Somatic Embryogenesis: The Molecular Network Regulating Embryo Formation

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Abstract

Somatic embryogenesis in plants is a process by which embryos can be produced from somatic cells cultured under specific conditions. A key initial step is represented by the ability of some cells within the explants to dedifferentiate, i.e., reacquire a "young" or immature state, and then redirect their fate into an embryogenic pathway, demarked by precise changes in gene expression. While the initial morphological patterns of somatic embryo formation can be quite different and difficult to categorize, developing somatic embryos can be assigned similar stages ascribed to zygotic embryos. These similarities allow the utilization of somatic embryogenesis as a model system to investigate physiological and molecular events governing zygotic embryogenesis. The aim of this chapter is to provide a general overview of somatic embryogenesis, by describing and analyzing several in vitro embryogenic systems, and to decipher the molecular network responsible for the generation of somatic embryos.

Keywords

Auxin • Embryogenesis • Microspore-derived embryos • Somatic embryos • Root apical meristem • Shoot apical meristem

14.1 Introduction

In flowering plants, embryogenesis is demarked by the fusion of the haploid gametes, i.e., egg and sperm cells, which through a double fertilization process form a single-celled zygote and an endosperm cell. The subsequent development of the zygote is referred to as embryogenesis, during which the zygote forms a fully developed embryo through very precise apical-basal and radial cell

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A. Mujib (ed.), *Somatic Embryogenesis in Ornamentals and Its Applications*,
DOI 10.1007/978-81-322-2683-3_14

division and differentiation patterns (De Smet et al. 2010). A fully developed embryo generally consists of one or more cotyledons surrounding a shoot apical meristem (SAM), an embryonic axis, and a root apical meristem (RAM).

Plant embryogenesis is characterized by three unique features that have immense implications on the elaboration of the different embryonic tissues and organs. Firstly, unlike animal cells, plant cells do not migrate during morphogenesis. Therefore, the final shape of the organism is the mere result of cell division and expansion. Secondly, the plant embryo is not a miniature plant, as it lacks many tissues and organs which are formed during post-embryonic development. Thirdly, the final stage of embryogenesis is characterized by an imposed desiccation period required for the termination of the embryogenic program and the initiation of germination. The time and modality of the desiccation process is species specific and results in a drastic reprogramming of gene expression (Elhiti and Stasolla 2013).

Recapitulation of embryogenesis can also occur in the absence of fertilization through the generation of asexual embryos. Through this process, referred as apomixis, embryos can develop from unfertilized egg cells or cells of the maternal tissue (Nogler 1984). Formation of asexual embryos can also be achieved via in vitro culture through gametophytic or somatic embryogenesis. Somatic embryogenesis can be theoretically initiated from all cells within the sporophyte, except gametic cells, while gametophytic embryogenesis involves the formation of haploid embryos from either the male or female gametophyte (Bhojwani and Razdan 1996; Raghavan 2000).

As hinted above, somatic embryogenesis has acquired relevance in the study of plant embryogenesis for several reasons. Firstly, it allows the synchronous development of embryos which are exposed and easily accessible. This is in contrast to zygotic embryogenesis, where the embryos are encased in the maternal tissue and often impossible to excise. This characteristic becomes problematic especially for collecting a suitable number of zygotic embryos for physiological and/or molecular studies. In addition somatic embryos are similar to their zygotic counterparts, and therefore knowledge acquired in vivo can be transferred in vitro (Yeung and Meinke 1993). As a consequence, several studies dealing with somatic embryogenesis at cellular, tissue, and molecular levels are currently available (Willemsen and Scheres 2004). Finally, generation of embryos in culture allows the targeted manipulations of environmental and/or culture conditions which would be difficult, if not impossible to perform in vivo. The selective addition or removal of specific chemicals in the medium is often used as a strategy not only to optimize culture conditions but also especially to understand the nature of the environment inductive for the proper development of the embryos.

Despite the existence of many similarities between somatic and zygotic embryogenesis, it must be noted that the two processes are also characterized by substantial differences which must be considered in comparative studies. Unlike zygotic embryogenesis, formation of somatic embryos is dependent upon the competence that some somatic cells have to change their developmental fate. This change involves an extensive and poorly understood reprogramming of gene expression which is unique of in vitro systems (Feher et al. 2003). Another relevant consideration is the fact that in vitro conditions are not fully optimized and therefore the "embryonic environment" created in vitro is different from the seed environment. As such, differences in embryo physiology and storage product depositions are often observed between the two systems. Finally, there are instances of in vitro-produced embryos able to germinate without a dormancy period which is often needed in vivo (Elhiti and Stasolla 2013).

Taken together, these considerations suggest that in vitro embryogenesis can indeed be used as a model system to study plant embryogenesis, but with the due care of acknowledging potential differences with zygotic embryogenesis.

14.2 Plant In Vitro Embryogenesis Systems

Over the past years, in vitro embryogenic systems have been developed for many plant species, including *Arabidopsis* and *Brassica napus*. While not a relevant crop, *Arabidopsis* has been

used quite extensively for in vitro studies due to the available genetic information which facilitates molecular and genetic analyses. Knowledge on *Arabidopsis* can also be transferred to *Brassica napus*, as the two species are related. The in vitro systems for the two species are very different, as somatic embryogenesis is used for *Arabidopsis*, while microspore-derived embryogenesis (androgenesis) is used for *Brassica*. It must also be mentioned that, unlike canola embryos which develop directly from immature microspores, somatic embryos in *Arabidopsis* arise from a callus derived from the explant. As such, this system is often referred as indirect somatic embryogenesis.

14.2.1 *Arabidopsis* Somatic Embryogenesis System

Although reports of *Arabidopsis* somatic embryogenesis from mature tissues are available, somatic embryos are more easily produced from immature explants, such as zygotic embryos (Mordhorst et al. 1998). Dissected zygotic embryos, preferably at the bent cotyledon stage of development, are cultured in a medium containing the auxin 2,4-dichlorophenoxyacetic acid (2,4-D), considered the inductive signal required for the dedifferentiation process of the somatic cells within the explants. Under these conditions, embryogenic callus is generated by the apical regions of the zygotic embryos and in particular from the adaxial side of the cotyledons. Removal of the auxin induces the formation of somatic embryos.

14.2.2 *Brassica napus* Microspore-Derived Embryogenesis System

A key event during androgenesis in *Brassica napus* is the redifferentiation step in which the genetic program of the immature microspores is redirected toward the embryonic pathway. This redirection is triggered by several treatments including elevated temperatures (usually 32 °C; Keller and Armstrong 1979), colchicines (Zhao et al. 1996), gamma irradiation (Pechan and Keller 1989), ethanol (Pechan and Keller 1989), low temperatures (Kasha et al. 1995), change in pH (Barinova et al. 2004), and sucrose starvation (Touraev et al. 1996). The first sign of dedifferentiation of the microspores, as reviewed by Telmer et al. 1992, involves changes in cytoskeletal organization.

Simmonds and Keller (1999) observed that the pre-prophase band which is composed by arrays of microtubules tends to localize in the middle region of induced microspores. This positioning ensures the symmetric cell division of the microspore, which demarks the completion of the inductive events and the acquisition of the embryogenic competence (Yeung 2002). This is in contrast to the gametophytic developmental pathway which is initiated with an asymmetric mitotic division of the microspore. Of the two daughter cells originating from the microspore, one is committed to form the suspensor of the embryo, while the other, referred to as the pro-embryogenic cell, will generate the embryo proper. The whole process is accomplished within 3 days. Through a series of anticlinal and periclinal divisions, the pro-embryogenic cell gives rise to a cluster of cells demarking the globular stage of embryogenesis after 5 days in culture. Within 7–9 days in culture, a globular embryo is produced characterized by a well-developed protoderm, the precursor of the epidermis. During the following days, the growth of the embryo is characterized by the formation of two cotyledons and a morphologically visible SAM and RAM. A detailed description of the histodifferentiation events occurring during microspore-derived embryogenesis is available (Yeung et al. 1996).

14.3 Genetic Components of In Vitro Embryogenesis

The mechanisms by which plant somatic embryogenesis is accomplished are quite complex but somehow conserved among plant species (Elhiti et al. 2013b). Simplified "molecular" steps of the in vitro embryogenic process have been reviewed by Elhiti et al. (2013a), and they are referred to as embryonic induction and development.

The embryonic induction leads to the formation of embryogenic tissue and is further subdivided in dedifferentiation, acquisition of totipotency, and commitment. During the dedifferentiation step, cells within the explants must lose their pre-acquired fate; this is accompanied up by the acquisition of totipotency which enables the cells with the potential to differentiate into any cell type. The concept of totipotency is often associated to that of "stemness" as stem cells are indeed totipotent. The new developmental fate acquired by the totipotent cells is regulated by extrinsic factors, which in culture are often determined by the presence of plant growth regulators. During somatic embryogenesis, the fate of the totipotent cells is redirected or "committed" toward the embryogenic pathway.

The different phases of embryogenesis are accompanied by major "molecular" reprogramming. As described by Elhiti et al. (2013a), somatic embryogenesis encompasses two developmental stages: (1) embryonic induction and (2) development. The embryogenic induction stage is further subdivided into three main phases: (a) dedifferentiation, (b) expression of totipotency, and (c) commitment of induction phase. Hereafter we will describe the genetic networking during each stage of somatic embryogenesis. These initial phases are followed up by the "development" step which is characterized by the growth of the embryos which is often achieved in the absence of plant growth regulators (Sugiyama 1999; Elhiti 2010). The following sections will provide an updated description of the molecular events underlying the induction and development phases.

14.4 Genetic Network of Early Embryogenesis

14.4.1 Genetic Networking Regulating the Induction Phase

During this stage, the genetic program of the somatic cells under culture condition is reprogrammed by either applications of exogenous hormones or stresses (Feher et al. 2003). The induction stage of somatic embryogenesis is very difficult to study at molecular levels because of the lack of clear cytological markers permitting the identification of those clusters of somatic cells undergoing reprogramming in gene expression leading to the acquisition of the embryogenic fate. As such, gene network modeling and bioinformatic analyses are the only means to identify candidate genes required during the three different phases (dedifferentiation, acquisition of totipotency, and commitment) of the induction step (Elhiti et al. 2013a).

14.4.1.1 Dedifferentiation

The dedifferentiation of somatic cells, which in culture is often promoted by auxins, possibly involves a major reprogramming in gene expression. Microarray analyses in *Arabidopsis* have identified *LATERAL ORGAN BOUNDARIES DOMAIN 29* (*LBD29*) as a key developmental gene controlling cell dedifferentiation processes both in vitro and in vivo (Liu et al. 2010). *LBD29* has been identified as a downstream target of the auxin response factors ARF7 and ARF19 (Feng et al. 2012), and lbd29 cells show a reduced sensitivity to auxin and are unable to dedifferentiate. These observations suggest that the native function of this gene is necessary for dedifferentiation and reinforce the notion that auxin acts as the inductive signal (reviewed by Elhiti et al. 2013a). Other possible candidate genes participating in the dedifferentiation step are *KRYPTONITE* (*KYP*)/*SUVH4*, a gene encoding H3 lysine 9 methyltransferase, which, if mutated, reduces the formation of embryogenic tissue, and POLYCOMB REPRESSIVE COMPLEX 1 (PRC1) which has a repressive effect on the ability of cells to dedifferentiate upon the imposition of inductive signals (Bratzel et al. 2010).

14.4.1.2 Totipotency

A key characteristic of plant cells is their inherent ability to retain all the genetic information required to alter their development fate even once fully differentiated (Birnbaum and Alvarado 2008). If expressed by appropriate environmental conditions, this ability, referred to as totipotency, allows the regeneration of the whole organism, as exemplified during somatic embryogenesis

(Verdeil et al. 2007). Despite extensive efforts to identify key elements required for the expression of totipotency, our knowledge on the molecular regulation of this process is very scarce. Independent studies suggest that epigenetic changes play an important role in totipotency (Costa and Shaw 2007; Birnbaum and Alvarado 2008). Furthermore, *Arabidopsis* mutant analysis showed that the concomitant knockout of *CURLY LEAF* (*CLF*) and *SWINGER* (*SWN*), genes encoding two polycomb repressor protein 2 (PRC2) proteins, results in the spontaneous production of embryogenic callus in culture in the absence of plant growth regulators which are normally required for callus formation. Based on these observations, the involvement of PCR2 proteins in the manifestation of totipotency cannot be excluded (Chanvivattana et al. 2004). Two other genes possibly implicated with the manifestation of totipotency are *PICKLE (PKL)* and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 (SERK1)*. It has been suggested that the function of *PICKLE* is to repress totipotency since embryogenic tissue and ultimately somatic embryos are produced spontaneously from *Arabidopsis pkl* roots in the absence of the inductive signals (Aichinger et al. 2009). As *PKL* encodes a putative CHD3 (chromatin helicase DNA binding protein 3), the authors suggest a possible implication of chromatin remodeling processes in totipotency. *SERK1*, which encodes a leucine-repeat receptor protein kinase, is highly expressed during early embryogenesis (Hecht et al. 2001). Ectopic expression of this gene favors the formation of embryogenic tissue and encourages somatic embryo production indicating an involvement in embryogenic competence (Hecht et al. 2001). Using gain-of-function screening approach Zuo et al. (2002) revealed that a shoot apical meristem-related gene, *WUSHEL*, is also expressed in *Arabidopsis* explants during the early inductive phases of somatic embryogenesis in specific domains giving rise to embryogenic cells. In the same study it was observed that overexpression of *WUS* in *Arabidopsis* roots, leaf petioles, stems, or leaves is sufficient to induce somatic embryogenesis. It must be noted, however, that the ability to produce somatic embryos is retained in *WUS* tissue (Zuo et al. 2002), thus suggesting the existence of complex and possible multiple pathways regulating embryogenesis in vitro.

Generation of *Brassica* microspore-derived embryos is dependent upon the ability of immature microspores to lose their gametophytic fate and acquire an embryogenic fate. Transcription studies have identified *LEAFY COTYLEDON1* and *LEAFY COTYLEDON2* (*LEC1*, *LEC2*) as potential candidates mediating this developmental switch and molecular markers for embryogenicity (Malik et al. 2007), a function which appears to be retained across species. A significant repression in *Arabidopsis* somatic embryo production was indeed observed in *lec* mutants (Harada 2001). It must be noted that during somatic embryogenesis, *WUS*, *LEC1*, and *LEC2* share similar expression profiles (Elhiti and Stasolla 2011). Taken together the authors speculated that *WUS* and *LEC* genes may be involved in the acquisition of totipotency possibly through parallel mechanisms. Genetic studies, including the analyses of *wus*/*lec1*/*lec2* triple mutants, might be needed to unravel the function of these genes in early embryogeny.

It is well known that *LEC* genes are required to promote the expression of *AGAMOUS-LIKE 15* (*AGL15*), encoding a MADS-domain protein (Zheng et al. 2009). Induction of *AGL15* strongly activates the gibberellin 2-oxidase *GA2ox6* which represses gibberellic acid synthesis. Therefore it cannot be excluded that *LECs* operate through the inhibition of gibberellins, which have been shown to act in an antagonistic fashion to auxin, the signal promoting the dedifferentiation of somatic cells.

The participation of auxin during the early embryogenic phases was also demonstrated in *Brassica napus* using *BABY BOOM*1 (*BBM1*), an AP2/ERF transcription factor (Boutilier et al. 2002). Ectopic expression of *BBM1* in *Brassica* seedlings results in the production of somatic embryos from leaf margins, while its overexpression in *Arabidopsis* produces cotyledon-like structures (Boutilier et al. 2002). Overall, the overexpression of *BBM1* was associated to other changes in leaf and flower morphology as well as

neoplastic growth. Furthermore, *BBM1* overexpressing explants were able to regenerate through organogenesis and embryogenesis without applications of exogenous plant hormones, an observation suggesting that *BBM1* may interfere with auxin sensitivity. The requirement of auxin signaling during early embryogeny was also demonstrated during *Arabidopsis* somatic embryogenesis. Elhiti et al. (2013b) demonstrated that the increased number of somatic embryos obtained by suppressing *GLB2*, a type 2 nonsymbiotic hemoglobin, was the result of elevated levels of auxins which accumulate at the sites of the explants where embryogenic tissue forms. The authors developed a model in which suppression of *GLB2* results in an increase in nitric oxide which represses the transcription factor MYC2, a repressor of auxin synthesis. Collectively, these studies demonstrated a solid link between the acquisition and manifestation of totipotency to auxin.

14.4.1.3 Commitment

It is believed that once somatic cells express their totipotency, specific signal cascades must be activated to promote cell division and encourage the acquisition of meristematic identity. Both events are crucial for the proper development of the embryos. Overall, the genes involved in this phase of somatic embryo induction may be divided into three main categories: genes participating in cell cycle, genes required for meristematic cell formation and regulation, and genes involved in several signal transduction cascades.

Genes Participating in Cell Cycle

Cell division in plants is controlled by complicated mechanisms which are governed by the expression of cyclin-dependent kinases (*CDKs*). *CDK*s are proteins influencing the entry time into the different phases of the cell cycle (Elhiti et al. 2013a). According to their internal motives, *CDK*s are classified into eight groups, *CDKA* through *CDKG* and *CDKL* (Zhang et al. 2012). Functional genetic analyses revealed that only *CDKA1* (also referred to as *CDC2A*) is involved in embryogenesis and its expression is induced by the plant growth regulators auxins and cytokinins (Nowack et al. 2006). In *Arabidopsis*, overexpression of *CDC2A* represses somatic embryogenesis, while a downregulation of the same gene enhances the number of somatic embryos produced (Hemerly et al. 2000). Another CDK possibly participating in embryogenesis is *CDKA*, the transcript levels of which increase during the early phases of somatic embryogenesis prior to declining as the embryos develop (Cortes et al. 2010).

It has been reported that PROPORZ1 (PRZ1), a putative *Arabidopsis* transcriptional adaptor, mediates cell proliferation through auxin and cytokinin signaling (Sieberer et al. 2003). Compared to WT tissue, in which ectopic cell proliferation is observed in the presence of both auxin and cytokinins, *prz1* tissue is able to produce callus when cultured with either auxin or cytokinin (Sieberer et al. 2003). Based on these observations, the authors suggested that PRZ1 mediates cell proliferation and differentiation by affecting the behavior of cell cycle regulators. Another possible component of the mitotic machinery with a possible involvement on somatic embryogenesis is histone H3-11, a mitosis-specific phosphorylation protein. Hendzel et al. (1997) suggested that histone H3-11 is particularly required during the inductive phases of embryogenesis, an observation consistent with the high levels of *histone H3-11* transcripts measured in alfalfa tissue subjected to 2,4-D treatments which stimulate embryogenic tissue formation (Kapros et al. 1992).

Genes Involved in Meristematic Cell Formation

The competent cells formed in culture on the explants respond to an inductive signal, usually provided by specific culture addenda such as growth regulators, and become meristematic cells. Elhiti et al. (2010) proposed that meristematic cell formation in culture is regulated by similar mechanisms involved in the formation and maintenance of the meristematic cells within the SAM in vivo. Proper SAM homeostasis relies on two classes of genes: those promoting cell division and those favoring cell differentiation. Members of the former class are *SHOOTMERISTEMLESS* (*STM*) and

WUSCHEL (*WUS*), while promoters of cell differentiation are *CLAVATA1* and *CLAVATA2 (CLV1* and *CLV2*). Using *Brassica* and *Arabidopsis*, it was demonstrated that while the constitutive expression of *STM* induces embryo formation in culture, overexpression of *CLV1* represses the production of embryos (Elhiti et al. 2010). This contrasting behavior in vitro is analogous to that observed during the maintenance of the SAM in vivo.

A lot of attention has also been directed toward the interaction of *WUS* and *CLV1*, the function of which has been well documented. In the SAM the role of *CLV1*, a transmembrane receptor serine/threonine kinase with leucine-rich repeat (Clark et al. 1997), is to promote the differentiation of meristematic cells by repressing *WUS* expression through a complex signaling model involving other CLV proteins (Dodsworth 2009). In this model *CLV3* produced by the apical cells of the SAM binds to CLV1/CLV2 receptor kinase complexes located in the subapical cells and through the activation of downstream signaling components downregulates *WUS* which is expressed in the "organizing center" (Dodsworth 2009). In *Arabidopsis* somatic embryogenesis system, the expression of *WUS*, induced by auxin, is first visible in those domains of the explants giving rise to the embryogenic tissue (Su and Zhang 2009). Chen et al. (2009) also demonstrated a cytokinin-mediated activation of *WUS*. The WUS-CLV interaction was shown to occur during *M. truncatula* somatic embryogenesis where the two genes competitively modulate the formation of embryogenic tissue formation (Chen et al. 2009). It must be noted, however, that all the SAM-related genes described above are not necessary for somatic embryo formation, as their respective *Arabidopsis* mutants are still able to produce somatic embryos in culture (Mordhorst et al. 1998).

It is well known that *WUS* acts as a transcription factor repressing A-type *Arabidopsis* response regulators, thereby activating cytokinin responses contributing to meristem maintenance (Leibfried et al. 2005). Several studies suggest that *WUS* activity in vivo requires the expression of the ARGONAUTE (AGO) protein ZWILLE/AGO10 (Tucker et al. 2008). AGO proteins are central elements of the RNA interference (RNAi) pathway and mediate the repression of target mRNA through mRNA degradation or translational inhibition (Mallory and Vaucheret 2010). Specifically, ZLL/AGO10 blocks the accumulation of microRNA165/microRNA166 in the stem cell niche of the SAM by sequestration mechanisms preventing the degradation of microRNA165/microRNA166 targets' transcripts of *HD-ZIPIII* transcription factor (Knauer et al. 2013). Based on these observations, it might therefore be interesting to ascertain the participation of AGO proteins in the initial phases of embryogenesis.

Genes Involved in Signal Transduction Cascade

The CLV signaling described in the first section is modulated by downstream components, the function of which, although not tested during in vitro embryogenesis, might participate during in vitro embryogenesis. Two intermediary modulators of CLV signaling are a kinase-associated protein phosphatase (KAPP) and a rho-like GTPase (Rop) (Song et al. 2006). These two proteins interact directly with *CLV1* forming a 450 kDa active signaling complex. KAPP functions in vivo as a negative regulator of the CLV signaling through direct dephosphorylation of *CLV1*, while Rop is assumed to transduce the CLV signal into the nucleus (reviewed by Elhiti et al. 2010). Future studies assessing the involvement of these two proteins during in vitro embryogenesis might further validate the notion that the formation of meristematic cells in vitro uses signaling systems governing SAM homeostasis.

Besides KAPP and Rop, SHEPHERD, a HSP90-like protein predicted to be required for correct folding of CLV complex (Ishiguro et al. 2002), and POLTERGEIST (*POL*), a nuclear-localized protein phosphatase 2C (PP2C) which acts downstream within the CLV transduction (Carles and Fletcher 2003), can be additional candidates to be tested during in vitro embryogenesis. These proposed studies would verify the proposed notion that meristematic cell formation in vitro relies on similar mechanisms governing SAM formation and maintenance in vivo.

14.5 Genes/Gene Homologues Influence Embryo Development

14.5.1 Genetic Networking Controlling Somatic Embryo Development

The developmental phase of in vitro embryogenesis culminates with the formation of fully developed embryos, the growth of which occurs along two axes: an apical-basal axis and a radial axis. While the apical-basal growth ensures the proper positioning of the cotyledons surrounding the SAM, a hypocotyl, and a RAM, the radial growth specifies concentric layers of tissues: the stele, cortex, and epidermis. Precise coordination of these events is paramount for the accurate establishment of the embryo body. The development of many *Arabidopsis* mutants, as well as high-resolution molecular techniques, has aided our understanding on the molecular networks coordinating apical-basal and radial growth.

14.5.1.1 Establishment of the Apical-Basal Body Plan

The formation of apical-basal axis of a somatic embryo is responsible for the proper positioning of the SAM and RAM at the opposite regions of the hypocotyl. Among the processes ensuring this axis pattern are asymmetric cell division and preferential elongation along the desired axis (De Jong et al. 1993; Emons 1994). While asymmetric cell divisions are promoted by plant hormones that alter cell polarity by interfering with pH gradients or the electrical fields across membranes (Smith and Kirkorian 1990), cell expansion is associated with the composition of polysaccharides within the cell wall and specific hydrolytic enzymes (De Jong et al. 1993; Emons 1994; Fry 1995). The participation of asymmetric cell divisions and elongation for the establishment of the apical-basal axis during in vivo embryogenesis are manifested at the zygotic stage when the zygote elongates and undergoes an asymmetric division leading to the formation of small apical cells, precursors of the embryo proper, and larger basal cells forming the suspensor cell. The contribution of these two events is also crucial for the later stages of embryogenesis (Zhang and Laux 2011). Although the early phases of embryogenesis in vitro follow less precise patterns, the roles of asymmetric cell divisions and elongations are still apparent in some systems, including *Brassica* microspore-derived embryogenesis where the type of division observed in the microspore, i.e., symmetric or asymmetric, marks its developmental fate. Molecular analyses during the earliest phases of *Brassica* microspore-derived embryogenesis identified some potential genes possibly involved in this fate acquisition, including *FUSCA3*, *LEAFY COTYLEDON1* (*LEC1*), *LEC2*, *BABY BOOM* (*BBM*), *PINFORMED7* (*PIN7*), two *WUSCHEL*-related homeobox (*WOX*) genes, *WOX2*, *WOX8* and *WOX9*, and *ABSCISIC ACID INSENSITIVE3* (Joosen et al. 2007; Malik et al. 2007; Tsuwamoto et al. 2007). While the involvement of these genes in asymmetric cell division is known, more information is available for *PIN7* and *WOX2*. During the asymmetric cell division of the zygote, *PIN7* is preferentially localized in the basal cell, while expression of *WOX2* is restricted in the apical cell. A mutation in either of the two genes compromises the ability of the zygote to divide properly (reviewed by Elhiti and Stasolla 2013).

The participation of auxin for the execution of asymmetric cell divisions is well established, and a precise distribution of this growth regulator is also required for the specification of somatic cells embarking in the embryogenic pathway. According to Su and Zhang (2009), the formation of an auxin gradient within the *Arabidopsis* embryogenic tissue is crucial for inducing the stem cell formation through the regulation of *PIN1*. This regulation would also mediate the expression of *WUS* and other *WOX* genes required for the establishment of the apical-basal axis. Of note, the observation that *WOX8* and *WOX9* are also expressed during conifer embryogenesis possibly through auxin-mediated mechanisms (Palovaara and Hakman 2009) raises the possibility of a more general involvement of these groups of genes in embryo patterning.

The apical domain of a fully developed embryo consists of cotyledons and a SAM. Independent studies have shown that the establishment of the apical embryonic domains in vivo is specified by *GURKE* and *TOPLESS*. *GURKE* encodes an acetyl-CoA car-

boxylase and, if mutated, precludes the formation of cotyledons and the SAM (Baud et al. 2003). Knockout of *TOPLESS* results in the formation of a root in the apical pole, thus indicating that the function of this gene is to abolish the manifestation of the basal patterning in the apical domains (Szemenyei et al. 2008).

The central embryonic domain consists of a hypocotyl, the specification of which is regulated by *FACKEL*, *HYDRA1*, and *CEPHALOPOD* (Willemsen and Scheres 2004). With mutations in these genes, embryos form without hypocotyls in which the apical domain is directly connected to the embryonic root (Lindsey et al. 2003). These genes participate in the biosynthesis of sterols, suggesting an involvement of these compounds in hypocotyl formation. Not surprisingly, auxin is also required for the development of a functional hypocotyl. Mutations of *MONOPTEROS*, a gene encoding an auxin responsive factor, produce embryos lacking a hypocotyl (Schruff et al. 2006). In the same study it is speculated that the specific function of these genes might be related to the formation of a functional stele, as this is the most affected tissue in the mutants.

Other genes interfering with auxin signaling: *AUXIN-RESISTANT6* and *BONDELOS* are also required for the proper establishment of the central embryonic domain (Park and Harada 2008).

The embryonic basal domain includes the RAM which is composed of quiescent cells surrounded by the stem cells. During early phases of embryogenesis, expression of *PINFORMED1*, *4*, and *7*, all encoding auxin efflux carriers, are required for the formation of an auxin maximum at the basal domain, which is essential for the specification of the RAM (Willemsen and Scheres 2004). Mutations in auxin downstream components, such as *PLETHORA*, which is expressed in quiescent cells and encodes AP2 domain transcription factor, cause the mis-specification of quiescent cells and consequently the improper formation of the embryonic root (Aida et al. 2004). Analyses of these mutants showed that the effects of *PLETHORA* in the formation of embryonic root are mediated through interaction with *SCARECROW* and *SHORTROOT* (Aida et al. 2004). Furthermore, *HOBBIT*, a homologue of a subunit of the anaphase-promoting complex, is also required for proper localization of quiescent cells in embryonic root (Willemsen et al. 1998). Collectively, these studies show that the apical, central, and basal embryonic domains appear to be controlled by independent genetic mechanisms which are coordinated by a proper flow of auxin. The majority of these studies, however, have been conducted in vivo, and it is not clear whether similar mechanisms also operate in vitro, where tissue patterning is less organized and predictable.

14.5.1.2 Establishment of Embryonic Shoot Apical Meristem (SAM)

The establishment of the SAM is considered a key event during embryogenesis and encompasses three phases: the specification of apical domain (discussed in the previous sections), the formation of the stem cell niche, and the separation of the central and peripheral domains. The transcription factor *WUS* defines the organizing center of the meristem and is considered the initial marker for the specification of the stem cell niche. Localization studies in *Arabidopsis* demonstrate that *WUS* transcripts appear very early during somatic embryogenesis (Su and Zhang 2009). The main function of *WUS* is to maintain the stem cells in an undifferentiated state, thereby ensuring the proper maintenance of the apical region. As previously described, *WUS* is regulated by *CLV* feedback mechanisms through the interaction of *CLV1-3*. Another marker of the initial formation of the SAM is the homeodomain transcription factor *STM* which is also expressed in somatic embryos starting from the globular stage of development (Elhiti 2010). Downregulation of *STM* results in fusion in the embryonic cotyledons resulting in the production of trumpet-shaped embryos (Elhiti et al. 2010). The demarcation between the central and peripheral domains of the SAM is necessary for the proper positioning of the cotyledons relative to the SAM (reviewed by Elhiti and Stasolla 2013). This process is mediated by *CUP-SHAPED COTYLEDON* (*CUC1*, *2*, *3*), expressed at the boundary between the cotyledons and the SAM (Aida et al. 1999). Knockout of *CUC*

phenocopies the *stm* phenotype (trumpet-shaped embryo), suggesting that *STM* and *CUC* may share the same pathway. It has been observed that accumulation of *CUC* transcripts is regulated by microRNA164 (Zhang et al. 2006).

14.5.1.3 Establishment of the Embryonic Radial Pattern

Radial patterning results in the proper specification of the epidermis, cortical tissue, and vascular tissues. The first hint of radial pattern formation during in vivo and in vitro embryogenesis corresponds with the separation of the protoderm from the inner cells (Elhiti and Stasolla 2013). Expression analyses in *Arabidopsis* indicate that *ARABIDOPSIS THALIANA MERISTEM LAYER1* and *PROTODERMAL FACTOR2*, encoding transcription factors containing the START domain, are implicated in the radial specification of the protodermal layer (Abe et al. 2003). Other genes involved in radial patterning are *KEULE* and *KNOLLE*, as a radial axis is never initiated in the two mutants. While their function is not fully clear, it has been shown that *KNOLLE* encodes a syntaxin-like protein involved in secretary processes (Song et al. 2000). A mutation in this gene results in abnormal cytokinesis due to incomplete formation of the cell wall separating the two daughter cells (Song et al. 2000).

SHORT ROOT (*SHR*) and *SCARECROW* (*SCW*), encoding transcription factors of the GRAS family, are required for the proper specification of endodermal and cortical layers. Knockout of *SHR* results in absence of the endodermis, while *scw* mutants have a single file of cells in place of cortex and endodermis (Di Laurenzio et al. 1996). Localization studies indicated that *SHR* is expressed in the vascular tissue and translocated into the endodermal layer where *SCW* is expressed (Di Laurenzio et al. 1996).

14.6 Conclusions

Embryo formation in vivo is initiated with the fusion of the gametes, i.e., sperm and egg, resulting in the formation of the zygote. Through precise cell division and differentiation processes, the zygote produces a fully develop embryo, composed of an apical, a central, and a basal domain. Recapitulation of embryogenesis can also occur in vitro through somatic and gametophytic embryogenesis. Formation of in vitro embryos relies on similar genetic mechanisms operating during in vivo embryogenesis although the culture conditions are less stable and often not optimized. As a result, the molecular events controlling in vitro embryogenesis are less defined. Overall, somatic embryogenesis can be divided in two distinct phases: induction and development. The first phase requires the dedifferentiation of the somatic cells, the acquisition of totipotency, and the commitment to embark an embryogenic fate. These events, critical for the overall embryogenesis, do not occur in vivo and are therefore specific to culture systems. Independent studies have demonstrated the relevance of auxin for the inductive step and the participation of genes regulating SAM formation and maintenance. Removal of plant regulators is often required to initiate the development of the somatic embryos, and during this event, the embryo body is elaborated. Like the in vivo system, the tissue patterning of in vitro-produced embryos occurs through an apical-basal and a radial axis. Growth along the two axes is mediated by distinct genetic networks, although auxin seems to be implicated with both. As the developmental phases of in vitro embryogenesis are very similar to those observed in zygotic embryos, knowledge on the molecular mechanisms operating in the latter system are often transferred to the former.

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