# Chapter 2

# Somatic Versus Zygotic Embryogenesis: Learning from Seeds

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## Abstract

Plant embryogenesis is a fascinating developmental program that is very successfully established in nature in seeds. In case of in vitro somatic embryogenesis this process is subjected to several limitations such as asynchronous differentiation and further development of somatic embryos, malformations and disturbed polarity, precocious germination, lack of maturity, early loss of embryogenic potential, and strong genotypic differences in the regeneration efficiency. Several studies have shown the similarity of somatic and zygotic embryos in terms of morphological, histological, biochemical, and physiological aspects. However, pronounced differences have also been reported and refer to much higher stress levels, less accumulation of storage compounds and a missing distinction of differentiation and germination by a quiescent phase in somatic embryos. Here, an overview on recent literature describing both embryogenesis pathways, comparing somatic and zygotic embryos and analyzing the role of the endosperm is presented. By taking zygotic embryos as the reference and learning from the situation in seeds, somatic embryogenesis can be improved and optimized in order to make use of the enormous potential this regeneration pathway offers for plant propagation and breeding.

**Key words** Biochemistry, Comparative approach, Maturation, Morphology, Proteome, Storage reserves, Stress response, Transcriptome

## 1 Introduction

Somatic embryogenesis, a fascinating developmental pathway through which plants can be regenerated from bipolar structures derived from a single or a few somatic cells was first described more than 50 years ago in carrot by Reinert [1] and Steward et al. [2]. This regeneration pathway offers a great potential to be applied in mass propagation, genetic transformation by direct means or via *Agrobacterium tumefaciens* and as a source of protoplasts as well as for long-term storage of germplasm using cryopreservation. Also fundamental studies of early embryogenesis are easier to be performed with somatic than with zygotic embryos. However, up to now the exploitation of this pathway is limited by inherent

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problems that are observed in many different plant species, like asynchronous differentiation and further development of somatic embryos, malformations and disturbed polarity, precocious germination, early loss of embryogenic potential, and strong genotypic differences in the regeneration efficiency. On the other hand, such limitations are not found in zygotic embryos developing within seeds. Thus, this review aims at comparing these two types of embryogenesis by regarding zygotic embryogenesis as a reference as suggested for the first time for wheat by Carman [3]. The identification of the major differences could enable new approaches to optimize somatic embryogenesis. Available literature dealing with comparisons of somatic and zygotic embryos on morphological, histological, biochemical, and also transcriptomic and proteomic level will be summarized, with emphasis on our model plant, the ornamental species *Cyclamen persicum*.

### 1.1 Zygotic Embryogenesis

The zygote is formed after double fertilization has taken place which is leading to the formation of the embryo and the endosperm. Zygotic embryogenesis is a complex, highly organized process, that has been studied for a long time by histological approaches only [4]. Recently it has been supplemented by molecular genetic studies, mainly based on mutant analyses of *Arabidopsis thaliana* as excellently reviewed in 2013 by Wendrich and Weijers [5] and depicted in Fig. 1. Embryogenesis is divided into (1) embryogenesis *sensu strictu* (morphogenesis of embryo and endosperm) meaning the development of the zygote up to a cotyledonary stage embryo and (2) the subsequent maturation phase that starts with the switch from maternal to filial control [6] and finally (3) the phase of embryo growth and seed filling ending with a desiccation phase [7].

Embryogenesis *sensu strictu* starts with a loss of polarity directly after fertilization of the egg cell which is followed by re-polarization and elongation of the zygote [5]. The important first asymmetric cell division of the zygote results in a more elongated basal cell that gives rise to the suspensor and the hypophysis and a small apical cell that generates the embryo. The suspensor positions the embryo within the embryo sac, conducts nutrients to the developing embryo and is a source of plant hormones that are important for polarity establishment [8]. It is eliminated by programmed cell death between globular and torpedo stage in angiosperms and in late embryogenesis in gymnosperms [9].

Auxin is the predominant plant hormone that has been reported to be involved in polarity and pattern formation. Especially, the PIN (PIN formed proteins) dependent asymmetric auxin efflux regulates these processes in early embryogenesis *(*[10], reviewed in 2010 by De Smet et al., [11]). The role of other plant hormones, among which cytokinins and brassinosteroids were reported to be important in these processes, is not yet clearly resolved [11].

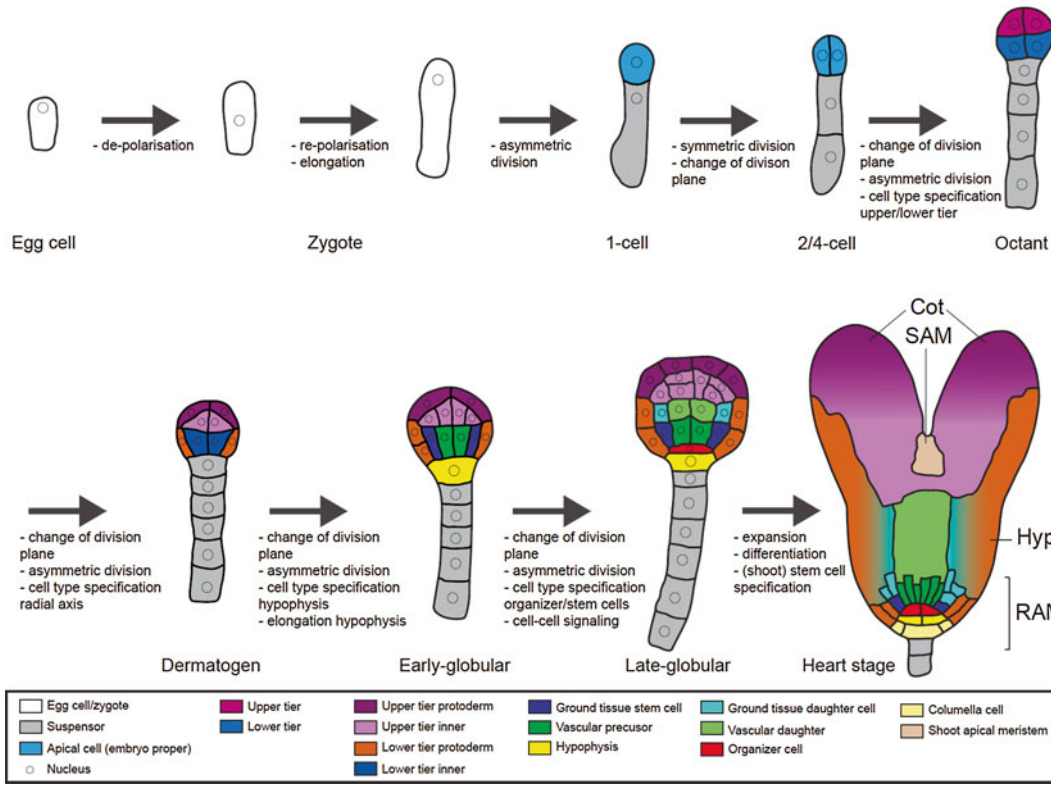


**Fig. 1** Morphogenetic processes during *Arabidopsis* embryogenesis. Schematic overview of *Arabidopsis* embryogenesis from the egg cell to the heart stage embryo, highlighting the morphogenetic processes required to progress from one stage to the next. The colors represent cells of (essentially) the same type (*see* color legend), based on marker gene expression and lineage analysis. *Cot* cotyledon, *SAM* shoot apical meristem, *Hyp* hypocotyl, *RAM* root apical meristem (reproduced from [5] with permission from New Phytologist)

Subsequent organized cell division in a symmetric way and only in one direction leads to the formation of the suspensor. In the apical cell division planes change in a strictly regulated way in *A. thaliana* and thereby establish two types of axes, defining upper and lower tiers and radially arranged cell types [5]. Most interestingly, the first cell divisions take place within the space provided by the apical cell. Thus, pattern formation occurs in the globular embryo by which the protoderm cells, vascular and ground tissue are defined. The last stage of embryogenesis *sensu strictu* is the heart stage being characterized by the presence of shoot and root apical meristems as well as early cotyledons. The key genes regulating morphogenesis of the embryo have been identified and encode transcription factors, receptor kinases, proteins involved in plant hormone signaling and micro RNAs pointing to the predominant

transcriptional control, and future research needs to focus on how these regulators hold their function in terms of cell biological implementations [5].

The later phase of seed development (maturation phase) comprises embryo growth, seed filling by deposition of storage reserves and finally desiccation. Mainly seed dormancy has attracted the attention of research in *A. thaliana* and other species (reviewed by Finch-Savage and Leubner-Metzger in 2006, [12]). Seed filling is of importance for many agricultural crops like rape seed or legumes as well (reviewed by Verdier and Thompson in 2008, [13]). At the end of seed development, the zygotic embryo is in a quiescent state which clearly separates embryogenesis from germination.

### 1.2 Somatic Embryogenesis

The term somatic embryogenesis already points to the pronounced morphological similarity of this vegetative regeneration pathway to zygotic embryogenesis. Somatic embryogenesis generally starts from a single cell or a group of cells of somatic origin and direct somatic embryogenesis is distinguished from indirect somatic embryogenesis in which a callus phase is passed through. The induction of embryogenic cells sometimes refers to all events that reprogram a differentiated cell into an embryogenic cell, but recently was divided into different phases, i.e., dedifferentiation, acquisition of totipotency, and commitment into embryogenic cells [14]. The first important difference compared to zygotic embryogenesis is the need for both, transcriptional and translational reprogramming of a somatic cell. Dedifferentiation of the somatic cells is the prerequisite to gain embryogenic competence and results in genetic reprogramming, loss of fate, and change into meristematic cells [15]. Stress due to wounding, separation from surrounding tissue, in vitro culture conditions, and also auxin are discussed to have a pivotal role in dedifferentiation [15]. Elhiti et al. [14] postulated that cells have to be cytologically separated for dedifferentiation as expression of genes responsible for secondary cell wall formation changed. Moreover, pronounced changes in the network that regulates the response to hormones have to take place. Twenty-five candidate genes being associated with the expression of cellular totipotency were identified by a bioinformatic approach using the CCSB (Center of Cancer Systems Biology) interactome database and *Arabidopsis* as a model for a molecular regulation network [14]. They cover functions in transcription, signal transduction, posttranslational modification, response to plant hormones, DNA repair and DNA methylation, and for the first time protein phosphorylation and salicylic acid signaling. The final step of the induction phase, the commitment into embryogenic cells, involves genes for signal transduction, microtubule organization, DNA methylation, regulation of transcription, apoptosis, and hormone-mediated signaling [14].

The establishment of polarity and a first asymmetric cell division has been observed in early somatic embryogenesis of carrot [16] and alfalfa [17]. By cell tracking experiments it was shown that carrot somatic embryos developed from different single suspension cells either via a symmetric or via an asymmetric first division [18], indicating that an asymmetric division is not decisive for proper somatic embryo development. However, as stated by Feher et al. [15], polarity, in terms of the transcriptional and biochemical status of the cell, is not necessarily expressed at the level of the morphology and symmetry of cell division. Therefore, early polarization is thought to be crucial in somatic embryogenesis as well as in zygotic embryogenesis, but needs to be set up by the cell internally following an external stimulus. The suspensor originating already from the first asymmetric division of the zygote is also formed in somatic embryos of conifers. It is supposed to support polarity and axis establishment in embryos and undergoes programmed cell death also in somatic embryos (reviewed by Smertenko and Bozhkov in 2014, [8]). In contrast, suspensor structures are often not so clearly detectable or completely missing in somatic embryos of plant species other than gymnosperms.

Due to the difficulty of identification of embryogenic cells, the early stages up to the globular embryo, and especially the precise sequence of cell divisions that can be described for *Arabidopsis* zygotic embryogenesis resulting in pattern formation have not often been recorded in somatic embryogenesis systems. Most studies that track the development of somatic embryos start with the globular stage [4]. Further development runs through the typical stages of angiosperm embryogenesis in dicots, namely globular stage, heart stage, torpedo stage, and cotyledonary stage. For a long time, markers for competent cells have been searched for, and most promising are Somatic Embryogenesis Receptor like Kinases (SERKs), that were identified to play a role in zygotic and somatic embryogenesis in *Daucus carota* [19] and *A. thaliana* [20]. They are involved in perception and transduction of extracellular signals and connected to brassinosteroid signaling [21], but their exact function is unknown up to now.

Maturation includes accumulation of storage reserves, growth arrest, and acquisition of desiccation tolerance and is, in case of somatic embryos, induced externally by increasing the osmotic pressure (lowering the osmotic potential) of the culture media (e.g. by addition of polyethylene glycol or increased sugar concentration) and application of abscisic acid (ABA) [22]. Germination requires similar conditions as in the respective zygotic embryos and completes this developmental pathway. Obviously, somatic embryos are completely lacking the effects of the surrounding seed tissues which provide physical (space) constraints and a specific and complex interaction of testa and endosperm supporting embryogenesis in an optimal way. For the induction of embryogenic cells,

external stimuli are mainly coming from the culture media, plant growth regulators, and culture conditions, but thereafter somatic embryogenesis is following an intrinsic autoregulatory developmental program [8]. Most likely, this process can be improved by mimicking conditions found in seeds.

## 2 Comparison of Somatic and Zygotic Embryos

### 2.1 Morphological and Histological Comparison

The fact that somatic embryogenesis was named after embryogenesis taking place in seeds clearly indicates a high degree of similarity of somatic and zygotic embryos. Many early studies were devoted to describe morphological aspects involving histological and microscopic investigations. Due to the typical stages both types of embryos pass through, globular, heart, torpedo, and cotyledonary stage, the parallels become obvious. Both kinds of embryos are bipolar structures from the beginning and do not have a vascular connection to maternal tissue which enables the discrimination of somatic embryogenesis and adventitious shoot regeneration.

The first cell division of the zygote is asymmetric while in somatic embryos this is not always the case (see above, [18]). Mathew and Philip [23] described the regeneration of *Ensete superbum* via somatic embryogenesis starting from single cells without the need of strong polarity establishment in these cells. However, all further stages that were compared in this histological approach revealed high similarity of somatic embryos to their zygotic counterparts in terms of structure of the embryonic apex or formation of cotyledons and hypocotyls. In many indirect somatic embryogenesis systems, the so-called proembryogenic masses, being clusters of small, dense cytoplasm rich embryogenic cells, give rise to the differentiating embryos, but their first divisions have not often been observed in detail, since the cell or the cell group from which the embryo originates is difficult to identify. While in gymnosperm somatic embryos the suspensor is a very prominent structure that in late embryogenesis undergoes programmed cell death [8], in many angiosperm systems suspensors are either absent or strongly reduced which might explain the difficulties in root formation reported for some species, especially due to the absence of the hypophysal cell [4].

Maize secondary somatic embryos derived from single primary somatic embryos or somatic embryos developing attached to callus cells, revealed malformations in the shoot meristem formation after direct regeneration of the single somatic embryos, while those that developed next to callus cells perfectly represented zygotic embryo development [24]. The authors discuss a possible role of the neighboring callus cells with similar functions as suspensor cells in the zygotic situation. Interestingly, in our model plant *C. persicum*

[25] embryogenic cultures are mixtures of embryogenic and nonembryogenic cells, and the differentiating somatic embryos are surrounded by a extracellular matrix resembling several cell wall layers (Douglas Steinmacher, Melanie Bartsch, and Traud Winkelmann, unpublished data). One possible explanation, for which further evidence is needed, could be that nonembryogenic cells undergo programmed cell death and thereby enable differentiation. In *Eucalyptus nitens* somatic embryos are only sporadically observed, but then appear on dark brown wounded callus cells [26]. An ultrastructural study not only recorded several analogies in cell and embryo structure when compared to zygotic embryos, it also identified a kind of waxy coat surrounding the somatic embryos which was supposed to originate from phenolic exudates [26]. Somatic embryos of *C. persicum* have three times larger cells than their zygotic counterparts, and their outer surface is more irregular than the smooth protoderm of zygotic embryos [27]. This observation indicates that the physical and chemical constraints of the surrounding tissue, the endosperm, may have an important influence on the cellular organization of zygotic embryos that is lacking in somatic embryogenesis systems (*see* also Subheading 3).

Maturation is a major bottleneck in somatic embryogenesis of several species including *Pinus pinaster* [28] and coffee [29]. Also loblolly pine somatic embryos did not reach full maturity and had lower dry weights than the zygotic ones [30]. Polyethylene glycol (PEG) which is often used in maturation media of conifers had clear effects on the morphology of somatic embryos of *P. pinaster* as numerous and larger vacuoles as well as larger intercellular spaces were induced by this treatment [28]. By the histological comparison of somatic embryos subjected to different maturation treatments (carbohydrates in various concentrations) protein bodies were found to appear earlier in somatic embryos, and to be more abundant in well-developed somatic embryos leading to the suggestion that storage protein accumulation could be regarded as a marker for embryo quality of *Pinus pinaster* [28]. The same authors observed starch accumulating in zygotic embryos in a gradient of higher concentrations at the basal end, whereas in somatic embryos the localization of starch granules strongly depended on the maturation treatment. However, irrespective of the maturation treatment, somatic embryos always contained higher amounts of starch than the zygotic ones again with significant differences between different kinds and concentrations of carbohydrates applied [28].

Another aspect, namely the water status, was studied in *Hevea brasiliensis* embryos [31]. In zygotic embryos the water content decreased sharply from 91 to 53 % within 1 week (14–15 weeks after pollination) and during the remaining maturation phase down to 42 %. In contrast, somatic embryos without maturation treatments had a water content of nearly 80 %, while those that had

been desiccated or cultivated on higher sucrose concentrations plus ABA still contained 71 % water but had much higher germination and conversion rates than the nontreated ones [31]. Also in date palm the zygotic embryos underwent dehydration with a water content of 80 % decreasing to 35 %, whereas somatic embryos had a water content of around 90 % throughout the whole development [32]. Both mentioned species still have high water content in the seed after desiccation. In species with true orthodox seeds and much lower water contents, the drop in water content and thereby the discrepancy between somatic and zygotic embryos can be expected to be even more pronounced.

Somatic embryogenesis is already commercialized in coffee, but its profitability is limited due to losses during conversion into plantlets. Thus, Etienne et al. [29] put special emphasis on studying this phase in the zygotic and somatic system. Differences were found in conversion time which took 22 weeks in somatic and 15 weeks in zygotic embryos, hypocotyl length being shorter in somatic embryos, a more spongy tissue in the somatic embryo axis, earlier differentiation of stomata in somatic embryos and less protein and starch in cotyledonary somatic embryos [29]. The water content of zygotic embryos increased strongly during germination starting from 28 % and reaching 80 % within 4 weeks, whereas the increase in somatic embryos was rather mild (water content from 70 to 85 %). Furthermore, the authors observed asynchronous germination in somatic embryos. It can be concluded that the phase of maturation which includes a growth arrest controlled by plant hormones (mainly ABA) and desiccation is obviously extremely important to allow the development of high quality somatic embryos that will germinate in high rates and in a synchronized way.

## 2.2 Biochemical Comparison

When screening the literature for studies comparing somatic and zygotic embryos on the biochemical level, mainly analyses of major storage compounds, i.e. storage proteins, carbohydrates, and lipids are found. Depending on the type of seed in a respective species, storage reserves may be found in the embryo itself and here mainly in the cotyledons or in the endosperm. Early studies in *Brassica napus* [33] and cotton [34] have shown that somatic embryos are able to accumulate storage proteins, but in much lower amounts (1/10 of that found in zygotic embryos in *B. napus*) and in earlier stages. In somatic embryos of alfalfa 7S globulin was dominant, while in zygotic embryos 11S globulin and 2S albumin were more abundant [35]. The processing and subcellular localization of 7S and 11S storage proteins in protein bodies was comparable in both embryo types, while 2S albumin in somatic embryos was detected in the cytoplasm, in contrast to zygotic embryos in which 2S albumins were localized in protein bodies [35]. Overall, also in alfalfa lower amounts of storage proteins were determined in somatic embryos, thus supporting the observations in *B. napus* and cotton. Thijssen et al. [36] visualized globulin (storage protein)

### 2.2.1 Storage Proteins

accumulation by fluorescence labeled antibodies in somatic and zygotic embryos of maize. Starting 10 days after pollination globulins were detected in the scutellum first and later in leaf primordia and roots. Lower amounts of intermediate globulin precursor proteins were found early in development of somatic embryos while mature globulins could be induced by a maturation treatment with ABA [36]. Date palm somatic embryos contained about 20 times lower amounts of total protein than zygotic embryos, a different protein composition, and were lacking glutelin, a storage protein with the typical accumulation and hydrolysis pattern in zygotic embryos [32]. In agreement with these studies are the observations in oil palm embryos in terms of earlier, but 80 times less production of 7S globulins in somatic embryos compared to zygotic ones [37]. A recent follow-up study [38] reported on early mobilization of storage proteins by proteases in somatic embryos, thus providing further evidence that the clear differentiation of the developmental phases of embryogenesis, maturation, and germination is lacking in somatic embryos. Instead there is an overlap of all three programs, since globulin synthesis still occurred during germination of somatic embryos and cystein proteases were active in all phases of somatic embryogenesis [38]. In order to gain insights into glutamine metabolism, a nitrogen compound that is important for embryogenesis, Perez-Rodriguez et al. [39] found cytosolic glutamine synthase 1a (GS1a) to be absent in zygotic, but present in somatic embryos of *P. pinaster* and *Pinus sylvestris* indicating the onset of precocious germination in late stages of somatic embryogenesis, since this gene is a marker for chloroplast differentiation. GS1b expression was detected in procambial tissues of both types of embryos with the level of expression correlating to the quality of somatic embryos [39]. Arginase expression in somatic embryos indicated that storage protein breakdown obviously started before germination [39]. Possibilities to improve storage protein accumulation by ABA treatment were shown for example for cocoa somatic embryos [40] or by increasing sucrose concentrations in maturation media for *Pinus strobus* [41] and cyclamen [42].

### 2.2.2 Carbohydrates

Cotyledonary white sprucesomatic embryos accumulated more starch, but less proteins and lipids than zygotic embryos in the same stage. This points to the fact that the conversion of starch into the energy rich storage compounds lipids and proteins did not take place in somatic embryos to the same extent [43]. According to this study, adjustment of in vitro culture conditions might be an option to improve this conversion during embryo maturation. Carbohydrates have important functions during plant development and growth as energy sources but also for osmotic adjustment, protein protection, and signaling molecules, and they have been analyzed in comparative approaches during somatic and zygotic embryogenesis. During maturation of cocoa zygotic embryos (*Theobroma cacao*) storage proteins and starch

accumulate, dehydration takes place and monosaccharides and sucrose decrease, while two oligosaccharides, raffinose and stachyose, increase [40]. In contrast, somatic embryos accumulated less protein and starch as detected in histological studies and they had higher levels of sucrose, xylose, and rhamnose [40]. A shift in carbohydrate composition was observed in Norway spruce for both, somatic and zygotic embryos, during later developmental stages with decreasing total carbohydrates and a higher sucrose:hexose ratio within time. However, only mature zygotic embryos contained raffinose and stachyose which play a role in desiccation tolerance [44]. After a maturation treatment with 3.75 % PEG 4000 the sucrose:hexose ratio in Norway spruce somatic embryos raised significantly from 0.88 to 6 which resembled more the ratio of 9.7 found in zygotic embryos, all in the early cotyledonary stage [45]. While in somatic embryos invertase and sucrose synthase were found in high activity during the proliferation and early maturation phase, invertase activity was low in developing zygotic embryos and sucrose synthase was first observed in the cell layer surrounding early zygotic embryos and later inside the embryos. From this the authors conclude that sucrose synthase plays an important role in the transition of the embryo from a metabolic sink to a storage sink [45]. The sucrose distribution within the embryo which is among other factors controlled by epidermal sucrose transporters was suggested to trigger starch accumulation during the maturation phase of *Vicia faba* zygotic embryos [46].

In the fruit tree *Acca sellowiana* that is native to South Brazil, total soluble carbohydrates per gram fresh mass were found to be about twice as high in zygotic compared to somatic embryos in the globular, heart, and torpedo stage, although the principal composition was the same. Especially for sucrose, fructose, myo-inositol, and raffinose (in the later stages of embryogenesis) zygotic embryos showed higher contents, even though somatic embryos were cultured in sucrose containing media. On the other hand starch contents of torpedo and cotyledonary stage somatic embryos exceeded those of their zygotic counterparts [47]. Also in pea changes in soluble sugar composition during maturation of zygotic embryos were observed with sucrose, galactinol, raffinose, verbascose, and stachyose being the most prominent in mature seeds. In contrast, pea somatic embryos contained much lower total soluble sugars being composed of fructose, glucose, myo-inositol, sucrose, raffinose, and galactinol, but lacking stachyose and verbascose. Most interestingly, irregular misshaped somatic embryos differed in their carbohydrate profiles from normal ones [48]. Taken together, these analyses on carbohydrates point to the fact that somatic embryos often contained lower total amounts of soluble sugars, in later stages show different monosaccharide:sucrose ratios and a lack or smaller amounts of raffinose and its derivatives that are considered to be important for desiccation tolerance. Thus, maturation obviously is the major bottleneck for somatic embryogenesis in several species.

#### 2.2.3 Lipids

Comparative lipid analyses in both types of embryos are hardly found in literature, except one report for *Prunus avium* [49]: the lipid profiles of somatic embryos resemble those of zygotic embryos with neutral glycerolipids and phosphatidylcholine being the major lipid classes. However, contents of these two classes of lipids in somatic embryos were comparable to those of immature zygotic embryos, which was in line with the observation that somatic embryos did not develop further, until they received a cold treatment that resulted in increased lipid levels.

#### 2.2.4 Polyamines

Polyamines (among which the commonly occurring spermidine, spermine, and putrescine) are assumed to play a role in embryogenesis [50] and they were quantified in somatic and zygotic embryos of Norway spruce [51]. If mature somatic embryos are contrasted to zygotic ones, the latter contained less spermidine, but more putrescine resulting in a much lower spermidine:putrescine ratio. This ratio as well as the higher absolute polyamine contents of somatic embryos may be connected to the lower germination ability of somatic embryos. However this assumption requires physiological explanations [51].

#### 2.2.5 Plant Hormones

In a comparison of plant hormone contents in somatic and zygotic larch embryos, 100 times higher concentrations of ABA were found in somatic embryos that were cultivated on medium containing the nonphysiological ABA concentration of 60 μM. During maturation the ABA content increased in somatic embryos while it declined in zygotic ones [52]. Among the cytokinins, only for isopentenyladenine differences were detected with much higher levels in zygotic embryos, whereas IAA contents were similar in both embryo types [52]. The set of enzymes detoxifying reactive oxygen species differed between zygotic and somatic embryos of horse chestnut [53]: catalases and superoxide dismutases showed different courses of expression and different isoforms, especially in the maturation phase that resembled more the germination phase in case of somatic embryos. These authors concluded that somatic embryos seem to be exposed to higher stress levels than their zygotic counterparts.

### 2.3 Comparison of Transcriptomes

While an increasing number of studies on gene expression during embryogenesis of either the somatic (e.g. soybean, [54]) or the zygotic type (e.g. loblolly pine, [55]), are available, only very few reports deal with transcriptomic comparisons of somatic and zygotic embryos. In *C. persicum,* Hoenemann et al. [27] compared zygotic and somatic embryos and also embryogenic and nonembryogenic cell lines using a cDNA microarray with 1216 transcripts. They observed an upregulation of oxidative stress response genes in somatic embryos, as for glutathione S-transferases, catalase, and superoxide dismutase. These genes were upregulated not only in early stages of somatic embryogenesis but also 3 weeks

after induction, pointing at lingered stress and/or the induction of secondary somatic embryos. The importance of pectin-mediated cell adhesion as a prerequisite for embryogenicity was proposed by these authors based on the higher abundance of several genes encoding pectin-modifying enzymes in embryogenic than in non-embryogenic cells. Moreover, a cationic peroxidase that prevents cell expansion was suggested to be important for early embryogenesis [27]. Thus, the early cell divisions that do not result in expansion in size in early zygotic embryogenesis could be realized in a similar way in somatic embryos.

Recently, next generation sequencing was applied in cotton to compare the transcriptome of three comparable stages of both somatic and zygotic embryos [56]. Among a total of more than 20,000 unigenes, 4242 were found to be differentially expressed in these six samples. Of the differentially expressed genes a higher number was upregulated in somatic embryos at all stages [56]. Especially, stress response genes including hormone-related genes (mainly ABA and jasmonic acid signaling), kinase genes, transcription factors, and downstream stress responsive genes—e.g. late embryogenesis abundant (LEA) genes, heat shock proteins—were found at higher expression levels in somatic embryos. Moreover, cotton somatic embryos were found to be metabolically more active than their zygotic counterparts as indicated by gene expression data, the number of mitochondria, bigger vacuoles, and more lipid droplets [56]. Stress on the one hand can be considered as an important trigger of embryo development which also occurs in the zygotic system during maturation to prepare the embryo for desiccation stress. On the other hand, if cells experience too much stress as it might be the case under in vitro conditions, this might disturb the developmental program or even lead to cell death.

### 2.4 Comparison of Proteomes

The proteome reflects the total set of proteins that is present in a defined tissue in a specific developmental stage under defined conditions and thus provides direct evidence of the biochemical and physiological status of these cells. A possible disadvantage of proteomic studies is that proteins of very low abundance such as important transcription factors may be not detected. Although the number of proteins that can be detected is limited if gel-based proteomics is used, the comparison of two proteomes can be visualized very well using 2D-SDS-PAGE (two-dimensional isoelectric focussing/sodium dodecylsulfate polyacrylamide gel electrophoresis). In our own comparative studies we used a gel-based proteomic comparison of somatic and zygotic embryos of *C. persicum*, the work-flow of which is depicted in Fig. 2 [57]. The first and essential step is to select the biological material that will allow a meaningful proteomic comparison; in our studies, the selection of comparable stages was based on embryo morphology [42, 58, 59]. Spots of interest being either more abundant or even specific for

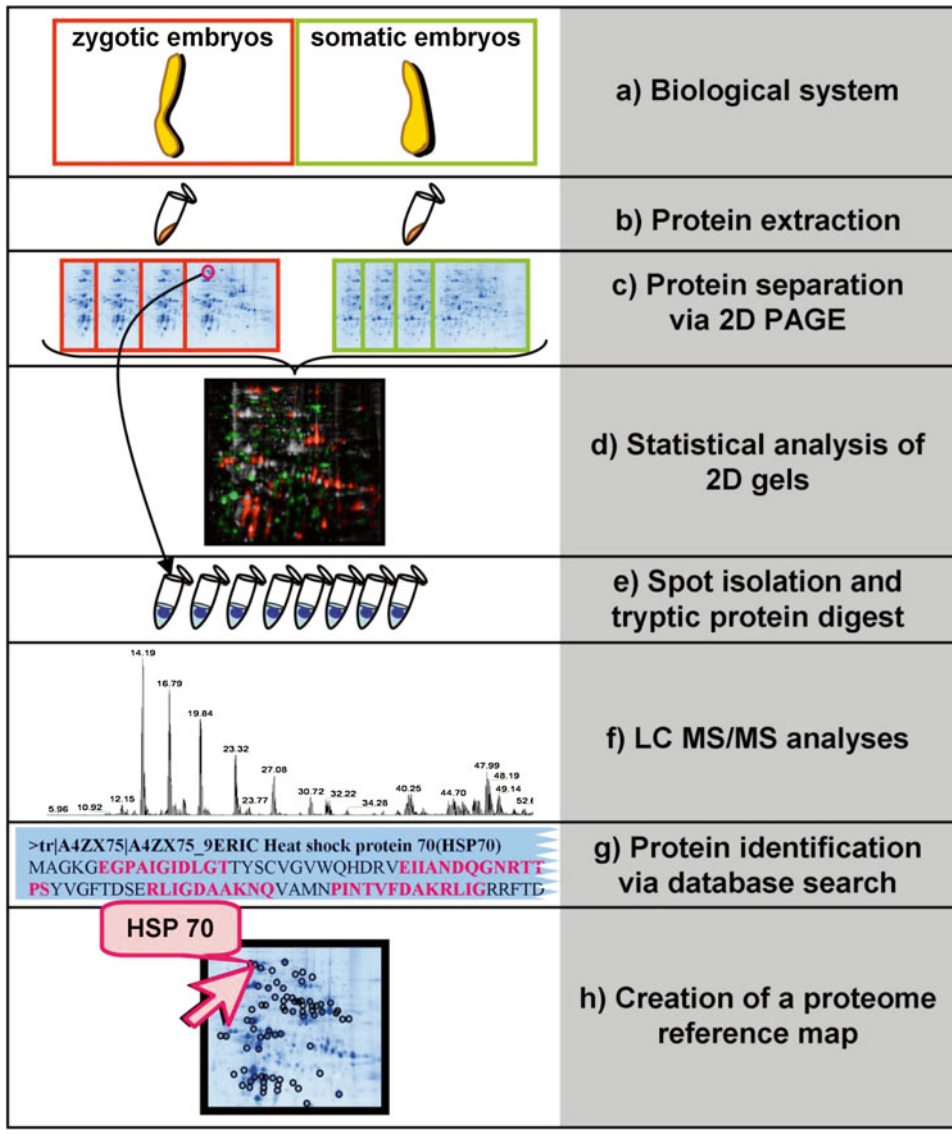


**Fig. 2** Workflow of a gel-based proteomic approach combined with mass spectrometry. The biological system represents one or more samples to be analyzed via a gel based proteomic approach. In the example given in this diagram, proteomes of zygotic and somatic embryos of *Cyclamen persicum* are analyzed and compared (**a**). Therefore, total proteins are extracted from each tissue (**b**) and separated via IEF-SDS PAGE (**c**). To perform statistical analyses with gels of different tissues, at least a set of three replicates for each tissue is required. Spots that differ significantly in abundance are labeled (*green* and *red*) in an overlay image of all gels analyzed (**d**). Protein of interest (e.g., differentially abundant proteins) are isolated from 2D gels and subsequently a tryptic protein digest is performed (**e**). The resulting peptides are separated via liquid chromatography (LC) before tandem mass spectrometry analyses (**f**). Protein identification is performed based on resulting peptide sequences (*pink*) via a database search matching to known sequences (**g**). Finally, a digital proteome reference map can be designed indicating all identified proteins (**h**). Using a gel-free shotgun approach, the steps (**c**) and (**d**) are replaced by digestion of a complex protein sample which is then further analyzed (reproduced from [57] with permission from author and Leibniz Universität Hannover)

one sample can then be eluted from the gel and subjected to mass spectrometry in order to identify the protein or proteins within this spot by comparison to databases. Finally, the obtained data can be combined to an interactive reference map which in our case was made publicly accessible and allows filtering spots by their abundance, metabolic function, or tissue specificity [60]. This technique was already applied in the '90s. Comparing somatic and zygotic embryos of *D. carota* torpedo shaped somatic embryos had a clearly distinct protein pattern from zygotic embryos and lacked the maturation specific proteins, namely two globulin-type storage proteins and a LEA protein [61]. In the gymnosperm species Norway spruce (*Picea abies*), similar protein patterns of zygotic and somatic embryos, the latter cultivated on maturation medium containing 90 mM sucrose and 7.6 μM ABA, were reported and both types were dominated by storage proteins [62].

Our model to study somatic embryogenesis is the ornamental plant *C. persicum*. In a pilot study, the proteomes of cyclamen somatic embryos grown in differentiation medium with 30 and 60 g/L sucrose were compared to zygotic embryos and endosperm [42]. When somatic embryos were differentiated in medium containing 60 g/L sucrose, 74 % of the protein spots were found in comparable abundance as in the zygotic embryos' proteome, while 11 % and 15 % were found in higher abundance in zygotic and somatic embryos, respectively. Enzymes of the carbohydrate metabolism, as well as heat shock proteins and a glutathione-S-transferase, were more abundant in somatic embryos. Thus, again evidence was presented for differences in stress response of both types of embryos. Furthermore, first insights into cyclamen seed storage protein accumulation and the synthesis of the storage carbohydrates xyloglucans were gained [42]. A follow-up study made use of the advances achieved in protein extraction, resolution, evaluation, more sensitive mass spectrometrical analyses and, most important, sequence information available in the data bases leading to higher identification rates even for this nonmodel organism [58]. In both embryo types glycolytic enzymes were identified as a high percentage of the identified proteins. In somatic embryos four protein spots showed six- to more than tenfold increased abundance, and the identified proteins within these spots were involved in oxidative stress defense: osmotin-like protein and antioxidant 1, peroxiredoxin type 2, and catalase. This finding is a clear indication that somatic embryos are much more stressed than zygotic ones [58]. The occurrence of truncated forms of enolases in zygotic embryos in relatively high amounts that disappear during germination suggested a new role of parts of this glycolytic enzyme as storage proteins [58]. We followed the original idea of taking the proteome of zygotic embryos as a reference for the optimized development of high quality somatic embryos: we could show that in somatic embryos a change of the proteome towards

the zygotic status was induced after the application of a maturation treatment with ABA [59]. After ABA treatment, the proposed new storage proteins ("small" enolases) appeared in the proteome of somatic embryos, thus resembling more the proteome of zygotic embryos (Fig. 3). Sghaier-Hammami et al. [64] found the total

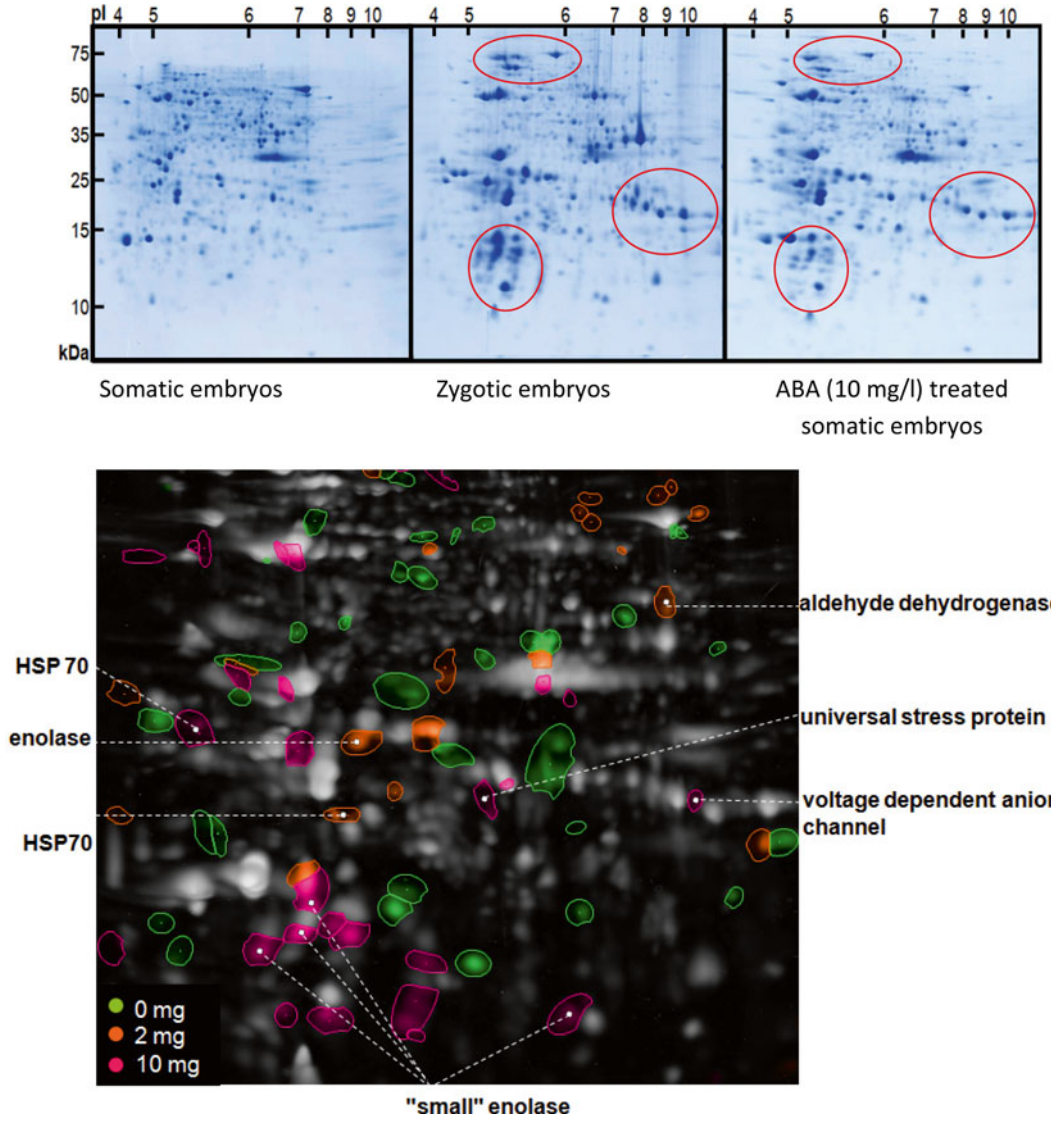


**Fig. 3** *Upper Part:* Comparison of protein gels of torpedo-shaped somatic embryos, zygotic embryos, and somatic embryos treated with 10 mg/L ABA for 28 days (taken from different studies [57, 63], *encircled* are parts of the gels which show high similarity in zygotic and ABA-treated embryos). *Lower Part:* Alterations in protein abundance of 56 days old somatic embryos after cultivation on medium containing 0, 2, and 10 mg/L ABA for 28 days. *Green labeled spots* are at least 1.5 times higher abundant in controls, *orange labeled spots* are at least 1.5 times more abundant in the 2 mg/L ABA treatment, and *pink labeled spots* are at least 1.5 times more abundant in the 10 mg/L ABA treatment (compared to control) (lower part of the figure reproduced from [63] with permission from the author and Leibniz Universität Hannover)

protein content as well as the number of spots to be higher in zygotic than in somatic embryos of date palm in a comparative 2-DE proteomic approach. Sixty percent of the protein spots differed in their abundance between the two embryo types, and out of 63 spots of differential abundance that were eluted from the gels, 23 were identified. Most of the proteins of higher abundance in somatic embryos were involved in the glycolysis pathway, citrate cycle, and ATP synthesis pointing to a higher energy demand, while in zygotic embryos a high abundance of storage proteins and stress-related proteins of the heat shock family indicated maturation and preparation of dehydration [64].

Also in cocoa, enzymes of the carbohydrate and energy metabolism were very prominent in torpedo stage somatic and zygotic embryos [65]. Interestingly, somatic embryos had a more active oxidative/respiration pathway while in zygotic embryos anaerobic fermentation might be the more important energy pathway. Again stress-induced proteins such as peroxidases, pathogenesis-related proteins, and glutathione S-transferase were more abundant in somatic embryos [65].

## 3 Role of the Endosperm

Somatic embryos lack an endosperm, which is not only a tissue that nourishes the developing embryo and the germinating seedling, but insulates the embryo from mechanical pressure and has important signaling function for embryo development, maturation and growth arrest, and finally germination timing [66]. Thus, for optimization of somatic embryogenesis a detailed look into the endosperm during seed development seems reasonable.

In order to develop optimal culture media for somatic embryo development in wheat, Carman et al. [67] analyzed minerals and primary metabolites of the endosperm during seed development. Maltose concentrations in the extracted kernel fluid increased between 6 and 18 days after pollination indicating that this product of starch hydrolysis is the major carbon source for the developing embryo. For the development of improved tissue culture media, the addition of free amino acids, the adjustment of phosphate and sulfur which were detected in relatively high concentrations in the kernel fluids probably because of their presence in phosphorylated sugars and amino acids, respectively, and the addition of maltose and short chain fructans were suggested [67]. Likewise in white spruce, somatic and zygotic embryos and the megagametophyte which is the haploid nourishing tissue of gymnosperms were analyzed with respect to their mineral contents [68]. The female gametophytes and zygotic embryos contained more phosphorus, potassium, magnesium, and zinc on a dry-weight basis than somatic embryos, whereas the female megagametophyte stood out due to

its high calcium content when compared to the embryo tissues [68]. However, if this information is going to be integrated into optimization of culture media, more data sets will be necessary for the mineral contents in different developmental phases, and also the forms in which the minerals are found in the respective tissue. Arabinogalactan proteins were identified in conditioned culture media of embryogenic cells by Kreuger and van Holst [69] and found to be essential for somatic embryo development [70]. Most interestingly, an endochitinase gene (EP3) which is involved in the generation of arabinogalactan proteins was expressed in carrot seeds by cells in the integuments and the protein localized in the endosperm and also in nonembryogenic cells of embryogenic cultures [70]. Also the formation of arabinogalactan proteins in the developing carrot seed was shown to be developmentally regulated [71]. In a review Matthys-Rochon [72] came to the conclusion that nonembryogenic cells within embryogenic cultures might take over some functions of the endosperm by secretion of signal molecules that control embryo development.

For *C. persicum* the proteomic analysis of the endosperm during seed development revealed a general shift from high molecular weight proteins to low molecular weight proteins and the accumulation of storage proteins (including "small" enolases) from 7 weeks after pollination when the endosperm is still liquid [73]. Furthermore proteins involved in synthesis of other storage compounds, namely lipids and xyloglucans were identified in the endosperm. Obviously, stress response including reactive oxygen species detoxification and ABA signaling also play a role in endosperm and embryo development [73].

## 4 Conclusions and Outlook

It can be concluded from the aforementioned literature that:

1. Somatic embryos are more exposed to stress than their zygotic counterparts,
2. Somatic embryos accumulate less storage compounds,
3. Somatic embryos do not undergo a proper maturation phase that would include a growth arrest but instead germinate precociously.

The role of stress which is on the one hand an important trigger of embryogenesis and, on the other hand, induces severe changes in the cellular metabolism; here especially the role of reactive oxygen species deserves further investigations. Obviously, particularly somatic embryogenesis is a process that only is successfully realized if the cells experience the right stress level at the right developmental time frame. Also programmed cell death which has

an impact in zygotic and somatic embryogenesis should be taken into consideration in coming research projects. The importance of the maturation phase for accumulation of storage reserves, and also for the clear distinction of differentiation and germination, has been noticed in many systems. Nevertheless, input is needed particularly to improve this phase of somatic embryogenesis in the future. At physical culture conditions, attention is not often paid, except at the oxygen concentration, for example in wheat embryogenesis [3, 74]. Here it has been shown that installing reduced $O_2$ levels, mimicking the situation found in seeds, improved growth and development of somatic embryos. However, the $O_2$ levels changed not only with time of development and spatially but also during the day due to photosynthesis [74]. Our own studies in cyclamen revealed hypoxic conditions in seeds at the position where the embryo is found about 5–6 weeks after pollination in unpublished measurements according to [75]. Thus, in vitro cultured somatic embryos which grow at ambient oxygen concentrations may establish too high or altered metabolic activity as indicated by some studies cited above (e.g. [64], [65]) and/or oxidation of plant growth regulators such as cytokinins, ABA, and indole acetic acid due to increased activity of oxidases as discussed by Carman and Bishop (2004) [74].

The "omics" tools (transcriptomics, proteomics, metabolomics…) will substantially improve in terms of sensitivity, resolution and identification, and affordable analyses of different genotypes over time and thereby enable us to gain deeper insights into plant embryogenesis and to optimize the in vitro protocols for somatic embryogenesis. Moreover, epigenetic regulation of embryogenesis by methylation/demethylation and histone modifications, post-transcriptional and posttranslational modifications should be studied in detail especially during the early phases. The role of specific micro RNAs as regulators of plant development including embryogenesis has to be elucidated, since Oh et al. [76] found differences in the abundance of five micro RNAs between somatic and zygotic embryos in loblolly pine. Although zygotic embryogenesis is more and more understood because of mutant analyses and molecular genetic studies of embryogenesis-related genes and both kinds of embryogenesis are studied in detail on a transcriptional and proteomic level, many aspects of the fascinating regeneration pathway of plant embryogenesis are still not explained. One interesting aspect for instance is the fact that somatic embryogenesis is highly dependent on the genotype, whereas zygotic embryogenesis is not. Especially for the recalcitrant genotypes improvements would be desirable by learning from seeds.

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