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Cellular Markers for Somatic Embryogenesis

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1. Introduction

Somatic embryogenesis (SE) is a process in which somatic cells under special conditions develop into embryos and - in the end - into a plant. That is why SE is a good model system for studying the genetic, molecular, physiological, biochemical, histological and cellular mechanisms underlying not only somatic but also zygotic embryogenesis and the totipotency of plant cells. SE begins with a transition of somatic cells to an embryogenic state and it can be induced under certain *in vitro* conditions. The mechanisms which determine SE induction - the transition of cells from the vegetative to the embryogenic state and the conditions underlying such changes - are the main questions of developmental biology (for a review see: de Jong et al., 1993; von Arnold et al., 2002; Fehér et al., 2003; Namasivayam, 2007; Yang & Zhang, 2010).

A description of the events taking place during SE requires the application of different scientific methods such as genetic, molecular or biochemical analysis and also histological studies of explant cells. Moreover, the morphological, histological and cytological analysis of SE is also an object of studies leading to an understanding of the basis of the totipotency, differentiation, dedifferentiation, redifferentiation and changes in cell fate (Quiroz-Figueroa et al., 2006). It could help us to understand the developmental processes taking place during plant growth and development, including pattern formation.

In this review we describe the cellular markers which can be used to identify different groups of cells within the explant during the process of SE. The aim of this review is to summarise information concerning the morphology and histology of explant cells, such as changes in the apoplast and symplast of explants, which can be used as markers to identify a cell/cells which changed their fate from the somatic to the embryogenic state.

2. Definitions

The first information about somatic embryo development in *in vitro* conditions was presented by Steward and co-workers (1958). From that moment on, this kind of plant propagation forced many scientists to study the mechanisms involved in changes from the

somatic to the embryogenic state and to improve the efficiency of this process as a method for plant propagation. Since during SE different processes leading to changes in cell fate are taking place, some important definitions concerning this phenomenon are reminded below.

Somatic embryogenesis is divided into direct and indirect embryogenesis (DSE and ISE respectively; Sharp et al., 1980; Evans & Sharp, 1981). In DSE, somatic embryos develop directly from the somatic cells of explants, and in ISE they develop from callus cells. Somatic embryogenesis is also divided depending upon the type of explants. If somatic embryos develop from primary explants it is called primary somatic embryogenesis; if they develop from primary somatic embryos, this is called secondary somatic embryogenesis.

In normal plant development, cells differentiate from an unspecialised to a mature state with the determined function. The term 'cell differentiation' can be interpreted as spatiotemporal and it focuses on the diverging path of differentiation among the constituent cells in a population (Romberger et al., 2004).

During SE, some explant cells change the direction of differentiation. For example, the epidermal cell is the "source" of the somatic embryo, and the parenchyma cell becomes a callus cell and afterwards develops into a somatic embryo. The processes by which cells can change their state of development are dedifferentiation, transdifferentiation and redifferentiation. It is well-documented that most of plant cells retain the possibility to dedifferentiate and as a consequence to change their fate (Grafi, 2004). Such changes are possible because plant cells are totipotent (or at least most of them are), where totipotency is the property of the cell which retains the potential for developing into a complete adult organism (Verdeil et al., 2007). For the most recent analysis of the definitions mentioned above, the article written by Sugimoto and co-workers (2011) is recommended.

According to Nagata (2010) and Grafi (2004), dedifferentiation is the process where differentiated non-dividing cells become meristematic. This concept explains many observations which had shown that cells divisions precede changes in the direction of their differentiation. During dedifferentiation, cells return to the undifferentiated, meristematic state. Transdifferentiation involves processes which lead cells or tissues from one differentiated state of development into a new one, and probably - first of all - such cells dedifferentiate and then redifferentiate along another developmental path (Thomas et al., 2003; Gunawardena et al., 2004). Redifferentiation is the ability of non-differentiated, meristematic cells to differentiate into a new direction, e.g., into new plant organs.

It is worth reminding ourselves of another definition concerning SE. According to Verdeil and co-workers (2007), the embryogenic callus is an undifferentiated, unorganised tissue enriched in embryogenic cells, and the embryogenic cell is a cell that requires no further external stimulus to produce a somatic embryo.

3. General description of SE

During SE, changes in explant tissues cause the development of the somatic embryo. Many studies have shown that somatic embryos are going through the same developmental stages as their zygotic counterparts, which in dicotyledonous plants are called the globular, heart, torpedo and cotyledonary stages (Fig. 1; sometimes such stages were named differently, as with, e.g., Quiroz-Figueroa et al., 2006).

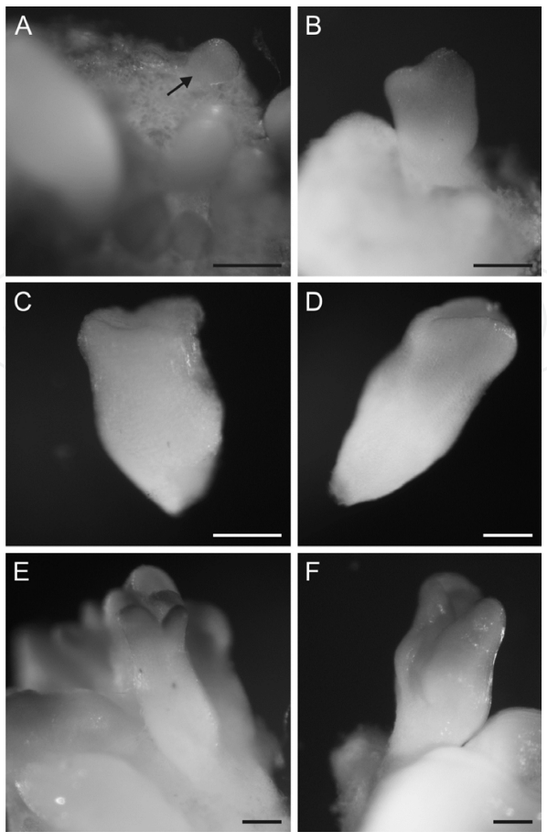

Fig. 1. Different developmental stages of somatic embryos from the example of *Arabidopsis* (A-globular; B-heart; C-torpedo; D-late torpedo; E and F-mature; bar = 200 μm).

Different parts of plant organs or zygotic embryos are used as an explant for the induction of SE. The literature describing this aspect of SE is huge and it is not possible to even mention here most of them. In some species, zygotic embryos are the best source of somatic ones and the explant organs involved in SE are cotyledons or shoot apical meristem (e.g. Canhoto & Cruz, 1996; Gaj, 2001; Kurczyńska et al., 2007; Raghavan, 2004; Rocha et al., 2011). Cultures of leafs, stems and roots parts are also efficient in SE induction (Mathews et al., 1993; Quiroz-Figueroa et al., 2002). In some cases, the production of protoplasts from different plant tissues or suspension cultures is the best for SE (Pennel et al., 1992; Quiroz-Figueroa et al., 2002).

Somatic embryos have a single-cell or multicellular origin. Analyses performed by Canhoto & Cruz (1996) on *Feijoa sellowiana* cotyledons of zygotic embryos, as an explant, showed that somatic embryos developed from a single protodermal cell or from a group of cells including sub-protodermis. Similar results were obtained during the histological analysis of somatic embryogenesis of *Arabidopsis thaliana*, where the single-cell and multicellular origins of somatic embryos were also detected (Kurczyńska et. al., 2007). Cork oak somatic embryos are of a multicellular origin or a single-cell origin depending on the explant cells which participated in the embryo's formation (Puigderrajols et al., 2001). The single- and multicellular origins of somatic embryos was also described (among others) in *Borago officinalis* (Quinn et al., 1989), *Camellia japonica* (Barciela & Vieitez, 1993), *Elaeis guinnesis* (Schwendiman et al., 1990) and

Theobroma cacao (Pence et al., 1980). The unicellular origins of somatic embryos was described (among others) in the leaf explant of *Coffea arabica* (Quiroz-Figueroa et al., 2002), coconut (Verdeil et al., 2001) and *Dactylis glomerata* (Trigiano et al., 1989). In some species, only the multicellular origins of somatic embryos were described, as, for example, in *Carya illinoinensis* (Rodriguez & Wetzstein, 1998) and *Passiflora cincinnata* (Rocha et al., 2011).

It is well-documented that dividing explant cells (e.g., in callus cultures) can follow different developmental pathways, such as organogenesis, SE or unorganised growth (Fehér et al., 2003). Distinguishing between somatic embryo and organ-like structural development within explants can sometimes be difficult. The most distinctive features in the histology of somatic embryos are the anatomically closed radicular end and the lack of a vascular connection with the maternal tissues (Fig. 2 A, B). Moreover, analysis of the distribution of starch in the radicular pole of the embryo showed that starch was present in both zygotic embryos and their somatic counterparts (Fig. 2 C). Using such a criterion it is much easier to distinguish somatic embryo formation from organogenesis, which can take place within the same explant.

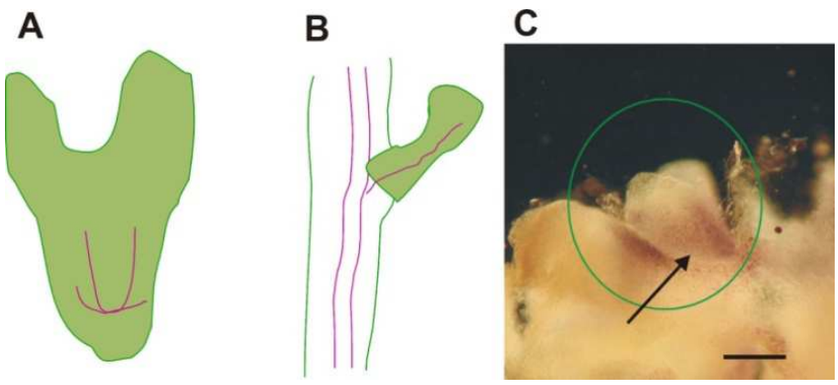

Fig. 2. Schematic differences in the histology of the basal region of the embryo (A) and buds (B; this resembles organogenesis) and starch distribution in the radicular pole of *Arabidopsis* somatic embryo (the red lines on A and B represent the vascular tissue; C – brownish colour after staining with Lugol solution marks starch; bar = 150 μm).

In the case of *Arabidopsis thaliana* (a system described by Gaj, 2001), somatic embryos develop via a DSE from explant cells located on the adaxial side in the cotyledon node (Fig. 3; Kurczyńska et al., 2007).

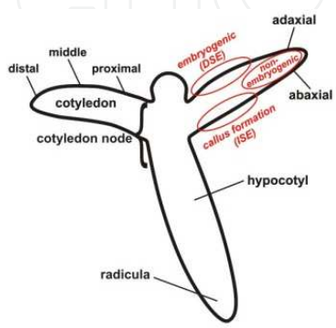

Fig. 3. Schematic representation of a longitudinal section through *Arabidopsis* explants. The location of the embryogenic and non- embryogenic regions is indicated.

From many observations and histological analysis, it appears that in this system only those cells located on the adaxial side of cotyledons undergo transition from a somatic to embryogenic state in the manner of DSE. Sometimes, if zygotic embryos are cultured in a different way, somatic embryos which developed from the callus were also detected (Fig. 4).

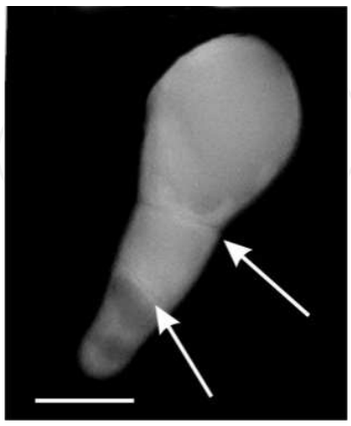

Fig. 4. Structure detected within the callus during SE in an *Arabidopsis* explants, which resembles a very early stage (a few cells) of a somatic embryo developed via an ISE (bar = 10 μm).

4. Changes in cell fate during SE

In the process of somatic embryogenesis, some somatic cells start to divide, becoming totipotent, and then enter the new pathway which is SE (Fehér et al., 2002). The most important question concerns the mechanisms underlying the changes (the transition) of the differentiated state of the plant cell into a totipotent and finally an embryogenic state (Fehér et. al., 2002). It was documented that during DSE somatic cells acquire their embryogenic competence through dedifferentiation (Harada, 1999; Fehér et al., 2003; Steinmacher et al., 2011). Such big changes in cell fate depend on the possibility of acquiring the ability to divide (Nagata, 2010). It is accepted that dedifferentiation is preceded by cell divisions (Fehér et al., 2002; Nagata et al., 1994; Wang et al., 2011) and it is postulated that existing developmental information must be changed so as to allow cells to respond to new signals (Fehér et al., 2002).

The transition from the somatic to the embryogenic state requires the induction of embryogenic competence (Verdeil et al., 2001). How should one recognise this stage of SE? The answer to this question is still far away, as it is very difficult to recognise the very early stages of somatic embryo development, starting from the changes in competence and transition from the somatic to the embryogenic state. Some studies were undertaken to answer this question and the results and the conclusions drawn from them are described below.

4.1 Cell division

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. In *Arabidopsis* explants, during DSE, the protodermal cell is involved in somatic embryo formation and divides

periclinally (Kulinska-Lukaszek et al., in press; Kurczyńska et al., 2007). Such a direction of cell division in the protodermal cell is unusual. In normal conditions, epidermal cells divide anticlinally (Considine & Knox, 1981) and periclinal division means that the phenotype of the protodermal cells was changed. This kind of division can be also called asymmetric (asymmetric does not necessarily mean that cells are of a different size after a division) because two daughter cells after periclinal division have a different neighbourhood; one of them still is in contact with the external environment while the other one is not. Other examples where unusual and asymmetric division was detected during SE were described in the case of the development of the secondary somatic embryos of *Trifolium repens* (Meheswaran & Williams, 1985), *Juglans regia* and *Medicago sativa* (Polito et al., 1989; Uzelac et al., 2007) and in the case of *Helianthus annuus* x *H. tuberossus* (Chiappetta et al., 2009).

4.2 Meristematic and embryogenic cells within explants

From many studies, it appears that the development of somatic embryos begins from the explant areas which are described as meristematic. Such a characteristic is typical not only for DSE but also for ISE.

The question now arises whether meristematic cells are histologically, morphologically and ultrastructurally equal to embryogenic ones? Next, how can we recognise meristematic and embryogenic explant cells?

Histological and ultrastructural analysis during the SE of pineapple guava showed that meristematic cells are rich in cytoplasm and containing many ribosomes, some amyloplasts and numerous mitochondria (Canhoto & Cruz, 1996; Canhoto et al., 1996). In this system, meristematic cells were similar on the ultrastructural level to embryogenic (proembryo) cells, with the only exception that the meristematic cells were more vacuolated. In the case of coconut, the meristematic cells were also characterised by dense cytoplasm, many ribosomes, reduced vacuole and a voluminous central nucleus with one or two nucleoli (Fig. 5 A; Verdeil et al., 2001). Cells with the same characteristics were described for *Carya* (Rodriguez & Wetzstein, 1998).

According to many studies, the most widely-described characteristic of the embryogenic cells involved in somatic embryo development are as follows: small cells with an isodiametric shape with dense cytoplasm, a nucleus located in the cell centre with a highly visible nucleolus and with small starch grains and vacuoles (Fig. 5 B; C; Canhoto & Cruz, 1996; Namasivayam et al., 2006; Verdeil et al., 2001). Pasternak and co-workers (2002) have also shown that embryogenic cells can be distinguished from non-embryogenic cells in the case of *Medicago* by the character of these cells. The embryogenic ones are characterised by their small size, with rich cytoplasm and filled with starch. The similar character of embryogenic cells was described in *Passiflora cincinnata* (Rocha et al., 2011) and cork oak (Puigderrajols et al., 2001). Cells with the same characteristics were described for the embryogenic parts of the explants of *Carya* (Rodriguez & Wetzstein, 1998). Nomura and Komamine (1985, 1995) have shown that isolated, small, cytoplasm-rich carrot cells have the ability to develop into somatic embryos. In carrot cultures, several phenotypes of cells capable for SE (embryogenic) were described (Toonen et al., 1994) but the efficiency of SE was highest in cells with a small size, a rich cytoplasm and which are spherical. The

comparison of the embryogenic and non-embryogenic parts of explants is much easier as the non-embryogenic parts of explants are highly vacuolated (Fig. 5 D).

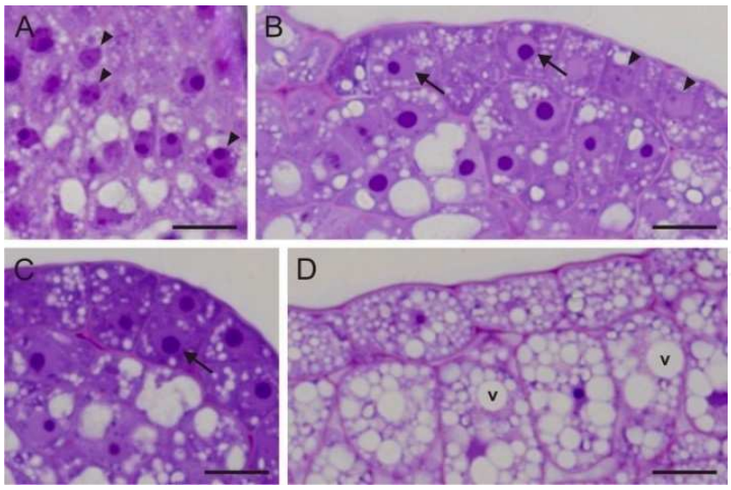

Fig. 5. Semi-thin sections through the *Arabidopsis* explant showing the examples of meristematic (A), meristematic and embryogenic (B), embryogenic (C) and non-embryogenic cells (D; the arrows point to embryogenic cells; arrowheads – to meristematic, note several nucleoli; V – vacuoles; sections stained with toluidine blue; bar = 10 μm; author – Izabela Potocka).

From the features of meristematic and embryogenic cells presented above, it appears that these differences are not distinct. According to Verdeil and co-workers (2007), some other features can be used for better distinguishing between meristematic and embryogenic cells, being the shape and the structure of the nucleus. In meristematic (in that case, the authors described the meristematic cells of shoot meristem) cells, the nucleus is spherical, with several nucleoli and heterochromatin (electron-dense areas under TEM) uniformly distributed within the nucleus. In the case of embryogenic cells, the nucleus is irregular in shape and contains one large nucleolus (Verdeil et al., 2007).

Some observations point to changes in the cell cytoskeleton which in embryogenic cells is organised in a different manner in comparison to non-embryogenic cells (Dijak & Simmonds, 1988; Dudits et al., 1991).

In conclusion: during the analysis of the cell morphology of explants during SE, one must remember that not all meristematic cells become an embryogenic cell, and not all embryogenic cells develop into somatic embryos. The direction of cell division within an explant can be a marker of cells which changed their direction of differentiation. The main features of embryogenic cells are their small size, low elongation rate, their small vacuoles, cells reach with cytoplasm, the high cytoplasm-nucleus ratio, changes in the nucleus and the nuclear envelope and their starch content.

5. Apoplast and symplast during SE

Between the somatic and embryogenic states of development, crucial processes called the transition and induction of embryogenic competence take place. This step is the most

important, but at the same time it is less understood (Verdeil et al., 2001). During this step, competent cells are those which are in a transitional state and which still require some stimuli to become embryogenic cells (Namasivayam, 2007). It is not clear how the embryogenic cells originate within the explants and what mechanisms control this process. Changes in cell fate and the direction of differentiation rely on the erasing of the genetic developmental program and switching on of a new one. How this is realised by explant cells is unclear. Some studies indicate that changes in the developmental program rely on physical isolation of a cell or a group of cells from the surroundings. This process may proceed by the isolation of the symplast and/or apoplast. The analysis of these plant compartments has shown that there are some features of the transition from the somatic to the embryogenic state on the cellular and histological level which allows the recognition of this developmental stage.

5.1 Changes in apoplast as a markers for SE

A unique feature of plants is the presence of a system of cell walls which is called 'apoplast'. For many years, apoplast has not been perceived as an important part of plant organisms. At present, it is no longer a dead part of the plant body but a temporally and spatially changing extracellular matrix. It is well-known that many processes depend not only on changes in the chemical and structural composition of the cell wall, but that the cell wall is a place where signal transduction takes place (Fry et al., 1993). If so, also process of SE was investigated from that point of view.

Studies with the secondary embryogenesis of *Brassica napus* have shown some features which should be convenient for the recognition of the transitional stage from the somatic to the embryogenic state (Namasivayam et al., 2006). It was shown that the explant epidermal cells involved in somatic embryogenesis were irregular in shape and size and covered by a layer of additional material deposited on their surface, while such material was not found in the non-embryogenic tissue (Namasivayam et al., 2006). What is interesting is that this material disappeared in the adult somatic embryos, suggesting that such a feature of embryogenic tissue could be a cellular marker for cells which changes their way of development. The staining of this material with AzurII/methylene blue suggested the presence of a mucilage/polysaccharide component (Namasivayam et al., 2006). A similar substance at the surface of the pre-embryogenic tissues was present in *Coffea arabica* (Sondahl et al., 1979), *Cichorium* (Chapman et al., 2000a, 2000b; Dubois et al. 1991, 1992), *Camellia japonica* (Pedroso & Pais, 1992, 1995), *Drosera* (Bobák et al., 2003; Šamaj et al., 1995), *Zea mays* (Šamaj et al., 1995), *Papaver* (Ovečka et al., 1997; Šamaj et al., 1994), *Pinus* (Jasik et al., 1995), *Citrus* (Chapman et al., 2000a) and coconut (Verdeil et al., 2001). The detected material was present only up to the globular stage of embryo development. Because of the time of its appearance and the location, it is postulated that this material is a cellular marker for the acquisition of embryogenic competency (Namasivayam et al., 2006). In some cases, this structure was called a 'supraembryonic network' (Chapman et al., 2000a, 2000b) or an 'extracellular matrix' (Namasivayam et al., 2006).

Another feature of apoplast during SE are the changes in the thickness of the cell wall (Fig. 6). Information about the necessity of the presence of the thick cell wall around developing somatic embryos showed that in some examples such an isolation is necessary (Dubois et al., 1991; Schwendiman et al., 1990; Verdeil et al., 2001). The thickening of the cell walls in the

explants' tissues was described for *Gentiana punctata* (Mikuła et al., 2004) and *Feijoa sellowiana*, where thick cell walls were detected around the proembryos (Canhoto & Cruz, 1996).

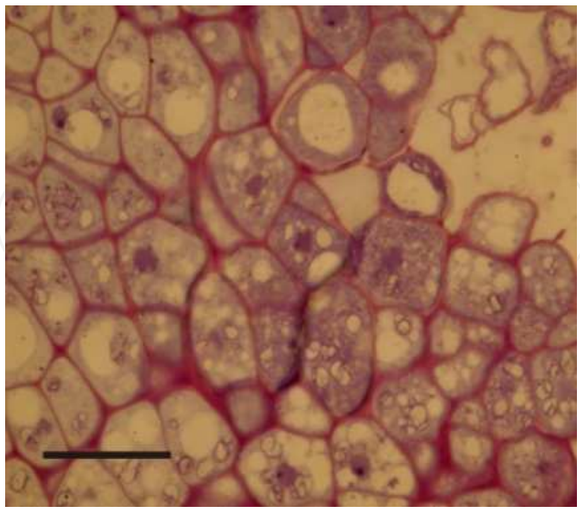

Fig. 6. Differences in wall thickness between cells within the explant through the example of *Arabidopsis* (PAS+toluidine blue staining; bar = 20 µm; author – Czekała).

It seems that the thicker cell walls surrounding the cell with a morphology which is typical for the embryogenic state is the result of the origin of these cells. Namely, if an embryo develops from the one cell and successive cell walls are formed within this mother cell, it is obvious that the cell wall at the surface of the proembryo is thicker, as is the older wall in such a complex. According to Williams and Meheswaran (1986), such isolation is necessary only if the embryogenic cells are surrounded by non-embryogenic ones.

5.1.1 Lipid transfer proteins

The lipid transfer proteins (LTPs) are proteins which can be divided into two classes, depending on the molecular weight. In *in vitro* conditions, it was shown that these proteins are able to transfer phospholipids between cellular membranes (Kader, 1997). The role of LTPs in the process of somatic embryogenesis was shown for the first time in the case of carrot embryos (Sterk et al., 1991). It is postulated that LTPs are involved in cutin biosynthesis and that they can be used as a cellular marker for the development of protodermis in somatic embryos (for a review, see Zimmerman, 1993). LTPs were also found in the extracellular proteins secreted by grapevine somatic embryos (Coutos-Thevenot et al., 1993). In *Arabidopsis* culture, LTPs were also observed outside the meristematic explant cells, which may indicate that LTPs can be used as a cellular marker during the transition from the somatic to the embryogenic state (Fig. 7).

Analysis of the presence of LTPs during somatic embryogenesis has rarely been performed, but studies on gene expression were more abundant and have shown that taking this expression pattern it is possible to distinguish between the embryogenic and non-embryogenic parts of a *Dactylis glomerata* suspension culture (Tchorbadjieva et al., 2005). Similar results indicating the role of LTPs in SE were performed on *Camellia* leaf cultures (Pedroso & Pais, 1995) and cotton (Zeng et al., 2006).

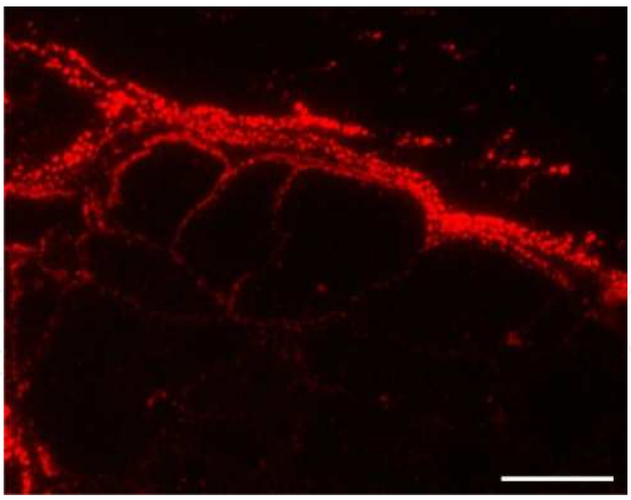

Fig. 7. The distribution of LTP1 epitopes (red dots) in the embryogenic area of *Arabidopsis* explant (LR White resin section-stained with the polyclonal anti-AtLTP1 antibody; bar = 10 μm; author – Potocka).

5.1.2 Arabinogalactan proteins (AGPs)

Arabinogalactan proteins are the group of extracellular and membrane-bound proteins which are very diverse in their composition and which are involved in many morphogenetic processes in plants, such as growth and development, cell expansion, cell proliferation and zygotic and somatic embryogenesis (Kreuger & van Holst, 1993; Qin & Zhao, 2006; for a review, see Seifert & Roberts, 2007). Many antibodies against different AGP epitopes have been introduced in order to study the role of this class of proteins in plant development. The role of AGP is postulated both during the early stages of embryogenesis and in the different developmental stages of the embryo (Stacey et al., 1990). It is also known that AGP secreted into the culture medium can promote the production of somatic embryos (Egertsdotter & von Arnold, 1995; Hengel et al., 2001; Kreuger & van Holst, 1993).

Developmental changes during somatic embryogenesis were described in detail in the case of *Daucus carota* and showed that cells "decorated" by the JIM8 antibody developed into somatic embryos, which suggests that this AGP epitope can serve as a cellular/wall marker for the very early transitional cell stage into an embryogenic pathway (Pennell et al., 1992).

The AGP epitope which was recognised by the JIM8 antibody was able to force the somatic cell of *Daucus carota* to produce somatic embryos, which points to the role of AGP in somatic embryogenesis (McCabe et al., 1997). Within the explant cells of *Arabidopsis*, only some of them during the culture period are characterised by the presence in their wall of AGP epitopes recognised by the JIM8 antibody (Fig. 8).

It was shown that the JIM4 monoclonal antibody can be an early marker for the development of somatic embryos (Stacey et al., 1990). Analysis with the use of the JIM13 antibody showed that in PEM (proembryogenic masses), in the case of *Picea abies* culture, this kind of AGP epitope was present in PEM cell walls and was not found in young somatic embryos, suggesting that this AGP epitope can be a good cellular marker for distinguishing between PEM and somatic embryos (Filonova et al., 2000).

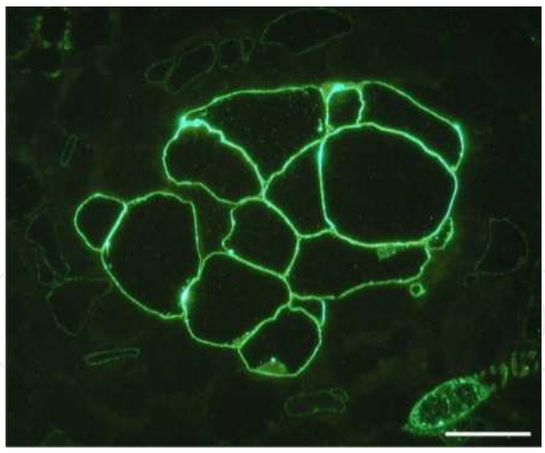

Fig. 8. A group of cells within an *Arabidopsis* explant with the presence of AGP epitope recognized by the JIM8 antibody (bar = 20 μm; author – Potocka).

5.1.3 Pectic epitopes

Pectins are the main component of the middle lamella and the primary cell wall. Pectins are acidic polysaccharides with a heterogeneous nature. The most important function of pectins is the attachment of cells.

During immunohistological studies of *Cichorium* SE with the use of the JIM5 antibody, the pectic epitopes recognised by this antibody were present in the supraembryonic network which covered the embryogenic parts of explant. It was postulated that unesterified pectic epitopes can be used as an early marker of SE (Chapman et al., 2000b).

Detected differences between the embryogenic and non-embryogenic calluses of *Daucus carota* in the amount of neutral sugars of pectin in comparison to the acidic parts of pectin are postulated as a marker for embryogenic cells (Kikuchi et al., 1995).

High levels of esterified pectins were detected during the embryogenesis of *Capsicum annuum* (Bárány et al., 2010), indicating that such a composition of cell walls is not only marker of cell proliferation but also an early marker of microspore reprogramming for embryogenesis.

In the *Arabidopsis* explants, the distribution of pectin epitopes recognised by the JIM5 and JIM7 antibodies was almost the same, but what is interesting in those parts of the explant which do not participate in embryogenesis is that neither pectin epitope was detected in the cells' walls (Fig. 9).

5.1.4 Callose

Callose is a (1→3)-β-D-linked homopolymer of glucose (Gibeaut & Carpita, 1994) present in different plant cells and what is most interesting is synthesized in response to wounding or other stress treatments (Fortes et al., 2002). However, the role of callose is not well-understood and - as was pointed out by Fortes and co-workers (2002) - in some tissues callose can prevent the absorption of water and in others it can enhance this process, which can also be important during SE.

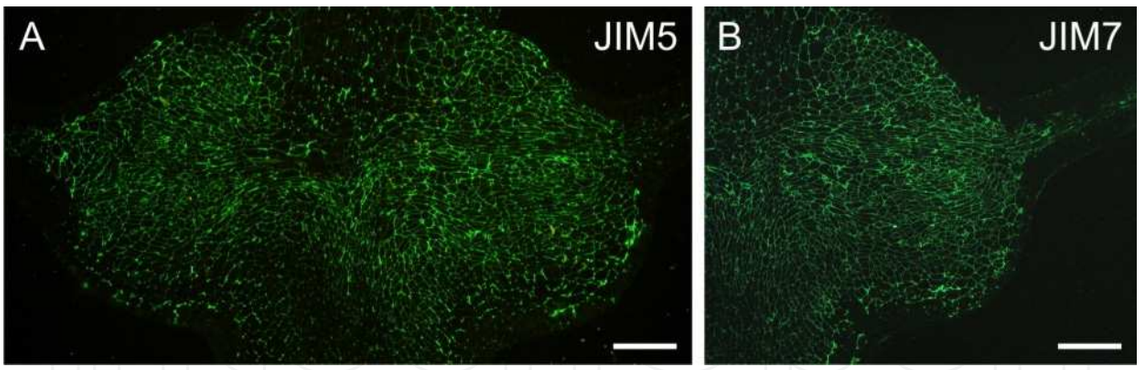

Fig. 9. The distribution of low- (left) and high-esterified (right) pectic epitopes within the *Arabidopsis* explant's cells (bar = 100 µm; author – Potocka).

The deposition of callose in the vicinity of plasmodesmata disturbs symplasmic communication (this will be described in detail below) between cells and - in this manner - influences the exchange of signals through plasmodesmata (Fig. 10 A). When callose is deposited in the cell wall it can interrupt the exchange of signals through the apoplast (Fig. 10 B).

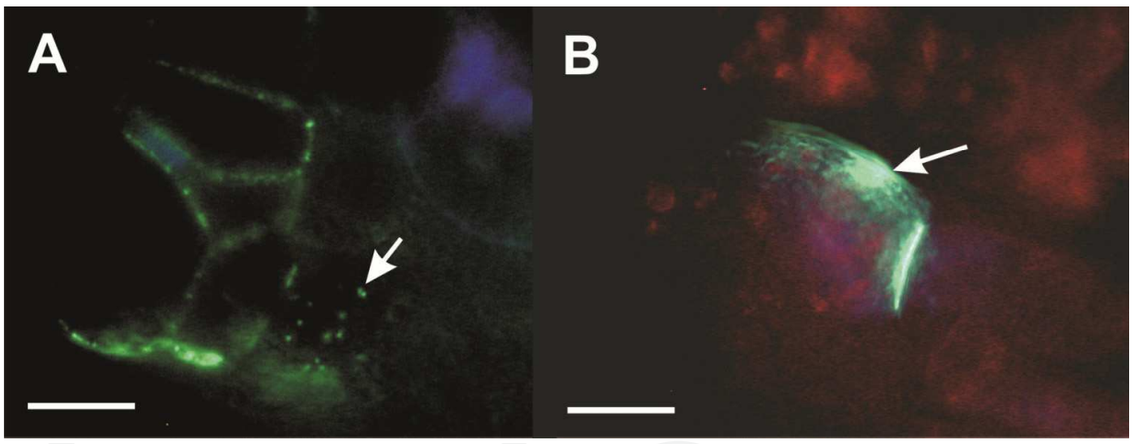

Fig. 10. The deposition of callose in the plasmodesmata regions, suggesting the closure of plasmodesmata only between some of the explants' cells (A), and in the cell wall, suggesting the isolation of neighbouring cells via apoplast (B) (*Arabidopsis* explants during SE; hand-cut sections stained with aniline blue; bar = 15 µm).

Studies with *Cichorium* and *Camellia japonica* showed that the deposition of callose is a prerequisite for somatic embryogenesis (Dubois et al., 1990; Pedroso & Pais, 1992). The same results were described for *Trifolium* (Meheswaran & Williams, 1985) and coconut (Verdeil et al., 2001).

Ultrastructural and histological studies on *Cichorium* during SE have shown that the first sign of SE is the deposition of callose in the cell wall (Verdus et al., 1993). Analysis performed on *Eleutherococcus senticosus* explants showed that after plasmolysis the amount of callose increased in comparison with untreated explants and - moreover - it was shown that callose is deposited between the plasma membrane and the cell wall (You et al., 2006).

5.1.5 Lipid substances

The deposition of lipid substances in the form of lamellae within the cell walls is postulated as being an important factor in the isolation of cells undergoing changes in their fate (Pedroso & Pais, 1992, 1995). It is postulated that apoplast isolation through the deposition of lipid substances is necessary for the abortion of the exchange of molecules through the cell wall. That is why this marker can be used in the detection of cells during the transition from the somatic to the embryogenic state. Unfortunately, there is not much information on the presence of lipid lamellae during the acquisition of embryogenic competence of explant cells.

Histological analysis of the series of a section of the *Arabidopsis* explant showed that within the callus cells some of them are isolated from the others by the lipid lamellae within the cell walls (Fig. 11). If this feature is characteristic of cells in their transition state then it requires further study.

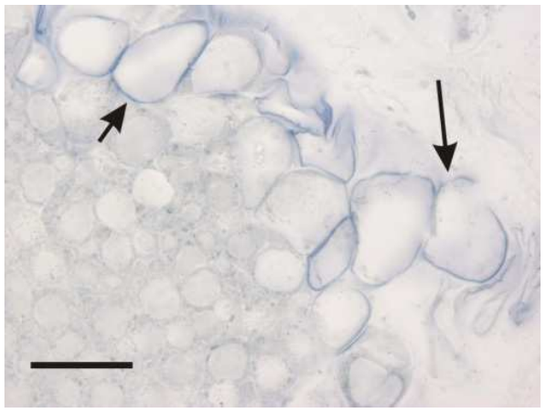

Fig. 11. The presence of lipid substances in some *Arabidopsis* explant cells during the process of SE (Sudan black staining; the arrows point to some of the cells with lipid lamellae in the wall; bar = 10 µm; author – Potocka).

In conclusion

The markers for the early stages of SE during the transition from the somatic to the embryogenic stage of cell development are present within the cell walls. These markers refer to the chemical composition of the extracellular matrix of a cell undergoing the process of transition, which involves changes in AGP and LTP, pectic epitopes, and callose and lipid substances deposited within the cell wall.

5.2 Changes in symplasm during SE

During SE, not only do changes in apoplast take place but changes also take place within the symplasm. Among the different mechanisms which control the process of plant development, including zygotic and non-zygotic embryogenesis (somatic embryogenesis and androgenesis), symplasmic communication/isolation is also postulated (Gisel et al., 1999; Kurczyńska et. al., 2007, Wrobel et al., 2011). This process is an important mechanism for the exchange of information between cells within a plant body. Such exchange of information is also a part of pattern formation within the plant organism, as it is known that

the process of cell differentiation relies on the cell's position (for a review, see Scheres, 2001). The exchange of information is important and it allows cells to realise the proper developmental program.

Symplasmic communication relies on a unique feature of plant organisms - the presence of plasmodesmata (PD) which links the cytoplasm of neighbouring cells and which creates the system called 'symplasm' (Romberger et al., 2004). It should be noticed that during plant growth and development, the connection through PD between cells changes and depends on the stage of development. As a result, plant organisms can be divided into symplasmic domains and subdomains (Zambryski & Crawford, 2000). Symplasmic domains present in the plant body can be permanent (for example stomata; Fig. 12 A). Symplasmic subdomains can be also temporal, which means that they changed spatially and temporally and may be composed of several cells or just one cell (Fig. 12). Analysis of the symplasmic tracer distribution within the protodermal cells of *Arabidopsis* explants showed that fluorochrome was present only in some cells (Fig. 12 B, C). What is interesting is that after the division of mother cell, only one of the daughter cells was filled with a fluorochrome, which suggests that communication between these cells is restricted (Fig. 12 B).

The main characteristic of PD is the upper limit of the molecules' size that can freely diffuse through PD, which is called the 'Size Exclusion Limit' (SEL). It was shown that SEL changed during the development because the PD diameter can be changed temporally, spatially and physiologically (Zambryski & Crawford, 2000). PD also can disappear during the development or may be created *de novo*. Thus, the limitation in symplasmic communication is a result of PD disappearance, lowering of their number or else the downregulation of SEL.

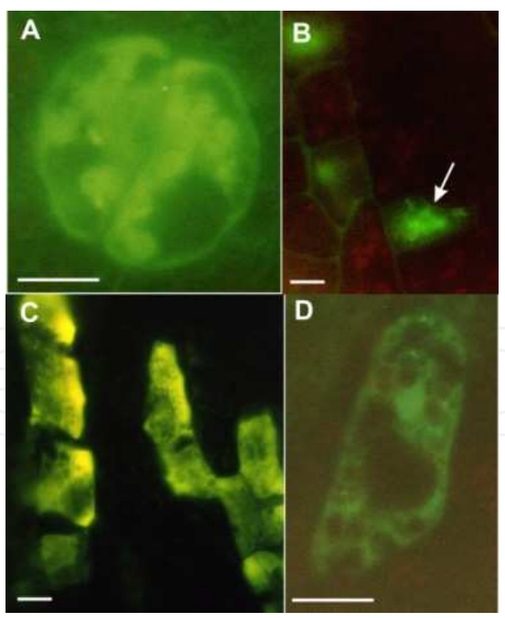

Fig. 12. The distribution of the symplasmic tracer (HPTS -8-hydroxypyrene-1,3,6-trisulfonic acid) within the protodermal cells of *Arabidopsis* explants. A – stomata as an example of the permanent symplasmic domain. B-C examples of the temporal symplasmic domains composed of a few cells (C) or in a single cell (D; as to B, note the unequal distribution of fluorochromes in the daughter cells after a division - arrow; bar = 10 μm).

As is known, molecules which can be exchanged between neighbouring cells through PD are not only ions, hormones, minerals, amino acids and sugars, but also proteins, transcriptional factors and different types of RNA (Kempers & van Bell, 1997; Lucas et al., 1993; Roberts & Oparka, 2003). This indicates that PD can regulate cell-to-cell movement and in this way participates in the regulation and coordination of plant development. It is known that PD plays an important role during the zygotic embryogenesis of *Arabidopsis thaliana* (Kim et al., 2002). Studies of the role of symplasmic communication during zygotic embryogenesis were based on the analysis of the movement of fluorochromes, dextrans conjugated to fluorescein and GFP (Green Fluorescent Protein) between embryo cells in different stages of their development. It appeared that the *Arabidopsis* embryo is one symplasmic domain up to the mid-torpedo stage (Kim et al., 2002). From that moment of development, the embryo is no longer a single symplast and the movement of symplasmic transport tracers of different molecular weights is restricted to different symplasmic domains and subdomains which correlate with the development of primary tissues and organs. This means that the downregulation of PD as the embryo develops is important for proper embryogenesis (Zambryski & Crawford, 2000). The studies mentioned above also indicate that disturbance in the normal permeability of PD leads to disorder in the development of *Arabidopsis*. The changes in PD permeability also took place when embryo changed its development from radial to bilateral symmetry (Kim & Zambryski, 2005). Detailed analysis of the GFP movement between cells also revealed the existence of subdomains which correspond to the establishment of the apical-basal axis of the *Arabidopsis* embryo (Kim et al., 2005b). These results clearly show that the regulation of embryogenesis is based (among others) on changes in symplasmic transport between embryo cells and they reveal the temporal and spatial correlation between the stages of embryo development and the formation of symplasmic domains and subdomains (Kim et al., 2002; Kim et al., 2005a; Kim et al., 2005b; Kim & Zambryski 2005; Stadler et al., 2005).

It is worth noting that there are some similarities between PD in plant organisms and the gap junctions in animal organisms. Namely, gap junctions play a control role during animal development (Warner, 1992).

The role of the disruption of symplasmic connection between cells which undergo different fate of differentiation has been postulated for many years. It is suggested that such a disruption allows those cells which are no longer connected by PD to differentiate in independent ways. Such physiological isolation is needed for reprogramming the cells. The question is: is the closing or decreasing of symplasmic communication a prerequisite for changing in direction of cell differentiation or is it the result of other changes which lead to the downregulation of symplasmic communication? The answer is not obvious. Some reports point to the first possibility while the other may suggest that it is a secondary cell reaction.

Symplasmic communication within explant cells during the initiation and development of somatic embryos was not intensively studied. Analysis of the distribution of CFDA (fluorescent tracer 5-(and-6) Carboxyfluorescein Diacetate) during the DSE in *Arabidopsis* explants showed the presence of the fluorochrome only in the protodermis and sub-protodermis of the explants, indicating that the downregulation of plasmodesmata connection within an explant took place (Kurczyńska et al., 2007).

Studies on the explants of *Panax ginseng* have shown that the disruption of plasmodesmata generated more somatic embryos than in normal conditions, indicating that cell-to-cell communication must be decreased for obtaining more efficient somatic embryogenesis (Choi & Soh, 1997). Similar results were obtained in *Morus alba* (Agarwal et al., 2004).

In the case of coconut, the cells forming the meristematic layer were connected by plasmodesmata, indicating that symplasmic communication between the cells in this layer is present (Verdeil et al., 2001). As somatic embryogenesis proceeds, the decreasing in symplasmic communication between proembryo and meristematic cells occurred, but plasmodesmata within the proembryo and embryo were present (Verdeil et al., 2001). This is an example that cells belonging to the same developmental stage - which is at the beginning of somatic embryo formation - are connected by plasmodesmata but are isolated from their neighbours.

Studies on the zygotic embryos of *Eleutherococcus senticosus* as explants showed that the disruption of plasmodesmata between explants cells promotes the formation of somatic embryos even on the medium without auxin (You et al., 2006). The interpretation of these results is as follows: the interruption of symplasmic communication stimulates the reprogramming of cells into cells competent for the embryogenic pathway (You et al., 2006).

In *Ranunculus*, analysis of the formation of somatic embryos showed that at the early stages of embryoid connection development by plasmodesmata between the embryoid and surrounding tissues were present, but in the latter stage the connection was disturbed (Konar et al., 1972). The isolation of competent cells during the formation of proembryos by disrupting plasmodesmata was also postulated by Yeung (1995). In *Gentiana punctata*, the disappearance of plasmodesmata during somatic embryogenesis was also detected (Mikuła et al., 2004).

Timmers and co-workers (1996), during the analysis of the level of calcium ions in the cells of *Daucus carota* culture, have also shown that an increasing level of these ions can cause the closure of the plasmodesmata between embryogenic cells and the proembryogenic mass.

The analysis of the presence of plasmodesmata in the callus cells of *Cichorium* shows the disappearance of connection by plasmodesmata during somatic embryogenesis, indicating that cells which will undergo new a physiological state are isolated from their neighbouring cells (Sidikou-Seyni et al., 1992). Similar results were described in the case of grasses, where the plasmodesmata connection existed only between cells belonging to the same group of cells creating aggregates (Karlsson & Vasil, 1986).

However, not all of the results described so far are in agreement with those presented above. In the case of *Pineapple guava* symplasmic, isolation was not detected during the formation of somatic embryos (Canhoto et al., 1996). Plasmodesmata were present between the cells of the embryo, but also between the embryo and the surrounding cells. This suggests that symplasmic isolation is not a prerequisite for somatic embryo formation (Canhoto et al., 1996). In other tissue culture systems, the same conclusion was drawn (Jasik et al., 1995; Thorpe, 1980; Williams & Meheswaran, 1986).

Symplasmic communication within somatic embryos is also not well-described. It was shown for barley androgenic embryos that the symplasmic barrier exists between protodermis and the underlying tissues up to the late globular stage, in the isolation of

meristematic cells of the embryo in the transitional and coleoptilar stage, and between the embryo proper and the scutellum and the coleorhizae at the mature stage of the embryo (Wrobel et al., 2011). In the case of *Arabidopsis*, symplasmic isolation was correlated with the morphogenesis of somatic embryos (Fig. 13; Wrobel, 2010). In the case of *Cephalotaxus harringtonia*, numerous plasmodesmata connecting the embryo cells were noticed (Rohr et al., 1997). Similar results were described when the secondary somatic embryos of *Eucalyptus globulus* were investigated (Pinto et al., 2008).

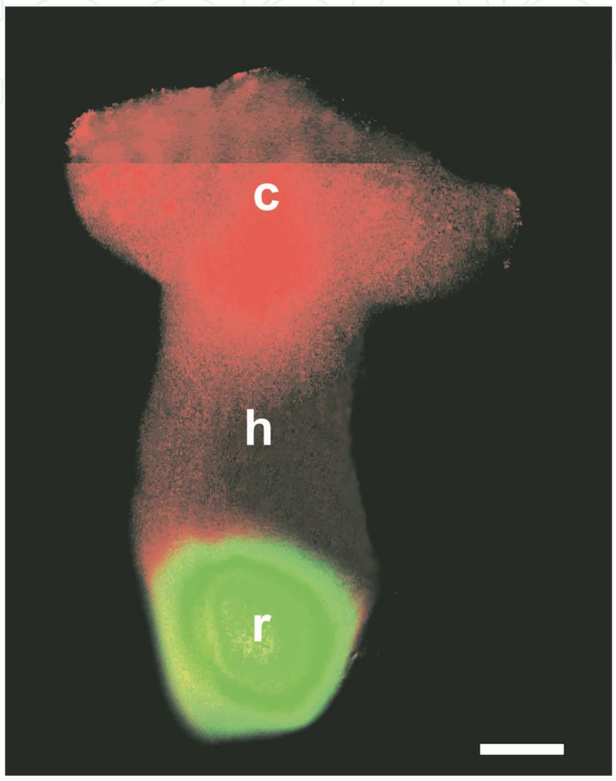

Fig. 13. The distribution of the symplasmic tracer (CMNB - caged fluorescein (fluorescein bis-(5-carboxymethoxy-2-nitrobenzyl ether, dipotassium salt) within the *Arabidopsis* somatic embryo, showing a border in symplasmic communication between the root meristem and other parts of the somatic embryo, which indicates that the symplasmic subdomains correspond with the main morphological parts of the embryo (fluorescence microscope; h – hypocotyl, c – cotyledon, r – root; bar = 150 µm; author – Wrobel, PhD thesis).

6. Conclusions

Knowledge of the cellular markers of somatic embryogenesis from the very early stages of changes in the direction of cell differentiation is important not only from a biotechnological point of view but also in helping in the understanding of the mechanisms underlying the changes in the direction of cell differentiation in general and the transition from the somatic to the embryogenic stage in particular.

It seems that promising cellular markers of cell fate changes exist at the ultrastructural and molecular level (Kiyosue et al., 1992; Pennell et al., 1992; Schmidt et al., 1997; Yeung, 1995).

The analysis of the cell wall's components and symplasmic communication during the changes in the direction of cell differentiation requires further study. Probably, both symplasm and apoplast are involved in the control of the synchronisation of cell division, histodifferentiation and primary organ development.

7. Acknowledgement

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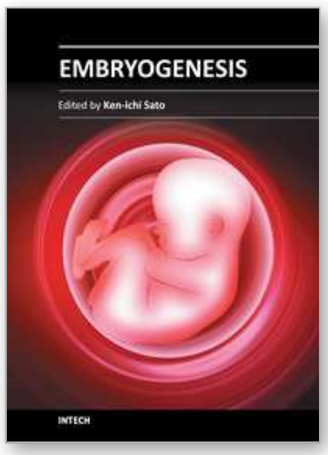

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