiated phase), (iii) regeneration of numerous embryos from these phase (embryogenic phase), and (iv) conversion of somatic embryos into mature embryos with the ability to regenerate healthy plants (Khalil et al. 2002; Gaj 2004; Ramírez-Villalobos and de García 2009). Either on semi-solid medium (calli) or in liquid medium (cell suspensions), the multiplication of embryogenic cells is a key step because it greatly and rapidly scales up the number of potential embryos that may be produced. Indirect SE with formation of a callus stage is a common experiential method use in bananas, while secondary somatic embryos/embryogenesis (SE_2) stimulated directly from the primary somatic embryo or through formation of a callus stage have been both reported (Khalil et al. 2002). Whereas, direct SE from split shoot tips have been recently reported (Remakanthan et al. 2014).

21.3 Initiation of Somatic Embryogenesis in Banana

The initiation of SE response is influenced by the developmental stage of the initial plant material, the genetic background of the stating banana plant material (Escalant et al. 1994; Youssef et al. 2010) and on the appropriate balance of plant growth regulators (PGR) in the medium perceived by the cells in culture. Indirect SE is induced by explant inoculation onto semi-solid or liquid medium in presence of exogenous PGR, predominantly auxin and sometimes cytokinin well-defined by the SE method employed (Krikorian and Scott 1995; Table 38.1). During indirect SE, dedifferentiation of the cells in the initial explant supports the formation of a mass of cells with embryogenic potential. From the earlier reports in the late 1980s, and thereafter, five main procedures for establishment of embryogenic callus and proliferation of ESC in banana are recognized. They rely on the type of explant in which SE is initiated for a large range of *Musa* genotypes tested, as follows: (i) immature zygotic embryos (IZEs), (Cronauer-Mitra and Krikorian 1988; Escalant and Teisson 1989; Navarro et al. 1997; Escobedo-GraciaMedrano et al. 2014; Maldonado-Borges et al. 2013; Krikorian and Scott 1995), and mature zygotic embryos (MZEs) (Uma et al. 2012), with modifications by the use of 2,4-dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-2-meth-oxybenzoic acid (dicamba) or 4-amino-3,5,6-trichloropyridine-2-carboxylic acid (picloram) as synthetic auxin (Novak et al. 1989; Escobedo-GraciaMedrano et al. 2014; Remakanthan et al. 2014). (ii) Rhizome slices and leaf sheaths (Novak et al. 1989), cultures of proliferating meristem called "scalps" (Dhed'a et al. 1991; Schoofs et al. 1998; Strosse et al. 2003), immature male (Ma 1991; Escalant et al. 1994; Côte et al. 1996; Grapin et al. 1996; Navarro et al. 1997), and female flowers (Grapin et al. 2000). More recently, proliferative male flower buds so-called "curds" (Pérez-Hernández and Rosell-García 2008) and male flower bract (Divakaran and Nair 2011) cultures, obtained in media supplemented with TDZ (thidiazuron), have been also used for initiation of SE. While, direct somatic embryos developed from

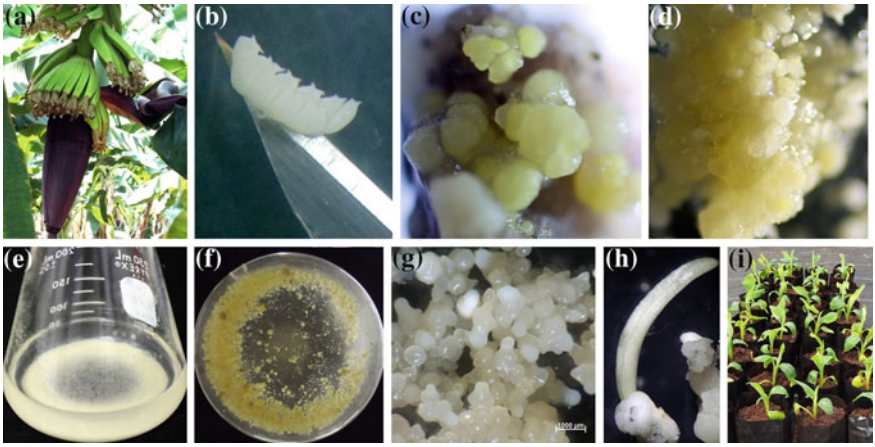

Fig. 21.1 Plant regeneration of *Musa acuminata* (AAA) cv. Grand Naine (subgroup Cavendish) via indirect somatic embryogenesis. **a** Inflorescence shown new opened female flowers and the distal male bud, **b** immature male inflorescence used to induce callus, **c** *yellow* friable callus obtained from immature inflorescence cultured on M1 medium for 3 months. **d** Homogeneous embryogenic callus with expression of globular somatic embryos, **e** homogenous cell suspension obtained after filtration of initial suspension (**f**) and subculture for 3 months in M2 medium. **g** Somatic embryos after 45 days in maturation medium, showing evidence of initiation of starch accumulation. **h** Germinated embryo after 8 days of culture on PGR-free medium, emergence of shoot is shown. **i** Emblings after 15 days of acclimation at the glasshouse

split shoot tips under a combination of the PGR picloram and 6-benzyladenine (BA), (Remakanthan et al. 2014). On the other hand, as shown by their higher SE response, immature zygotic embryos have greater competence for embryogenic callus formation compared to differentiated tissue that need to undergo dedifferentiation (Marroquin et al. 1993; Escobedo-GraciaMedrano et al. 2014), yet, immature embryos responded better than mature embryos (Uma et al. 2012). From the inflorescence male bud (Fig. 21.1a), immature male flowers (Fig. 21.1b) are used for induction of SE callus (Fig. 21.1c). This method has been successfully employed on a large range of *Musa* cultivars (Strosse et al. 2003); however, this kind of explant is only suitable for genotypes having a male bud, the alternative for banana cultivars lacking male buds is to either use female flowers or the scalp methods. The frequency of embryogenic responsive male flower buds varied between 0.5 and 37 %, a response which is dependent on the genetic constitution of the donor plant, its physiological status, and some seasonal factors which influence the response (Escalant et al. 1994). Among the reported genotypes, the cv Enano Gigante (AAA, Cavendish subgroup) showed lower, from 0.7 to 10 %, responses (Navarro et al. 1997; Youssef et al. 2010). An average of 8 % embryogenic callus response has been reported for the scalp method for a range of genotypes (Strosse et al. 2003). Whereas, 12.5 and 25 % of embryogenic response was found using the method of proliferative male flower buds", under semisolid and liquid inductive medium, respectively, (Pérez-Hernández and Rosell-García 2008). Thus, the

mentioned SE protocols suitable for different purposes i.e., banana germplasm conservation, and massive propagation, needs assessment on a larger pool of samples in order to adjust them for each banana genotype. In general, for indirect SE the embryogenic reaction of the diverse banana explants to the different media (Table 21.1) follow three or four main patterns of callus development (Strosse et al. 2004; Youssef et al. 2010). Variation in callus formation response to the components of the induction media depend on the genotype (Strosse et al. 2006). Formation of fast/growing yellowish withe callus is undesirable because the callus oxidized rapidly and shows no embryogenic structures. In the scalps method, a positive embryogenic response occurs on 3–8 months old induced explants (Strosse et al. 2004), while the same response can take nearly 2–3 months in the male inflorescence, zygotic embryo (Escalant et al. 1994; Navarro et al. 1997) and curds (Pérez-Hernández and Rosell-García 2008) methods. The appearance of individual embryos under the different embryogenic method used is a promising indication of the embryogenic competence and plasticity of the starting material. In particular, it seems that direct SE is possible, though the plant conversion rates need to be improved (Remakanthan et al. 2014). As for indirect SE, the presence of a friable white callus, consisting of only early stage globular somatic embryos (Fig. 21.1d) and non-organized cell clusters, is the most wanted callus for the initiation of embryogenic cell suspension (ECS) cultures (Fig. 21.1d), from either of the cited methods (Ma 1991; Escalant et al. 1994; Côte et al. 1996; Grapin et al. 1996; Navarro et al. 1997; Marroquin et al. 1993; Escobedo-GraciaMedrano et al. 2014).

21.4 Proliferation of Embryogenic Callus and Initiation of Cell Suspension Cultures

After the formation of the embryogenic callus (3–5 months) in an auxin-supplemented semisolid medium, the callus is multiplied by a monthly refreshment of the medium (MA1, for immature male flower methods and MI medium for the immature zygotic embryo method), except that the concentration of the sole auxin 2,4-D is reduced to 4.5 μM. Whereas for the scalp method, both auxin and cytokinin plant growth regulators (PGR) are use and maintained in the culture medium (Table 21.1). The existing protocols for somatic embryogenesis using embryogenic cell suspension (ESC) cultures have the potential to produce non-chimeric plants. Thus, establishment and maintenance of embryogenic cultures with high capacity to generate somatic embryos is an important and desired step in any protocol for plant regeneration at a massive level and transformation success. Banana ECS cultures are established by transferring embryogenic callus (Fig. 21.1d) into M2, MA2 liquid medium (M1 and MA1, without gelling agent) for male flower method, or the MI (Marroquin et al. 1993) or S1 and S2 liquid media for the immature-zygotic-embryo method (Table 21.2); most of these media are added with L-glutamine and malt extract, respectively, (Côte et al. 1996; Strosse et al. 2003; Escobedo-GraciaMedrano et al. 2014; Jafari et al. 2015). Under such

Table 21.1 Composition of culture media frequently use to initiate indirect somatic embryogenesis in *Musa* spp.

Components		Somatic embryogenesis initiation media			
	MA1	ZZss	SST	MI	MTDZ
MS macro-elements	MS	½ MS	MS	½ MS	MS
MS micro-elements	MS[a]	MS	MS	MS	MS
KH_2PO_4 (mM)				1.47	
Vitamins	MS	MS	MS	MW[b]	MS
Biotin (μM)	4.1				
2,4-D (μM)	9–18.1	4.5		4.5[c]	
NAA (μM)	5.4				
IAA (μM)	5.7				
Picloram (μM)			4.14	9[c]	
Zeatin (μM)		1			
BA (μM)			0.22		
TDZ (μM)					0.36–4.5
L-Glutamine (μM)	684				
Ascorbic acid (mg L^{-1})		10			
Sucrose (mM)	87	87	87	175	87
Gelrite (g L^{-1})	2	3		2	
Agar type II (g L^{-1})	7		7		7
pH	5.7	5.8	5.7	5.8	5.8
Reference	Ma (1991), Escalant et al. (1994)	Strosse et al. (2003)	Remakanthan et al. (2014)	Marroquin et al. (1993), Navarro et al. (1997), Escobedo-GraciaMedrano et al. (2014)	Divakaran and Nair (2011)

MA1 Immature flower method, *ZZss* scalps method, *SST* split shoot tips, *MI* immature zygotic embryos method, *MS-TDZ* bract method for diploid cultivars
[a]Murashige and Skoog (1962)
[b]Morel and Wetmore (1951)
[c]Either 2,4-D or Picloram

Table 21.2 Composition of culture media frequently used for the establishment of embryogenic cell suspension cultures in *Musa* spp.

Plant material (genome group)	*M.a.* cv. Grand Naine (AAA)	*Musa* spp. (AA, AB, AAA, AAB, ABB)	*M. a.* Colla (AA) ssp. *malaccensis*	cv. Mas (AA)
Components/medium	Ma2/M2	ZZI	MI/S1, S2	M2b
MS[a] macro-elements	MS	½ MS	MS	½ MS
MS[a] micro-elements	MS	MS	MS	MS
Vitamins	MS[a]	MS[a]	MS[a]	D[b]
Biotin (μM)	4.1		4.1	
2,4-D (μM)	4.5	4.5	4.5[**]	5
Picloram (μM)			2.1[**] (7.5[*])	
IAA (μM)				
Zeatin (μM)		1		1
L-Glutamine (μM)	684		684	
Malt extract (mg L^{-1})	100		100	
Ascorbic acid (mg L^{-1})		10		10
Sucrose (mM)	131	87	87	58
pH	5.3	5.8	5.3	5.8
Reference	Ma (1991), Côte et al. (1996), Grapin et al. (1996, 2000)	Strosse et al. 2003, Strosse et al. (2006)	Marroquin et al. (1993)[*], Escobedo-GraciaMedrano et al. (2014)[**]	Dhed'a et al. (1991), Jalil et al. (2003)

Ma2, M2, M2b immature flower method, ZZI, scalps method; immature zygotic embryo, MI[*]. S1 or S2[**]
[*]Marroquin (1993)
[**]Escobedo-Gracia Medrano (2014)
[a]Murashige and Skoog (1962)
[b]Dhed'a et al. (1991)

media, the ECS cultures multiply (Fig. 21.1e, f), with refreshment of the medium every one to two weeks. It is important to note that one of the main difference with the scalp method is that the concentration of exogenous PGR (2,4-D and zeatin) is kept the same throughout the induction and multiplication phases (ZZss and ZZl) and neither glutamine nor malt extract are added to the medium(Strosse et al. 2003). Whereas, for the flower method, with the onset of embryogenic callus formation at high auxin concentration, reduction of auxin concentration is essential for proliferation of somatic embryogenic callus and expression of somatic embryos (Ma 1991; Côte et al. 1996; Jafari et al. 2015). In spite of extensive advances in SE, low embryo germination and a loss of ECS morphogenetic competence are still the bottlenecks of SE procedures in various banana cultivar and embryogenic systems (Schoofs et al. 1999).

21.5 Development and Maturation of Somatic Embryo

The development of the embryo (zygotic and non-zygotic) follows a sequence of organized events, which comprise active cell division that contribute to the formation of an undifferentiated globular shape embryo with a defined protodermis, followed by cellular differentiation and enlargement of the embryo. Maturation, the final stage of embryo development is distinguished by overall cell expansion and accumulation of reserve substances of the embryo. In general, at this stage, repression of germination and the acquisition of desiccation tolerance, is frequent in the zygotic embryo maturation, and often is lacking in somatic embryo terminal development (Merkle et al. 1995; de Moura Vale et al. 2014). In several edible bananas genotypes, the basis for the low quality and quantity of the somatic embryos, and the poor regeneration and conversion to vigorous plants is frequently the least studied aspects in banana (Krikorian and Scott 1995). In this regard, the quality and quantity of banana somatic embryos have shown to be influenced by medium pH, the so-called ECS acidogenic growth hypothesis (Chung et al. 2015). Five types of cell aggregates has been recognized in banana ECS cultures of *Musa* AAA cv. Grande naine, each type can have a significant effect on the quality of the embryo established during the development–maturation stage, and likewise can promote embryo germination (Domergue et al. 2000). The pH of buffered media during ECS cultures have an effect on the type of cell aggregates developed. Addition of L-glutamine and malt extract which is a mix of amino acids, carbohydrates and vitamins, in the culture medium during the initial step of embryogenesis and embryo development until the maturation step is a general practice in banana protocols (Tables 21.2 and 21.3). These compounds rapidly incorporated into the carbon skeletons for metabolism and protein synthesis. The addition of the amino acids L-glutamine, alanine or L-glutamic acid have shown to strongly stimulate the development of somatic embryos in carrot (Higashi et al. 1996). The effect of L-glutamine, and to a lesser extent proline, in somatic embryo development has proven to enhance the efficiency of banana (*Musa acuminata* cv. Berangan) regeneration (Husin et al. 2014). In the absence of exogenous PGR, histodifferentiation of developing embryos in *M. a.* ssp. *malaccensis* proceed well under limited water availability and reached the stage of maturity (coleoptilar stage) perceived by the typical white-opaque-color of the embryo and exhibit the cotydelonary slit (Maldonado-Borges et al. 2013; Escobedo-GraciaMedrano et al. 2014). One of the most important factors during somatic embryo maturation is the impact of water stress (Attree et al. 1991). During this stage with the gradual loss of water, the embryos initiate the process of desiccation tolerance (Bomal et al. 2002), which gives the embryo the capacity to survive under water-limiting conditions (Bewley et al. 2013; Dekkers et al. 2015). During this state of "dehydration," the embryo is stimulated to accumulate reserve proteins, carbohydrates and lipid compounds (Klimaszewska et al. 2000; de Moura Vale et al. 2014). Available banana protocols control the water availability to the developing somatic embryos by using either higher concentration of gelling gum and/or filter paper or both

Table 21.3 Comparison of the composition of six culture media used for the development and maturation of somatic embryos of *Musa* spp.

Components/Plant material (genome group)	cv. Grand Naine (AAA)	cv. Grand Naine (AAA)	Several banana and plantain genotypes	cv. Dwarf Brazilian (AAB)	*M. a.* ssp. *malaccensis* (AA)
Macroelements	SH[a]	SH	½ MS	MS	MS
Microelements	SH	MS	MS	MS	MS
KH_2PO_4 (mM)					1.47
Vitamins	MS	MS	MS	MS	MW
Ascorbic acid					
Myo-Inositol (mg L^{-1})					
Biotin (μM)	4.1	4.1		4.1	
ANA (μM)	1.1	1.1		5.4	
Kinetin (μM)	0.5	0.5		2.3	
Zeatin (μM)	0.2	0.2		0.9	
2-iP (μM)	0.7-1.0				
Malt extract (mg L^{-1})	100	100		100	
Glutamine (μM)	680			680	
Proline (mM)	2				
Sucrose (mM)	131	131	87.6	131	87.6
Lactose (mM)	28				
Phytagel (g L^{-1})				2.6	
Gelrite (g L^{-1})	3	2	3		2
Filter paper No. 1	+	–	+	–	+
pH	5.3	5.8	5.8	5.8	5.8
Medium	Ma3/M3	MM	RD1	SK8	MM
Reference	Ma (1991), Côte et al. (1996), Navarro et al. (1997)	Navarro et al. (1997)	Strosse et al. (2003)	Khalil and Elbanna (2004)	Escobedo-GraciaMedrano et al. (2014)

[a]Schenk and Hildebrandt (1972)
[b]Morel and Wetmore (1951)

(Table 21.3), under this circumstances accumulation of starch in during embryo maturity is indicated by the white-opaque color (Fig. 21.1g) around 45 days of culture (Côte et al. 1996; Chung et al. 2015), as is in other plant species (Márquez-Martín et al. 2011). Molecular evidences from transcribed expressed sequences have suggested that an early responsive to dehydration proteins (ERDs) are involved in embryo maturation. The *ERD* genes are defined as genes rapidly activated during drought stress and involved in Abscisic acid mediated developmental and stress responses (Maldonado-Borges et al. 2013).

21.6 Somatic Embryo Derived Plants (Emblings)

Plant recovery by germination of somatic embryo with emergence of normal root and shoot (Fig. 21.1h) is achieved on media germination with added or without PGR, which depends on the genotype and the previous culture procedure followed during embryo development (Table 21.4). Following the acclimatization and hardening of emblings (Fig. 21.1i), conversion rates are estimated and the values are compared with the percentages of embryo germination. The percentage of somatic embryo germination recorded for some genotypes fluctuates between 3 and 46 % in triploid Cavendish (AAA) bananas. These values can reach up to 91 % when somatic embryos differentiated from ECS cultures of type 2 cell aggregates (Domergue et al. 2000). Higher rates (90–95 %) found in the triploid (AAB) cv Dwarf Brasilian (AAB) and the seminiferous *M. a.* ssp. *malaccensis* (AA), have in common that embryo development passed through a differentiation–maturation phase. In the case of somatic embryos obtained by the direct SE obtained from split shoot tips (*M. a*. AAA, cv. Grand Naime), a 2–3 % of embryo conversion has been reported (Remakanthan et al. 2014). Although several SE protocols have been described for different bananas genotypes, the comparisons of results as to the percentage of somatic embryo germination and emblings conversion rates is sometimes difficult, because the presented data do not always make a distinction between both situation. The emblings conversion rates vary from 13 % in the edible (AA) Pisang mas banana, 13–25 % for Grand Nain of the Cavendish subgroup (AAA), 66.7 % in the highland African banana (AAA) (Namanya et al. 2004), and 100 % on the wild *M. a.* ssp. *malaccensis* (AA), respectively, (Table 21.4). With regard to non-conventional breeding (genetic transformation) for the pest and disease problems of banana and plantains worldwide, high germination and conversion rates underscore the fact that SE is essential in the development of in vitro regeneration systems which are a critical step for development of resistant varieties (Ghag et al. 2014a). So far, the data indicates that continuous work is needed for the development and optimization of SE protocols of many different cultivated clones.

21.7 Somaclonal Variation in Somatic Embryo Regenerated Plants

The in vitro culture environment, viz., type and concentration of applied plant growth regulators (PGRs), genetic background of the explant, and total number and duration of subcultures, can affect the properties of plants regenerated by somatic embryos (Konieczny et al. 2012). All these factors can contribute to the generation of genetic and epigenetic variation (Bairu et al. 2011) revealed in the phenotype, a phenomenon so-called somaclonal variation (SV) (Larkin and Scowcroft 1981). SV might be a pre-existent genetic variation in the explant due to changes in chromosome numbers, i.e., polyploidy and aneuploidy, chromosome structure

Table 21.4 Medium composition commonly used for somatic embryo germination in *Musa* spp.

Compounds/Plant material (genome group)	cv. Grand Naine(AAA)	cv. Grand Naine (AAA)	cv. Grand Naine (AAA)	cv. Dwarf Brazilian (AAB)	cv. Mas (AA)	*M. a.* ssp. *malaccensis* (AA)
Macro and mico-elements	MS	MS	MS	MS	MS	MS
KH_2PO_4 (mM)		1.47	1.47			1.47
Vitamins	MW	MS	MS	MS	MW	MW
AIA (μM)	1.1	11.4	1.14		1.1	
BA (μM)	0.2	2.22	0.22–1.0		0.8	
Sucrose (mM)	87	175	87	87	87	87
Gelrite (g L^{-1})	3	2	2	2	2	2
pH	5.7	5.8	5.8	5.8	5.7	5.8
	Ma4/M4	MG	GM	SK10	M4	MG
% Germination	3–20		35–46	90–96		90
% Conversion		13-25			13	100
Reference	Côte et al. (1996)					
	Navarro et al. (1997)	Youssef et al. (2010) com. Per. López-Gómez	Khalil and Elbanna (2004)	Jalil et al. (2003)	Escobedo-GraciaMedrano et al. (2014)	

(D'Amato 1990), or induced during the in vitro culture (Evans et al. 1984), in addition, mutations and epigenetic changes can take place at the DNA sequence level (Roux et al. 2004; Bairu et al. 2006; De-la-Peña et al. 2015). Banana SV have been reported to be associated with long-term cultures or cultures that involve a callus phase or high rates of multiplication treatments (Roux et al. 2004; Bairu et al. 2006). The decline in regeneration capacity of ECS cultures have been associated with cytogenetic instabilities in triploid (AAA, genome) Cavendish bananas, off-type regenerants from long-term Bluggoe suspension cultures (ABB, cooking banana) and subsequent loss of regeneration potential (Schoofs et al. 1998; Roux et al. 2005). Cytogenetically, regenerants from short-term SE showed genetic stability as compare to higher DNA amount found in emblings derived from longer-term cultures (Escobedo-GraciaMedrano et al. 2014). The genetic stability/instability evaluated by morphological and agronomical parameters have shown that variation is around 0.3–3.6 %, and molecular marker recorded a low variation (1.4–1.6 %) within the natural variation found in the mother plant used as explant source (Table 21.5). On the other hand, somaclonal variants are currently been used as a source for the selection of Fusarium resistance clones (Ghag et al. 2014b).

21.8 Banana Genetic Transformation Using ECS Cultures

In vitro culture of a wide range of commercial banana cultivars through SE using ECS cultures has been essential in developing reliable procedures for gene transfer in banana. Several groups working with different biotechnological approaches have succeeded in developing genetic transformation protocols taking advances of the available SE systems for the improvement of bananas (Crouch et al. 1998). These works have evaluated reporter genes, and transformation methods, i.e., DNA delivery by Agrobacterium, or particle bombardment using biolistic gun device and introduction of DNA into regenerable, ECS-derived protoplasts by electroporation (Sági et al. 2000). These procedures depend on the efficient regeneration of emblings from cells for delivery of genes that may confer resistance to biotic or abiotic stresses (Table 21.3). Protocols for ECS transformation by particle bombardment (Becker et al. 2000; Sagi et al. 1995) and co-cultivation with *Agrobacterium* are available for both, dessert and cooking bananas (Ganapathi et al. 2001a). The *Agrobacterium*-mediated transformation method may be more widely applicable as it offers advantages over direct gene transfer methodologies like particle bombardment and electroporation (Arvanitoyannis et al. 2008). The currently technology for banana transformation uses ECS cultures initiated form either scalps or immature male flower as starting plant material (Table 21.6), which have been applied to a range of banana cultivars and genotypes in which new genes (trans or cis) are successfully introduced. These approaches that include the expression of genes encoding plant, fungal, or bacterial hydrolytic enzymes

Table 21.5 Somaclonal variation in banana plants regenerated by in vitro somatic embryogenesis

Plant materials (genome composition)	Tissue/source of variation	Method of detection	Percentage (%) of variation	Reference
Musa acuminata (AAA) cv. Enano Gigante	Embryogenic culture and dicamba	Morphology	0.5 and 3.6 % with 22.6 μM and 31.7 μM dicamba, respectively	Shchukin et al. (1997)
M. a. (AAA) cv. Gran Naine	Embryogenic culture	Morphology	0.5–1.3 % of plants with variegated or deformed leaves; 0.5–2 % with fasciated-leafed	Côte et al. (2000)
Musa spp.	Embryogenic suspension cultures and age of culture	Flow cytometry	Different ploidy levels and DNA content related with loss of regeneration capacity	Roux et al. (2005)
Banana hybrid cultivar FHIA-18	Embryogenic suspension cultures	Morphology	0.13 % of variation morphological characters	Gómez Kosky et al. (2006)
M. a. (AAA) cv. Enano Gigante	Embryogenic culture	AFLP	1.4 %	Youssef et al. (2011)
M. a. (AAA) cv. Williams	Embryogenic culture	AFLP	1.6 %	Youssef et al. (2011)
M. a. (AA) Colla ssp. *malaccensis*	Embryogenic culture/short and long term 2,4-D exposure	Flow cytometry	Genetic stability in short-term regenerants/instability with higher DNA content in long-term culture regenerants	Escobedo-GraciaMedrano et al. (2014))
M. a. x *M. balbisiana* (AAB)	Embryogenic culture and age of culture	cDNA-RAPD	Somaclonal variants with low lipoxygenase A expression related to *Fusarium* resistance	Ghag et al. (2014b)

Table 21.6 Genetic transformation in different banana cultivars using embryogenic cell suspension (ECS) systems initiated from tissues of different origin

Cultivar (genome group)	Tissue (origin)	Expressed gene	Gene transfer technique	Modified trait	Transgenic plants	References
Williams (AAA), Three Hand Planty (AAB), Bluggoe (ABB)	ECS (scalps)	GUS	PB		+ + +	Sagi et al. (1995)
Three Hand Planty (AAB)	ECS (scalps)	Antimicrobial peptides	PB	Resistance to *Fusarium oxysporum* f. sp. *cubense* and *Mycospaerella fijiensis*	+	Remy et al. (1998)
Rasthali (AAB)	ECS (shoot tips)	GUS	AG		+	Ganapathi et al. (2001a)
Rasthali (AAB)	ECS (shoot tips)	MSI-99, a magainin analogue	AG	Resistance to *F. oxysporum* f. sp. *cubense* and *M. musicola*	+	Chakrabarti et al. (2003)
Rasthali (AAB)	ECS (shoot tips)	Hepatitis B antigen, HBsAg (pHBS, pHER, pEFEHBS)	AG	Edible vaccine against hepatitis B	+	Kumar et al. (2005)
Rasthali (AAB)	ECS (shoot tips)	Sm-AMP-DI gene (a Stellaria media defensin)	AG	Resistance to *Fusarium oxysporum*	+	Ghag et al. (2014a)
Gran Nain (AAA)	ECS (male flowers)	BBTV DNA-5 gene and the BBTV DNA-1 internal gene both encode proteins that may be involved in virus replication	PB	Virus Resistance	+	Becker et al. (2000)

(continued)

Table 21.6 (continued)

Cultivar (genome group)	Tissue (origin)	Expressed gene	Gene transfer technique	Modified trait	Transgenic plants	References
Gran Nain (AAA)	ECS (male flowers)	Protein engineered rice Cystatin (OcIdeltaD86)	AG	Resistance to nematode *Radopholus similis*	+	Atkinson et al. (2004)
Gran Nain (AAA)	ECS (male flowers)	Endochitinase gene ThEn-42 from Trichoderma with grape stilbene synthase (StSy) and superoxide dismutase gene Cu,Zn-SOD from tomato	PB	Tolerance to fungal diseases (*Botrytis cinérea*)	+	Vishnevetsky et al. (2011)
Gros Michell (AAA)	ECS (male flowers)	Rice chitinase genes	AG	Resistance to *M. fijiensis*	+	Kovács et al. (2013)

PB Particle bombardment, *AG Agrobacterium*

(Vishnevetsky et al. 2011), or the production of edible vaccine against hepatitis B (Kumar et al. 2005) are examples of topics in *Musa* research.

21.9 Conclusions

With regard to nonconventional breeding (genetic transformation) for pest and disease problems of banana and plantains worldwide, high germination of somatic embryos and emblings conversion rates underscore the fact that SE is essential in the progress of in vitro regeneration systems which are a critical step for development of resistant varieties. So far, the data indicates that continuous efforts are needed for the optimization of SE protocols of many different clones cultivated, the assessment of SV of emblings derived from different SE procedure is an important aim to underpin basic knowledge of the physiological, biochemical and molecular process that underlie the SE process and SV.

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Chapter 22
Somatic Embryogenesis in *Jatropha curcas*

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Abstract *Jatropha curcas* is an economically important member of the Euphorbiaceae family with numerous uses as a food source or fertilizer, as well as in the production of bioactive compounds and biodiesel. Propagation by seeds results in variation in the biochemistry of the plant, including oil productivity and other important compounds. In contrast, plant tissue culture offers the alternative approach of clonal propagation, which yields numerous genetically homogeneous plants. Although several studies associated with tissue culture in *J. curcas* have been published, the extensive genetic diversity of this semidomesticated plant makes it necessary to reevaluate and improve the established protocols with several genotypes. The application of herbicides with plant growth regulator activity could be useful for inducing somaclonal variation, which could then result in the addition of new agronomical traits. However, continuing studies in genetic diversity, molecular marker-assisted breeding, the production of secondary metabolites, and oils in in vitro cultures such as calli, suspension culture, and hairy roots are nec-

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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_22

essary to exploit the full potential of *J. curcas*. In this chapter, we will discuss recent studies of *J. curcas* plant tissue culture, as well as new research topics that will improve the efficiency of somatic embryogenesis.

22.1 Introduction

Embryogenesis is the process of formation of the embryo; its formation is a result of double fertilization event in the ovule, which gives two different structures. First one is zygotic embryo and second is endosperm; together they develop into a seed for propagation. In next generation, mature embryo germinates and gives rise to different tissues and organs and finally a whole plant. Alternatively, embryogenesis can take place without the involvement of fertilization. The origins of asexual embryos are diverse; apomictic embryos are formed from an unfertilized egg cell or from maternal tissue. It is also possible to obtain in vitro embryos from microspores and pollen grains, the androgenic embryos. Somatic embryos are developed from somatic cells in several species such as in leaves of the genus *Kalanchoe* (Garcês et al. 2007). Single plant cells can also be induced to develop an embryo by in vitro tissue culture methods. These asexual embryos grow into a new plant without seed coat or endosperm tissue. Somatic embryogenesis is the basis of cellular totipotency, which is unique to plants (Loyola-Vargas et al. 2008).

Somatic embryogenesis and plant tissue culture have been very useful tool for plant researchers, and they have multiple applications. (1) Massive propagation of genetically uniform plants, (2) Production of virus free plants, (3) Source explants in plant genetic engineering for crop improvement, (4) Embryo rescue and propagation of dangerous extinction plants, (5) Plant generation from single cell called protoplast, (6) In synthetic seed technology, (7) Germplasm collections, and (8) Production of secondary metabolites.

Any parts of a plant can be cultured; the basic procedure for producing somatic embryo involves the use of synthetic medium supplemented with a source of carbon, with plant growth regulators (phyto regulators), such as auxins, cytokinin, abscisic acid (ABA), or combination of two or several growth regulators; including synthetic herbicides can be added such as 2,4-dichlorophenoxy acetic acid (2,4-D) or picloram.

22.2 Factors Associated with Somatic Embryogenesis

Molecular studies during embryonic induction and development are an important prerequisite to improve the understanding and use of somatic embryogenesis. In vitro development of embryos depends on many factors which have the influence to a great extent. These factors include type of explant, age, genotype, and supplements medium and physical culture conditions (Gaj 2004). The main factors

involved in stimulating embryogenesis or any other morphogenetic process and the type of response (either direct or indirect) depend on: the source and physiological state (the ability to respond) of the explant, the major and minor salts of the synthetic medium (components), the type, concentration—doses-, and exposure time of the phyto regulators employed, status of endogenous phytohormones in explants (Loyola-Vargas et al. 2008). Plant growth regulators such as auxins and cytokinins have been used widely as a supplement to induce and control the cell cycle. In this cue, the 2,4-D and its high efficiency induce an embryonic response in highly responsive species and genotypes (Jiménez 2005). The role of endogenous hormones during somatic embryogenesis is fundamental, and the up- or down-regulation of genes in response to novel developmental signals is followed by changes in plant metabolism and physiology.

In order to continually adapt and survive, plants rely on their inherent phenotypic plasticity (Sultan 2000). Plants have a capacity of unlimited growth to overcome the loss from harmful conditions like abiotic and biotic stresses. During stress conditions, the meristematic tissues (i.e. stem cells) of plants are able to differentiate and give rise to new tissues and organs (Scheres 2007). Mostly biotic and abiotic stresses affect the plant growth by posing local damage which is overcome by tissue replacement and regenerates the plant parts. In addition, plants have an exceptional ability to entirely regenerate from a single cell. Different plant species have a different level of ability to induce this phenomenon and can be widely exploited for the propagation of economically important species (Loyola-Vargas and Vázquez-Flota 2006). Plants cells are more flexible in comparison to animal cells as they can be manipulated easily by dedifferentiation and redifferentiation under certain controlled conditions. Although many plant tissue culture techniques are available for vegetative propagation, direct embryo induction and development of complete plants by somatic embryogenesis are the most highly investigated types of plant regeneration. Nevertheless, somatic embryogenesis could be used to greater effect if the underlying molecular mechanism was better understood in different plant species.

There is still much debate over somatic embryo induction regarding how only a small group of differentiated cells from restricted genotypes in the same explant tend to reacquire the embryonic cell fate.

Since many years, researchers are working to unveil the cause of this mechanism. On the basis of their findings, they conclude that somatic embryogenesis may be a developmental stress response and that depends on the genotype capacity to respond to corresponding stress conditions (Dudits et al. 1991; Quiroz-Figueroa et al. 2006). The theory is already widely accepted, but the exact molecular mechanism controlling the embryo induction remains unknown and needs to be investigated for better exploitation and welfare (Fehér 2015). Finally, Genes related to various stress responses like salinity, heavy metals, wound responsive, water stress, etc., play an important role in the somatic embryogenic induction and suggest an adaptation process (Dudits et al. 1995).

22.3 Somatic Embryogenesis and Application in *Jatropha curcas*

Jatropha curcas is native of the American tropics, most likely Mexico and Central America, and is cultivated in tropical and subtropical regions around the world. In the last decade, *Jatropha curcas* has gained attention as an alternative source of renewable energy. Its cultivation is simple and it can grow in terrain ranging from waste/marginal lands to poor or salty soils with sand, gravel or stones. It has a great potential for adaptation and it can be propagated by seeds, although cuttings are a better approach since they grow faster. Yields are significant after 3 years of planting, although harvesting may begin after 12 months. Once planting is complete, yields can be collected for up to 30 years, the normal life expectancy of this species. Average seed production is between 3.5–4.0 t/ha and can reach up to 12 t/ha after 5 years depending on soil, nutrient and rainfall conditions (Openshaw 2000). Seed oil content can range from 8–54 % which gives an great opportunity for utilization of *Jatropha* oil as an alternative energy source (Ovando-Medina et al. 2011). For example, *Jatropha curcas* seed oil can be processed to produce a good quality biodiesel fuel that is readily usable in diesel engines. Briefly, this process involves transesterification, which is a simple chemical reaction that neutralizes the free fatty acids present in fatty substances. Glycerin settles down at the end of the process and gives a byproduct that is also very useful in many industrial purposes. The biomass residue, known as press cake, can be used as feedstock in power plants, and also as fertilizer since it contains nitrogen, phosphorus and potassium. Similarly, *Jatropha curcas* can be used in jet fuel production, and several recent exploits have succeeded in using varying blends of *Jatropha curcas* oil with conventional jet fuel (Belot 2009). In light of these data, *Jatropha curcas* has rightfully been referred to as a wonder plant and a promising biofuel crop, gaining much interest for the international research community. Researchers must now generate and ensure the availability of high-quality planting material. For instance, increasing the seed oil content is necessary to obtain higher and more economical yields. The most common method to obtain *Jatropha curcas* plantlets is by seed germination, which can be severely limited by poor seed viability, low germination percentage, inadequate rooting in growth plants in small pots and the delayed rooting of seedlings. Vegetative cuttings have also been utilized, although cuttings do not develop tap root systems (Openshaw 2000)

Micropropagation through the induction of multiple shoots or somatic embryogenesis has been used widely to obtain large quantities of high-quality healthy plant material. Two types of somatic embryo differentiation can be induced in *Jatropha curcas*. Research groups (Table 22.1) have made progress to develop efficient somatic embryogenesis protocols and healthy plant material. Here, we summarize several recent and important reports in this field.

Table 22.1 Recent plant tissue culture protocols of somatic embryogenesis in *Jatropha curcas* published

Medium	PGR (mg/L)	Explant	Type of somatic embryogenesis	Reference
MS	Picloram (1)	Embryo axis and Cotyledon	Indirect	Nindita et al. 2014
MS	2,4-D (0.5) and BA (5)	Leaf and Shoot tips	Direct	Medipally et al. 2014
MS	BA (0.5) and IBA (0.09)	Root, hypocotyl and leaf	Indirect and Direct	Galaz-Ávalos et al. 2012
MS	2,4-D	Immature zygotic embryos	Indirect	Cai et al. 2011
MS	Kin (0.5) and IBA (0.52)	Leaf explant	Indirect	Jha et al. 2007

MS = Murashis and Skoog medium, PGR = Plant growth regulator

22.4 Recent Advances in Somatic Embryogenesis Studies in *Jatropha Curcas*

Nindita et al. (2014) optimized a culture medium for the induction of indirect somatic embryos by using explants such as the embryo axis and cotyledons. *Jatropha curcas* seeds were collected from different regions of Indonesia. Explants were cultured in MS supplemented with different concentrations of the plant growth regulators 2,4-D (1–3 mg L^{-1}) and picloram (1–5 mg L^{-1}) for 45 days in the absence of light. Different types of calli varying in color and shape (Fig. 22.1) were obtained in these experiments. The two regulators were 100 % successful at inducing calli after 12 weeks, although the somatic embryo response was different. Only picloram (1 mg L^{-1}) was able to induce somatic embryogenesis in the embryo axis and cotyledons. In this study, the explants were found to be a major limiting factor for somatic embryogenesis, and the callogenesis and embryogenic responses were related to the concentration and type of plant growth regulator. It is important to reevaluate the protocol established in this study with other genotypes, which will be highly useful for future studies associated with *Jatropha curcas* breeding programs.

Recently Rudiyanto and Ermayanti (2014) developed an improved and simple method to induce somatic embryogenesis in *Jatropha* by using a combination of poly ethylene glycol (PEG) and sucrose. In this report, the germination (formation) of somatic embryos was depending on the concentration of sucrose and PEG treatments. On the medium containing sucrose at 20, 30, 40, and 50 g L^{-1} in combination with 0, 2.5, and 5 % of PEG, the embryos grew well. Increasing concentration of PEG more than 5 % adversely affects the growth of somatic embryos. In the medium containing 10 % of PEG at any concentration of sucrose and PEG at 15 % in combination with 20 and 30 g L^{-1} of sucrose, the colors of embryos were brown-yellow and had a small size of germinated embryos, while at

15 % PEG and 40 and 50 g L^{-1} of sucrose no embryo was germinated. In conclusion, medium supplemented with 20 or 30 g L^{-1} of sucrose with 5 % of PEG gave normal embryos on bipolar features with hypocotyl and epicotyl grew well and recorded the best combination with optimal somatic embryos development in *J. curcas*.

Manipulation of culture conditions during different developmental stages is important to change cell fate (Stasolla et al. 2003). Fitch (1993) reported the significant role of sucrose in culture medium to induce the somatic embryo in *Carica papaya*. Sucrose in medium contributes to enhancing photosynthetic capacity of embryos at very low concentration (0.2–0.4 %) whereas a high concentration of carbon sources affects the post-embryogenic development but not embryogenic induction (Rybczynski et al. 2007). PEG is known to stimulate water stress and has been used for somatic embryogenesis in various plant species. These data suggest a crosstalk between plant stress and morphogenetic signaling (Potters et al. 2007).

Medipally et al. (2014) reported an efficient method of direct embryo induction and development of plantlets from leaf and shoot tips of *J. curcas* recently. Wild plants were identified at Venkatapur village, Sircilla Mandal, Karimnagar district, Andhra Pradesh, India, and seeds were collected. Seeds were germinated, and explants were collected from 9-month-old plants. Explants were inoculated into sterile culture tubes containing MS medium supplemented with 3 % (w/v) sucrose, phyta-agar as gelling reagent and combinations of plant growth regulators. For somatic embryo induction, MS basal media was used with 2, 4-D (0.5 mg L^{-1}), BA (0.5 mg L^{-1}), Glutamine (684.2 μm), and citric acid (520.5 μm) [Medium-A]. Later, cultures were transferred to medium B [MS medium supplemented with BA (2 mg L^{-1}), Glu (684.2 μm) and citric acid (520.5 μm)] for conversion of somatic embryos to plantlets. Well-developed shoots were transferred to half strength MS supplemented with IBA (2 mg L^{-1}), Glu (684.2 μm) and citric acid (520.5 μm) [Medium-C] for root development. Each treatment was subcultured in every 10–12 days in a fresh medium. Fully developed plantlets were then hardened to pots and gradually exposed for acclimatization to the glasshouse and later to the field. Karimnagar genotype has been chosen in this study because this is the second highest oil producing genotype in India and also its oil has good biochemical characteristics suitable for biodiesel conversion (Naresh et al. 2012).

An efficient method was done in this regard for regeneration of *J. curcas* from somatic embryogenic calli based on suspension cultures (Cai et al. 2011). In this report, embryonic regeneration was optimized by minimal use of exogenous growth regulators in order to reduce somaclonal variations and modification of inorganic nitrogenous sources. Immature zygotic embryos were used as explants, and direct somatic embryogenesis was achieved at high frequency with 0.1–0.2 mg L^{-1} 2,4-D. Three different germplasm lines were collected from India, China, and Indonesia and tested for somatic embryogenesis. Developing fruits were surface sterilized, and zygotic embryos were extracted and used as explants. Zygotic embryos were selected on the basis of size, longer than 0.5 cm embryos were dissected into hypocotyl and cotyledons explants. Small embryos were very fragile, therefore, cultured intact. The enlarged and hardened embryos were cut at the

endosperm-embryo junctions and cultured. Subculture was done every 2–3 weeks. The explants were subsequently transferred to DGA medium for 1–2 month to induce somatic embryogenesis. Indirect somatic embryogenesis was achieved when endosperm tissue and immature embryos between 0.5 and 1.0 cm in length were cultured in a medium with 2,4-D, preferably at 5–10 mg L^{-1}, followed by a shift to a hormone-free medium supplemented with glutamine and asparagine. Production of secondary embryos was improved by supplementing KNO_3, glutamine, and asparagine. Growth regulator 2,4-D (0.1–0.2 mg L^{-1}) and polyethylene glycol (5–10 % w/v) were used to maintain the embryogenic callus in liquid culture medium. Regeneration of soil ready plants took as short as 3 months using the suspension cultures. Over 95 % of the regenerated trees were able to flower and set seeds with no discernable morphological abnormality. This regeneration method is expected to facilitate the development of a more efficient transformation system for *J. curcas*. In this interesting study, somatic embryogenesis was achieved efficiently by using immature zygotic embryos as explants and thousands of plants were generated. This protocol can be very useful for various approaches in genetic engineering experiments to develop improved varieties of *Jatropha*. This regeneration method has potential to serve as a great alternative for micropropagation and generation of quality planting material in large scale.

Jha et al. (2007) reported the development of embryogenic callus from leaves of *Jatropha*. Leaf explants were cultured on MS basal medium supplemented with 9.3 μM kinetin to obtain callus. These calli were subcultured with different combinations kinetin and IBA to obtain globular somatic embryos. The response was recorded and 2.3 μM kinetin with 1.0 μM IBA was selected as the most effective inducer of somatic embryogenesis. These mature somatic embryos were extracted and cultured on half strength MS medium in order to get plantlets. Plantlets were transferred to the fields effectively with 90 % of survival after 12–16 weeks. This method has been efficient and time saving with high-quality homozygous planting material (Fig. 22.1).

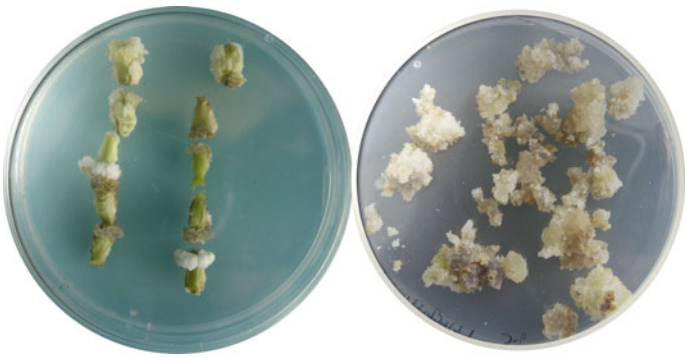

Fig. 22.1 Callus with different embryogenic responses, non-embryogenic, and embryogenic

22.5 Conclusion

Propagation of *Jatropha curcas* by seeds can result in oil concentration variations between 8–54 % (Ovando-Medina et al. 2011). In contrast, seed harvesting and quality screening is difficult and less efficient, and the lower oil content in seeds has disappointed producers after establishing the plants. Any significant amount of plant material that yields high-quality oil content has the potential to fulfill the current market requirement. Clonal propagation by somatic embryogenesis (Fig. 22.2) shoot buds (Fig. 22.3) will be important to rectifying the lack of a massive elite cultivar in *Jatropha curcas*.

Somatic embryogenesis, which was first reported in carrot callus cells more than five decades ago (Steward et al. 1958), is of great interest to researchers due to its capability of producing morphologically and developmentally similar plantlets.

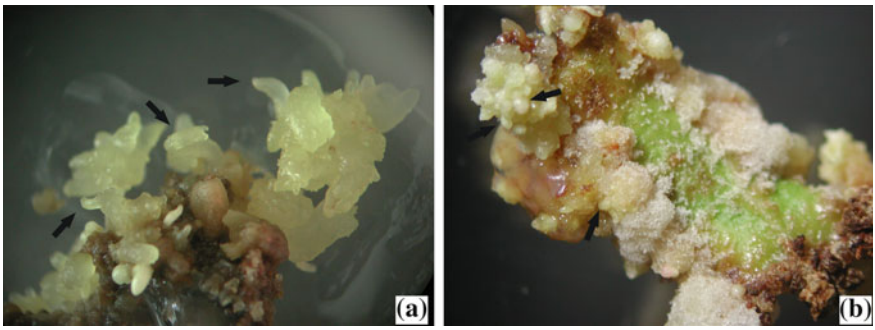

Fig. 22.2 *Jatropha curcas* embryogenic calli. The *arrows* indicate the presence of embryogenic structures. Pictures are a gift from the laboratory of Víctor M. Loyola-Vargas from Centro de Investigación Científica de Yucatán

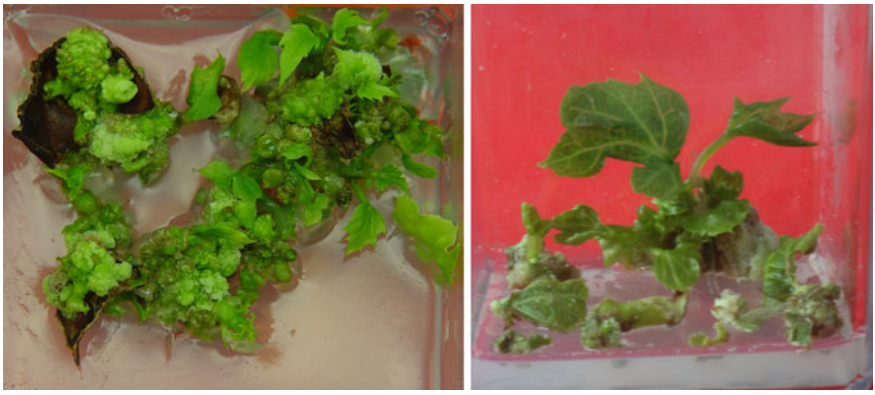

Fig. 22.3 Shoot bud system in *J. curcas*

Subsequently, this phenomenon was recognized for its potential in regeneration and micropropagation of important plants, as well as the study of early regulatory and morphogenetic events in plant embryogenesis. Research over the past decades from different explants and various plant species has helped reveal the pathway of this phenomenon, making it a highly useful system to study differentiation of a single somatic plant cell into a whole plant (Nomura et al. 1985). The establishment of suspension cell cultures has enabled the study of different physiological and biochemical characteristics such as the effects of plant growth regulators, growth parameters, protein and DNA synthesis, nutrient uptake, and responses towards the surrounding environment.

22.6 Perspectives

Jatropha curcas, a wide-spread tropical perennial plant, is an economically important member of the Euphorbiaceae family. *Jatropha* has a fast growth rate (even on degraded lands), is resistant to drought and disease, and is not grazed by animals (Pandey et al. 2012). In addition, this species has numerous uses in soil reclamation, erosion control, living fences, green manure, soap production and insecticides, and is furthermore a source of oil for biodiesel and a raw material for the pharmaceutical and cosmetic industries. Although there is a high occurrence of natural genetic variation within *Jatropha curcas*, its domestication will require concerted research efforts in its genetic diversity, adaptability to different environmental situations, the evaluation of agronomical traits, and conservation of diverse germplasms. There are two *Jatropha curcas* genotypes based on phorbol-ester accumulation. One is toxic, whereas the other is non-toxic and only found in Mexico. In natural environments, productivity can vary considerably from less than 100 kg to more than 10,000 kg of seeds per ha. This variation is important for future selection and breeding programs. Coupling these programs with the development of agronomical practices is essential for the economic exploitation of *Jatropha curcas* (Becker 2009). Plant tissue culture and micropropagation are important tools in modern biotechnology that make it possible to regenerate tissues, organs or whole plants under controlled nutritional and environmental conditions. Currently, plant tissue culture is used not only to obtain clones, but also to understand plant physiology and morphology, and to introduce new traits by tissue culture and Agrobacterium-mediated genetic transformation, for the production of secondary metabolites plant products at the commercial level. Molecular techniques such as next-generation sequencing, proteomics, metabolomics and lipidomics could be introduced to identify superior germplasms in marker-assisted breeding, and may be used to produce an unlimited number of genetically similar plants in a relatively short period of time and in a continuous process (independent of the season and space requirements). In addition, certain types of callus cultures can be achieved by tissue culture with different growth regulators, which can produce clones that differ from the parent plants due to somaclonal variability, leading to the

development of improved varieties. Somatic embryogenesis and other types of morphogenesis can vary according to their nutritional supplements (Saad et al. 2012). The culture medium generally contains the following components: macronutrients; micronutrients; a source of carbon; vitamins; nitrogen supplements such as amino acids; and plant growth regulators (with or without a gelling agent). The use of other carbohydrates such as glucose or fructose for somatic embryogenesis in *Jatropha curcas* has not been greatly explored. Vitamins are essential for optimal growth in every cell, and the most frequently supplemented types in other plant tissue culture systems are nicotinic acid, thiamin, pyridoxine, and myo-inisitol, which could improve the somatic embryogenic response or morphogenesis in *Jatropha curcas*. Amino acids provide a source of nitrogen that is more rapidly assimilated by plant tissues and cells than inorganic nitrogen sources (Saad et al. 2012) and which enhance cell growth (Torres 1989).

To establish somatic embryogenesis or morphogenesis in a new accession of *Jatropha curcas*, it is recommended to test the protocols using basal media such as MS, LS or B5 (with full-strength or reduced salts) combined with auxin or cytokinins as a plant growth regulator (Saad et al. 2012). Plant materials derived by micropropagation techniques provide several advantages over the traditional methods of propagation through seed, cutting or grafting. Indeed, the tissue culture techniques in *Jatropha curcas* described in this chapter represent a new horizon for plant biology research Table 22.1.

Acknowledgments The authors are grateful to the National Council on Science and Technology (CONACyT) for financing this research through project No. 146409 and No. 250738.

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Part III
Applications and Techniques

Chapter 23
The Uses of Somatic Embryogenesis for Genetic Transformation

Neftalí Ochoa-Alejo

Abstract Direct or indirect somatic embryogenesis protocols used for efficient *Agrobacterium*-, particle bombardment-, and chemical-mediated genetic transformation are revised in this chapter. Reported protocols for genetic transformation of important annual crops (corn, sorghum, rice, soybean, wheat, and sugarcane, among others) as well as perennials (*Pinus*, *Picea*, *Vitis*, *Hevea*, citrus, coffee, and several more), model plants (*Nicotiana* and *Daucus*), or pharmacologically attractive plants (opium poppy) are summarized. In general, a description of protocols developed with vectors bearing reporter and selectable gene markers is presented, and also the integration and expression of foreign genes for the protection of plant species against viruses, bacteria, fungi, and insects, or to enhance tolerance to herbicide or salt, and for producing recombinant proteins are described.

23.1 Introduction

Tissue culture techniques are fundamental for plant genetic transformation as an alternative for genetic or metabolic engineering of plant species and also for gene functional studies. Organogenesis is the most common morphogenetic process used for genetic transformation because it is the easiest way of regenerating plants in vitro from a number of species; however, somatic embryogenesis is theoretically most efficient than organogenesis since a larger number of plants can be generated once the whole process is standardized. The regeneration of plants in vitro via somatic embryogenesis has some distinct characteristics from those of organogenesis such as single-cell origin, the consequent low frequency of chimeras, and the production of a high number of regenerants. Considering that somatic embryos

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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_23

are formed without any fertilization event, they are genetically identical to the parent tissue and are therefore clones. Somatic embryogenesis is generally of two types: one is indirect somatic embryogenesis where the embryos form from callus phase; the other one is direct somatic embryogenesis where the embryos are formed from organized tissue without an intervening callus stage. Somatic embryogenesis is a very important tool in plant biotechnology and can be applied in a number of ways; for example, in genetic transformation studies, for studying molecular, regulatory, and morphogenetic events in plant embryogenesis, and for the production of large-scale plants from embryogenic lines. Utilization of direct and indirect somatic embryogenesis as efficient systems for genetic transformation of diverse plant species is presented.

23.2 Direct Somatic Embryogenesis Systems Used for Genetic Transformation

23.2.1 Agrobacterium-Mediated Transformation

Soybean is a very important leguminous crop worldwide. Breeding programs through modern techniques using plant genetic transformation should be of high value for improvement of this crop species. Immature zygotic embryos (5–8 mm in length) of soybean (*Glycine max* Merril.) from six cultivars were used to get immature cotyledons as explants for recovering transgenic plants through genetic transformation. Three *Agrobacterium tumefaciens* strains bearing the pCAMBIA1305.1 plasmid containing the *uidA* gene encoding the β-glucuronidase, the hygromycin resistance gene (*hpt*) for selection, and a maize chitinase gene were used for infection (Ko et al. 2003). It was observed that the cultivar, the *Agrobacterium* strain (KYRT1) and the orientation of the explant on the culture medium (the adaxial side in contact with the surface of the medium) were critical factors for an efficient transformation protocol (final transformation frequency of 1.1–1.7 %) by direct embryogenesis. GUS expression and Southern blot analyzes confirmed the stable integration of the introduced genes.

Tobacco, besides its commercial importance, has been a model for plant genetic engineering manipulation for a long time. An efficient protocol for in vitro plant regeneration is a fundamental requirement for genetic engineering looking for the expression of foreign proteins of interest such as those for vaccines. Pathi et al. (2013) described a protocol for direct somatic embryogenesis and its application for *Agrobacterium*-mediated genetic transformation of tobacco (*Nicotiana tabacum* L., *Nicotiana tabacum* cv. Xanthi, *N. tabacum* cv. Petit Havana and *Nicotiana benthamiana*). Explants from fully expanded leaves of 45-day-old aseptic seedlings were used for infection with *A. tumefaciens* strain LBA4404 harboring the plasmid

pCAMBIA1301 with the reporter *uidA* gene and *hpt* as selectable marker under the control of 35S promoter. Explants were immersed in an *Agrobacterium* suspension for 25–30 min in agitation, blotted on filter paper and cocultured for 3 days in the dark at 23–25 °C and then cultured on a preselection medium and later on regeneration MS medium (Murashige and Skoog 1962) with B5 vitamins (Gamborg et al. 1968) supplemented with 2.5 mg L^{-1} BA (benzyladenine) + 0.2 mg L^{-1} indoleacetic acid (IAA) + 2 % sucrose + 250 mg L^{-1} cefotaxime and 30 mg L^{-1} hygromycin. Incubation proceeded under a 16 h light/8 h dark photoperiod at 26 °C. Developed hygromycin-resistant somatic embryos were transferred onto a medium without growth regulators to promote elongation and rooting. Transformation efficiency was higher than 95 %. Foreign gene integration in the transgenics was confirmed by histochemical GUS assays and by PCR amplification of *uidA* gene using genomic DNA.

23.2.2 Particle Bombardment-Mediated Transformation

Girijashankar et al. (2005) established a genetic transformation protocol using particle bombardment and a direct somatic embryos regeneration system of sorghum (*Sorghum bicolor*) to recover transgenic plants expressing a synthetic *cry1Ac* gene from *Bacillus thuringiensis* to confer resistance to the spotted stem borer (*Chilo partellus*). Shoot apices of the genotype BTX623 from 7-day-old seedlings were incubated on MS basal medium with 0.4 M of both mannitol and sorbitol for 4 h and they were cobombarded with tungsten microparticles coated with pJS108 (with *bar* and *uid*A genes under the control of the 35S and the rice *actin1* gene promoters, respectively) and p*mpiC1cry1Ac* (*cry1Ac* gene under the control of the *mpi* gene wound-inducible promoter). Bombarded shoot apices were incubated overnight and then subcultured first on MS medium with 5 μM thidiazuron (TDZ) + 4 mg L^{-1} BA + 0.1 mg L^{-1} naphthaleneacetic acid (NAA) for somatic embryo stimulation and further on MS medium supplemented with 4 mg L^{-1} BA and 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and incubated under 16 h/8 h photoperiod for somatic embryo induction. Meristematic masses showing multiple buds were subcultured on MS medium with 4 mg L^{-1} BA and 0.1 mg L^{-1} NAA for somatic embryos germination. Finally, shoot elongation was promoted on MS medium with 1 mg L^{-1} BA and 0.5 mg L^{-1} indolebutyric acid (IBA). Plant regeneration through somatic embryogenesis starting from the bombarded shoots took about 16 weeks with a three-step selection pressure using 1, 2, and 2.5 mg L^{-1} BASTA. Regenerated plants were transferred to pots and grown under greenhouse conditions. The transgenic plants were characterized by RT-PCR amplification of *cry1Ac* using genomic DNA, Southern blot analysis to detect *cry1Ac* integration, and ELISA to determine Cry1Ac protein. Moreover, regenerated plants exhibited insect resistance (<50 % reduction in leaf feeding) when leaf disks were assayed in the presence of neonate larvae of spotted stem borer. Transformation efficiency was 1.5 %.

Snyman et al. (2006) published an optimized embryogenic sugarcane system for particle bombardment-mediated genetic transformation. Immature leaf roll disks with preemergent inflorescence of sugarcane cultivars 88H0019, NCo376, N12, and N27 (*Saccharum officinarum*) were surface sterilized, sliced transversely into sections, and these explants were cultured on MS medium with 0.6 mg L^{-1} 2,4-D and 0.5 mg L^{-1} casein and incubated in the dark at 28 °C. Subcultures were carried out at 2-week intervals. For transformation, the explants were pretreated for 4 h on semisolid MS medium supplemented with 0.2 M sorbitol and 0.2 M mannitol and bombarded with tungsten microparticles coated with plasmids pEnuKN and pHR1 harboring the *nptII* and the *CP4* (5-enolpyruvyl-shikimate-3-phosphate synthase conferring glyphosate resistance) genes. After tungsten microparticle bombardment, the explants were placed again on the medium with osmoticum for 4 h and transferred to the initiation medium and maintained for 3 days in the dark. Bombarded explants were cultured in the dark on the same medium supplemented with 45 mg L^{-1} geneticin as a selector. Explants were cultured for 6–8 weeks on the selective medium and then 6–8 weeks under a photoperiod of 16 h/8 h on the regeneration medium (same medium but without 2,4-D) with geneticin. Regenerated plants were cultured on half-strength MS medium with 5 g L^{-1} and geneticin in Magenta vessels for 4 weeks and then planted in pots and maintained in a greenhouse. Southern blot analysis of the *CP4* gene as the probe confirmed the transgenesis. Regenerated transgenic plants also exhibited resistance to glyphosate in the greenhouse.

An improvement in the efficiency of wheat (*Triticum aestivum*) microparticle bombardment transformation method was achieved by changing the content of ammonium nitrate in the culture medium to increase direct somatic embryogenesis formation frequency (Greer et al. 2009). Basically, scutellar tissue was exposed to elevated ammonium nitrate levels during culture. Aseptic immature scutella were bombarded with gold microparticles carrying a mixture of DNA with *bar*, *LUCIFERASE* and *ANTHOCYANIN* cassettes and cultured on DSEM control medium containing 1× level of nitrogen (22.91 mM = 2.06 mM NH_4NO_3 + 18.79 mM KNO_3), and on a medium with approximately 3× N (62.56 mM = 31.28 mM NH_4NO_3) and another one with *ca.* 6× N (125.10 mM = 62.55 mM NH_4NO_3). Explants were incubated in the dark at 23 °C for 14 days. Primary somatic embryos were excised and transferred onto the medium with 1× N (SEM) for further growth. Genomic DNA from T_0 regenerated plants was used for PCR analysis of *bar* gene; anthocyanin was detected by microscope observation. Gene expression of *bar* was carried out by RT-PCR in the BASTA tolerant regenerated plants. Transformation efficiency calculated as the number of PCR-positive plants in relation to the number of cultured scutella was higher as the level of N increased (1.7–12.1 %).

23.3 Indirect Somatic Embryogenesis Protocols Used for Genetic Transformation

23.3.1 Agrobacterium-Mediated Transformation

Agrobacterium tumefaciens-mediated genetic transformation of cotton (*Gossypium hirsutum*) was achieved using the line Coker 310FR, which readily regenerate plants through somatic embryogenesis (Chaudhary et al. 2003). Hypocotyls or cotyledonary leaves from 7-day-old seedlings were used as the explant sources. The explants were cocultured with *A. tumefaciens* strain GV3101 containing a binary vector with the *nos-nptII* gene and 35S *gus*-int gene, and successively cultured on semisolid MS medium with 3 % glucose and 100 μg L^{-1} 2,4-D + 500 μg L^{-1} kinetin (Kin) (named MST1) for 30–35 days to induce callus under selective conditions imposed by the presence of 50 mg L^{-1} kanamycin, two cycles of 30–40 days on MSOT2 medium (MST1 without 2,4-D) with 50 mg L^{-1} kanamycin to produce the embryogenic callus, which in turn was transferred to MSOT3 (MSOT2 with extra 1.9 g L^{-1} KNO_3) with 25 mg L^{-1} kanamycin and cultured for 45 days to get somatic embryos. These somatic embryos were cultured 1–2 cycles of 30–50 days on MSOT3 without kanamycin and reduced to a thin layer to allow slow desiccation of the embryos to promote shoot formation; then they were cultured once more on MSOT3 fresh medium for 15 days for shoot development. Regenerated transgenic shoots were finally excised from the embryo explant and grown on MS medium with 2 mg L^{-1} IBA or were grafted on wild-type rootstocks to get entire plants that were transferred to soil. Percentages of callus producing embryos ranged from 8.2 to 17.4, whereas the percentages of embryos forming shoots varied from 48.6 to 62.7 %. Southern blot and GUS analysis in the regenerated plants confirmed the transformation events.

Sairam et al. (2003) reported a protocol for somatic embryo regeneration from callus of maize (*Zea mays*) using shoot meristems from six hybrids and an inbred line as the source of explants. Callus tissue showing somatic embryos was produced on the explants cultured on MS medium with 5 mg L^{-1} 2,4-D after 3–4 weeks of incubation. Inbred line R23 showed the highest capacity to form callus tissue (higher than 80 %) and plant regeneration. When calli bearing somatic embryos were cultured on MS medium with myoinositol and glycine (100 mg L^{-1}), supplemented with BA (10 mg L^{-1}) and Kin (1 mg L^{-1}), they formed green plantlets that were adapted to greenhouse conditions. *Agrobacterium*-mediated transformation was carried out by independent coculture of shoot meristems with bacterial suspensions of three different strains [EHA105 (considered supervirulent), LBA4404, and GV3101] for 3 h and then incubated in the dark for 3–4 days. The infected shoot meristems were cultured on the callus induction medium containing carbenicillin (500 mg L^{-1}) and cefotaxime (250 mg L^{-1}), incubated in dark conditions and subcultured at 15-day intervals. Selected calli with somatic embryos were cultured on the previously described medium to promote further plant development. Southern blot analysis, GUS histochemical and PCR analysis of regenerated plants derived

from LBA4404 or EH105-infected shoot meristems or by the green fluorescent protein (GFP) expression analysis of plants from GV3101-infected shoot meristems confirmed their transgenic character. Interestingly, the percentage of genetic transformation efficiency was dependent on the plant material and the bacterial strain of *A. tumefaciens* (LBA4404>GV3101>EHA105). This protocol substantially reduced the time (4–6 weeks instead of 4–6 months) and increased the efficiency (60–87 % transference of T-DNA to shoot meristems after infection) to get transformed plants in comparison with those where immature zygotic embryos or inflorescences of maize are used as explants. Moreover, the regenerated transformed plants seemed to be derived from single-cell transformation events since all of them expressed GFP or GUS uniformly.

According to Charity et al. (2005) a consistent and stable expression of the *nptII*, *uidA*, and *bar* genes was achieved with an embryogenic tissue of *Pinus radiata* cultured on a nurse tissue after infection with the EHA105 strain of *A. tumefaciens* bearing the supervirulent helper plasmid pTOK47, which contains additional copies of the virulence genes *vir*B, *vir*C, and *vir*G. The binary vectors pCAMBIA3300 and pCAMBIA3301 were used to generate the binary vectors pKEA and pGUL containing the *bar* gene conferring resistance to phosphinothricin controlled by the 35S promoter; pKEA also contained a 35S-*uidA* gene cassette with an intron sequence. *A. tumefaciens* was transformed by electroporation with the created vectors and used to transform embryogenic tissue from two genotypes of *Pinus radiata* that was cultured on a medium with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BA 7 days before the *A. tumefaciens* infection. For this, the embryogenic tissue was suspended in liquid medium with no growth regulators and was inoculated with *A. tumefaciens* in the presence of acetosyringone (1 mg L^{-1}) and incubated for 1.5 h. The infected cells were collected with a Nybolt filter and then transferred onto the nurse culture on semisolid medium. The nurse culture consisted of four pieces of embryogenic tissue in a 3 × 3 cm^2 and a fifth piece in the center. After 5 days of coculture, the tissue was collected by filtration on a Nybolt filter and washed to remove the excess of bacterial cells. Rinsed cells were then suspended in liquid medium with timentin (200 mg L^{-1}) to inhibit bacterial growth and samples were distributed on Whatman No 1 filters, which were placed on the nurse system and cultured for 5–7 days. The filters were transferred to semisolid medium to allow plant regeneration in the presence of 200 mg L^{-1} timetin and five to ten mg L^{-1} geneticin as a selector. Regenerated transgenic plants were analyzed for *nptII*, *uidA,* and *bar* expression, and the presence of *nptII*, *uidA* in the genomic DNA by PCR, Southern blot, and also for their resistance to ammonium glufosinate.

Blanc et al. (2006) established an efficient *Agrobacterium*-mediated transformation of embryogenic callus tissue from clone PB 260 of *Hevea brasiliensis* Müll Arg. Tissues from immature fruits were used as the starting material to induce friable callus on a medium developed for *Hevea* containing 4.5 μM Kin, 4.5 μM 2,4-D, 30 μM $AgNO_3$, 12 mM $CaCl_2$, and 234 mM sucrose. Cultures were transferred at 15-day intervals on a maintenance medium (MM) with a composition similar to the initiation medium, but with 9 mM $CaCl_2$, 1.34 μM BA, 1.34 μM 2,4-D, and 0.5 μM abscisic acid (ABA). Friable callus masses were precultured on

a $CaCl_2$-free maintenance medium with 4.5 μM BA and 4.5 μM 2,4-D for 15 days before infection with *Agrobacterium* (strain EHA 105 harboring the plasmid pCAMBIA2301 with the *uidA* and *nptII* genes under the control of the 35S promoter). Precultured calli were cocultured with the *Agrobacterium* suspension for 2–12 days and then cultured on the MM medium with 500 mg L^{-1} ticarcillin to eliminate the bacteria. After two subcultures at 3-week interval, ticarcillin concentration in the medium was reduced to 250 mg L^{-1} and increasing levels of paromomycin (from 50 to 100 mg L^{-1}) were added as a selector at the third to the sixth subculture. Resistant calli were analyzed for GUS activity, and those that gave positive reaction were further cultured on the MM medium with 0.44 μM 2,4-D + 0.44 μM 2,4-D with no $AgNO_3$, 60 g L^{-1} maltose, 20 g L^{-1} sucrose, and 250 mg L^{-1} ticarcillin to promote plant regeneration. Selected resistant calli were subcultured twice in a temporary immersion culture system (RITA) at 1-month interval in the same medium but with no growth regulators neither ticarcillin (DEV) for somatic embryos development. Somatic embryos were subcultured on semisolid DEV medium in glass tubes to promote germination. Resulting plants (372) were developed and acclimatized (93) under greenhouse conditions. Leaves from the acclimatized plants showed GUS positive reaction.

Embryogenic cultures of *Vitis rotundifolia* established from in vitro grown shoot cultures were used to develop a transformation protocol using *A. tumefaciens* infection (Dhekney et al. 2008). Leaf explants from in vitro shoots were inoculated on Nitsch and Nitsch medium (Nitsch and Nitsch 1969) with 9 μM 2,4-D and 4.4 μM BA and incubated at 26 °C in dark conditions to induce callus formation. Callus tissue was subsequently cultured on MS without glycine and supplemented with 10.7 μM NAA and 0.9 μM BA under the light. Proembryogenic tissues were then cultured on MS medium lacking glycine but supplemented with 3.03 g L^{-1} KNO_3 and 0.364 g L^{-1} NH_4Cl, 60 g L^{-1} sucrose, 1.0 g myoinositol, and 0.5 g L^{-1} activated charcoal. This proembryogenic tissue was inoculated in liquid medium (MS minor salts + B5 major salts, 400 mg L^{-1} glutamine, 60 g L^{-1} sucrose and 4.5 μM 2,4-D) to establish cell suspensions. Proembryogenic cells were cocultured for 8 min with a suspension of *A. tumefaciens* strain EHA105 bearing a plasmid with a translational fusion between *GFP* gene and *nptII* under the control of a double promoter CsVMV (Cassava Vein Mosaic Virus). After coculture, the excess of bacterial suspension was eliminated with filter paper, and the proembryogenic cells were transferred to Petri dishes with a double layer of filter paper soaked with liquid medium and incubated for 3 days in the dark at 26 °C and subsequently cultured on regeneration medium with kanamycin (75–100 mg L^{-1}). GFP was used to follow the transformation process from the infection of proembryogenic tissue with *A. tumefaciens* till the regenerated plants. Molecular analysis (PCR and Southern blot) complemented the characterization of the transgenic plants.

Opium poppy (*Papaver somniferum* L.) is a plant of pharmacological interest due to its medicinal uses (narcotic analgesics). Application of biotechnological approaches requires the development of in vitro plant regeneration and transformation systems (Facchini et al. 2008). A genetic transformation protocol was developed using root explants from 7-day-old aseptic seedling precultured for

3–4 weeks on a medium constituted by the MS salts and the organic components of White medium (White 1967) with 2.5 μM 2,4-D and 2.5 μM BA, 0.5 g L^{-1} myoinositol and 2.5 % sucrose for callus induction. These explants were wounded with a scalpel blade in the presence of strain EHA105 of *A. tumefaciens* with the pCAMBIA3301 plasmid bearing the *pat* (phosphinothricin acetyltransferase) gene under the control of a tandem repeat of 35S promoter and the reporter *uidA* gene for β-glucuronidase driven by a single 35S promoter. Coculture with *A. tumefaciens* proceeded for 8 h at 75 rpm on a rotary shaker in the B5 medium with 1 % glucose, 2 % sucrose, 50 μM ATP, 50 μM $MgCl_2$, 2.5 μM 2,4-D, and 2.5 μM BA. After coculture, the tissues were rinsed with cocultivation medium 300 m L^{-1} timentin to eliminate the bacteria and were incubated for 7 days in the dark. These treated tissues were cultured on the callus induction medium supplemented with 10 mg L^{-1} phosphinothricin and 300 mg L^{-1} timentin. Proliferating callus was subcultured at 4-week intervals for 3 months to select herbicide-resistant callus. Portions of callus tissue were transferred onto a medium containing 5 μM 2,4-D for 4–6 weeks to induce globular callus which was then incubated on the callus-inducing medium without growth regulators and with 3 % sucrose and 0.5 g L^{-1} glutamine to form somatic embryos in the dark and 24 °C. Somatic embryos at the cotyledonary stage were treated with 500 μM IBA for 5 h and incubated with a photoperiod of 16 h light/8 h dark at 15 °C and subsequently cultured on filter paper placed on the surface of MS semisolid medium supplemented with 2 % sucrose in Magenta boxes to promote conversion into plantlets. Characterization of regenerated transgenic plants included the expression of both the gene conferring herbicide resistance and the reporter one. Additionally, an enzyme assay of PAT activity was carried out with tissues of the regenerated plants and also the histochemical staining of GUS.

Cryptomeria japonica D. Don, known as sugi, is a forest tree of economical importance in Japan because the wood is used for construction and timber. Genetic engineering is looking for the manipulation of pollen production to avoid sugi pollinosis, an allergenic disease; this requires the establishment of efficient protocols for in vitro plant regeneration and transformation. Seeds from immature cones of *C. japonica* were surface sterilized, and the megagametophytes with precotyledonary zygotic embryos were dissected and cultured on half-strength MS medium with 10 μM 2,4-D (induction medium; IM) to induce embryogenic tissue (Taniguchi et al. 2008). After 4 weeks the tissue was subcultured at 2-week intervals on the maintenance medium (MM) consisting of the IM supplemented with a reduced level of 2,4-D (2 μM), 1 g L^{-1} casein hydrolyzate, 0.5 g L^{-1} glutamine and 50 % of the inorganic nitrate salts of the IM and the incubation proceeded in the dark at 25 °C. Vector UbiP-sGFP(S65)/HygR was used to transform the *A. tumefaciens* C58/pMP90 strain, which was utilized to infect by coculture (2 days) embryogenic tissues grown on the MM for 1 week. After coculture the embryogenic tissue was washed with liquid MM, collected on a filter paper, cultured on MM containing 250 mg L^{-1} carbenicillin for 3 days, and transferred to fresh medium every 2 weeks. Once the tissue grown was cultured on the MM with 250 mg L^{-1} carbenicillin and 25 mg L^{-1} kanamycin or 5 mg L^{-1} hygromycin

(selection medium; SM), subcultures proceeded every 2 weeks. Recovered colonies were grown on maturation medium (Smith 1996) with two g L^{-1} activated charcoal, 100 μM ABA, 150 g L^{-1} polyethylene glycol 4000 and 30 g L^{-1} maltose, and were incubated in the dark at 25 °C for 8 weeks. Germination of somatic embryos was induced on a modified Gresshoff and Doy medium (Okamura and Kondo 1995) supplemented with ten g L^{-1} sucrose and incubated under a 16 h light/8 h dark photoperiod at 25 °C. Regenerated plants were transferred to pots and maintained in the greenhouse. GFP was visualized by fluorescence microscopy in the regenerated plants, whereas the *sgfp* and *hpt* genes were amplified by PCR using the genomic DNA to verify their integration; moreover, Southern blot analysis confirmed the integration of the *sgfp* gene. The transformation efficiency of this protocol was eight events per gram (fresh weight) of embryogenic tissue.

Genetic transformation of plants has been used to express genes encoding bacterial toxins as an approach for vaccine production (Kim et al. 2009); this is the case of the cholera toxin B subunit (CTB) produced by *Vibrio cholerae*. Hypocotyl explants from axenic carrot seedlings were precultured on MS medium with 1.0 mg L^{-1} 2,4-D and after 2 days of incubation were cocultured for 2 days with *A. tumefaciens* strain LBA4404 harboring the vector pMYO53 consisting of *sCTB* gene with an endoplasmic reticulum retention signal peptide under the control of 35S promoter. Infected explants were transferred onto the same medium supplemented with 50 mg L^{-1} kanamycin and 300 mg L^{-1} cefotaxime. Subcultures on fresh medium proceeded every 4 weeks. Somatic embryos produced from the embryogenic callus were cultured on MS medium without 2,4-D containing the mentioned antibiotics. Regenerated plantlets (5–7 cm in length) were grown in pots with soil. The presence of *sCTB* gene in the transgenic plants was confirmed by PCR in the genomic DNA, and the toxin was detected in the mature roots by immunoblot and quantitatively analyzed by ELISA. The affinity of the expressed sCTB protein for its receptor (GM_1-ganglioside) was demonstrated by GM_1-ELISA.

Tapia et al. (2009) published the use of proembryogenic masses of grapevine (*Vitis vinifera*; 'Thompson Seedless') cultured in a semiautomatized airlift bioreactor as an efficient tool to recover transgenic plants. Proembryogenic and embryogenic masses were established and maintained on X5 and DM mediums (a modified B5 medium), respectively, and incubated at 21 °C. Liquid immersion cultures were started with 100 mg of proembryogenic masses inoculated into 250 mL flasks with 80 mL of induction medium (IM), B5 macronutrients, MS micronutrients and organic additives + 4.5 μM 2,4-D, 400 mg L^{-1} glutamine, 0.5 g L^{-1} activated charcoal, and 60 g L^{-1} sucrose. Once established, these cultures were subcultured three times at 14-day intervals and then inoculated into liquid maintenance medium (MM) with the same composition as IM, but with 1 μM BA instead of 2,4-D, and they were subcultured under the same schedule. Batches of 2.5 g of embryogenic masses were used for airlift (2 L operative volumetric value) characterization and genetic transformation experiments. The genetic transformation was conducted with *A. tumefaciens* EHA105 harboring the pCAMBIA vector bearing the *gfp* gene or a modified pCAMBIA2300-34S-Chi33 with the *chitinase 33* gene and using 2.5 g of cells

cultured for 7 days in the airlift bioreactor. Regenerated plantlets were analyzed by PCR of the *nptII* gene. Transformation efficiencies using the airlift bioreactor were 56 % PCR-positive plantlets from 33.6 % regenerated plantlets in the case of pCAMBIA2202, and 34.4 % out of 52.4 % for those of pCAMBIA2300-34S-Chi33.

Genetic improvement of citrus species requires the conjunction of conventional techniques and genetic transformation. In this regard, Dutt and Grosser (2010) developed a genetic transformation protocol for several citrus cultivars even some of them considered recalcitrant. Embryogenic cultures were initiated using unfertilized ovules dissected from surface-sterilized fruits of the following species and cultivars: *Citrus sinensis* Osbeck cvs. 'Hamlin', 'Valencia' and OLL8; *C. reticulata* Blanco cvs. 'Ponkan' and 'W Murcott'; *C. amblycarpa* (Hassk.) Ochse; and *C. depressa* Hayata cv. 'Shekwasha'. These unfertilized ovules were cultured on an embryogenic callus-inducing medium based on the MT medium (Murashige and Tucker 1969) with five mg L^{-1} Kin, 500 mg L^{-1} malt extract, and 50 g L^{-1} sucrose. After induction, embryogenic callus was maintained on the induction medium without Kin and malt extract. Embryogenic cell suspensions were established with about five grams of callus inoculated in 25 ml of liquid medium to allow cell proliferation. Cell suspensions after three 2-week subcultures were cocultured for 10 min with *A. tumefaciens* EHA105 [pCAMBIA1300 (with *hptII* as selectable gene marker) or pCAMBIA (with *nptII* gene) harboring the *egfp* gene driven by the 35S promoter were used as vectors for genetic transformation], then blotted dry on filter paper and plated on a medium with maltose substituting sucrose and supplemented with 100 μM acetosyringone and incubated in the dark at 25 °C for 5 days. Transformed embryogenic cells were selected by culturing in a liquid medium with 25 mg L^{-1} hygromycin B or 100 mg L^{-1} kanamycin and 400 mg L^{-1} timentin. The embryogenic cultures were incubated under photoperiod (16 h light/8 h dark) at 28 °C and subcultured every 2 weeks. After the second subculture, cells were inoculated on semisolid medium with a layer of liquid medium supplemented with 25 mg L^{-1} hygromycin B and 400 mg L^{-1} timentin. Developing embryos were observed under a fluorescence microscope to identify those EGFP positives. Globular embryos were cultured on 0.22 μm cellulose acetate membrane filters on semisolid medium to promote further growth. Transgenic embryos were matured, germinated, and the plantlets were rooted and transferred to soil and grown under greenhouse conditions. Shoots that did not form roots in vitro were micrografted onto Carrizo or Orange 16 rootstocks. Transgene integration in the citrus genome was demonstrated by PCR amplification of *htpII* gene in the genomic DNA, by Southern blot using a DIG-labeled *egfp* probe and by the fluorescence of EGFP protein. In general, an average of transgenic embryo production ranged between 15 and 53 per callus, and recovery of transgenic plantlets was dependent on the cultivar.

Papaya ringspot virus (PRSV) and *Papaya leaf distortion mosaic virus* (PLDMV) infections very often affect papaya (*Carica papaya* L.) production. An alternative approach to face this problem is to develop resistant cultivars to these viruses using genetic engineering techniques. An untranslatable chimeric construct (pYP08) with the truncated PRSV and PLSMV coat protein genes coupled with the

3' nontranslated region of PLDMV was used to transform embryogenic cultures of papaya cultivars 'Tainung No 2,' 'Sunrise,' and 'Thailand' with *A. tumefaciens*. Shoot tips from 1-year-old hermaphrodite papaya plants were surface sterilized and cultured to induce multiple shoots, which were dissected and grown on MS medium with 0.5 mg L^{-1} IBA for 1 week in dark conditions to induce adventitious rooting (Kung et al. 2010). Treated shoots were transferred to perlite with half the volume of MS basal medium to stimulate the development of adventitious roots, which were the source of explants for somatic embryogenesis. Segments of roots were cultured on the induction medium (IM4) consisting of MS medium supplemented with 1 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BA and incubated at 28 °C in the dark. Tissues were subcultured every month. The somatic embryos produced from root tips of adventitious roots were inoculated onto the embryo-inducing medium with 4 mg L^{-1} 2,4-D and subcultured three times (monthly intervals) to increase the embryogenic tissues for genetic transformation. Portions of three to four grams of embryogenic tissue were wounded with carborundum in a vortex and infected with a suspension of *A. tumefaciens* for 5 min. Excess of bacteria was eliminated, and the treated tissues were cultured for 20 days on induction medium (IM4) supplemented with 500 mg L^{-1} carbenicillin and then subcultured initially on the same medium with 50 mg L^{-1} kanamycin for 30 days and 100 mg L^{-1} for 60 days. After selection, the tissues were cultured on the same medium but without kanamycin to stimulate somatic embryo development (embryo maturation). Matured somatic embryos were cultured for a week on the shoot induction medium (MS with 0.5 mg L^{-1} IBA) and subsequently transferred to vermiculite with ½ volume of MS basal medium to promote rooting. Regenerated plants were analyzed by PCR to confirm the genomic integration of the foreign viral genes and also of the *nptII* gene; Southern blot analysis using an *nptII* probe demonstrated its integration. Moreover, transgenic plants were evaluated for their resistance to PRSV or PLDMV and they showed different levels of resistance. The transformation efficiency was variety dependent and ranged between 3.7 and 37.6 % with an average of 31.2 %.

Coffee is an important industrial crop whose genome has been already published. In order to apply genetic engineering approaches to crop improvement, it is imperative to develop efficient regeneration and transformation protocols. According to Ribas et al. (2011), an efficient system for *Agrobacterium*-mediated genetic transformation was established using a long-term *Coffea arabica* (L.) embryogenic callus cultures. Aseptic leaf explants from var. Caturra trees were used to establish the embryogenic cultures on half-strength MS medium with 2.26 μM 2,4-D + 4.92 μM IBA and 9.84 μM 2-isopentenyladenine (2iP). After 1 month, callus tissue was cultured on the embryogenic callus induction medium with 4.52 μM 2,4-D and 17.76 μM BA for 6–8 months until regeneration of yellow embryogenic callus in the dark at 27 °C. *A. tumefaciens* LBA1119 harboring the vector pBIN35SGFP with the *gfp2* reporter gene driven by the 35S promoter was used for genetic transformation. The binary vector pMDC32 carrying the *hptII* hygromycin resistance gene was used as the selectable marker. Embryogenic calli from 7-month-old cultures were cocultured with *A. tumefaciens*, and after

decontamination, the cultures were subcultured twice at 4-week intervals on regeneration medium with 17.76 μM BA + 50 mg L^{-1} hygromycin and decreasing levels of cefotaxime (125, 250 mg L^{-1}) and also twice on maturation medium with 1.35 μM BA, 100 mg L^{-1} hygromycin, and 125 mg L^{-1} cefotaxime, and further subcultures were on the same medium without hygromycin and cefotaxime to allow plant development. Transgenesis was followed by GFP analysis during selection and regeneration processes. Regenerated plants were acclimatized and analyzed. Leaf tissue from 60 regenerated plants was analyzed by PCR to detect the *hpt* gene in the genomic DNA, and the positive ones were also analyzed by Southern blot. This method was highly efficient for recovering transgenic plants since from 560 cocultured calli, 462 produced yellow resistant calli representing an efficiency of 82.5 %, and several plantlets were regenerated from each resistant callus.

Sweet potato (*Ipomoea batatas* Lam.) produces starchy storage roots used as a source of food, animal feed, and industrial raw materials. Aseptic apical or axillary buds from 13 cultivars were cultured on embryogenic callus-inducing medium with 2 mg L^{-1} 2,4-D under light (Yang et al. 2011). Generated callus embryogenic tissues from ten cultivars were used to establish cell suspensions in a liquid medium and maintained by subcultures in fresh medium twice a week for 4–24 weeks. Embryogenic cell cultures from seven cultivars were tested for genetic transformation capacity. For this goal, the *A. tumefaciens* strain LBA4404 carrying a binary vector pCAMBIA1301 with the *uidA* gene interrupted by an intron and the *htp* gene conferring resistance to hygromycin under the control of 35S promoter was used for transformation assays. Embryogenic calli from cell cultures were cocultured with an *A. tumefaciens* suspension under vacuum for 10–20 min, then they were dried on filter paper and transferred onto a filter paper placed on the semisolid coculture medium with 200 μM acetosyringone and incubated for 4–7 days under 16 h light/8 h dark photoperiod and at 25 °C. Calli were rinsed before transfer to the selection medium containing 10 mg L^{-1} hygromycin and 200 mg L^{-1} cefotaxime. Hygromycin-resistant calli or somatic embryos were cultured on fresh medium with 200 mg L^{-1} cefotaxime to regenerate plants. Resistant calli were analyzed for histochemical GUS assay. Regeneration efficiency after the selection was cultivar dependent, and high capacity was observed in three cultivars. 379 plant lines were randomly selected from six cultivars and tested for rooting in the presence of hygromycin, among them 350 (92.4 %) showed root formation. GUS assay and Southern blot analysis confirmed the transgenic events.

Sweet chestnut or European chestnut (*Castanea sativa* Mill.) is a tree species of economic importance distributed in Europe. Ink disease caused by *Phytophthora cinnamomi* and *Phytophthora cambivora* affects this species in a destructive manner. In an approach to develop resistant plants a native gene encoding a thaumatin-like protein (*CsTL1*) was isolated from cotyledons of *C. sativa* and was expressed constitutively in this recalcitrant species through an *Agrobacterium*-mediated transformation system using embryogenic cultures (Corredoira et al. 2012). Leaf explants from shoot cultures were used to generate the embryogenic line C12-H1, whereas zygotic embryos were the explants source to establish the embryogenic lines Cl-3 and Cl-9. Embryogenic cultures were generated and maintained on

half-strength MS medium with 0.44 μM BA + 0.49 μM NAA + 3 mM glutamine and incubated under 16 h light/8 h dark photoperiod at 25 °C light/20 °C dark. Small clumps of somatic embryos at the globular or early torpedo stages were precultured on the proliferation medium without growth regulators for 24 h and then infected for 30 min with a suspension of *A. tumefaciens* EHA105 with the vector pK7WG2D-TAU bearing *CsTL1* and *EGFP* genes driven by independent 35S promoter, and *nptII* gene for kanamycin resistance under the control of *Nos* promoter. After coculture, the explants were blot-dried on filter paper and cultured on proliferation medium with growth regulators in the dark and at 25 °C. The explants were rinsed with a solution of 500 mg L^{-1} cefotaxime for 30 min, dried on filter paper and inoculated onto Petri dishes with proliferation medium supplemented with 150 mg L^{-1} kanamycin, 200 mg L^{-1} cefotaxime and 300 mg L^{-1} carbenicillin. After 12 weeks under selection conditions, putative somatic embryo transformants were observed for the fluorescence of GFP. Transformation efficiency is given by the percentage of initial explants that exhibited positive fluorescence which was dependent on the cell line tested and ranged between 7.1 % for the Cl-3 cell line and 32.5 for the Cl-9 line. One hundred and twenty-six independent transformed lines were selected. Analysis of *CsTL12* expression by qPCR revealed differences among the transgenic lines with a maximum of 13.5-fold increase. Southern blot and PCR analysis confirmed the integration of *CsTL1* gene into the genome of selected lines. Transgenic plants were recovered after maturation and germination of transformed somatic embryos.

Rice (*Oryza sativa*) is undoubtedly one of the major cereal crops whose genome is publicly available for comparative genetic studies. Genetic transformation is a key tool for gene function in rice and also for crop improvement programs. Bevitori et al. (2014) characterized the induction of embryogenic calli from three Brazilian rice cultivars (BRS Primavera, BRS Bonança, and BRS Caiapó) by histological analysis and scanning electron microscopy (SEM) as an approach to establish a suitable transformation system. Embryogenic units (EUs) from 2-week-old cut proliferated calli were used to test the suitability for *Agrobacterium*-mediated transformation. Dehulled sterilized seeds were the explants to generate callus tissue on an induction medium (IM) based on the N6 medium formulation (Chu et al. 1975) supplemented with 2.5 mg L^{-1} 2,4-D, 500 mg L^{-1} proline, 100 mg L^{-1} myoinositol, 6 mM glutamine, 0.1 mM glycine, 2 mM asparagine, and 30 g L^{-1} maltose. Explants were incubated at 28 °C in the dark. Calli formed in 2 weeks on the scutellum were removed and prepared for SEM and histological observation. After 4 weeks of incubation, embryogenic nodular units (EUs1) released from the primary calli formed on the embryo scutellum were subcultured on IM medium for 10 days and then prepared for SEM observation. Production of embryogenic units from proliferated 2-week-old calli (EUs2) was established with individual pieces of EUs1 cut into four pieces; each piece was cultured on fresh IM medium for 2 weeks. EUs2 were infected for 15 min with *A. tumefaciens* LBA4404 harboring the vector pCAMBIA1305.1 containing the reporter *uidA* gene and the selectable marker *hptII* gene driven by the 35S promoter. Additionally, this vector included a *gomesine* gene from *Acanthoscurria gomesiana* hemocytes driven by the maize

ubiquitin gene promoter. Gomesine is a peptide that strongly affects bacterial growth, as well as the development of filamentous fungi and yeast. Calli selected in the presence of hygromycin were stained for histochemical GUS assays. Plant regeneration of putative transgenics from EUs occurred at 58 % frequency. Insertion of *gomesine* gene was demonstrated in 85 % of the regenerated plants by PCR amplification using genomic DNA.

Fu et al. (2015) explored the possibility of recombinant protein production in cultures of somatic embryos from alfalfa (*Medicago sativa*) via genetic engineering. Cholera toxin B subunit (*ctb*) and human interleukin 13 (*hI L-13*) (a secreted T cell-derived cytokine, which has an important role in the regulation of inflammatory and immune responses in humans) and *uidA* genes were independently introduced into alfalfa somatic embryos by an *Agrobacterium*-mediated transformation protocol. Initially, petiole explants from aseptic seedlings of alfalfa genotype N4.4.2 with high somatic embryogenesis capacity were independently infected with *A. tumefaciens* GV3010/pMP90 with vector pCAMBIA2301 bearing each gene of interest driven by a dual-enhancer 35S promoter and *nptII* gene as selectable marker. The construct with *hI L-13* was fused to *gfp* gene. After infection, the petiole explants were cultured on SH2 K medium (Schenk and Hildebrandt 1972) for embryogenic calli induction in the presence of kanamycin and subsequently inoculated onto BOi2Y semisolid medium (Blaydes 1966) with kanamycin for transgenic embryo generation. Developed embryos were cultured on MS medium with kanamycin for germination and rooting and finally transferred to half-strength MS medium for plant development. Regenerated plants were analyzed by PCR amplification of *nptII* and *uidA* genes to confirm their integration into the genome. Transformation efficiency was very high since 38 out of 41 analyzed plants gave positive for the presence of *ctb*, whereas 14 out of 15 were positives for *hI L-13* and 12 out of 12 gave positive for *uidA*. Explants from regenerated transgenic plants with *ctb*, *hI L-13,* and *uidA* genes were used to induce somatic embryos, which were analyzed by Western blot and ELISA, and fluorometric or histochemical assay, respectively, for their capacity to produce the corresponding proteins. In general, somatic embryos derived from transgenic plants accumulated higher recombinant protein levels than vegetative tissues (roots, stems, and leaves). These results indicate that transgenic somatic embryos could be a good alternative system for the production of heterologous proteins of interest at high yields.

Cassava (*Manihot esculenta* Crantz) is a tropical crop important for staple food production. Genetic improvement and functional genomics of cassava have been limited for low-efficiency transformation protocols. Nyaboga et al. (2015) investigated the influence of factors that favor plant regeneration and genetic transformation of the recalcitrant cultivar TME14 preferred in Africa. They observed that production of friable embryogenic callus from axillary buds and immature leaf lobes of aseptic plantlets was promoted using DKW medium (Driver and Kuniyuki 1984), crushing of organized embryogenic structures (OES) through a metal mesh (1–2 mm pore size), washing of crushed OES tissues and exposure of somatic embryos to tyrosine (12 mg L^{-1}) in the presence of 12 mg L^{-1} picloram. On the other hand, genetic transformation efficiency was increased using low *Agrobacterium* cell

density for coculture (0.25 OD_{600}), whereas cocentrifugation of friable embryogenic callus (FEC) with *Agrobacterium* enhanced 1.5-fold transient gene expression of *uidA*. Germination of paramomycin—(gradual increase from 30, 40 and 50 mg L^{-1}) resistant somatic embryos on a culture medium with BA (2 weeks with 0.1 and 2–4 weeks with 0.4 mg L^{-1}) promoted 70 % shoot formation, and also variations of the frequency of subcultures of cotyledonary embryos on elongation medium improved shoot formation. Using FECs infected with *Agrobacterium* LBA4404 carrying the vector pCAMBIA2301 approximately 80 transgenic lines per milliliter of settled cell volume were regenerated under selective conditions. GUS assays and expression of *uidA* by RT-PCR demonstrated the transgenic character of regenerated plants. Integration of *uidA* gene was confirmed by PCR and Southern blot.

23.3.2 Particle Bombardment-Mediated Transformation

Transgenic peanut plants were regenerated from cotyledons of immature peanut (*Arachis hypogaea*, cultivars Luhua 9, and YueYou 116) zygotic embryos (30–50 days after pollination) precultured for 1–5 days on a semisolid induction medium, treated with the induction medium with 0.4 mannitol for 3 h and then bombarded with gold microparticles carrying the pCAMBIA-11301 plasmid with a chimeric *hpt* and a chimeric intron-*gus* gene (Deng et al. 2001). Hygromycin-resistant callus tissue was selected on a semisolid culture medium containing the MS medium salts (Murashige and Skoog 1962), B5 vitamins, and supplemented with 10–40 mg L^{-1} 2,4-D and 10–25 mg L^{-1} hygromycin. Calli resistant to hygromycin were subjected to a further selection process in liquid medium with 20 mg L^{-1} 2,4-D and 20 mg L^{-1} hygromycin. Somatic embryos appeared 10 days after bombardment, and hygromycin-resistant transgenic plants were regenerated on 1.6 % of the bombarded cotyledons with a 0.3 transgenic somatic embryo mean per cotyledon. Integration of foreign DNA in the regenerated hygromycin-resistant plants was confirmed by PCR (intron-*gus* gene) and Southern hybridization of the *hgh* gene. Additionally, GUS activity in leaves of the regenerated plants confirmed the transgenic character.

Kumar et al. (2004) reported an interesting system to enhance salt tolerance in carrot through the overexpression of the *betaine aldehyde dehydrogenase* gene (*BADH*) via plastid genetic engineering. Transgenic plants were regenerated after microparticle bombardment of embryogenic callus cultures established from stem explants inoculated on MS medium + B5 vitamins supplemented with 3 mg L^{-1} 2,4-D and 1 mg L^{-1} Kin. Carrot chloroplast transformation vector (pDD-*Dc-aad*A/*BADH*) was directed to the 16S/*trn*I-*trn*A/23S spacer region of the chloroplast genome by homologous recombination. Transgenic carrot cell lines expressing the *BADH* gene selected in increasing levels of spectinomycin (150–500 mg L^{-1}) could be visually recognized by the green color when compared to nontransformed cells. Transformation efficiency was very high (13.3 %). Transformed cells also showed better growth (sevenfold more) and accumulated 50- to 54-fold more betaine in a culture medium with 100 mM NaCl than nontransformed cells.

Transgenic plants were regenerated on MS medium plus B5 vitamins containing 0.2 mg L^{-1} Kin, and they exhibited better growth and darker green color under high salt conditions (up to 400 mM NaCl) than nontransformed plants. Transgene integration into the plastid genome was demonstrated by PCR. Southern blot analysis revealed that transgenic plants regenerated after two subcultures of cell lines on selective medium with 350 mg L^{-1} spectinomycin showed heteroplasmy (presence of wild-type and transformed chloroplast genome), whereas those recovered after eight to ten subcultures of the cell lines were homoplasmic.

Athmaram et al. (2006) described a protocol for the genetic transformation of peanut or groundnut (*Arachis hypogaea*) using immature zygotic embryos bombarded with tungsten microparticles coated with a binary plasmid carrying *BTVP2* gene (pCAMBIABTVP2) encoding the major antigenic determinants of Bluetongue (BT) disease caused by a double-stranded RNA virus (*Orbivirus* genus), which affects primarily sheeps, but also goats, cattle, and wild ruminants. Pods of the cv. JL24 were harvested 50 days after pollination and were surface sterilized; immature zygotic embryos were dissected under sterile conditions and were cultured on a callus induction medium (MS + B5 vitamins and 2,4-D at 5–50 mg L^{-1}) under dark conditions at 28 °C. After 5 days of culture, the immature embryos were treated with inducing medium supplemented with 0.4 M mannitol as osmoticum for 3 h and then bombarded and cultured under selective conditions imposed by the presence of 40 mg L^{-1} kanamycin. Resistant callus tissues were cultured in liquid induction medium with 40 mg L^{-1} kanamycin to get somatic embryos. The transformation efficiency using the number of regenerated somatic embryos was 12.8 %. GUS expression assays, Southern hybridization with a *BTVP2* gene probe, and RT-PCR analyzes of *BTVP2* gene expression confirmed the transgenic character of the regenerated somatic embryos.

An embryogenic system was used to overexpress (sense) or repress (antisense) a class I *KNOX* homeobox gene (*HBK3*) as an approach to demonstrate its role in the development of somatic embryos of Norway spruce (*Picea abies*) (Belmonte et al. 2007). The embryogenic line 95:88:22 was maintained on a medium with 9 μM 2,4-D and 4.4 μM BA. This embryogenic line was cotransformed by bombardment with microparticles coated with the plasmid pUTV45-HBK3 sense or antisense combined with pUbi-BAR (resistance to BASTA under the control of ubiquitin promoter). Transformed cells were recovered in the presence of one mg L^{-1} BASTA in the culture medium. Somatic embryos from the selected transformed cells were matured and germinated and then analyzed by RNA blot hybridization for the *HBK3* expression. A total of four sense and four antisense sublines were recovered. Insertion of sense *HBK3* was demonstrated by PCR analysis. A significant increase in conversion of proembryogenic masses into somatic embryos was observed in the sense *HBK3* transformed sublines, whereas no conversion was observed in those proembryogenic masses transformed with antisense *HBK3* demonstrating its role in the embryogenic process. An increase in somatic embryo formation >20 % was recorded for the sense *HBK3*-transformed sublines.

The embryogenic capacity of some plant materials can be improved by traditional breeding techniques to allow further genetic transformation approaches.

G. max cultivar Jack, which is efficient for somatic embryogenesis, was used to introduce this characteristic into the breeding Line QF2 exhibiting poor embryogenic competence and additionally null mutations of the major storage proteins glycinin and β-conglycinin (Kita et al. 2007). Backcrossed lines were selected on the basis of their capacity to produce somatic embryos and the absence of the mentioned storage proteins. Cotyledons from immature zygotic embryos (4–5 mm in length) were inoculated on MS medium + B5 vitamins supplemented with 40 mg L^{-1} 2,4-D and were incubated for 4 weeks at 25 °C under a 23 h light/1 h dark photoperiod. Embryogenic cell suspensions were established, and aliquots were placed in a Petri dish with semisolid medium (MS salts + B5 vitamins and 20 mg L^{-1} 2,4-D) and then bombarded twice with gold microparticles coated with the plasmid pUHG harboring the *hpt* gene and *gfp* gene both under the independent control of the 35S promoter. PCR, Southern blot, and GFP visualization in regenerated plants confirmed the integration of the foreign genes; additionally, seeds from the transgenic plants were analyzed, and they lacked glycinin and β-conglycinin.

23.3.3 Chemically Mediated Transformation

Protoplasts from a nucellus-derived embryogenic cell suspension line (B6–68) of Valencia orange (*Citrus sinensis* Osbeck) were chemically transformed (polyethylene glycol treatment) with a plasmid carrying a *thermostable pectin methylesterase* gene fused to the *gfp* gene under the control of a double 35S promoter (Guo et al. 2005). The aim of this work was to investigate the effect of overexpression of this gene on the quality of the orange juice. An embryogenic line expressing GFP was identified and visually separated from the nontransformed tissue and was cultured on semisolid MS medium with 500 mg L^{-1} malt extract + 0.15 M maltose and covered with a thin layer of liquid medium to induce somatic embryogenesis. At this step the transgenic proembryos formed were cultured on acetate membranes (0.22 μm) placed on the induction semisolid medium with 0.15 M maltose to promote embryo formation then they were cultured on a germination medium, and finally on the induction medium supplemented with 1,500 mg L^{-1} malt extract, 0.01 mg L^{-1} 2,4-D, and 3 mg L^{-1} BA to induce adventitious shoot formation. Regenerated transgenic shoots were grafted in vitro on Carrizo citrange (*Citrus sinensis* Osbeck X *Poncirus trifoliate* (Raf) rootstocks to accelerate transgenic plant recovery. Transgenic plants were analyzed for the integration of the foreign and the reporter genes into the genomic DNA by PCR and also by Southern blot confirmation of the presence of *GFP* gene.

23.3.4 Conclusions

Genetic transformation of plant species depends on the establishment of efficient in vitro regeneration protocols. Efficient somatic embryogenesis systems have been developed for important crop species, and they have been found appropriated for highly efficient genetic transformation methods using *Agrobacterium tumefaciens* or particle bombardment. The most common or often used system for plant genetic transformation is the indirect somatic embryogenesis combined with *Agrobacterium* infection and in a minor proportion that with particle bombardment followed by direct somatic embryogenesis/particle bombardment and *Agrobacterium* coculture. Genetic transformation based on somatic embryogenesis is still limited to those species showing high embryogenic capacity. However, since embryogenic responses are dependent on the genotype and on the environmental factors, more efforts should be focused to find the right in vitro culture conditions to overcome recalcitrance as it was described for citrus species (Dutt and Grosser 2010) and cassava (Nyaboga et al. 2015). Alternatively, embryogenic capacity in recalcitrant plants can be improved by traditional genetic techniques (Kita et al. 2007) or by the genetic manipulation of specific master genes involved in the control of embryogenesis as reported for Norway spruce (*Picea abies*) (Belmonte et al. 2007).

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Chapter 24
Somatic Embryogenesis in Temporary Immersion Bioreactors

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Abstract Somatic embryogenesis is a very useful micropropagation technique, due to its high multiplicative capacity that offers the potential for large-scale propagation using temporary immersion bioreactors. The temporary immersion system is mainly based on the contact of plant tissue with culture medium by certain cycles of immersion, avoiding the problems of hyperhydricity, malformed embryos, and low conversion rates, which occur in continuous immersion systems. The automation of some or all of the phases of the process of somatic embryogenesis in a bioreactor could reduce labor and gellant costs and increase micropropagation efficiency, allowing high-quality plantlets to be obtained through more efficient and controlled protocols. This chapter describes the different types of temporary immersion bioreactors that have been used to increase or scale somatic embryogenesis in different plant species.

24.1 Introduction

Micropropagation through different techniques is an effective alternative biotechnological method for obtaining pathogen-free plants and cloning elite materials. However, the cost of in vitro micropropagated plants is still high compared to those naturally propagated, and more efficient and cost-effective methods are needed for large-scale production for commercial planting. Somatic embryogenesis coupled with the use of immersion bioreactors might offer such an option.

Somatic embryogenesis (SE) is a morphogenic process by which somatic embryos are generated from somatic cells. Somatic embryos can be produced in liquid media in flasks and in bioreactors by culturing embryogenic suspensions, such as coffee (Ducos et al. 2007a), conifer (Gupta and Timmis 2005), and cyclamen (Winkelmann et al. 1998). However, the embryogenic suspension cultures have

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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_24

some problems, such as the need to transfer the later-stage embryos to semi-solid medium and the requirement of constant movement, which prevents the development of plants from vitrification and anoxia, due to the continuous contact of explant with the liquid medium and the lack of aeration (Etienne et al. 1997).

An alternative solution to these problems is the use of temporary immersion systems (TIS). The advantage of TIS is related to the gentle air vent in the bioreactor and the intermittent contact of explant with the liquid medium (Teisson and Alvard 1995). Since the first temporary immersion bioreactor developed for micropropagation by Harris and Mason (1983), various models for this purpose have been created to date. Georgiev et al. (2014) describe some of the temporary immersion bioreactors mostly used in the micropropagation of different species.

In this paper, we describe how bioreactors are used in the somatic embryogenesis of different species and their influence on production, development, quality, and germination of somatic embryos.

24.2 Temporary Immersion Systems

Temporary immersion systems (TIS) are dynamic systems that alternate between a stage in which the plant tissues are immersed in liquid culture and an aerated stage (Etienne and Berthouly 2002; Berthouly and Etienne 2005). These systems are designed to provide an optimal environment, achieve better utilization of nutrients and gas transfer, and achieve low mechanical stress in order to reduce physiological disorders, and preserve the morphological integrity of the plant in in vitro cultures (Georgiev et al. 2014).

TIS combine the positive effect of the liquid medium but avoid continuous immersion, providing adequate aeration and nutrient mixture (Etienne and Berthouly 2002). In addition, hyperhydricity that may arise in such systems can be overcome by changing the immersion times and frequency (Etienne and Berthouly 2002; Watt 2012). The physiologically more important advantage of TIS is the efficient gas exchange between plant tissue and the gas phase within the container (Teisson and Alvard 1995).

TIS employ bioreactors specially designed for this purpose, which allow the scaling of micropropagation processes, such as embryogenesis and organogenesis, in several species (Berthouly and Etienne 2005; Etienne et al. 2006), allowing the automation of all or some phases of both processes (Berthouly and Etienne 2005; Etienne et al. 2006; Watt 2012, Georgiev et al. 2014). Appropriately designed temporary immersion bioreactors (TIB) can be fast, economical, and efficient multiplication systems that generate high-quality plants that survive transplantation to ex vitro conditions (Berthouly and Etienne 2005; Robert et al. 2006).

The efficient use of temporary immersion systems for micropropagation requires the study of the morphogenesis of plants in liquid medium and the mechanisms that control the development of the embryo or organ (Ziv 2010). Furthermore, the optimum conditions of each of the phases of micropropagation to be carried out in

the bioreactor must be established, including immersion time, the volume of liquid medium, container volume, or forced ventilation, which depend on the characteristics of the bioreactor used (Ducos et al. 2007b).

Several types of temporary immersion bioreactors for the micropropagation of plants of commercial interest currently exist. The differences between each of these are mainly due to the following: (1) size and shape of the container, (2) computerized immersion control system or a simple timer, (3) use of a peristaltic pump, an air pump or the mechanical motion of the container to displace the liquid, (4) whether the culture medium is recycled, and (5) separation or incorporation of the medium in the general container (Etienne and Berthouly 2002).

TIB usually used in micropropagation are constructed with plastic or transparent glass vessels and are illuminated by an external source. The most common types are those with a power input, i.e., (1) pneumatic, (2) air and gravity, and (3) mechanical (Georgiev et al. 2014). The TIB in the first group are the twin-flask bioreactor (Escalona et al. 1999) and RALM bioreactor (Biorreactores RALM, Ralm Industria e Comércio ltda., Braz, http://ralm.nuvemshop.com.br/laboratorios-e-biofabricas/biorreator-de-imersao-temporaria-ref-bio-001-2010/). The second group consists of the ebb-and-flow bioreactor (Ducos et al. 2007b), RITA bioreactor (Teisson and Alvard 1995), thermo-photo-bioreactor (Zuñiga Navarro et al. 2013), bioreactor of immersion by bubbles (Scheidt et al. 2009), SETIS (Vervit, Belgium, distributed by Duchefa Biochemie, The Netherlands), PLANTIMA bioreactor (A-Tech BioscientificCo., Ltd., Taiwan), PLANTFORM bioreactor (Plant Form AB, Sweden and TC propagation Ltd., Ireland), and the Box-in-Bag bioreactor (Ducos et al. 2010). In the third group are the Rocker systems (Harris and Mason 1983), rotating drum bioreactor (Akita and Ohta), WAVE bioreactor (Eibl and Eibl), and BioMINT bioreactor (Robert et al. 2006).

24.3 Somatic Embryogenesis in Temporary Immersion Bioreactors

Unlike in organogenesis, in somatic embryogenesis, whole plants are obtained without the requirement for cut stages (multiplication stages). Plant regeneration via ES includes five steps: (a) induction of embryogenic culture, (b) proliferation of the embryogenic culture, (c) prematuration of somatic embryos, (c) maturation of somatic embryos, and (d) development of the plants (Arnold et al. 2002). The processes carried out in this type of system should be optimized for each species and for each phase of the process (Etienne et al. 2006). The phases of ES in which TIB were used are detailed in the sections below.

24.3.1 Induction of the Embryogenic Culture

TIB are mostly used in the embryogenic culture phases of the proliferation, maturation, and germination of embryos; there are few studies on its use in the

induction phase (Table 24.1). However, the culturing of explants directly in the container of the bioreactor has been reported in *Saccharum* spp using leaf disks (Snyman et al. 2011), *Camptotheca acuminata* with hypocotyl segments (Sankar-Thomas et al. 2008), and in *Agave fourcroydes* and *Agave tequilana* using layers of stem (Monja-Mio and Robert, data no reported), all with the aim of initiating embryogenic culture.

24.3.2 Proliferation of the Embryogenic Culture

TIB influence the proliferation of the embryogenic callus. In *Elaeis guineeensis,* a seven times increase of initial embryogenic callus was reported when an immersion time and frequency of 3 min/3 h was used in the RITA bioreactor (Marbun et al. 2015). In the same species, using an inoculum of 0.5 g of embryogenic callus, a frequency of 3 min/6 h and a Nalgene filter unit TIB, biomass was increased two times (Sumaryono et al. 2008).

24.3.3 Maturation of Somatic Embryos

High production and improved embryo development have been observed in various species using TIB (Cabasson et al. 1997; Etienne et al. 1997; Etienne-Barry et al. 1999; Tahardi et al. 2003; Niemenak et al. 2008).

In *H. brasiliensis*, during the embryonic development stages and during the stages of maturation, drying and germination, TIB gained further advantages over the semi-solid medium (3–4 times: 400 embryos/g fresh weight under the best conditions (Etienne et al. 1997).

In *C. deliciosa*, it was observed that on the semi-solid system, 60 % of somatic embryos developed to the cotyledonary stage but were hyperhydric; in the liquid system, the continued growth in suspension culture hindered the formation of the protoderm, and the somatic embryos were unable to develop beyond the globular state. In the temporary immersion system, 66 % of the somatic embryos developed to the cotyledonary stage and were morphologically similar to nucellar embryos (Cabasson et al. 1997).

In *C. arabica*, the complete development of embryos was achieved after four months to from 200 mg of embryogenic mass per bioreactor; each unit produced 8,000 torpedo stage embryos (Etienne-Barry et al. 1999).

24.3.4 Germination and Conversion into Plantlets

It has been observed that the use of TIB positively affects the germination of somatic embryos (Etienne et al. 1997; Etienne-Barry et al. 1999; Tahardi et al.

Table 24.1 References for somatic embryogenesis in a temporary immersion bioreactor

Species	TIB used	Container volume	Phase of SE	Inoculum	Volume of medium	Time and Frequency	Production	References
Musa spp	Nalgene™ filter units modified	–	Multiplication of the embryogenic cultures for SSE	0.250 g of translucent embryogenic culture (150 somatic embryos)	–	1 min/6 h	6000 somatic embryos	Escalant et al. (1994)
Citrus deliciosa Ten	Nalgene™ filter units modified	500 mL	Embryo development	–	250 mL	1 min/4 h	66 % of the somatic embryos produced were cotyledonary	Cabasson et al. (1997)
Hevea brasiliensis	Nalgene™ filter units modified	1 L	Production of somatic embryos	1 g fresh weight of callus per apparatus	250 mL	1 min/12 h	400 embryos/g fresh weight	Etienne et al. (1997)
			Maturation, desiccation and germination	Somatic embryos obtained in 330 mg of embryogenic callus	250 mL	15 min/6 h	Root development (+60 %) and epicotyl emergency (+35 %)	
Coffea arabica L.	RITA®	1 L	Somatic embryo regeneration	200 mg of embryogenic masses	200 mL	1 min twice a day	8,000 torpedo-shaped somatic embryos/bioreactor	Etienne-Barry et al. (1999)
			Germination of somatic embryos	1,600 somatic embryos	200 mL	5 min twice a day	66 % of germinated embryos	
Camellia sinensis (L.) O. Kuntze	Nalgene™ filter units modified	250 mL	Multiplication of somatic embryos for SSE	20 globular stage somatic embryos (1 g)	100 mL	1 min/6 h	Twenty four-fold (474.67 embryos)	Akula et al. (2000)

(continued)

Table 24.1 (continued)

Species	TIB used	Container volume	Phase of SE	Inoculum	Volume of medium	Time and Frequency	Production	References
C. sinensis	Nalgene™ filter units modified	500 mL	Somatic embryo development	200 globular embryos (4 g)	125 mL	3 min/6 h	Increased by 88 embryos (1.4-fold) and 5.761 g of biomass (2, 4-fold)	Tahardi et al. (2003)
C. arabica	RITA®	1 L	Somatic embryo regeneration	200 mg of aggregates of embryogenic suspension	200 mL	1 min/4 h	3,081 embryos	Albarrán et al. (2005)
Coffea canephora	Glass bottle temporary immersion bioreactor	10 L	Pre- germination of somatic embryos	30–60 g of torpedo stage embryos	4.5 L	5 min/12 h	Depending on the clones, between 3,700 to 10,700 pre-germinated embryos are collected per TIB. From some vessels, as high as 20–25,000	Ducos et al. (2007b)
C. acuminata	RITA® and Dual vessel system	1 L	Induction	10 hypocotyl segments	200 mL	1 min/6 h	8 of ten segments in DVS and 6 of ten in RITA formed callus	Sankar-Thomas et al. (2008)
			Plant regeneration	25 embryos	200 mL	1 min/4 h	58 % of regenerant were obtained in RITA and 30 % in DVS	
							DVS: 30 %	
Elaeis guineensis Jacq.	Nalgene™ filter units modified	–	Embryogenic Callus Proliferation	0.5 g embryogenic calli	75–100 mL	3 min/6 h	Increased 2 times	Sumaryono et al. (2008)
Theobroma cacao L.	Twin-flask bioreactors	1 L	somatic embryo multiplication	400–500 mg of embryogenic calli	300 mL	1 min/6 h	159 embryos	Niemenak et al. (2008)
C. arabica L. cvs. Caturra and Catuaí	RITA®	1 L	Development of somatic embryos from suspension cultures	250 mg fresh weight of suspension cultures	200 mL	1 min/8 h	25 somatic embryos	Gatica-Arias et al. (2008)

(continued)

Table 24.1 (continued)

Species	TIB used	Container volume	Phase of SE	Inoculum	Volume of medium	Time and Frequency	Production	References
			Germination of somatic embryos and conversion into plantlets	20 somatic embryos	200 mL	1 min/8 h	100 % Germination of embryos and 45 % conversion of embryos into plants	
C. canephora P.	Glass Jars	10 L	Pregermination of somatic embryos	519 g of fresh weight of torpedo stage embryos	5 L	5 min/12 h	18,576 pre-germinated embryos and 42 % embryo-to-plantlet Conversion Rate	Ducos et al. (2010)
	Box-In-Bag	10 L		943 g of fresh weight of torpedo stage embryos	5 L	6 min twice a day	26,794 pre-germinated embryos and 57 % embryo-to-plantlet conversion rate y	
Kalopanax septemlobus	Modified column type bioreactor	2 L	Somatic embryo germination and plantlets conversion	Cotyledonary somatic embryos (500 per bioreactor)	500 mL	30 min in six times per day	85 % of embryos successfully produced plantlets	Kim et al. (2011)
Saccharum spp.	RITA®	1 L	Somatic embryo induction and germination stages	30 Leaf disks	–	1 min/12 h	18,368 plants/leaf roll	Snyman et al. (2011)
Bactris gasipaes	Twin flasks	–	Induction of secondary somatic embryos	250–300 mg isolated somatic embryos	250 mL	3 min/6 h	48.8–64.2 % of the explants showed high embryogenic capacity	Steinmacher et al. (2011)
Quercus robur	RITA®	1 L	Embryo proliferation for SSE	45 embryo clusters (500 mg)	125 ml	1 min/8 h	1,500 somatic embryos	Mallón et al. (2012)
Leucojum aestivum	RITA®	1 L	Regeneration of plants	1 g globular somatic embryos	200 mL	5 min/2 h	156.2 plants per 1 g of embryos	Ptak et al. (2013)

(continued)

Table 24.1 (continued)

Species	TIB used	Container volume	Phase of SE	Inoculum	Volume of medium	Time and Frequency	Production	References
Quercus suber L.	RITA®	1 L	Proliferation phase	1.5 g of proliferative tissues (embryogenic calli and embryo clusters)	200 mL	1 min/4 h	13.709 g final mass. Its favored the production of cotyledonary embryos	Pérez et al. (2013)
						1 min/6 h	13.386 g final mass. It enhanced the production of proliferative stages	
B. gasipaes Kunth	RITA®	1 L	Multiplication of somatic embryo clusters	1 g fresh mass of somatic embryo clusters (SEC)		3 min/3 h	7.4 g final fresh mass	Heringer et al. (2014)
	Twin flask						2 g final fresh mass	
	Twin flask modified						4.3 g final fresh mass	
	RITA®	1 L	Somatic embryo conversion	Green somatic embryos derived from 0.2 g SEC			315.7 plantlets	
	Twin flask						78.7 plantlets	
Phoenix dactylifera L.	Plantform	–	Somatic embryo development	500 mg initial callus	400 mL	1 min/4 h	193 embryos	Al-mayahi (2015)
E. guineensis Jacq.	RITA®	1 L	Proliferation of embryogenic callus	0.3 g of embryogenic callus with globular stage embryos	150 mL	3 min/3 h	Increased 7 times	Marbun et al. (2015)

2003). In *H. brasiliensis*, temporary immersion greatly stimulated root development (+60 %) and the emergence of epicotyl (+35 %) (Etienne et al. 1997). In *C. sinensis*, embryos derived from TIB had a higher germination rate (46.4 %) than those derived from semi-solid system (25.4 %); similarly, the conversion rate of embryos that germinated to plants was higher in embryos derived from TIS (87.7 %) than embryos derived from a semi-solid system (38.3 %) (Tahardi et al. 2003). In *C. arabica*, using the RITA bioreactor, the conversion time of embryos to plants was 4 weeks compared to 6 weeks in semi-solid medium (Gatica-Arias et al. 2008). In *C. canephora*, TIB allowed 95 % maturation from the torpedo stage to the cotyledonary stage (Ducos et al. 2007b, 2010).

In *C. arabica*, the direct sowing of germinated embryos resulted in a highly successful conversion of embryos to plants (Etienne-Barry et al. 1999; Albarrán et al. 2005). The production of somatic embryos or young plants in a liquid medium that can be directly transferred to the greenhouse or the nursery is a way of reducing the production costs of somatic embryogenesis (Etienne-Barry et al. 1999).

24.3.5 Induction of Secondary Somatic Embryogenesis

In some species, it has been observed that TIB promotes the development of secondary somatic embryos as *Musa* spp (Escalant et al. 1994), *C. sinensis* (Akula et al. 2000), *B. gasipaes* (Steinmacher et al. 2011), and *Q. robur* (Mallón et al. 2012). Microscopic observations of the *Musa* spp embryogenic cultures verified that multiplication occurs from epidermal cells of primary somatic embryos (Escalant et al. 1994).

In *C. sinensis*, a high rate of multiplication of secondary somatic embryos (24 times the initial inoculum) was obtained using TIB, and this was six times more than that obtained on the semi-solid system (Akula et al. 2000). In *Musa* spp, after 2 months of culture, the production of secondary somatic embryos was 1,375 in TIB and 450 on the semi-system; in the latter, the embryos became compact, white calluses, while the embryos derived from TIB presented the same appearance and maintained embryogenic proliferation, reaching a production of 6,000 embryos in 6 months (Escalant et al. 1994).

24.4 Culture Parameters Involved in Efficiency of TIB

The efficiency of TIB in the embryogenesis of any plant will depend on the optimization of culture conditions for each phase of the SE and for each species. Among these parameters are the inoculum density, the volume of the medium used, dipping cycles (immersion time and frequency), volume and design of the bioreactor.

24.4.1 Inoculum Density

The inoculum density varies with the phase in which TIB are used (Table 24.1). In *Coffee arabica*, it was observed that the culture density of 1,600 embryos/bioreactor positively affected the morphology of the embryo (elongation of the embryo axis); each embryo showed a better conversion rate after direct seeding in greenhouses (Etienne-Barry et al. 1999).

24.4.2 Volume of Culture Medium

The volume of the culture medium varies according to the type of bioreactor used in the ES and has a range from 100 mL to 5 L. In 1 L bioreactors, the volume of the medium used is typically 200 ml, while in the 10 L bioreactors, the medium volume is 5 L (Table 24.1). There are no reports on the influence of the culture medium volume on the production of somatic embryos.

24.4.3 Cycles of Immersion (Time and Frequency of Immersion)

The immersion cycle (time and frequency of immersion) is the parameter most critical for system efficiency (Berthouly and Etienne 2005); this parameter determines the absorption of nutrients and exposure to growth regulators, affecting production and quality of the embryos (Etienne and Berthouly 2002). Teisson and Alvard (1995) found that the nutrient uptake was mainly from the film of the medium retained in plant tissues by capillarity between cycles of immersion; the film does not limit gas exchange or give rise to a risk of hyperhydricity.

Immersion cycles depend on the species and the bioreactor used (Etienne and Berthouly 2002). In coffee, it was observed that the production and quality of the embryos are stimulated by increasing the frequencies of short periods of immersion (1 min every 4 h), while if the long frequencies were used, production decreased (Albarrán et al. 2005). Short immersion times also stimulated the production of embryos in other species such as *Musa* spp. (Escalant et al. 1994), *H. brasiliensis* (Etienne et al. 1997), *C. deliciosa* (Cabasson et al. 1997), *C. sinensis* (Akula et al. 2000), *Thebroma cacao* (Niemenak et al. 2008), and *C. arabica* (Gatica-Arias et al. 2008) (Table 24.1).

Immersion cycles also influence the secondary somatic embryogenesis. In coffee, it was observed that short immersion times with long frequencies (1 min every 24 h) promoted the formation of secondary somatic embryos while long periods of

immersion with shorter frequencies (15 min each 6 h) led to the development and germination of the embryos (Berthouly et al. 1995).

24.4.4 Volume and Design of TIB

One factor that significantly influences the efficiency of bioreactors is its design. Designs used in micropropagation involve factors such as size, the composition of the container (polycarbonate, polyethylene, polypropylene, or glass), operating system, container orientation (vertical or horizontal), form of sterilization, etc. (Georgiev et al. 2014). In the ES, one of the most commonly used bioreactors is the RITA, possibly due to the design of its container (Table 24.1).

In *Campotheca acuminata*, a higher percentage of well-developed plantlets were obtained in the RITA bioreactor than in the twin-flask bioreactor; apparently, the presence of the polyurethane foam in the upper chamber that offers support to the embryos and the RITA container design positively affected development (Sankar-Thomas et al. 2008). In *B. gasipaes*, a better response was achieved using the RITA bioreactor than using the twin-flask bioreactor (modified and unmodified), both in the multiplication of the cluster of somatic embryos and in converting them (Heringer et al. 2014). For the pre-germination of coffee embryos, bioreactors called Glass Jar (Ducos et al. 2007a, b) and box-in-bag (Ducos et al. 2010) have been used successfully. Ducos et al. (2010) found that horizontal bioreactors (box-in-bag) may be more convenient than vertical bioreactors because they provide a greater surface per volume. Furthermore, produced plantlets can be directly passed from laboratory to the greenhouse.

The container volume varies according to the type of TIB used in this type of process and has a range of 250 mL to 10 L (Table 24.1).

24.5 TIB Used in Somatic Embryogenesis

24.5.1 TIS Using Nalgene Filter Units

Alvard et al. (1993) developed a simple system using autoclavable filter units (Nalgene) (Fig. 24.1), which tested satisfactorily in banana meristem proliferation. This type of container has been successfully used in different species such as ES *Musa* spp (Escalant et al. 1994), *C. deliciosa* (Cabasson et al. 1997), *H. brasiliensis* (Etienne et al. 1997), *C. sinensis* (Akula et al. 2000; Tahardi et al. 2003), and *E. guineensis* (Sumaryono et al. 2008). This type of system is the previous version of the commercial RITA bioreactor. Nalgene filter units have been used in this type of SIT, ranging from 250 mL to 1 L (Table 24.1).

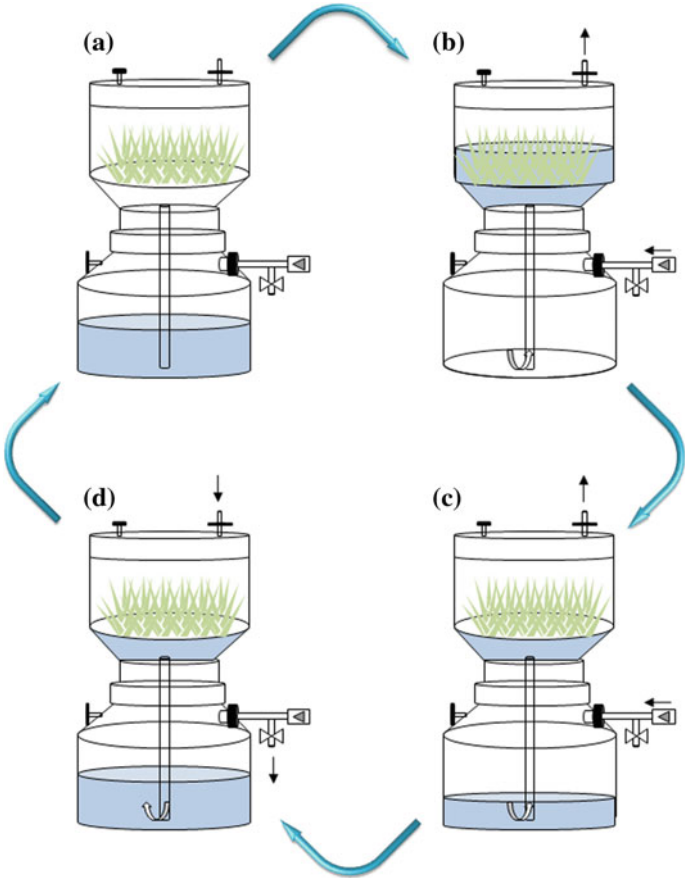

Fig. 24.1 Technological design and operational principle of Nalgene filters in a bioreactor system. The culture medium is in the lower compartment (**a**). Putting this compartment under pressure forces the solution into the *upper* compartment and immerses the plantlets there (**b** and **c**). When the pressure is released, the medium flows back into the lower compartment (**d**). The use of solenoid valve in the air circuit accelerates the return of the medium to the lower compartment. Alvard et al. (1993)

24.5.2 RITA (Recipient for Automated Temporary Immersion System)

RITA TIB was developed by the BIOTROP CIRAD laboratory in Montpellier, France (Alvard et al. 1993; Teisson and Alvard 1995) (Fig. 24.2) and is the most used bioreactor in the ES of different species. They have been successfully used in species such as *C. arabica* (Etienne-Barry et al. 1999; Albarrán et al. 2005; Gatica-Arias et al. 2008), *C. acuminata* (Sankar-Thomas et al. 2008), *Saccharum* spp (Snyman et al. 2011), *Q. robur* (Mallón et al. 2012), *L. aestivum* (Ptak et al. 2013), *Quercus suber*

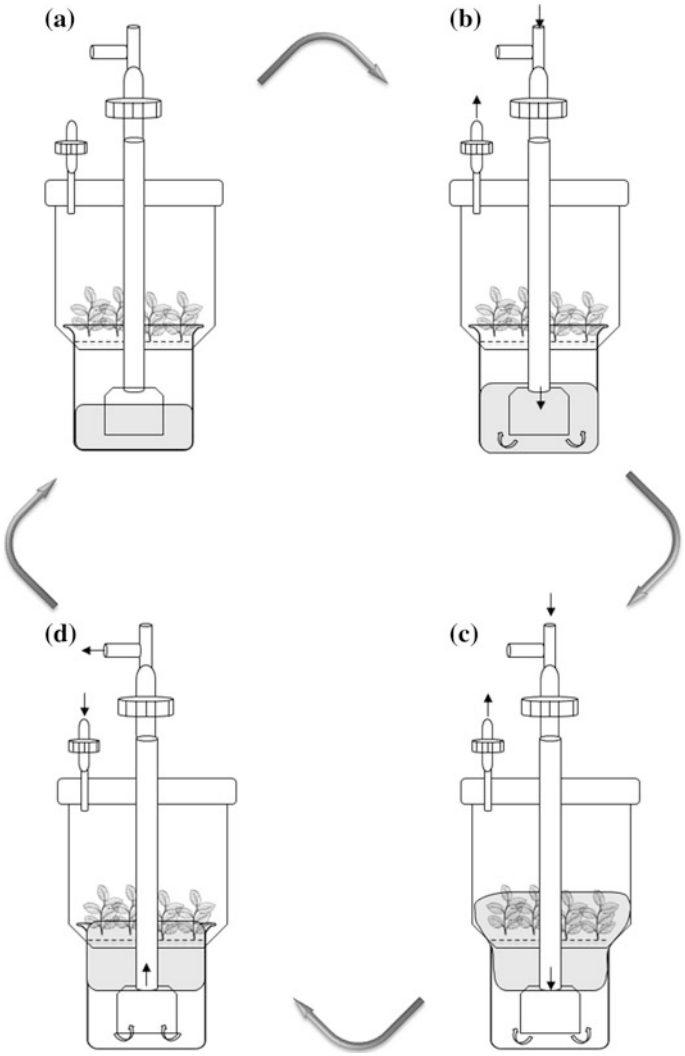

Fig. 24.2 Technological design and operational principle of the RITA system: **a** period of exposure; and **b** dislocation of liquid medium. Air pressure is applied to the bottom compartment through the central pipe. The liquid medium is moved to the *upper* compartment; **c** period of immersion; and **d** draining out the nutrient medium. The air flow is stopped, and the medium flows back to the bottom compartment due to gravity. Teisson and Alvard (1995)

(Pérez et al. 2013) and *B. gasipaes* (Marbun et al. 2015). Considering that the bioreactor described above is also considered to be a RITA TIB, the number of reports on this system has increased.

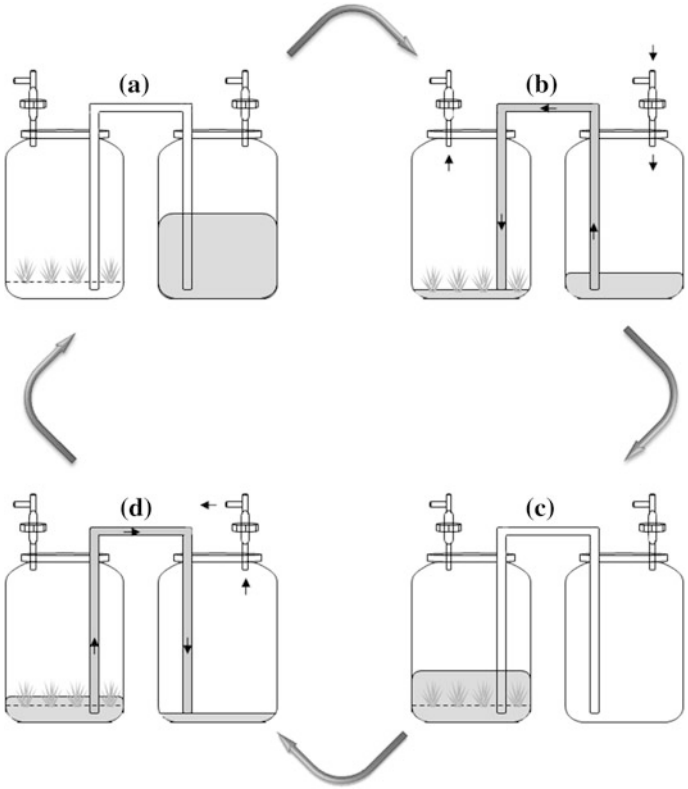

Fig. 24.3 Technological design and operational principle of the twin-flask system: **a** period of exposure. The whole volume of liquid medium is located in the medium storage tank. Air lines of both containers are closed and the solenoid valves are opened to the atmosphere; and **b** dislocation of liquid medium from medium storage tank to culture chamber. The air line of the cultivation chamber is closed, and the airline of the medium storage tank is opened. The overpressure moves the medium into the cultivation chamber; **c** period of immersion. The propagules are immersed into the medium liquid. The medium storage tank is empty. Air lines for both containers are closed and the solenoid valves are opened to the atmosphere; **d** draining out the nutrient medium back to the culture medium tank. The air line of cultivation chamber is opened, whereas the air line of medium storage tank is closed. The overpressure moves the medium back into the medium storage tank. Escalona et al. (1999)

24.5.3 Twin-Flask Bioreactor

Another type of temporary immersion bioreactor used in the ES is the twin-flask bioreactor (Escalona et al. 1999) (Fig. 24.3). Its use in the ES of *T. cacao* (Niemenak et al. 2008), *C. acuminata* (Sankar-Thomas et al. 2008) and *B. gasipaes* (Steinmacher et al. 2011; Heringer et al. 2014) has been reported.

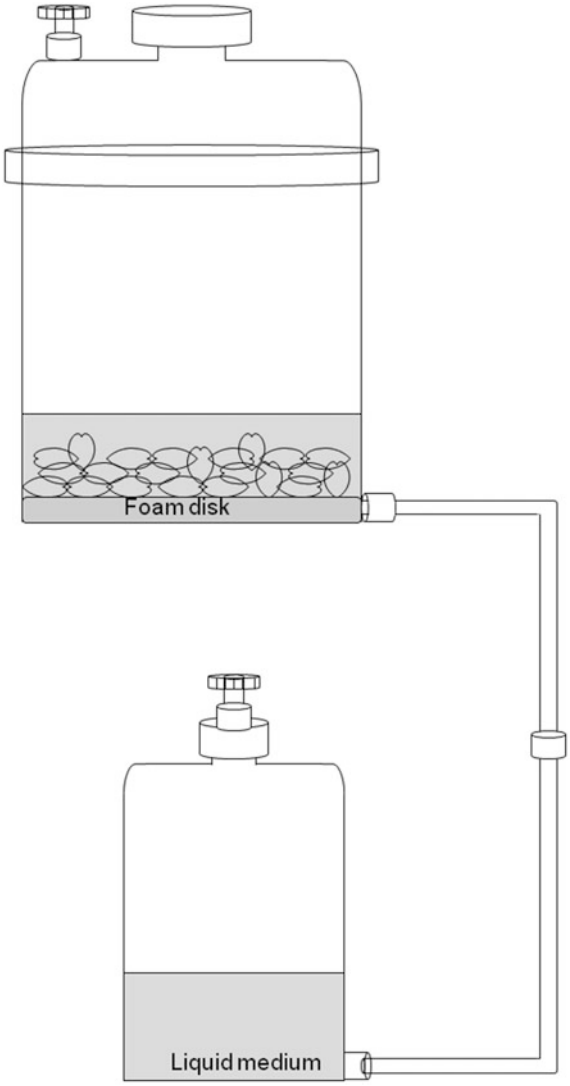

Fig. 24.4 Technological design and operational principle of glass jar temporary immersion bioreactor. Diagram of the bioreactor system. Air pressure is applied to the medium storage tank (*bottom*) and the liquid medium moves into the culture chamber. After the immersion period, the nutrient medium is drained out by switching off the air pressure and the medium flows back to the medium storage tank due to gravity. The main characteristic of this bioreactor is a polyurethane foam disk laid on the bottom of the 10 L glass jar (*top*). Between the immersion periods, this disk isolates the embryos from the thin liquid medium layer that remains in the vessel. It retains approximately 1 L of liquid medium inside the vessel and therefore maintains a sufficient relative humidity (85–90 %). Ducos et al. (2010)

24.5.4 Glass Bottle 10 L

This type of bioreactor is similar to the twin flask. Its main feature is the polyurethane foam disk placed on the bottom of the 10 L bottle; between periods of immersion, the disk isolates embryos of the layer of the liquid medium remaining in the container (Ducos et al. 2007b, 2010) (Fig. 24.4). This type of bioreactor has

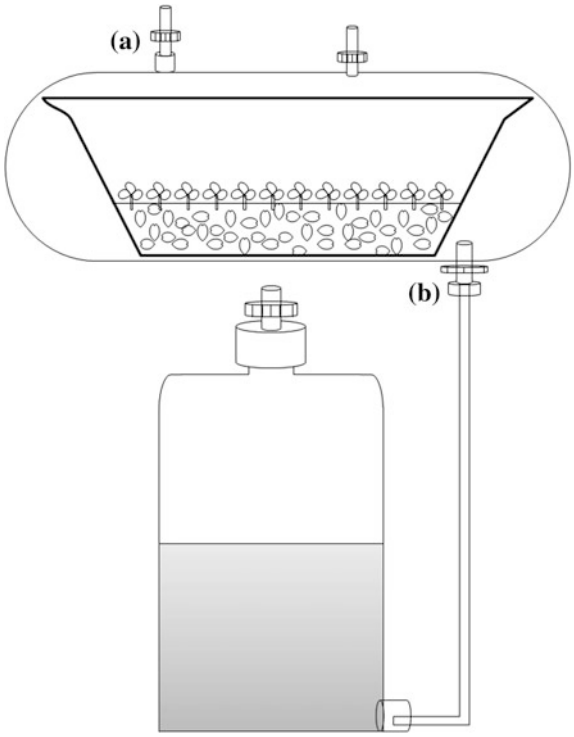

Fig. 24.5 Technological design and operational principle of a box-in-bag temporary immersion bioreactor. The diagram system shows the operational functions. The polyethylene bag has two polyethylene ports molded into the film. One port is placed above the bag (**a**); it is used for the inoculation step and then for the air outlet. The other port is located below of the device (**b**) and is used for the air inlet and medium entrance and exit. The bag covers a rigid box made of a transparent and ionizable plastic, for instance, polycarbonate. Foam disks of polyurethane are fixed in the bottom of the bag; one of the foam disks is located just above the port B, which functions as both the medium inlet and an air entrance inlet. A funnel made of silicone tubing is fixed through this disk to permit the rapid introduction of the medium inside the box at the beginning of the immersion periods. Ducos et al. (2010)

been used in scaling embryo pre-germination of coffee (state conversion torpedo to cotyledonary state) (Ducos et al. 2007b).

24.5.5 Box-in-Bag

This is a horizontal type of bioreactor that has also been used in the pre-germination of coffee embryos (Ducos et al. 2010) (Fig. 24.5).

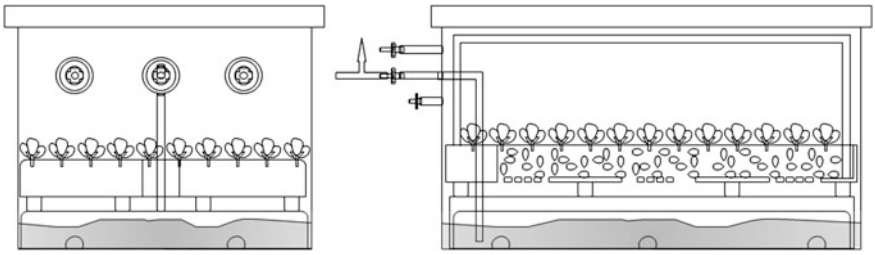

Fig. 24.6 Technological design and operational principle of a PLANTFORM bioreactor system. The bioreactor consists of a body made of polycarbonate that is transparent and can be heated to 120 °C. Gas exchange is controlled through three inlets/outlets anchored to the side by nuts and clamps through holes in the body and sealed with heat resistant silicon O-rings. The middle filter is connected to a plastic tube on the inner chamber and goes through the basket. An inner chamber with three grooves on the long side and two grooves on short side is placed at the *bottom* of the bioreactor. It is designed in a way to allow nutrients to rise efficiently when a pressure is applied. A basket containing the plant material is placed above the inner chamber. The small holes in the basket are placed in three rows, which allow the nutrients to flow efficiently through the basket. From http://www.plantform.se/db/technical_improvement.pdf Image modified from Georgiev et al. (2014)

24.5.6 Plantform

This bioreactor (Fig. 24.6) has been used in the organogenesis of different species (Welander et al. 2014) and has also been observed in the SE of *P. dactylifera* (Al-Mayahi 2015).

24.5.7 BioMINT

The BioMINT™ is a TIB with higher capacity and greater ease of use at a cost far below that of other commercial bioreactors (Robert et al. 2006) (Fig. 24.7). It presents a more suitable design for the micropropagation of certain plants and has been used in the propagation of species of *Agave* (Robert et al. 2006), *Capsicum chinense* (Bello-Bello et al. 2010), and *Cocos nucifera* (Andrade-Torres 2011). In addition, it has been successfully used in the induction of ES *A. fourcroydes* and *A. tequilana* (Monja-Mio 2013).

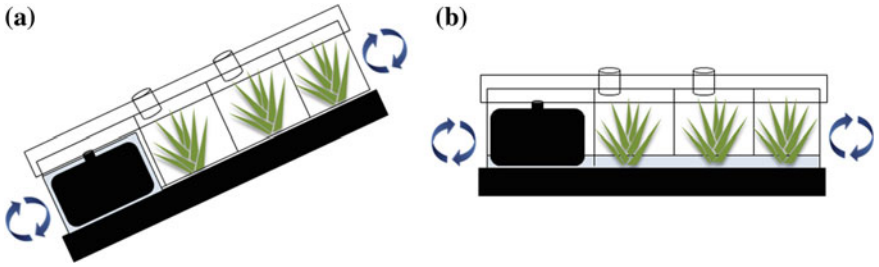

Fig. 24.7 Technological design and operational principle of the BioMINT system: The BioMINT unit consists in three main parts made of polycarbonate; all are fully autoclavable. The cover has two open ends to allow for passive atmosphere exchange or forced ventilation. The main body is a translucent rectangle with three removable accessories divided into three different sections in the bioreactor, each section has nine channels (2 mm each one) in the bottom that let the liquid medium culture pass through the compartments and avoid being mixed with the explants or plantlets. The other part is a black box. This container protects the medium nutrients that are susceptible to the light action from the light. The liquid culture medium is displaced from one side to the other by gravity when the bioreactors change their inclination. In **a**, the BioMINT is in standby in 34° angle and all the medium culture is cover by the *black box*, meanwhile in **b**, the immersion period is started and the BIOMINT has a 180° angle or horizontal position to allow the liquid to immerse the plantlets. The system is controlled by an electronic sequential temporizer. Robert et al. (2006)

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Chapter 25
Application of Somatic Embryogenesis to Secondary Metabolite-Producing Plants

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Abstract Plants display an amazing biosynthetic capacity. To date, around 200,000 different chemical compounds have been isolated from them. Only a relatively few of these compounds are common to all plant species, since they are involved in basic or primary cell processes, such as energy metabolism. However, the broader plant chemical diversity corresponds to those compounds showing a restricted distribution among only a few taxonomically related species and which are not involved in primary metabolic pathways. These compounds are called secondary or specialized metabolites and they have important roles in numerous plant-environment interactions. Aside from these functions, secondary metabolites, and the plants bearing them, represent highly regarded commercial products given their pharmaceutical, flavoring, aromatic, coloring, and poisonous properties. In here, we present some selected examples of secondary metabolite-producing plants for which efficient protocols of somatic embryogenesis have been developed. The review covers mainly plants producing fine chemicals, used either in pharmaceutical or food industries. As shown, the development of somatic embryogenesis procedures could respond to two main goals: the genetic transformation of a given plant species, or the massive propagation of selected materials. Furthermore, the use of such protocols for the generation of diversity through indirect embryo formation is also presented.

Abbreviations

2,4-D 2,4-diclorophenoxyacetic acid
ABA Abscisic acid
BA 6-benzyladenine

FAVF is recipient of a CONACYT grant (National Council for Science and Technology, México) (CB-2012-0181880).

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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_25

GA Gibberellic acid
Kin Kinetin
NAA Naphthalenacetic acid
TDZ Thidiazuron

25.1 Introduction

Plants are frequently regarded as chemical factories due to their sophisticated biosynthetic capacity. It is estimated that over 200,000 chemicals have been isolated from plants and that up to 20 % of the plant genome could be devoted to the production, packing, and mobilization of these compounds, collectively known as *secondary* or *specialized* metabolites (Scheible et al. 2004; Hartmann 2007). This estimation also includes genes involved in the perception of the environmental cues that trigger the process of synthesis as a response leading to adaptation to new conditions. Plant chemical diversity is derived from the gene plasticity associated to non-fundamental cell processes. Genes involved in secondary metabolism do not show the tight conservation existing in those involved in primary biochemical routes, such as energy metabolism or the synthesis of cellular components. Genes ascribed to secondary metabolism often originate from divergence of those involved in reactions of primary metabolism (Pichersky and Lewinsohn 2011). As a consequence, every plant species presents a unique chemical blend, different from the others.

Nonetheless, even when plant secondary metabolism can display such wide diversity, products often show a restricted distribution. Commonly, only a few plants that are taxonomically related display similar products. Moreover, secondary metabolites accumulate in low amounts in plant tissues, which seem to be directly related to their physiological activities. Secondary metabolites play critical roles in plants' interactions with other organisms, mainly herbivores and microbes, often exerting toxic effects on them. In fact, some plants can be toxic to other plants, and even to themselves; hence, different mechanisms operate to strictly control the synthesis, storage, and distribution of secondary metabolites. Interestingly, only low doses of secondary metabolites are needed to exert their effect, given their affinity to specific cell receptors and thus, prompting a physiological response. This is the result of their structures mimicking those of the actual ligands (Hartmann 2008).

Besides their important role in nature, secondary metabolites, and the plants bearing them, represent highly regarded commercial products. Secondary metabolites have pharmaceutical, flavoring, aromatic, coloring, and poisonous properties. Consumers are willing to pay premium prices for secondary metabolites obtained from natural sources that do not display the health hazards that some chemically obtained products display. One can satisfy hunger with different types of staples, but only caffeine produces such a stimulant effect on us.

25.2 Plant Cell Cultures and Secondary Metabolites

Plant secondary metabolites are found in a number of products that are daily consumed around the world. Some of them include stimulant beverages such as coffee, cocoa, tea, and other brewed infusions; spices, such as pepper, clover, nutmeg, peppermint, and cinnamon; flavorings, such as garlic, saffron, and ginger; and pharmaceuticals, such as opium and belladonna, among others. Other industrial products containing plant secondary metabolites or their derivatives are pesticides (pyrethrins from *Chrysanthemum* or thiophenes from *Tagetes*) and textile dyes (as the natural indigoes from the *Indigofera* genus), to name two of them. Based on their continuous demand and economic value, cell culture technology was applied to the production of secondary metabolites since its early development (Vázquez-Flota and Loyola-Vargas 2003). Earlier approaches focused on the use of cell suspensions in a similar fashion as yeast strains producing antibiotics and other fine chemicals. However, plant cells' biosynthetic capacity was frequently lost or reduced in undifferentiated cultures that lack the required specialization for the synthesis and storage of these metabolites. However, despite the lack of commercial success, in vitro cell cultures have been fundamental to the understanding of mechanisms controlling secondary metabolism (Hartmann 2007, 2008).

Somatic embryogenesis applied to secondary metabolite-producing plants has been directed, either to massive propagation of elite materials, or as one of the strategies involved in genetic improvement. As happens for other economically important plants, interest in the genetic improvement of those producing secondary metabolites mainly focuses on increasing yields per area. This can be achieved by either promoting a higher total accumulation of the product of interest or by reducing the accumulation of less valuable or undesirable byproducts. In both cases, the biosynthetic pathways leading to the synthesis of these products should be known well enough to identify critical steps or regulatory mechanisms controlling the processes. Identification of genes involved in the control of production of secondary metabolites allows their isolation and introduction by genetic transformation techniques. The value of embryogenic cultures of these species is therefore evident. However, recalcitrance is a common problem associated to such species (both for transformation and embryogenesis). This may be explained in terms that most of them are non-model plants (Facchini and De Luca 2008). Although primary embryogenesis is frequently a success, embryo maturation and conversion to entire plants are critical and limiting steps to obtain transformed mature plants (Facchini et al. 2008).

In here, we present some selected examples of secondary metabolite-producing plants for which efficient protocols for somatic embryogenesis have been developed. The review covers mainly plants producing fine chemicals, used either in pharmaceutical or food industries. It should be mentioned that the hormonal treatments applied at the different stages of the in vitro process were kept in the same concentration units (either as mg/L or μM) as in the original reports.

25.3 Somatic Embryogenesis in Plants with Medicinal Applications

25.3.1 Alkaloid-Producing Plants

Alkaloids are basic compounds that include a tertiary nitrogen atom, usually as part of a heterocyclic structure. Most alkaloids are synthesized from amino acids and display powerful toxic effects at low doses given their high affinity to cell receptors, displacing the actual ligands (Hartmann 2007). Due to their complex chemical structures, which often present one or more chiral centers, alkaloids are commonly obtained from various natural sources, rather than from chemical synthesis. Moreover, alkaloid-producing plants are frequently collected in wild areas, which can result in discontinuous supplies to pharmaceutical manufacturers. For these reasons, the development of biotechnological tools for the improvement of alkaloid-producing plants is highly desirable. Three of the most commonly pharmaceutically employed alkaloids are those produced by *Catharanthus roseus*, *Papaver somniferum*, as well as, the tropane alkaloids produced by certain members of the Solanaceae family.

25.3.2 Catharanthus roseus *(Apocynaceae)*

This plant, also known as Madagascar periwinkle, remains as the only commercial source of the antineoplastic dimeric alkaloids vinblastine (VLB) and vincristine (VCR) widely used to treat different types of tumors. In the past, numerous attempts to produce these therapeutically valuable agents using cell culture technology were done without clear results. These unsuccessful attempts have been related to the high tissue and cell specialization involved in their synthesis, which is lost during the induction of in vitro cultures (Facchini and De Luca 2008). Moreover, one of the subunits required for dimer formation (catharanthine) is not accumulated inside the tissues, but rather excreted and kept in the leaf covering wax, excluding it from vindoline, the other moiety needed for obtaining these alkaloids (Yu and De Luca 2013).

The morphogenic response of *C. roseus* cultured in vitro has been found to be genotype-related (Lee et al. 2003). Moreover, differential tissue responses to in vitro culture have been reported (Dhandapani et al. 2008). Although a good embryogenic response was obtained with anthers (Kim et al. 1994) and immature zygotic embryos (Kim et al. 2004), low efficiencies in embryo germination were observed in both cases. Dhandapani and coworkers (Dhandapani et al. 2008) found that the use of TDZ (7.5 μM) on explants from mature zygotic embryos of *C. roseus* "Little Bright Eye" cultivar produced a noticeable embryogenic response (up to 49 %). Embryo maturation and plantlet germination were also accomplished using TDZ (2.5 μM) and IBA (2.4 μM), respectively. In this work, though other explants were employed, including cotyledons, hypocotyls, and petioles, the best embryogenic

response was recorded with zygotic embryos (Dhandapani et al. 2008). Interestingly, hypocotyl explants showed a high embryogenic response when exposed to NAA (Junaid et al. 2006, 2007; Aslam et al. 2011). After formation, embryo maturation and germination to entire plantlets can be achieved in a liquid phase by replacing culture media supplemented with GA and BAP plus IBA, respectively (Junaid et al. 2007). Other complete liquid-phase protocols using hypocotyl derived explants, from the induction of primary embryogenic calli to the formation entire plantlets, have been described (Junaid et al. 2007).

As mentioned above, a high degree of cell organization is required for the formation of the *Catharanthus* alkaloids. Both VLB and VCR contents increased as embryos progressed from calli to their mature stage and during early plantlet development (Aslam et al. 2009, 2011). It is noteworthy to mention that treating embryos with low temperatures at some point through the process markedly promoted alkaloid accumulation, perhaps as a response to the desiccation that the treatment induced (Aslam et al. 2011). Contents of other *Catharanthus* alkaloids, such as ajmalicine and serpentine, also increased as embryo formation proceeds in cell suspensions (Favretto et al. 2001).

Alkaloid accumulation has been studied in mature *Catharanthus* plants obtained through somatic embryogenesis, using hypocotyl explants as starting materials (Filippini et al. 2000). Comparison to the original plant materials revealed no variations in alkaloid contents in leaves and stems, while a noticeable accumulation in flowers of plants derived from somatic embryos was recorded. Similar results were obtained for both pink and white flower cultivars (Favretto et al. 2001).

25.3.3 (Papaveraceae)

Opium has been used for medicinal and recreational purposes since the dawn of civilization. Records of opium usage can be traced as far as 1400 BC and all the major antique Eastern civilizations did know of its properties and made ample use of them (Schivelbusch 1992). Opium is the dried latex from the opium poppy (*Papaver somniferum*) obtained by milking unripened seed capsules through blade-made incisions. Latex, in turn, is the cytoplasm of the laticifer cells, which are part of the highly specialized internal secretory system of this plant. Opium is composed of a number of alkaloids; however, its narcotic effects are mainly due the opiate alkaloids morphine, codeine, and noscapine. The biosynthetic pathway leading to the formation of *Papaver* alkaloids is complex and different cell types are involved (Facchini and De Luca 2008). Therefore, as it frequently occurs in these cases, the biosynthetic capacity is lost in undifferentiated cell cultures (Facchini et al. 2012).

Despite its long relationship with mankind and extensive manipulation, this plant presents a relatively narrow genetic base. As a consequence, efforts for its genetic improvement by traditional means (Levy and Milo 1997) have encountered limited success. Hence, attempts to generate variation using biotechnological tools have

been envisioned for decades (Facchini et al. 2008). Reports of the regeneration of shoots of *P. somniferum* and *P. orientale* plants from embryogenic suspension showed that alkaloid profiles (morphine, codeine and thebaine) were similar to those from seed-germinated plantlets of the same size and morphology (Schuchmann and Wellmann 1983). Similar results were obtained by Day and coworkers when comparing *P. bracteatum* plants derived from somatic embryos and seed-grown plants (Day et al. 1986). These examples indicate that alkaloid synthesis in *Papaver* is relatively stable after passage through in vitro cultures.

Most protocols involve indirect embryogenesis using young developing tissues, such as hypocotyls from approximately two-week-old seedlings, as initial explants. A combination of auxin (NAA or 2,4-D) and cytokinin (BAP or Kin) is employed to induce the embryogenic cultures at doses ranging from 1 to 2.5 mg/L. Transition from embryonic calli or suspensions to somatic embryos and further maturation is achieved by eliminating auxins from culture media (Ovecka et al. 1997; Chitty et al. 2003; Frick et al. 2004). Although shoots readily developed from the mature embryos, root formation has shown to be a limiting step (Ovecka et al. 1997). Roots from developing seedlings can also be used as starting material with good results (Facchini et al. 2008; Pathak et al. 2012). As for hypocotyl-derived calli, hormones should be removed to promote embryo maturation (Facchini et al. 2008). ABA and GA (between 0.1 and 0.2 mg/L) can be added to the culture medium to promote maturation (Pathak et al. 2012).

A highly efficient protocol of indirect embryogenesis was combined with *Agrobacterium tumefaciens*-mediated genetic transformation to confer herbicide resistance to *P. somniferum* by introducing the phosphinothricin acetyltransferase (*pat*) gene (Facchini et al. 2008). Explants (excised root tissues) were transformed prior to the induction of the embryogenic cultures. Rooting of cotyledonary embryos was improved by dissecting individual embryos from clusters and avoiding direct contact with the agar medium using filter paper. Moreover, short exposures to a high IBA concentration (100 mg/l for less than 6 h) and incubation at low temperatures (<20 °C) were also beneficial for the process (Facchini et al. 2008).

Somatic embryogenesis and *Agrobacterium*-mediated transformation has also been used to generate plants over-expressing the first to last step of morphine biosynthesis (codeinone reductase; *PsCor1.1*) (Larkin et al. 2007).

Protocols for direct somatic embryogenesis have been described using hypocotyls and seedling cotyledons (Kassem and Jacquin 2001).

25.3.4 Plants Producing Tropane Alkaloids

Tropane alkaloids are formed either from arginine or ornithine. There are more than 200 of these alkaloids, some of them with pharmacological interest due to their anticholinergic effects caused by binding to the muscarinic acetylcholine receptors at the nervous central system. These alkaloids are common in the Solanaceae and

Erythroxylaceae families (Jirschitzka et al. 2013). Examples of plants producing this type of alkaloids are members of the *Datura* genus, *Atropa belladonna* (the deadly nightshade), and *Hyoscyamus niger* (henbane).

Datura metel (the devil's trumpet) is a shrub, native to India and southern China. It is used in the treatment of a number of ailments, given its anthelmintic, anticancer, antispasmodic, hypotensive, and antiviral properties. Unrestricted gathering of this plant has resulted in a severe reduction of its population (Nithiya and Arockiasamy 2007). As a strategy to preserve this important medicinal plant, a massive propagation procedure using somatic embryogenesis has been developed. Root explants from seedlings were exposed to 4 mg/L BAP resulting in the direct formation of embryos after two weeks. Shoot elongation and root formation were achieved using BAP, GA_3, and IBA (2, 1 and 1 mg/L, respectively) in the culture media. Over 100 entire plantlets per root explant were obtained through this method (Nithiya and Arockiasamy 2007). Another approach for its clonal propagation was the use of hypocotyl sections of androgenic embryos, derived from anthers (Wijesekara and Iqbal 2013).

Hyoscyamus niger. This plant produces hyoscyamine, a powerful anticholinergic agent employed as muscle and gastric spasms relaxant. Scopolamine, which is also found in this plant, has similar medicinal uses, but with stronger side effects (Jirschitzka et al. 2013).

A highly efficient method for plant regeneration through somatic embryogenesis has been described using mature zygotic embryos. One week of exposure to 1 mg/NAA of pre-soaked embryos (16 h in water) resulted in 80 % embryo formation directly on the hypocotyl segment (Tu et al. 2005). Several waves of embryo formation occurred; however, after the first round, an unsynchronized development was observed. Plant formation occurred without transfer of embryos to fresh media; although, auxin elimination promoted their development (Tu et al. 2005). Since high numbers of morphologically normal embryos were obtained, this method has been proposed to be used for the transformation of this valuable medicinal plant (Tu et al. 2005).

25.3.5 Other Select Cases of Medicinal Plants

Terminalia chebula. This is a tree, common to sub-Himalayan forests, that has spread to different parts of India. Seeds are a valuable source of ellagitannic acid, highly demanded in the tannery industry. These also contain the hexapeptide cherbuin, which displays significant antispasmodic activity. Dried fruits are used in different medical preparations as adjuvants and they are prescribed in Ayurvedic medicine against a number of diseases, including leprosy, jaundice, epilepsy, and hiccough. Tree propagation is made by seeds, but low germination rates have been determined. Therefore, biotechnological approaches such as multiple shoot cultures (Shyamkumar et al. 2003) and somatic embryogenesis protocols have been developed (Anjaneyulu et al. 2004).

Seed cotyledons and mature zygotic embryos of *T. chebula* were used as explants on MS medium supplemented with 30 g/L sucrose and 2,4-D and Kin (1 and either 0.01 or 0.1 mg/L, respectively) for callus induction. After 6–8 weeks, somatic embryos developed on the same media composition and their maturation was achieved by increasing sucrose to 50 g/L. A good embryo to plantlet conversion (nearly 50 %) was achieved with 0.5 mg/L BA (Anjaneyulu et al. 2004). Interestingly, an *Agrobacterium*-mediated transformation protocol for this species has been described (Shyamkumar et al. 2007), which combined with embryogenic lines, will allow the genetic modification of this important Indian medicinal tree.

Paris polyphylla. This is a shrub, commonly known as "Chonglou" in Chinese, which is widely used in several Chinese medical preparations to treat conditions that include parotiditis and certain types of tumors. Rhizomes accumulate steroidal saponins with antitumor effects, as well as anthelmintic and antifungal properties (Xiao et al. 2009). Due to effectiveness of using rhizome for medicinal treatments, entire plants are collected from wild areas in Asian forests, endangering populations both in China and India. Using immature zygotic embryos as initial explants, direct somatic embryogenesis has been achieved using agar solidified 0.5× MS media. A good embryogenic response (higher than 30 %) was obtained using hormone-free media (Raomai et al. 2014). Moreover, when primary embryos were isolated and cultured on the same media, secondary, morphologically normal, embryos were formed. This response, however, was reduced after few subcultures, but pretreatment with 1 M mannitol for 12 h re-established the embryogenic potential (Raomai et al. 2014). Cotyledonary *Paris* embryos germinated to form entire seedlings, showing shoots, rhizomes, and radicles when they were exposed to 0.5 mg/L GA_3. Complete transition to healthy and vigorous plantlets required an exposure to 0.05 mg/L BA and 0.1 mg/L NAA (Raomai et al. 2014).

Thymus hyemalis. This is a herb, native to the West Mediterranean region, known as winter thyme. It belongs to the Lamiaceae family and produces essential oils in the leaves with antifungal, antibacterial, and insecticide properties, as well as antioxidant activity. Leaves also produce diterpenes such as thymol, carcavol, and borneol (Tepe et al. 2011), as well as other metabolites such as flavonones, rosmarinic acid, and triterpenes. Due to its wide use as a medicinal plant, as well as food preservative and seasoning, it is collected from wild populations, mainly in the Southeastern Iberic Peninsula (Jordán et al. 2006). In order to avoid plant over-extraction from its environment, a procedure for its massive in vitro propagation through somatic embryogenesis was formulated. Nodes of in vitro maintained shoots were used to generate embryogenic calli by exposure to 1.8 and 0.5 μM 2,4-D and NAA, respectively. Over 85 % of the explants formed embryogenic calli, which were allowed to develop for four weeks. Embryos were formed on calli exposed to 4.44, 0.54, or 4.65 μM of BA, NAA or Kin, respectively (Nordine et al. 2014). Embryo germination was achieved in hormone-free medium. Although over 90 % of the embryos formed shoots, rooting was not as efficient; nevertheless, it was induced ex vitro, by transplanting them to a 2:1 mixture of peat and vermiculite (Nordine et al. 2014). The complete process, from callus induction to nursery-established plantlets, took around three months to complete. Thus, this

scheme has been proposed for genetic improvement through gene transformation-embryogenesis scheme or to be integrated in a conservation program to avoid plant-over extraction from nature (Nordine et al. 2014). It has not been yet established if the patterns of secondary metabolites in plants derived from somatic embryos are comparable to those arising from seed germination.

25.4 Somatic Embryogenesis in Plants Used as Food Seasonings

Colorants, either dyes or pigments, represent a valuable market. In 2010, it was worth 1.8 billion dollars and has been globally increasing by 4.5 % annually. Natural colorants represent about one third of the total market (0.66 billion dollar), but it is increasing at a 6.7 % annual rate. Some projections estimate that by 2020 natural colorants' market will be worth around 2.0 billion dollars (see Caro et al. 2012). The food industry is the main consumer of natural colorants, followed by soft drinks and alcoholic beverages. Public concerns about possible health hazards associated to food colored with synthetic compounds have increased due to reports linking red and yellow additives to cancer and children hyperactivity (Nigg et al. 2012; Potera 2010). Although there is still some controversy, major manufactures of processed food products have turned to natural colorants, charging premium prices to consumers willing to acquire more natural products. Higher demands of natural colorants result from volumes required to replace the synthetic ones, but also from the larger quantities of these compounds used to obtain similar shades due to their sensitivity to storage and manipulation (Delgado-Vargas et al. 2000).

In the following paragraphs, selected examples of somatic embryogenesis of plant used to produce natural dyes are presented. Since, as it was mentioned above, main health concerns refer to red and yellow-orange shades, plants producing these colorants have been included, namely saffron and annatto for reds and curcuma and marigold for yellows. Horticultural plants, such as beet, berries, carrots, and chili peppers, which may also use as colorant sources, were not considered.

25.4.1 Crocus sativus *(Iridaceae)*

Saffron is the spice derived from the dry styles of *Crocus sativus* flowers. It is highly appreciated due to its intense red color and bitter taste and aroma, caused by the accumulation of three major carotenoids: crocetin glycosides, picrocrocin, and safranal (Carmona et al. 2006). Important medicinal properties, as an anticonvulsant, antidepressant, and anticancer agent, have also been claimed for this plant (Akhondzadeh et al. 2005). Saffron can reach high prices based on the difficulties encountered for its cultivation and also by the large amount of flowers (about

120,000) required to obtain 1 kg of the spice (Carmona et al. 2006). Since *Crocus* is male sterile, plants do not set seeds and are propagated using vegetative corms, which are produced in a reduced number (three to four per plant) in each growing season. Moreover, crop improvement is limited to the selection and vegetative propagation of naturally occurring variants (Carmona et al. 2006). The use of mass propagation techniques, such as somatic embryogenesis, could certainly help to overcome these limitations.

An efficient method to produce *Crocus* cormlets that will be used as propagation materials was developed, using leaf bases as initial explants. Direct formation of embryos was observed on MS medium supplemented with 2.5 μM TDZ and 2.0 μM picloram (Devi et al. 2014). Secondary embryo formation occurred on the same medium, but only after reducing picloram to 1.0 μM. These embryogenic cultures retained its capacity for over three years. A high conversion rate was recorded when embryos were cultured on media containing TDZ and picloram. Shoots, formed from these embryos, were propagated in BAP and NAA MS media (27 and 1 μM, respectively). Interestingly, in vitro formed shoots produced cormlets similar in size and behavior to those formed by field plants (Devi et al. 2014).

Retention of embryogenic capacity of *Crocus* (over 10 years) was also reported by Blazquez and coworkers in corm-derived cultures maintained on 2,4-D and BAP (Blazquez et al. 2009). Differentiation of somatic embryos from these cultures was achieved switching auxin to NAA (Blazquez et al. 2009).

25.4.2 Bixa orellana*(Bixaceae)*

The annatto plant is a perennial woody shrub from where the red pigment bixin is obtained. Bixin is an apocarotenoid accumulated in the seed arile and is used in the food industry to color different products, such as cheeses, charcuterie, and sauces (Rivera-Madrid et al. 2006). Problems with the production of bixin relate to genetic variation among populations resulting from the conventional heterozygous seed propagation of the plant (Rivera-Madrid et al. 2006). Propagation through somatic embryogenesis (Paiva Neto et al. 2003a) and other in vitro culture techniques (Paiva Neto et al. 2003a; Da Cruz et al. 2014) represents an interesting alternative to overcome this limitation (see also Monteiro Matos et al.; Chap. 14, this book).

Immature zygotic embryos exposed to a combination of 2,4-D and Kin (2.3 and 4.5 μM, respectively) on MS media supplemented with 1.0 g/L of activated charcoal turned to directly formed embryos after 25 days (Paiva Neto et al. 2003a). Mature embryos did not respond to any of the hormonal treatments assayed. Moreover, embryogenic response was related to the genotype employed (Paiva Neto et al. 2003a, b). Although a good primary embryogenic response was observed (close to 70 % of the explants formed embryos), transition to mature stages was very poor, with less than 5 % of the embryos reaching the cotyledonary

phase (Paiva Neto et al. 2003a). Interestingly, although *Bixa* embryos displayed both root and shoot meristems and a well-formed vascular system, very low number of them developed to form complete plants (Paiva Neto et al. 2003a).

25.4.3 Curcula longa *(Zingiberaceae)*

This plant from the Zingiberaceae family is also known as turmeric and frequently called the golden spice for its characteristic yellow-orange color. It is a common ingredient of sauces, mustards, soups, and butter, just to mention a few (Gang and Ma 2008). Color is the result of phenolic compounds accumulated in the rhizomes, collectively known as curcuminoids [i.e., curcumin (1,7-bis (4-hydoxy-3-methoxyfenil)-1,6-heptadiene-3,5-dione), demethoxy-curcumin, and bismethoxy-curcumin]. Rhizomes are also used for medicinal purposes as an antihelmintic and antiviral, and most recently, for its antioxidant and anticancer activities (Gang and Ma 2008). Turmeric is propagated vegetatively through underground rhizomes, which result in a relative narrow genetic base. In fact, cultivars with highest curcumin contents are susceptible to fungal and bacterial infections, as well as to nematode infestation.

Indirect somatic embryogenesis from young inflorescences of *C. longa* (He and Gang 2014) has been reported and this method resulted in embryogenic suspensions' which were used in genetic transformation procedures. Use of Gamborg's B5 medium supplemented with 5 and 30 mg/L of NAA and BAP resulted in the formation of friable calli that later gave way to embryogenic suspensions. One week-old calli were co-cultivated with an infective *Agrobacterium tumefaciens* strain for 20 min and thereafter maintained on MS medium with 0.5 mg/L Kin and 30 mg/L NAA for embryo maturation. Shoot formation was achieved on a medium with similar composition, but reducing Kin to 0.3 mg/L (He and Gang 2014). Rootless shoots developed when Kin was eliminated and NAA concentration reduced to 10 mg/L (He and Gang 2014). Although this procedure was highly efficient, passage through callus phase might introduce undesirable genetic instability. Using leaf bases as initial explants, a direct embryogenic process was described by Raju and co-workers (Raju et al. 2015). Preincubation of explants in the dark on solid MS medium supplemented with 4.5 μM 2,4-D followed by a change to liquid media with 1.3 μM BAP and light exposure. Over 90 % of the explants formed primary embryos, from which secondary embryos readily arose by doubling BA concentration (Raju et al. 2015). Embryo maturation and further germination was achieved on half-strength MS media with 1.4 μM GA_3, although it was not indispensable. Over 80 % of the embryos produced entire plants (Raju et al. 2015).

Procedures for the somatic embryogenesis of other *Curcuma* species, such as *C. amada* and *C. caesia* (Raju et al. 2013), have also been reported.

25.5 Concluding Remarks

Plants that produce secondary metabolites represent a valuable natural resource. They can be considered either for commercial purposes, in order to obtain active principles for pharmaceutical formulations and other products, or as part of the heritage of ancient cultures around the world. Biotechnological approaches based on these plants are, therefore, focused on three main goals: increasing products yields, efficient propagation, and preservation of rare varieties. Somatic embryogenesis could be applied to all those objectives. In this way, the availability of embryogenic cell lines might allow the development of genetic transformation protocols designed to introduce regulatory or limiting step genes. Moreover, indirect embryo formation (after callus tissue was formed) may generate genetic variation in those cases where vegetative traditional propagation may have reduced the genetic base. However, once elite materials have been selected, clonal propagation is required in order to keep the genetic stability of those traits. Somatic embryogenic propagation offers the means to achieve such end.

On the other hand, secondary metabolite-producing plants are frequently collected in wild forests, which, in some cases are endangering wild populations. Propagation through somatic embryogenesis may help to restore such populations.

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Chapter 26
Histology and Histochemistry of Somatic Embryogenesis

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Abstract The seminal reports of somatic embryogenesis in the umbellifers *Oenanthe aquatica* by Harry Waris in 1957 (Krikorian and Simola, Physiol Plant 105:348–355 (1999)) and carrot (Steward et al., Am J Bot 45:693–703 (1958)) paved the way for current studies on the mechanisms involved in the transition of somatic cells to the embryogenic state for many species (Fehér et al., Plant Cell Tiss Org 74:201–228, 2003; Elhiti and Stasolla, Plant embryo culture: methods and protocols, Humana Press, New York, 2011; Fehér, Biochim Biophys Acta 1849:385–402, 2015). Somatic embryogenesis has been a focal point of research in plant development. This process relies on somatic cell totipotency (i.e., the capacity to regenerate the entire plant from single somatic cells), and it has been long used in biotechnological breeding techniques as an efficient system for regenerating plants in a large-scale basis. Also, because it is a unique system which includes a large number of events—such as physiological reprogramming of explants as well as

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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_26

changes in the gene expression and cell division patterns, and in cell fate (Fehér, Acta Biol Szeged 52:53–56, 2008; Rose et al., Plant developmental biology-biotechnological perspectives. Springer, Heidelberg, 2010)—somatic embryogenesis has also become an appropriate method for studying the morpho-physiological and molecular aspects of cell differentiation. The comprehension of the developmental events during the induction phase as well as the development of somatic embryos is essential to regulate each stage of the somatic embryogenesis developmental program efficiently. Additionally, it may be useful for the development of efficient protocols for somatic embryogenesis induction and validation in genetic transformation systems (Fehér et al., Plant Cell Tiss Org 74:201–228, 2003; Yang and Zhang, Crit Rev Plant Sci 29:36–57, 2010; Rocha and Dornelas, CAB Rev 8:1–17, 2013; Mahdavi-Darvari et al., Plant Cell Tiss Org 120:407–422, 2015). Anatomical and ultrastructural studies have contributed to the better understanding of the basic cellular mechanisms involved in the acquisition of competence and histodifferentiation of somatic embryos (Canhoto et al., Ann Bot 78:513–521, 1996; Verdeil et al., Trends Plant Sci 12:245–252, 2001; Moura et al., Plant Cell Tiss Org 95:175–184, 2008; Moura et al., Sci Agric 67:399–407, 2010 ; Almeida et al., Plant Cell Rep 31:1495–1515, 2012; Rocha et al., Protoplasma 249:747–758, 2012; Rocha et al., Plant Cell Tiss Org 120:1087–1098, 2015; Rocha et al., Protoplasma 111:69–78, 2016). In addition, histochemical methods have enabled the monitoring of the mobilization and synthesis of reserve compounds during embryogenic development. This way, the dynamic and fate of cells committed to the somatic embryogenesis can be supported by microscopy techniques. The formation of an embryogenic callus and the subsequent differentiation of somatic embryos can be analyzed over time, and the cytological changes that have occurred during these processes can also be of great value, by associating the observed cytological changes with the expression patterns of several genes from the initial explant through competence acquisition to the formation of somatic embryos. Somatic embryogenesis has been intensively studied over the past decades. A range of descriptive studies using light and electron microscopy has provided a detailed characterization of histocytological events underlying the progression from somatic cells to the formation of embryos. Here, we review recent studies that have advanced our understanding of the anatomical and ultrastructural changes that characterize the somatic embryogenesis developmental pathway.

26.1 General Aspects of Somatic Embryogenesis Pathway

Somatic embryogenesis is the process by which somatic cells, including haploid cells under appropriate inducing conditions, divide, and differentiate into an entire plant, similarly to zygotic embryogenesis. Under in vitro conditions, the supplementation with plant growth regulators (PGRs) on the culture medium as well as stress factors play a central role in mediating the signal transduction cascade leading to somatic embryogenesis induction (von Arnold et al. 2002; Yang and Zhang 2010; Rocha and Dornelas

2013). Based on the requirements and effects of the exogenous PGRs, the somatic embryogenesis process is generally divided into two phases: (i) induction, in which differentiated somatic cells acquire competence and proliferate as embryogenic cells; (ii) expression, in which embryo morphogenesis proceeding independently of exogenous PGRs (Jiménez and Bangerth 2001; Namasivayam 2007). Some authors subdivide the induction phase into two steps: competence acquisition (somatic cells acquire competence to assume a new developmental fate) and cell determination (competent cell or tissue becomes committed to embryo formation in response to PGR supplement). Although the somatic embryogenesis pattern of some species does not strictly follow the established stages, the first step in the process (i.e., the acquisition of competence) is certainly conserved and denoted as a key step to this developmental program (Karami et al. 2009; Yang and Zhang 2010).

Somatic embryogenesis has been induced by different types of explants (e.g., cotyledons, leaves, inflorescences, stem segments, roots, protoplasts, zygotic embryos, microspores). It may occur directly from explant or indirectly after the formation of a callus stage; these processes are known as direct and indirect somatic embryogenesis, respectively. Previous studies have hypothesized that both processes are extremes of one continuous developmental pathway wherein callus represents a reprogramming step of unorganized tissue that precedes embryo formation (Fehér et al. 2003; Yang and Zhang 2010). However, recent reports have suggested that callus formation is the differentiation of pericycle-like cells present in the organ toward root meristem-like tissue and not a process of reprogramming to an undifferentiated state, as previously thought (Sugimoto et al. 2011). The mechanisms behind the induction of each developmental pattern (direct and indirect) remain poorly understood.

In general, direct embryogenesis has been induced from a culture of microspores, ovules, and zygotic embryos (Germana 2003; Paiva Neto et al. 2003; Malik et al. 2007). Indirect embryogenesis has been more often reported from different types of explants and used in biotechnological breeding methods (Jin et al. 2005; Li et al. 2006; Yang et al. 2007). Somatic embryogenesis can also be induced from somatic embryos developed from primary explants. In this case, it is called secondary somatic embryogenesis.

26.2 Structural Changes Involved in the Somatic Embryogenesis Program

26.2.1 Early Somatic Embryogenesis

As reported above, early somatic embryogenesis involves somatic cells acquiring competence and the proper induction step, in which competent cells become committed to following the embryogenic program and proliferate as embryogenic cells. The initiation of the embryogenic pathway is restricted to cells that are able to respond to a particular induction condition leading to the reprogramming of gene expression and changes in cell fate generating embryogenic cells (Nomura and Komamine 1985; Quiroz-Figueroa et al.

2002, 2006). Most cell-tracking studies of the somatic embryogenesis process have demonstrated that the morphogenesis responses typically originate from procambial cells (Schmidt et al. 1997; Somleva et al. 2000; Wang et al. 2011; Almeida et al. 2012) or from epidermal and/or subepidermal cells (Canhoto et al. 1996; Rodriguez and Wetzstein 1998; Moura et al. 2008; Rocha et al. 2012, 2015, 2016). Procambial cells are pluripotent vascular stem cells and can be easily linked to the capacity of inducing different developmental programs, such as somatic embryogenesis (De Smet et al. 2006; Atta et al. 2009; Sugimoto et al. 2010; Wang et al. 2011). Contrastingly, the mechanisms related to the plasticity of epidermal cells and their capacity to produce totipotent cell lineages that give rise to embryos still remain elusive.

26.2.2 Acquisition of Competence and Cellular Division Pattern

The acquisition of competence is mediated by adaptive mechanisms to the stress imposed by in vitro culture conditions, resulting in the reprogramming of the gene expression and cell division patterns and leading to changes in the cellular fate (Fehér et al. 2003; Fehér 2005). Competent cells may have different morphological structures depending on the species and culture conditions. In general, the competent state has been attributed to cells that exhibit meristematic features during the induction phase, such as small size, isodiametric shape, dense cytoplasmic, large, prominent and conspicuous nuclei and nucleoli (Fehér et al. 2003; Quiroz-Figueroa et al. 2006; Namasivayam 2007; Yang and Zhang 2010). Our understanding of the cytological changes involved in the acquisition of competence comes largely through ultrastructural studies. Microscopy analysis during the somatic embryogenesis of *Feijoa sellowiana* showed that somatic cells that acquired meristematic features had dense cytoplasm containing many ribosomes, numerous mitochondria with dense matrix, and some amyloplasts with small amounts of starch grain accumulation (Canhoto et al. 1996). In coconut, the meristematic cells were also characterized by dense cytoplasm, small and poorly developed vacuoles, and a voluminous central nucleus with one or two nucleoli (Verdeil et al. 2001). Similar characteristics were also observed in the somatic embryogenesis process of *Acrocomia aculeata* wherein epidermal cells became meristematic cells showing the same features described above (Moura et al. 2008).

In a case study with *Passiflora cincinnata*, histological and ultrastructural analyzes during the somatic embryogenesis induction from mature zygotic embryo explants showed that the first alterations have occurred in the peripheral layers of the cotyledons (Rocha et al. 2012). Epidermal cells (Fig. 26.1a, b) expanded in a continuous process and assumed columnar shape after successive anticlinal divisions. These cells had dense cytoplasm, large nuclei, evident nucleoli, and also showed periclinal divisions (Fig. 26.1c), initiating callus formation on the explant surface (Fig. 26.1d, e; refer to Rocha et al. 2012 for further details). The meristematic features observed in

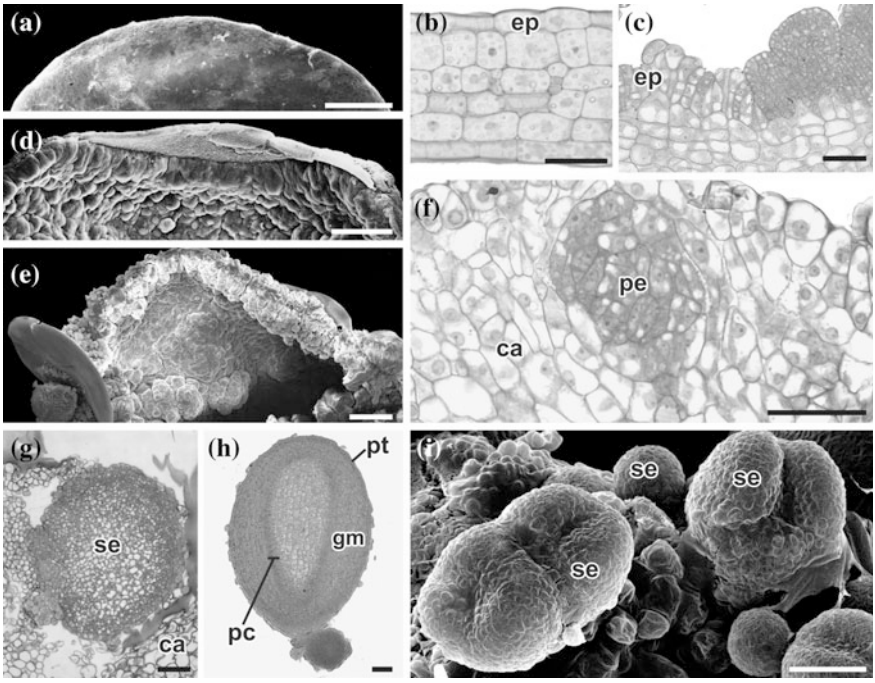

Fig. 26.1 Somatic embryogenesis of *Passiflora cincinnata*. Light (**b**, **c**, **f**, **h**) and scanning electron microscopy (**a**, **d**, **e**, **i**). **a** Cotyledon abaxial surface of the initial zygotic embryo explant. **b** Histological organization of initial zygotic embryo explant. **c** Explant after 7–10 days of culture showing anticlinal and periclinal divisions of elongated epidermal cells. Note that part of epidermal cells is already showing typical meristematic features. **d**, **e** Development of embryogenic callus. **f** Differentiation of proembryos (pe) in the periphery of callus (ca). **g** Development and exposure of a somatic embryo. **h** Somatic embryo (se) at late stages of development. Note the presence of protoderm (pt); ground meristem (gm), and procambium (pc). **i** Embryogenic cluster showing somatic embryos at different developmental stages. *Abbreviation* ep, epidermis. *Bars* **a**, **d** (0.5 mm); **b**, **c** (50 μm); **e** (1 mm); **i**, **g**, **h** (100 μm)

the epidermal cells once they assumed a columnar shape (Fig. 26.1c) were considered by the authors to be the first indication of competence acquisition during the somatic embryogenesis process. The cellular division pattern of columnar epidermal also corroborates that expanded epidermal cells may represent competent cellular state in this regeneration system. These cells showed periclinal divisions after acquiring meristematic features, denoting an unusual cellular division pattern for this tissue and also meaning that the phenotype of the epidermal cells was changed. From studies on the explants of different species, it appears that the direction of cell division can be a marker of cells undergoing changes in cell fate (Sussex and Kerk 2001; De Smet and Beeckman 2011; Almeida et al. 2012; Kurczyńska et al. 2012). In Arabidopsis somatic embryogenesis induction, the epidermal cells involved in somatic embryo formation also showed periclinal cell division pattern (Kurczyńska et al. 2007). The importance of cellular division pattern to the embryogenic developmental process is

highlighted with the occurrence of asymmetric divisions. This kind of division originates two daughter cells that fates diverge due to the subsequent interaction with neighboring cells (Heidstra 2007; Verdeil et al. 2007; ten Hove and Heidstra 2008). It does not necessarily mean those cells are of a different size after a division. For an asymmetric division to occur, coordination among the locations of cell division and cell expansion is necessary (Fowler and Quatrano 1997). The occurrence of unusual and asymmetric division during somatic embryogenesis was described for *Medicago sativa* (Uzelac et al. 2007), *Bactris gasipaes* (Almeida et al. 2012), and *Araucaria angustifolia* (Steiner et al. 2016).

26.2.3 Cellular Proliferation—Embryogenic Calli Features

Once meristematic competent cells have been formed (Fig. 26.1c), they continue to proliferate (Fig. 26.1d, e) and form proembryogenic clusters (Fig. 26.1f). However, not all meristematic cells become embryogenic cells. Somatic embryos can differentiate either directly from the explant or indirectly after a callus phase.

During the indirect somatic embryogenesis system, both embryogenic and non-embryogenic regions are present in the calli (Fig. 26.2). It is usually easy to distinguish the embryogenic and non-embryogenic zones on the basis of morphological structure, cellular characteristics, and color (Carvalho et al. 2013). Embryogenic clusters present yellow or dark-yellow color, nodular features, and smooth surface whereas cells are generally characterized by the small size, isodiametric shape, dense cytoplasm, numerous mitochondria, evident stained nuclei and nucleoli, small vacuoles, and a higher metabolic activity. Conversely, non-embryogenic regions are generally described as rough, friable, and translucent with disorganized cellular system (Fig. 26.2) constituted by different cell shapes and

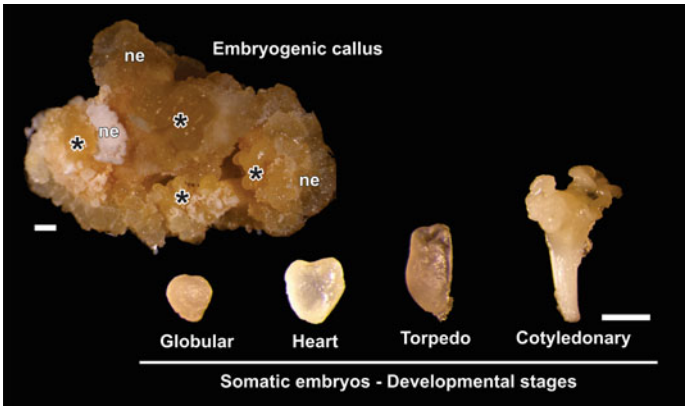

Fig. 26.2 Somatic embryogenesis of *Passiflora cincinnata*. Embryogenic callus and the stages of embryo development. Note the presence of embryogenic (*) and non-embryogenic (ne) regions in the callus. *Bars* 10 mm

highly vacuolated cells, with few organelles that can be interpreted as signals of low metabolic activity (Jiménez and Bangerth 2001; Yang and Zhang 2010; Carvalho et al. 2013).

26.2.4 *Late Somatic Embryogenesis—Origin and Developmental Stages of Somatic Embryos*

Somatic embryos originate from a single polarized cell (unicellular) or a group of cells (multicellular). Embryos originated from a single cell usually present a coordinated cell division patterning during their development, which has facilitated the comprehension of the cellular events undergoing the embryogenic process (Quiroz-Figueroa et al. 2006). The presence of a suspensor-like structure connecting the embryos to the maternal tissue has also been shown in the embryos with unicellular origin. In contrast, embryos with a multicellular origin show a complex cell division patterning. The mechanisms involved in the embryogenic initiation are not clear and the suspensor-like structure is not morphologically distinguishable from the maternal tissue in most of the multicellular systems reported (Quiroz-Figueroa et al. 2006). Histological studies in different species have described both unicellular (Canhoto et al. 1996; Verdeil et al. 2001; Quiroz-Figueroa et al. 2002; Rojas-Herrera et al. 2002) and multicellular (Rodriguez and Wetzstein, 1998; Moura et al. 2010; Rocha et al. 2012; 2015) pathways. The occurrence of both formation patterns in the same embryogenic system has also been reported (Puigderrajols et al. 2001; Kurczyńska et al. 2007; Moura et al. 2008; San-José et al. 2010; Almeida et al. 2012).

Microscopy studies have characterized proembryogenic stem-like cells as small sized with an isodiametric shape, dense cytoplasm, and high nucleus/cytoplasm ratio (Fig. 26.1f), revealing that most of ultrastructural characteristics are similar to those described for meristematic competent cells. However, cytological features of the nucleus have proposed to distinguish between meristematic and embryogenic cells (Verdeil et al. 2007; Kurczyńska et al. 2012). In meristematic cells (described from shoot meristem), the nucleus is spherical and contains several nucleoli and heterochromatin uniformly distributed within the nucleus. In the case of embryogenic cells, the nucleus usually shows an irregular shape with only one large nucleolus (Verdeil et al. 2007). In *Passiflora edulis,* differences in the structures of the nucleus were also observed during somatic embryogenesis (Rocha et al. 2016). At the beginning of the process, protodermal cells that acquired meristematic features showed nuclei with conspicuous nucleoli and heterochromatin distributed within the nucleus. Later, cells recognized as proembryogenic stem-like cells were described as those containing a central nucleus with one nucleolus and small heterochromatic regions located at the periphery of the nucleus (Rocha et al. 2016). The authors also described the presence of numerous rough endoplasmic reticulum cisternae concentrically arranged, characteristic that is commonly observed in embryogenic cells (Canhoto et al. 1996).

Somatic embryo development encompasses the same embryogenic stages of zygotic embryogenesis: globular-shaped, heart-shaped, torpedo-shaped, and cotyledonary stages in eudicots (Figs. 26.1g–i; 26.2); globular scutellar and coleoptilar stages in monocots. The mature somatic embryos resemble zygotic embryos morphologically and physiologically. Both exhibit apical–basal and radial polarity, possess the apical shoot and root meristems, and contain the typical embryogenic organs cotyledons, hypocotyl, and radicle. Histologically, the primary tissues protoderm, ground meristem, and procambium are also identified in the somatic embryos (Fig. 26.1g, h).

26.3 Histochemical Evidences During Somatic Embryogenesis

Storage reserves may have an important role during in vitro morphogenesis, and histochemical analysis has been used to correlate the mobilization and synthesis of storage compounds with the development of somatic embryogenesis pathway (Cangahuala-Inocente et al. 2004, 2009; Moura et al. 2010; Rocha et al. 2012; Almeida et al. 2012; Silva et al. 2015). Analyzes performed by Rocha et al. (2012) and Silva et al. (2015) on passion fruit cotyledons of mature zygotic embryos used as an initial explant of two different species, indicated the presence of storage reserve. Protein and oil bodies were evidenced by the positive reaction to xylidine Ponceau and Sudan black (Fig. 26.3a), respectively. During the somatic embryogenesis induction, proteins, and lipids were consumed (Fig. 26.3b) supporting the idea that reserve compounds are necessary for cellular reorganization and differentiation. The authors also reported the presence of starch, as revealed by the positive reaction to periodic acid–Schiff's reagent, during embryogenic process (Fig. 26.3c, d). This compound was not observed as a storage reserve in the initial zygotic embryo explant (Fig. 26.3c), indicating the occurrence of de novo synthesis (Rocha et al. 2012; Silva et al. 2015).

The accumulation of storage reserves is a key process of zygotic embryogenesis, providing the energy required for subsequent germination and seedling establishment. In somatic embryos, the accumulation of similar storage compounds has also been reported, although differences in timing of accumulation and proportion between individual types of nutrients were observed (Moura et al. 2010; Pinto et al. 2010; Jariteh et al. 2015). A comparison between macaw palm zygotic and somatic embryos, using a complete histochemical approach showed that zygotic embryos present high quantities of protein and lipids, stored in protein and lipid bodies, respectively. In contrast, these compounds were weakly detected or completely absent in somatic embryos, which was associated with the low conversion of these embryos into plants (Moura et al. 2010). Similar results were also reported in *Eucalyptus globulus*, where the reserves of somatic embryo cotyledons differed from those of their zygotic embryo counterparts (Pinto et al. 2010). The authors also

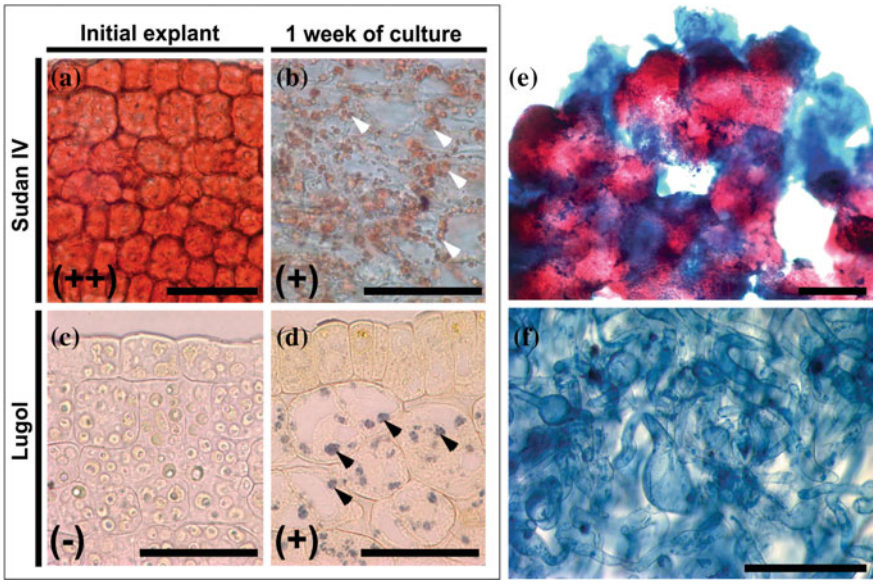

Fig. 26.3 Histochemical analysis. **a**–**d** Study of *Passiflora cincinnata* cotyledons at the early stages of somatic embryogenesis induced from mature zygotic embryos explants. **a**, **b** Transverse sections of initial explants (**a**) and after 1 week of culture (**b**) subjected to Sudan IV test. A positive reaction for lipids is evidenced by the *orange color*. **c**, **d** Transverse sections of initial explants (**c**) and after 1 week of culture (**d**) subjected to Lugol test. Starch grains positively stained shows a *purple stained color* (*black arrowheads*). **e**, **f** Evans blue/acetocarmine histochemical test. Embryogenic/meristematic showed an intense *red stained* with acetocarmine (**e**). Non-embryogenic cells *stained blue* (**f**). *Abbreviations* (++) abundance, (+) presence, (−) absence. *White arrowheads*, oil bodies. Bars = **a**–**d** (50 μm); **e**, **f** (100 μm)

related reserve profile of somatic embryos with the low germination rates, which reinforce the importance of reserves in the embryogenic process.

Histochemical monitoring was also used to determine the essential factors involved in the embryogenic differentiation allowing the recognition of regions and/or tissues with high energetic activity (Kouakou et al. 2007; Pinto et al. 2011; Rocha et al. 2012). Tests with acetocarmine and Evan's blue have been successfully used to differentiate embryogenic/meristematic and non-embryogenic regions present in the calli (Durzan 1998; Steiner et al. 2016; Silva et al. 2009). In general, cells with embryogenic/meristematic features such as small, isodiametric and dense cytoplasm, and high nucleus/cytoplasm ratio stained an intense red with acetocarmine (Fig. 26.3e). Non-embryogenic cells stained blue (Fig. 26.3f). Histochemical tests for proteins as xylidine Ponceau and naphthol blue–black have also been used to detect potential morphogenic regions in the explant. Cells with intense staining by xylidine Ponceau or naphthol blue–black may suggest a high incidence of RNA synthesis and high metabolic activity (Stein et al. 2010; Almeida et al. 2012).

26.3.1 Changes in Apoplast During Somatic Embryogenesis

26.3.1.1 Arabinogalactan Proteins and Pectins

Arabinogalactan proteins (AGPs) are an umbrella term for a large class of proteoglycans widely distributed and ubiquitous throughout the plant kingdom (Nothnagel 1997; Seifert and Roberts 2007). At the subcellular level these compounds are associated with secretory pathways, plasma membranes, and cell walls, as well as being secreted into the culture medium (Šamaj et al. 2000; Showalter 2001). Classical AGPs contain a domain responsible for attaching the protein backbone to a glycosylphosphatidylinositol (GPI) membrane anchor. Other classes of AGPs include lysine rich AGPs, AG peptides, FLA (fascin-like AGPs), and chimeric AGPs (Seifert and Roberts 2007).

AGPs have been described in distinct processes of cell differentiation, cell expansion and division, and have been proposed to be integral parts of the signaling cascade of plant growth regulators (i.e., gibberellin (Suzuki et al. 2002; Mashiguchi et al. 2008), abscisic acid (van Hengel et al. 2004)). Additionally, AGPs may act as molecular markers for specific cells (i.e., during reproductive development (Coimbra et al. 2007)), as well as for morphogenetically competent cells (Šamaj et al. 1999), with putative mechanical and physiological roles during the signaling cascade and plant cell polarization. A more general role of AGPs as a cell-cell signaling molecule during the induction and development of somatic embryos has also been proposed.

Early in the development of somatic embryos in vitro polarization is established (Šamaj et al. 2006). Both the cytoskeleton and cell walls appear to play an essential regulatory role during this process (Šamaj et al. 2006). It has been shown that AGPs can indirectly interact with the microtubule and actin in the cells (Sardar et al. 2006; Driouich and Baskin 2008), playing a role during polarized growth. AGPs are also interlinked with pectins (Immerzeel et al. 2006). Pectins are mostly localized in the middle lamella and primary cell wall and are, like AGP, branched molecules (Carpita and Gibeaut 1993). These interactions of AGPs with pectin and microtubule support the hypothesis of a continuum between cytoskeleton, plasma membranes, and cell walls (Kohron 2000; Baluska et al. 2003). Therefore, a multifaceted biological function of AGPs is expected and although it is far from being completely understood (Chapman et al. 2000a, b; Seifert and Roberts 2007), their characterization offers the opportunity of identifying regulatory mechanisms of somatic embryogenesis.

Different techniques might be used for detection and studying the role of AGPs during plant development. The use of Yariv reagent (ßGlcY), a synthesized chemical antibody that specifically binds AGPs molecules is a reliable method to study the localization and the role of AGPs during plant morphogenesis (Chapman et al. 2000a, b; Steinmacher et al. 2012). In a previous study, ßGlcY was applied as a staining dye and showed the characteristic red color of the AGPs-ßGlcY complex over the globular somatic embryos, with an intense staining found in the apex of

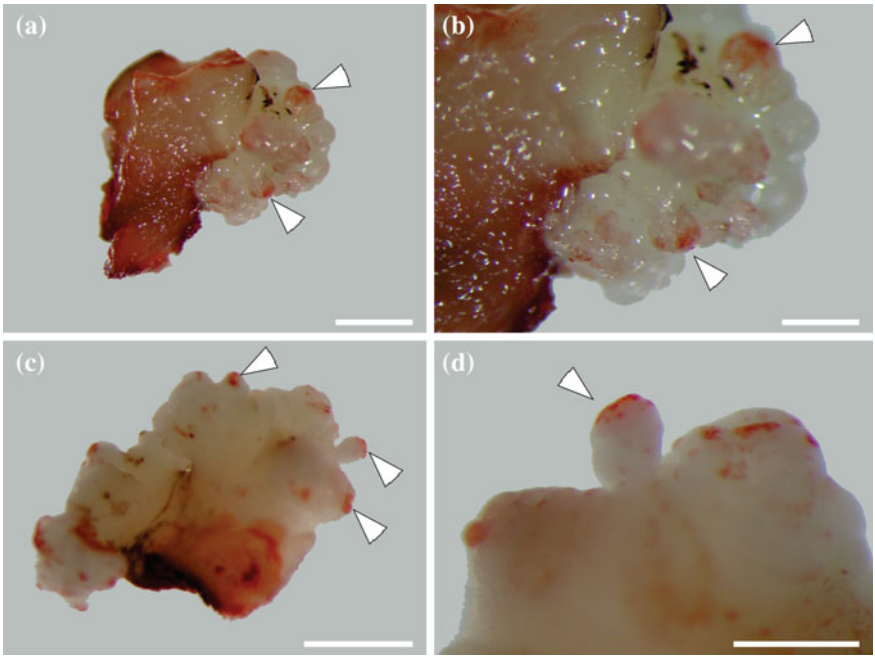

Fig. 26.4 Clusters of somatic embryos stained overnight with ßGlcY solution. (**a–d**) An intense *red color* in specific sectors of the explant as well as on the protoderm of the globular somatic embryos was observed (*arrowheads*). *Bars* **a–c** (5 mm); **d** (2 mm)

(pre) globular somatic embryos (Fig. 26.4), revealing that AGPs were secreted in a polarized way (Steinmacher et al. 2012). Yariv reagent (ßGlcY) also binds to and aggregates AGPs molecules causing its loss-of-function, reducing somatic embryo formation in *Chicorium* sp. (Chapman et al. 2000a, b) and *Bactris gasipaes* (Steinmacher et al. 2012). The inclusion of ßGlcY into the culture medium also showed the inhibition of cell division in suspension-cultured *Rosa* sp. cells (Serpe and Nothnagel 1994) and 50 μM ßGlcY could completely inhibit cell division in *Brassica* sp. microspores (Tang et al. 2006). In *Daucus carota* a stage-specific response to ßGlcY was observed, with root growth being promoted in the early stages and overall growth reduced in late stages (Thompson and Knox 1998).

The presence of ßGlcY in the culture medium also resulted in morphological alterations during the development of somatic embryos. Apparently, a more pronounced effect is observed in the protoderm cells which turn loosened and bulged, as shown in *Bactris gasipaes* somatic embryos (for details see Steinmacher et al. 2012) and *Brassica* sp. somatic embryos (Tang et al. 2006). Other responses might also occur, as in tobacco, where disturbing AGPs by application of ßGlcY increased the symmetrical division rate in zygotes (Qin and Zhao 2006). These observations support the indirect interaction between AGPs, microtubules, and actin filaments (Sardar et al. 2006; Driouich and Baskin 2008). However, continuous contact with

ßGlcY is necessary to exert its effect. On solid culture medium the development of *Bactris gasipaes* somatic embryos was affected by ßGlcY only in those areas in contact with the culture medium (Steinmacher et al. 2012). When applied to *Arabidopsis thaliana* seedlings, ßGlcY was not able to enter the stele, and its effect was observed only in the root epidermal cells (Willats and Knox 1996).

The ßGlcY reagent can also be used for quantification of secreted AGPs into the culture medium, through a technique named radial gel diffusion (van Holst and Clarke 1985), which consists in the comparison of the diffusion potential in agarose-gels of solutions of isolated AGPs at unknown amounts with standards at pre-defined amounts (usually Arabic gum). Results showed that the amount of AGPs secreted into the culture medium have a possible correlation with the development of somatic embryos (Saare-Surminski et al. 2000; Steinmacher et al. 2012). Secreted AGPs also have a profound effect on the in vitro plant cells responses, as a specific set of AGPs from conditioned culture medium or from immature seeds could increase or even fully restore somatic embryo formation (McCabe et al. 1997; van Hengel et al. 2001). Results also pointed to a non-species-specific response because conditioned culture medium from one species could increase the embryogenic response in other species (Kreuger and van Holst 1993; Ben Amar et al. 2007). The increase in somatic embryogenesis induction with the inclusion of AGPs re-isolated after an endochitinase treatment has also been described (van Hengel et al. 2001). In the plant model *Gossypium hirsutum* the increased rate of somatic embryos induction was statistically greater with the inclusion of the extracellular AGPs fraction into the culture medium (Poon et al. 2012). Furthermore, it is known that a specific set of AGPs could have an inhibitory effect on somatic embryogenesis (Toonen et al. 1997) as has been documented with AGPs extracted from *G. hirsutum* non-embryogenic calli, which inhibited somatic embryogenesis when incorporated into the culture media of the same species (Poon et al. 2012).

Immunolocalization techniques are one of the best methods to identify and to precisely locate polymers in situ within complex tissues. These methods generally use monoclonal antibodies that were developed from complex cell-wall-derived materials (Knox 2008). During the onset and differentiation of somatic embryos different expression pattern of AGPs within the cells and development of somatic embryos have been observed. This is especially linked to protoderm and shoot meristem differentiations, as observed in *B. gasipaes* (Steinmacher et al. 2012), *A. thaliana* (Hu et al. 2006), *D. carota* (Stacey et al. 1990) and in *E. pulcherrima* (Saare-Surminski et al. 2000). This suggests specific roles of AGPs during the coordinated development of somatic embryos.

Somatic embryo development is frequently associated with the formation of an extracellular matrix surface network (ECMSN—also known as a supraembryogenic network) covering the (pre-) globular somatic embryos. Numerous studies revealed that it is composed of AGPs, peptidic substances, proteins, and lipophilic substances (Chapman et al. 2000a, b; Konieczny et al. 2005; Namasivayam et al. 2006; Popielarska-Konieczna et al. 2008a, b; Steinmacher et al. 2012; Pilarska et al. 2013). It appears to be an evolutionarily conserved characteristic, described in

gymnosperms (Šamaj et al. 2008) and angiosperms (Chapman et al. 2000a, b; Verdeil et al. 2001; Bobák et al. 2003; Steinmacher et al. 2012; Pilarska et al. 2013). Different roles for the ECMSN have been proposed, including cell adhesion and control of morphogenesis of a specific group of cells (Šamaj et al. 2006; Popielarska-Konieczna et al. 2008a, b). Oligosaccharides released from ECMSN might act as signaling molecules involved in the regulation of developmental processes (Eberhard et al. 1989; Darvill et al. 1992), and the ECMSN is related to cuticle formation, which in turn would play a protective role (Popielarska-Konieczna et al. 2008a, b). Detailed analyzes with a specific MAb Jim13 in *B. gasipaes* revealed its association with the endomembrane and secretory vesicles of the protoderm cells and its presence on the ECMSN (Steinmacher et al. 2012). In *Chicorium* sp., immunogold localization of different AGPs epitopes (including MAb Jim13) evidenced its localization also in the outer cell walls of globular somatic embryos and ECMSN (Chapman et al. 2000a, b). A chimeric AGP and a non-specific lipid transfer protein (nsLTP) named Xylogen, with a cell–cell signaling role during xylem differentiation, have been described in *Zinnia* sp. (Motose et al. 2004). This chimeric AGP was recognized by the monoclonal antibody (MAb) Jim13 and was shown to play a fundamental role in xylem differentiation, linking the AGPs to the roles of nsLTPs, revealing multiple functions of a single macromolecule regulated in an orchestrated manner.

The composition of the ECMSN has revealed differences between different plant groups (Pilarska et al. 2013), including differences in the pectin fraction (Konieczny et al. 2007). The presence of highly-methyl esterified pectin (recognized by MAb Jim7) is associated with the ECMSN in monocot species (Šamaj et al. 2006; Konieczny et al. 2007; Steinmacher et al. 2012). On the other hand, the ECMSN in eudicot species also showed the presence of low-methyl esterified pectins recognized by MAb Jim5 (i.e., chicorium (Chapman et al. 2000a, b), kiwi (Popielarska-Konieczna et al. 2008a) and *Trifolium nigrescens* (Pilarska et al. 2013)).

26.3.2 Lipid Transfer Proteins

Kader (1975) described the lipid transfer proteins (LTPs) 40 years ago. From that moment, available data increased and now our knowledge about its structure, localization, gene expression, and biological function is significantly larger (Carvalho and Gomes 2007; Liu et al. 2015). LTPs are small peptides divided into two families called LTP1 (10 kDa molecular weight) and LTP2 (7 kDa molecular weight; for details see Carvalho and Gomes 2007). LTPs are involved in different biological processes like pollen adherence (Park et al. 2000), plant signaling (Maldonado et al. 2002), adaptation to various environmental stresses, both biotic and abiotic (Liu et al. 2015; Safi et al. 2015), cutin synthesis (Domínguez et al. 2015), seed development and germination (for details see Liu et al. 2015), and somatic embryogenesis (Sterk et al. 1991; Potocka et al. 2012; Smertenko and Bozhkov 2014;). Involvement of LTPs in embryogenesis is postulated because it

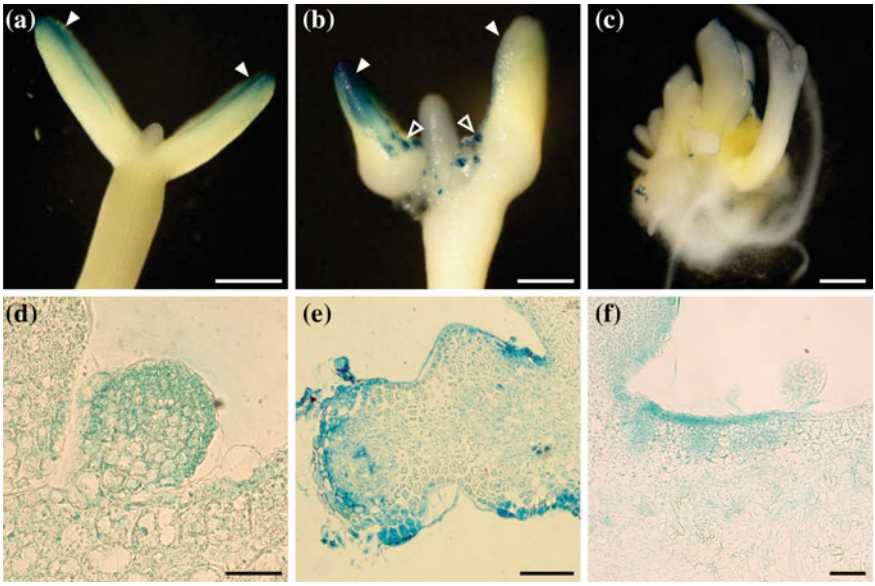

Fig. 26.5 Morphological and histological analysis of GUS distribution in explants of transgenic plants carrying GUS reporter gene driven by promoter of *LTP1* and spatial pattern of *LTP1* promoter activity in some developmental stages of the culture. **a** Whole mount of explant cultured for 4 days, GUS staining in the distal parts of cotyledons (*solid arrowheads*). **b** Whole mount of explant cultured for 6 days, staining in both the distal (*solid arrowheads*) and proximal (*empty arrowheads*) parts of cotyledons which are engaged in somatic embryogenesis. **c** Mature somatic embryos. **d** Histological section through the explant and somatic embryo showing high level of the promoter activity in the embryo cells. **e**, **f** Sections through the cotyledons of the explants with intense staining in the peripheral tissues, day 8 (**e**) and day 21 (**f**) of culture. *Bars* **a**–**c** (200 μm); **d**, **f** (20 μm); **e** (100 μm)

was shown that LTPs are secreted into the medium, participate in the formation of a protective layer of the embryo, and the high level of *LTP* gene expression was observed during the embryo development (Potocka et al. 2012). Such data are consistent with the hypothesis that LTPs are engaged in the processes of embryogenesis including the somatic ones (for details see Kader 1996).

LTP gene expression in the *Arabidopsis thaliana* explants cultured according to the method described by Gaj (2001) differed in various parts of explants during the culture period (Fig. 26.5). Stereomicroscopic observation and histological analysis revealed *AtLTP1* gene expression in distal parts of explant cotyledons, especially at the early stages of the culture (Fig. 26.5a). In more advanced cultures, the expression of this gene was detected in explant regions committed to the somatic embryogenesis process (Fig. 26.5b; Kurczyńska et al. 2007, 2012). In explants with the well visible somatic embryos (SE) the expression almost disappeared (Fig. 26.5c). In SE *LTP* gene expression was different in different developmental stages and was detected from the globular to mature stage (Fig. 26.5c, d). This

expression was mostly located at the periphery of explant tissues (Fig. 26.5e, f). In all studied cases, the gene expression was correlated with the explant cells that changed the direction of their differentiation (Fig. 26.5a, b, e, f). *LTP* gene expression was studied only in a few cases in connection with embryogenesis. For *Arabidopsis* zygotic embryogenesis it was shown a position-specific expression of the *AtLTP1* gene. In wild-type embryos this gene was expressed in the protoderm and initially in all protodermal cells, whereas in more mature embryos *AtLTP1* expression was confined to the cotyledons and the upper end of the hypocotyl (Vroemen et al. 1996). Some similarities between zygotic and somatic embryos in connection with *LTP* gene expression are clearly visible. In the case of *Daucus carota* in situ hybridization showed the expression of the *EP2* gene in protodermal cells of somatic and zygotic embryos (Sterk et al. 1991). In an androgenic culture of *Brassica napus* (Tsuwamoto et al. 2007) and *Hordeum vulgare*, *LTP* gene had a similar expression pattern to that of an LTP known to be a marker of the early stages of the carrot somatic embryogenesis (Vrinten et al. 1999).

The cellular/tissues distribution of LTP was described only in the case of *A. thaliana* during post-embryonic development, and such histological analysis at the electron microscopy level showed the presence of this protein within the cell walls of epidermal cells of different organs (Thoma et al. 1993). Other studies described the LTP distribution during *A. thaliana* somatic embryogenesis (Potocka et al. 2012). Studies on the distribution of lipid transfer protein 1 (LTP1) epitopes during somatic embryogenesis of *A. thaliana* showed the correlation with the morphogenic events during this process (Potocka et al. 2012). It was shown that in protodermal cells of the cotyledon exhibiting features typical of embryogenic cells, LTP was present in the anticlinal and inner periclinal walls and in the cytoplasm. However, in protodermal cells of the cotyledon exhibiting features typical of meristematic cells, LTP was detected only in the cytoplasm (Potocka et al. 2012). The described differential distribution of this protein within the explant is postulated as a marker for embryogenic cells.

Additional analysis of the spatiotemporal distribution of LTP, recognized by an anti-AtLTP1 antibody in explants of *A. thaliana* subjected to somatic embryogenesis induction, showed more differential distribution of this protein between cells with different developmental programs within the explant (Fig. 26.6). At the start of the cultures, LTPs were present abundantly in the outer periclinal walls of protodermal cells of the explant (Fig. 26.6a). Another distribution pattern is characterized by a punctate presence of LTP within cytoplasm of some explant cells and on the plastids envelope (Fig. 26.6b). The most distinctive/repeated pattern of LTPs distribution during the culture was their presence in the surface parts of the explant, and also their extracellular localization (Fig. 26.6c). Within an explant, apart from the meristematic and embryogenic cells, the cells which can be named competent (Rocha et al. 2016) are also present, and are distinguished from cells with other developmental programs, as a less intensive labeled (Fig. 26.6d). However, this difference is because of the extensive labeling of plastid envelope which is

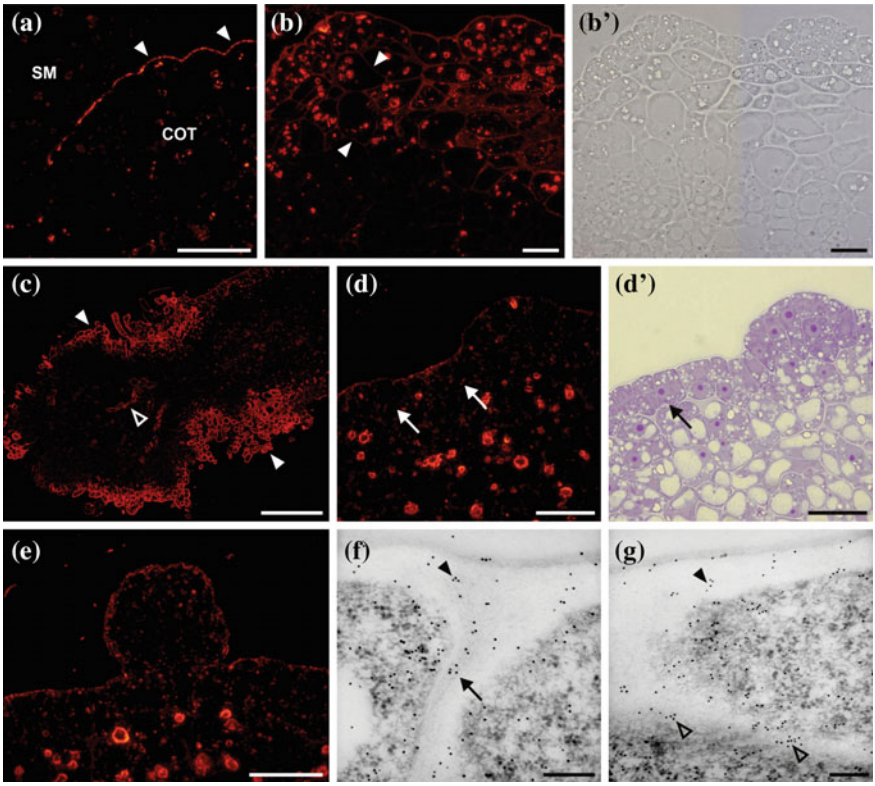

Fig. 26.6 Immunolocalization of LTP1 epitopes during somatic embryogenesis in *Arabidopsis thaliana*. **a** 4-day cultured explants, labeling in the outer periclinal walls of the protoderm (*solid arrowheads*) and in the plastid envelope. **b** Cells in the adaxial part of the cotyledon (day 14 of culture), labeling in the cell walls (*solid arrowheads*) and in various cell compartments. **b'** Phase contrast view of the section shown in (**b**). **c** Cotyledon of the explant cultured for 14 days, labeling restricted to the peripheral cells (*solid arrowheads*) and tracheary elements (*empty arrowhead*). **d** LTP1 epitopes in the embryogenic competent cells (*arrows*, day 7 of culture). **d'** Section neighboring to the one in (**d**), stained with Toluidine Blue O, showing cytological characteristics of cells. **e** Globular somatic embryo, labeling present in the outer periclinal cell walls of the protoderm and in the cytoplasm. **f**, **g** Immunogold localization of LTP1 epitopes in the adaxial protodermal cells of the cotyledons, gold particles visible in anticlinal (*arrow*), outer periclinal (*solid arrowheads*) and inner periclinal (*empty arrowheads*) walls (day 9 of culture). *Abbreviations* SM, shoot apical meristem; COT, cotyledon. *Bars* **a**–**b**, **d**, **e** (20 μm), **c** (100 μm), **f**, **g** (200 nm)

abundant in bordering cells. Labeling was also observed in developing somatic embryos (Fig. 26.6e).

Differences in labeling abundance and distribution between embryogenic and nonembryogenic regions of explants were also studied in detail with the use of immunogold electron microscopy, and the labeling was stronger in both the outer periclinal and anticlinal walls of the protodermal cells of the explant (Fig. 26.6f, g, and Potocka et al. 2012).

26.4 Perspectives and Conclusions

Histochemical and histological techniques are instrumental and have contributed significantly to assessing and better characterizing morphogenic events that lead to efficient in vitro somatic embryogenesis systems. As stated by Yeung (1999) indeed a good anatomical and histochemical-based work provide insightful clues to support further experimental hypothesis. Once reliable and reproducible somatic embryogenesis protocols are in hands it is important to characterize the developmental events during the induction, transition and formation of somatic embryos linked structural changes that take place in the explants. An understanding of embryogenic initiation, origin of somatic embryo is critical to scientific and biotechnological applications. During the past decade a lot of progress has been made on the cellular and molecular-based studies involved in somatic embryogenesis induction. What is expected is also an ever-growing advanced microscopy technique to monitor deeply and efficiently cellular changes to evolve alongside with such a fast and ever-growing knowledge and scenarios on molecular aspects that govern somatic embryogenesis. To gain better insights into the mechanisms of somatic embryogenesis, the integration of cellular and molecular analysis are necessary to provide critical new information through the embryogenic program with spatial and temporal approaches.

Acknowledgments This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Brazil), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (Brazil), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) (Belo Horizonte, MG, Brazil), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (São Paulo, SP, Brazil), and Ministry of Science and Higher Education (Poland; Grant Number 1S-0115-001-1-01-01-05).

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Chapter 27
Laser-Assisted Microdissection to Study Global Transcriptional Changes During Plant Embryogenesis

Ricardo A. Chávez Montes, Joanna Serwatowska and Stefan de Folter

Abstract Next-generation sequencing platforms are able to provide a comprehensive overview of gene activity in a particular biological sample. When coupled to techniques that isolate specific cell types, or the RNA from specific cell types, NGS can provide a detailed and granular view of the transcriptional activity in an organ or tissue. Laser-assisted microdissection is a microscopy-based technique that allows the isolation of discrete cell types from their tissular context while maintaining RNA integrity. In this chapter we will present an overview of the LAM technique, and present the few works that have undertaken transcriptomic analyses of plant embryos. While zygotic embryos, embryogenic calli or somatic embryos from different plant species have been studied using microarrays or high-throughput sequencing, LAM-based transcriptomics has only been applied to the Arabidopsis zygotic embryo and seed.

27.1 Introduction

Organisms such as higher plants are composed of diverse cellular types, each defined by a particular transcriptomic, proteomic, and metabolomic profile. An essential step toward unraveling this complexity will be to obtain a complete, cell specific atlas of gene expression for all cell types of a particular organism. Indeed, gene expression is essential for our understanding of the transcriptional, biochemical, or physiological pathways that underlie each cell population. Next-generation sequencing (NGS) techniques provide the answer to the first part of the equation: they are able to provide a comprehensive view of gene expression for a particular biological sample. NGS can then be complemented with techniques

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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_27

aimed at obtaining discrete cell populations, the combination of both resulting in highly specific tissue or cell type gene expression profiles. Several techniques have been developed that can be used to obtain cell type-specific transcriptomes: cell sorting of isolated protoplasts from fluorescent reporter lines (Birnbaum et al. 2003, 2005; Iyer-Pascuzzi and Benfey 2010), translating ribosome affinity purification (TRAP) (Mustroph et al. 2009, 2013; Zanetti et al. 2005), or isolation of nuclei tagged in specific cell types (INTACT) (Deal and Henikoff 2010, 2011) (reviewed in Palovaara et al. (2013)) have been used to characterize the transcriptomic profiles of discrete cell populations. Unfortunately, these techniques have requirements that cannot be met for many plant species: not all tissues are a suitable source material for protoplasting, and TRAP or INTACT require plants that can be transformed in order to obtain the corresponding fluorescence or epitope-tagged proteins. Laser-assisted microdissection (LAM) has therefore established itself as a popular technique that avoids such limitations, can be used with different plant species, and provides sufficient material for downstream transcriptomic analyses (Day et al. 2005; Gautam and Sarkar 2015; Kerk et al. 2003; Nelson et al. 2006). In Arabidopsis, LAM-based transcriptomic studies have been reported for the shoot apical meristem (Brooks et al. 2009), inflorescence and floral meristems (Mantegazza et al. 2014), megaspore mother cell and nucellus tissue (Schmidt et al. 2011; Tucker et al. 2012), female gametophyte (Wuest et al. 2010), ovule primordium (Matias-Hernandez et al. 2010), and seed (Belmonte et al. 2013). Nowadays, it is even possible to do metabolomics on LAM-captured cells (reviewed in Fang and Schneider (2014)).

27.2 Overview of the Laser-Assisted Microdissection Technique

LAM is a microscopy-based technique that enables the isolation of specific cell types from their tissular context. Although there is no one protocol that can be applied to all plant cell types, all procedures follow the same basic steps (Fig. 27.1):

(1) Tissue fixation
(2) Tissue embedding in a solid matrix
(3) Tissue sectioning
(4) LAM
(5) RNA purification and downstream analysis.

During all these procedures precautions are taken toward one sole purpose: to preserve RNA integrity. In the following sections, we will present the most common methodology used for each of these steps, with emphasis on throughput and the preservation of RNA quality. For more in depth focus on the technical aspects of the technology, we refer the reader elsewhere (Gautam and Sarkar 2015; Kerk et al. 2003; Ludwig and Hochholdinger 2014; Takahashi et al. 2010).

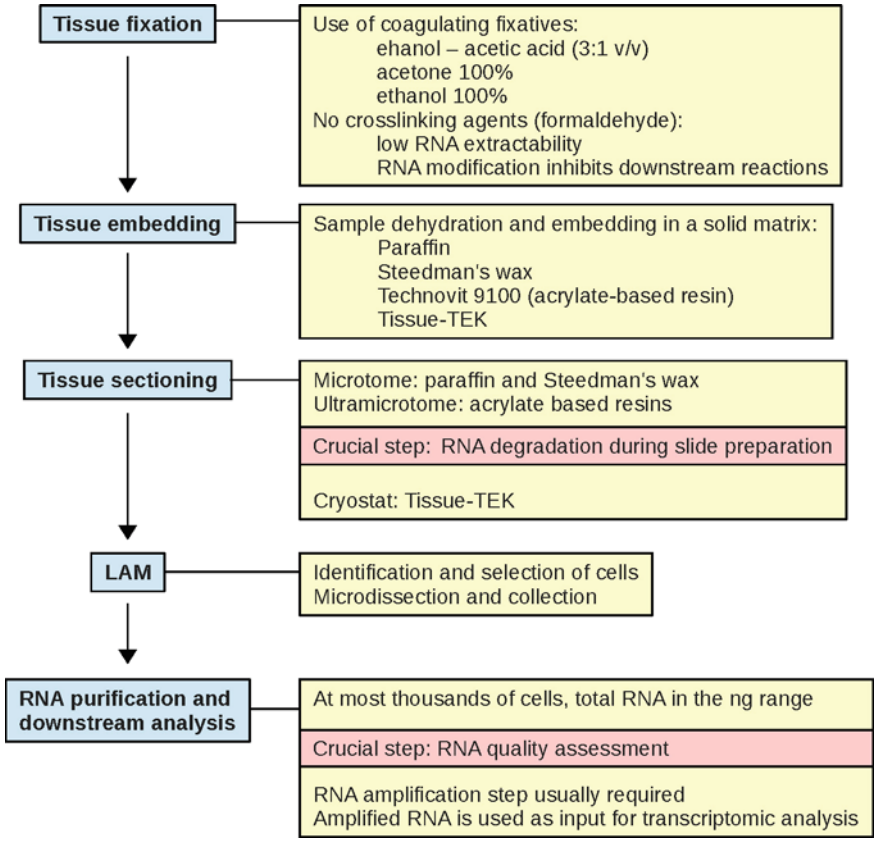

Fig. 27.1 Overview of the LAM technique

27.2.1 Tissue Fixation

In order to preserve RNA integrity for downstream applications, fixation of the tissue, the first step in embedding protocols, is done without cross-linking agents. Use of formaldehyde during fixation is better at preserving morphology, but RNA is less extractable and the modifications it suffers prevent its use downstream (Kerk et al. 2003; Nakazono et al. 2003; Yu et al. 2007). Most LAM-oriented tissue embedding protocols use as fixative an ethanol–acetic acid (3:1) mixture (Farmer's fixative; Kerk et al. (2003)), although 100 % acetone or 100 % ethanol can also be used (Jiao et al. 2009; Takahashi et al. 2010). 100 % acetone has been reported to slightly alter the morphology of some tissues (Yu et al. 2007).

27.2.2 Tissue Embedding

The most common approach in LAM-based techniques is embedding of the tissue in a paraffin matrix. After fixation with ethanol–acetic acid, tissue is dehydrated with a graded ethanol series. Acetone or ethanol fixations do not require dehydration steps. Ethanol is then replaced by paraffin solvent, either xylene or an equivalent reagent such as Histo-Clear (National Diagnostics) or Clearing Agent (Electron Microscopy Sciences). Xylene is then replaced by paraffin and blocks are prepared for tissue sectioning. A typical embedding protocol is as follows:

Tissue fixation (4 °C):

- Ethanol:acetic acid (3:1 v/v) 2 × 15 min under mild vacuum
- Leave in ethanol:acetic acid 3:1 overnight

Dehydration (4 °C):

- Ethanol 70 %: 1 h w shaking
- Ethanol 80 %: 1 h w shaking
- Ethanol 90 %: 1 h w shaking
- Ethanol 100 %: 1 h w shaking
- Ethanol 100 %: 1 h w shaking
- Leave in ethanol 100 %: overnight

Xylene (ambient temperature):

- Ethanol:xylene (3:1 v/v): 1 h
- Ethanol:xylene (1:1): 1 h
- Ethanol:xylene (1:3): 1 h
- Xylene (100 %): 1 h

Add a few beads of Paraplast and leave overnight
Paraffin (58−60 °C):

- Decant a few ml and replace with molten Paraplast every 1 h, then
- Leave in Paraplast 100 % overnight

Prepare blocks

The above listed procedure is not absolute: protocols from different laboratories will have minor variations in incubation times, the number of dilutions and the concentration of ethanol during fixing or dehydration, the number of steps for xylene to paraffin replacement, and so on. While paraffin is the most common embedding material used, there are also reports on the use of Steedman's wax (Gomez et al. 2009; Hogekamp et al. 2011), which has a melting point of around 37 °C. Finally, acrylate-based resins have also been used (Klink et al. 2013; Okada et al. 2013). In this case sections obtained in the next step are thinner and require the use of glass or diamond knives.

Although RNA integrity should be preserved during embedding (Jiao et al. 2009), this process takes several days to be completed, and thus potential for RNA

degradation exists. Inada and Wildermuth developed a microwave-based method that reduces the embedding process from days to hours (Inada and Wildermuth 2005). This method therefore minimizes RNA degradation, and is also useful for delicate samples that can lose morphological integrity during manipulation.

27.2.3 Tissue Sectioning

Once paraffin blocks are prepared, tissue is sectioned using a rotary microtome and placed on microscopy slides. Slides can be either plain glass slides or special purpose slides. Plain glass slides can be used with catapulting-based microscopes, while special purpose slides will be dependent on the laser microscope to be used. Sections can be from 8 up to 20 μ thick. Paraffin ribbons are floated on water, which is removed by heating at 42 °C or lower temperature to allow adhesion to the slide of the paraffin embedded sections. This step is of crucial importance for the preservation of RNA integrity: several laboratories have reported that water floating is the main, and likely sole, cause for RNA degradation, even when all material and reagents used are prepared RNAse free (Cai and Lashbrook 2006; Jiao et al. 2009; Takahashi et al. 2010; Tauris et al. 2009). To minimize RNAse activity, water with RNAse inhibitors can be used (Takahashi et al. 2010), floating the paraffin ribbons can be done on methanol instead of water (Mantegazza et al. 2014; Schmid et al. 2012), or a tape transfer system can be used (Cai and Lashbrook 2006). Cryosectioning, which is better at preserving RNA integrity, can be used as an alternative for this step (Nakazono et al. 2003; Tauris et al. 2009). However, cryosectioning requires a distinct embedding protocol, and further specialized equipment (Barcala et al. 2012; Zhang et al. 2012).

Although it is preferable to immediately process the resulting slides, it has been reported that non-deparaffinated slides can be stored for days at 4 °C. Slides are stored in the presence of desiccant in order to create an anhydrous atmosphere that will impede RNAse activity.

27.2.4 Cell Isolation

Slides that are ready to be processed must be deparaffinated prior to LAM. A typical deparaffination procedure involves immersing the slides in paraffin solvent and drying:

Xylene 100 %: 2 × 5 min
Xylene:ethanol (1:1 v/v): 2 × 5 min
Ethanol 100 %: 2 × 5 min
Air dry

Again, there are variations across protocols from different laboratories. For example, it is possible to air dry the slides immediately after the xylene 100 % step, or to deparaffinate the slides using multiple washes of 100 % ethanol at 32 °C (Hogekamp et al. 2011). Deparaffinated slides should be used immediately for LAM.

The details of the LAM procedure itself will vary slightly depending on the laser microscope to be used (Fig. 27.2), but the basic steps are: (1) identification of the cells to be isolated, (2) selection of the area to be collected using the microscope's software, and (3) dissection and recovery of the cells through LAM. Step 1 implies that the user will have sufficient knowledge on the histology of the tissue to be processed. Dissection and collection steps will vary with different LAM microscopes, and manufacturers will provide instructions for their respective systems. However, this step usually consists on the isolation of the cells through ablation of the surrounding tissue, followed by transfer of the isolated cells to a collection tube. A variant exists when plain glass slides and a catapulting-based system are used. In this case, it is possible to collect the cells using a series of catapulting shots across the area of interest, with or without previous ablation of the surrounding tissue.

It should be noted that identification and selection of the cells using the system's software is a time-consuming process, and while most systems allow for an automated dissection and collection process, sometimes user intervention cannot be completely avoided. For example, secondary wall containing tissues can require more than one laser pass to be successfully ablated. Incomplete ablation can result

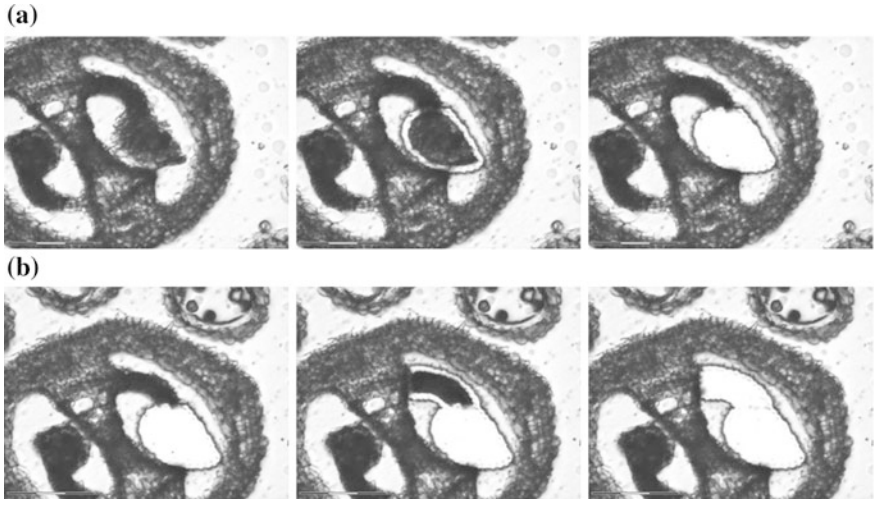

Fig. 27.2 LAM of different Arabidopsis carpel tissues using a PALM MicroBeam (Carl Zeiss, Germany): **a** capture of ovule cells before laser cut, after laser pulses, and tissue remaining after capture; and **b** capture of funiculus cells before laser cut, after laser pulses, and tissue remaining after capture. For this purpose, *Arabidopsis thaliana* buds were processed as described in Mantegazza et al. (2014), embedded in paraffin, sliced (8 µm thick paraffin sections), and deparaffinated in Histo-Clear 100 %. Microdissection was performed immediately. *Bars* represent 75 µm

in cells still being attached to the surrounding tissue, thus impeding transfer of the cells to the collection tube. Finally, since LAM can take up to several days, it usually freezes at −80 °C the isolated cells until sufficient material has been obtained for RNA extraction.

27.2.5 RNA Extraction and Downstream Analysis

Material obtained using LAM will be, at most, in the thousands of cells, and therefore RNA yield is usually in the nanogram range (see for example (Takahashi et al. 2010)). Commercial kits are available for extraction of such quantities of RNA, including the Arcturus' PicoPure RNA isolation kit, Stratagene's Absolutely RNA Nanoprep Kit, or Qiagen's RNeasy Micro kit. It is only at this point that RNA quality can be assessed using an Agilent Bioanalyzer platform. Furthermore, as RNA quantities are usually insufficient for direct downstream analysis, one or more rounds of amplification are undertaken. Amplified RNA is then used as input for the transcriptomics platform of choice. As shown in the next section, both microarrays and RNA-based sequencing (RNA-seq) have been used for such analyses. RNA-seq is preferable as it has higher sensitivity, is able to identify sequence variants or alternative splicing events, and does not have an absolute requirement for a reference genome (Schmid et al. 2012).

27.3 Plant Embryo Transcriptomics

High-throughput gene expression profiles of zygotic embryos, embryogenic calli, or somatic embryos have been obtained for several plant species (Table 27.1), although reports for cell type-specific transcriptomes are only available for Arabidopsis zygotic embryos or seeds (Belmonte et al. 2013; Casson et al. 2005; Spencer et al. 2007). Tens of thousands of genes have been detected in each species and, when comparisons are available, a strong overlap of expressed genes between different samples is observed. For example, Wickramasuriya and collaborators (Wickramasuriya and Dunwell 2015) obtained an RNA-seq based transcriptomic profile of whole Arabidopsis somatic embryos at different stages of development. Their results show that 24081, 25347, and 24944 genes can be detected at 5, 10, and 15 days of development, but, more importantly, that 21743 (85–90 %) of these genes are common to all stages, and are also expressed in an Arabidopsis leaf sample. While it could be argued that such an important overlap might be due to the use of whole embryos, LAM-based datasets lead to the same observation: Belmonte and collaborators (Belmonte et al. 2013) used LAM to isolate distinct Arabidopsis seed subregions at different stages of development. Using microarray hybridizations, between 6000 and 13000 mRNAs were detected in each subregion, which is likely an underestimation of the actual number of expressed genes due to the low

Table 27.1 Available plant embryo global transcriptomic profiles

Plant species	Sample	Cell type isolation	Transcriptomics technique	Genome available	Number of genes detected (total genes in reference)	References
Arabidopsis thaliana	Zygotic embryos	LAM	Microarray (Affymetrix)	Yes	12,870–15,461 (33,602)	Casson et al. (2005)
	Zygotic embryos	LAM	Microarray (Affymetrix)	Yes	13,454–17,479 (33,602)	Spencer et al. (2007)
	Seeds (embryo, endosperm and seed coat subregions)	LAM	Microarray (Affymetrix)	Yes	6,200–12,520 (33,602)	Belmonte et al. (2013)
	Somatic embryos	No	RNA-seq	Yes	24,081–25,347 (33,602)	Wickramasuriya and Dunwell (2015)
Dimocarpus longan	Embryogenic callus	No	RNA-seq	No (de novo assembly)	68,925 unigenes	Lai and Lin (2013)
Gossypium hirsutum	Embryogenic callus and somatic embryos	No	RNA-seq	Yes (NCBI unigenes)	12,230–12,895 (20671)	Yang et al. (2012)
	Zygotic and somatic embryos	No	RNA-seq	Yes (NCBI unigenes)	18,995–19,539 (NP)	Jin et al. (2014)
Picea balfouriana	Embryogenic calli	No	RNA-seq	Yes (*Picea abies*)	22,295 (26437)	Li et al. (2014)
Theobroma cacao	Somatic embryos	No	Microarray (custom, gene model-based)	Yes	10,288 DEG (28,752)	Maximova et al. (2014)
Zea mays	Zygotic embryo explants	No	RNA-seq	Yes	28,992 (39,456)	Salvo et al. (2014)
Eleutherococcus senticosus	Embryogenic cell cluster and somatic embryos	No	Microarray (custom, cDNA-based)	No (EST assembly)	NA	Zhou et al. (2014)
Cocos nucifera	Somatic embryos	No	RNA-seq	No (de novo assembly)	73,607 transcripts (NA)	Rajesh et al. (2015)

NP Data not provided by the authors. *DEG* Differentially expressed genes. *NA* Not applicable

sensitivity of this technique (Schmid et al. 2012). But more importantly, their results show that few mRNAs (at most 545 in the heart stage chalazal endosperm sample) are specific for a particular seed region. We expect that similar gene expression overlaps will be found for RNA-seq datasets of specific embryo cell types. Although differential expression and enrichment analyses can provide the first clues as to the molecular pathways present in each cell type, it is likely that that the resulting embryo gene expression atlas will need to be complemented with protein-DNA and protein-protein information in order to reconstruct the distinct gene regulatory networks involved in plant embryogenesis.

27.4 Conclusions

LAM is a microscopy-based technique that allows the isolation of discrete cell populations from their tissular context while preserving RNA integrity. RNA expression profiles can then be obtained through microarray or RNA-seq analyses, and thus a gene expression atlas for the specific cell types that compose of a tissue or an organ can be established. LAM has been used to obtain microarray-based profiles of the Arabidopsis embryo. These studies show that there is an important overlap of gene expression between the different regions that were sampled. RNA-seq data from specific embryo cell types will provide more precise information on the identity and abundance of the RNA species expressed during embryo development, and this information will be a crucial first step toward our understanding of the gene regulatory networks involved in zygotic and somatic embryogenesis.

Acknowledgments We thank the Mexican National Council of Science and Technology (CONACyT) for a postdoc fellowship to JS. This work in the de Folter laboratory was supported by the CONACyT grant CB-2012-177739.

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Erratum to: Plant Fidelity in Somatic Embryogenesis-Regenerated Plants

Hervé Etienne, Romain Guyot, Thierry Beulé, Jean-Christophe Breitler and Estelle Jaligot

Erratum to:
Chapter 8 in: V.M. Loyola-Vargas and N. Ochoa-Alejo (eds.), *Somatic Embryogenesis: Fundamental Aspects and Applications*, DOI 10.1007/978-3-319-33705-0_8

The book was inadvertently published with an incorrect tagging of given names and family names in the affiliation part of Chap. 8: The author names should be displayed as H. Etienne, R. Guyot, T. Beulé, J.-C. Breitler, E. Jaligot. The chapter and the book have been corrected.

The updated original online version for this chapter can be found at DOI 10.1007/978-3-319-33705-0_8

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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_28