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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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

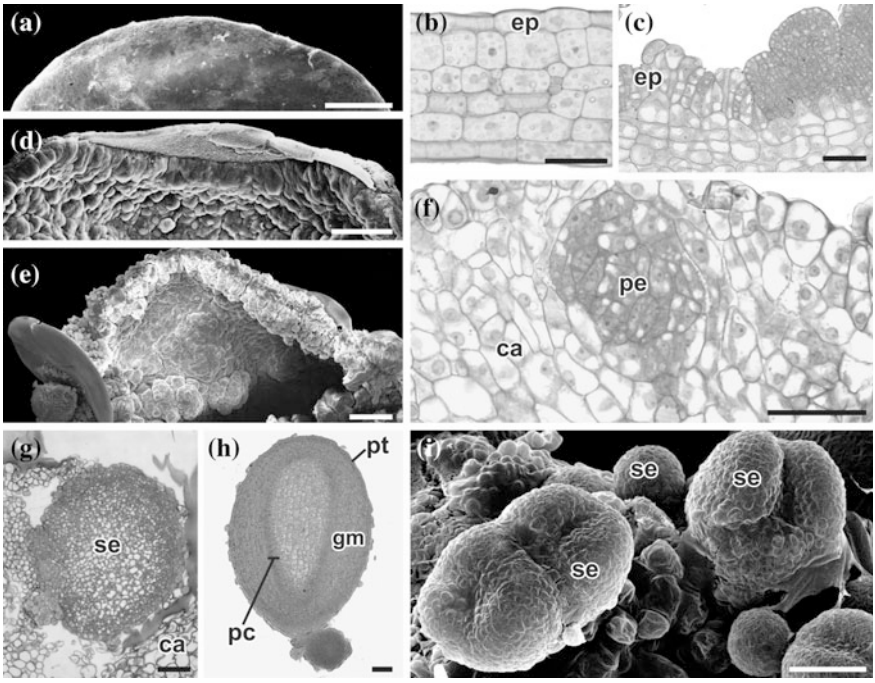

Fig. 26.1 Somatic embryogenesis of *Passiflora cincinnata*. Light (**b**, **c**, **f**, **h**) and scanning electron microscopy (**a**, **d**, **e**, **i**). **a** Cotyledon abaxial surface of the initial zygotic embryo explant. **b** Histological organization of initial zygotic embryo explant. **c** 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

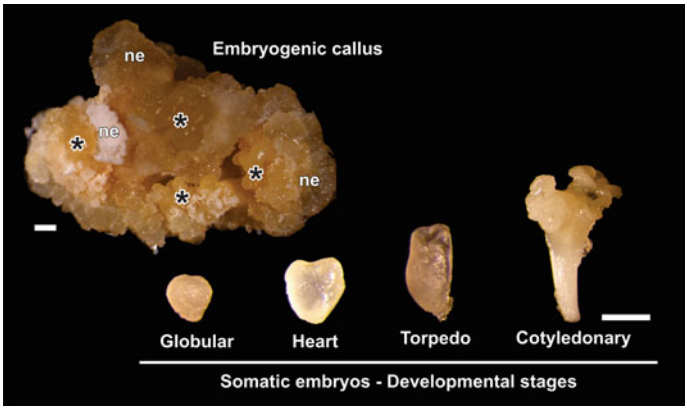

Fig. 26.2 Somatic embryogenesis of *Passiflora cincinnata*. Embryogenic callus and the stages of embryo development. Note the presence of embryogenic (*) and non-embryogenic (ne) regions in the callus. *Bars* 10 mm

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

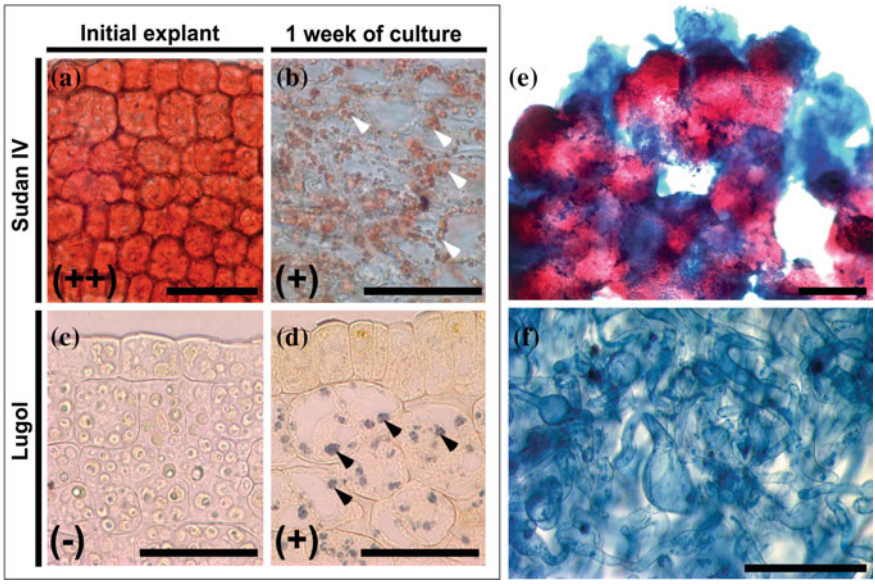

Fig. 26.3 Histochemical analysis. **a**–**d** Study of *Passiflora cincinnata* cotyledons at the early stages of somatic embryogenesis induced from mature zygotic embryos explants. **a**, **b** Transverse sections of initial explants (**a**) and after 1 week of culture (**b**) subjected to Sudan IV test. 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

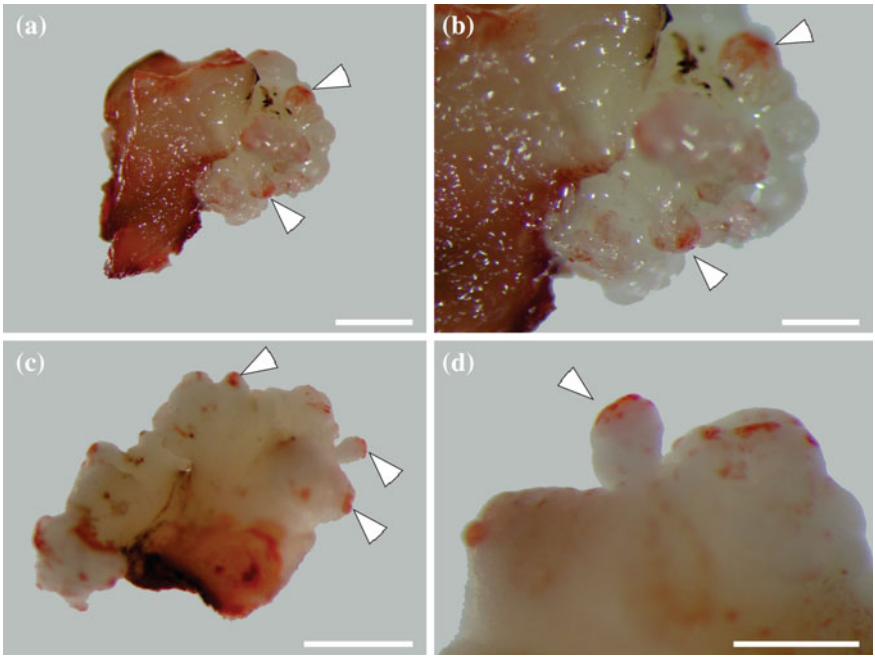

Fig. 26.4 Clusters of somatic embryos stained overnight with ßGlcY solution. (**a–d**) An intense *red color* in specific sectors of the explant as well as on the protoderm of the globular somatic embryos was observed (*arrowheads*). *Bars* **a–c** (5 mm); **d** (2 mm)

(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

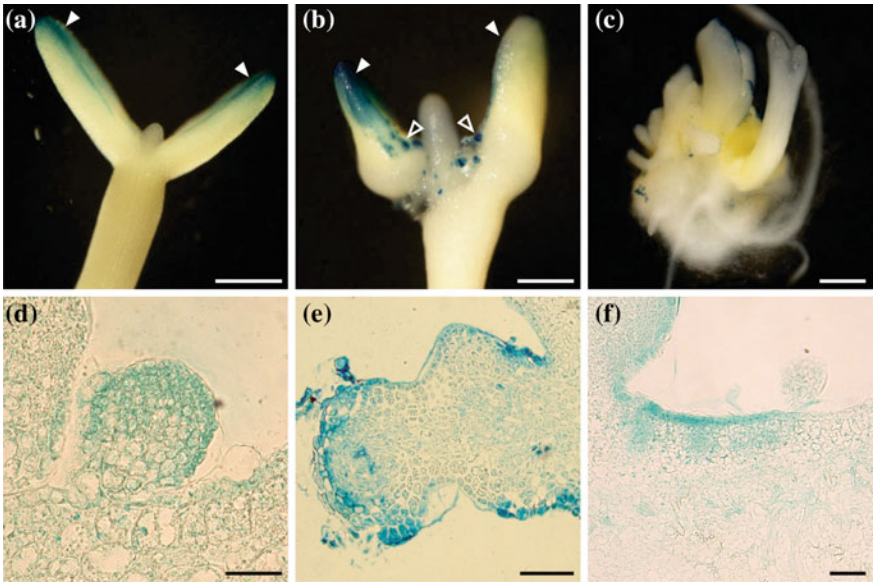

Fig. 26.5 Morphological and histological analysis of GUS distribution in explants of transgenic plants carrying GUS reporter gene driven by promoter of *LTP1* and spatial pattern of *LTP1* promoter activity in some developmental stages of the culture. **a** Whole mount of explant cultured for 4 days, 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

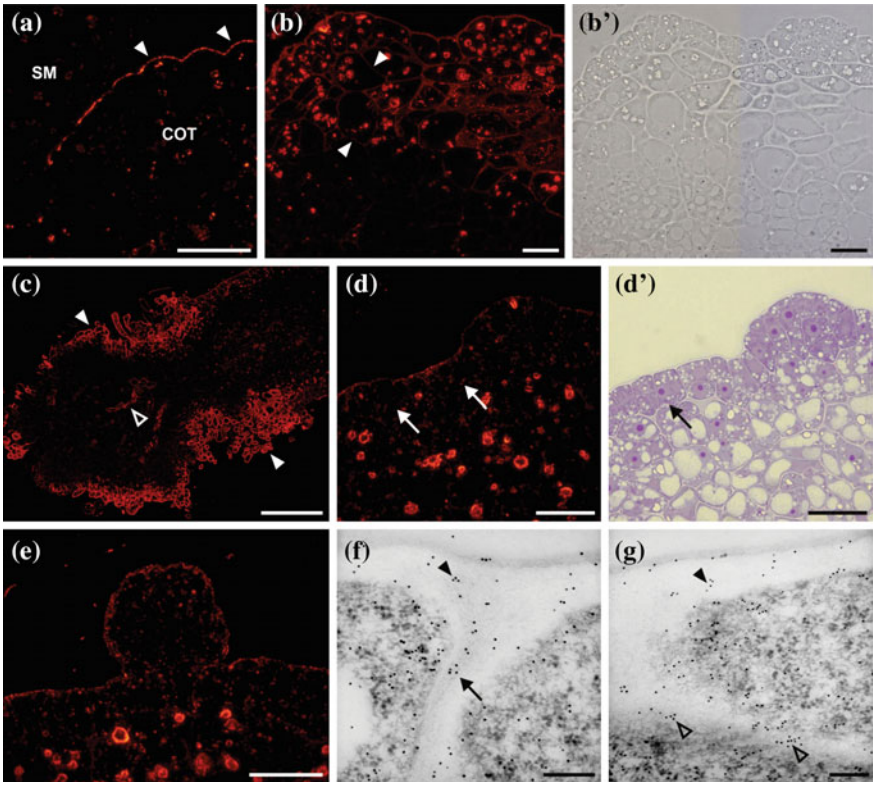

Fig. 26.6 Immunolocalization of LTP1 epitopes during somatic embryogenesis in *Arabidopsis thaliana*. **a** 4-day cultured explants, labeling in the outer periclinal walls of the protoderm (*solid arrowheads*) and in the plastid envelope. **b** Cells in the adaxial part of the cotyledon (day 14 of culture), 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.

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