

Chapter 6

Experimental and Practical Application of Fern Somatic Embryogenesis



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Abbreviations

| | |
|--------------|--|
| ABA | Abscisic acid |
| ACC | Subunit acetyl-CoA carboxylase |
| ACS | Acetyl-coenzyme A synthetase |
| APX | L-ascorbate peroxidase |
| CKs | Cytokinins |
| DAHPS1 | Phospho-2-dehydro-3-deoxyheptonate aldolase 1 |
| DHQ synthase | 3-dehydroquininate synthase |
| GDH | Glutamate dehydrogenase |
| GST | Glutathione S-transferase |
| IAA | Indole-3-acetic acid |
| MED37 | The mediator of RNA polymerase II transcription subunit 37 |
| RGP1 | UDP-arabinose mutase |
| RHM1 | UDP-4-keto-L-rhamnose-reductase RHM1 |
| SE | Somatic embryogenesis |

6.1 Introduction

Somatic embryogenesis (SE) is a process by which bipolar structures called somatic embryos develop from non-zygotic cells. It was first described for carrot callus cells 60 years ago (Reinert 1958; Steward et al. 1958). By 2015, this unique developmental process had already been recognized for many spermatophytes, but for only two cryptogamic species, i.e. *Lycopodiella inundata* (L.) Holub and *Huperzia selago*

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(L.) Bernh. ex Schrank. (Atmane et al. 2000; Szypuła et al. 2005). In 2015, the first report of SE in a fern was published for *Cyathea delgadii* Sternb., a plant species belonging to Monilophyta, a clade that contains the closest living relatives of spermatophytes (Mikula et al. 2015b). Although the life cycle of ferns is completely different from that of spermatophytes, the ability to switch the developmental program of mature cells to that of somatic embryo formation seems to be universal and needs to be explained (Kennedy and Norman 2005). The switch in cell fate, both in seed plants and cryptogamic plants, is characterized by changes in phytohormone content and abundance of protein, by patterns of gene expression, as well as structural rearrangement of explant cells. Determining the changes by which somatic cells are able to reset a new developmental pathway is important if we are to understand the capacity of plant cells for asexual embryogenesis. Our recent work shows that the use of *C. delgadii* is useful in understanding somatic-to-embryogenic transition.

6.2 Story of Somatic Embryogenesis in the Tree Fern *Cyathea delgadii*

The sequence of events leading to termination of zygotic embryogenesis under in vitro culture conditions (Fig. 6.1) provides not only information about sexual reproduction in *C. delgadii* but is also a source of experimental material for SE. It is worth emphasizing that maintaining cultures in darkness inhibits the development of gametophytes, and the latter are prevented from reaching sexual maturity. Under 16/8 h photoperiod conditions, gametophytes achieve maturity within 6 months following spore sowing on 1/2 strength Murashige and Skoog (MS) medium supplemented with 1% sucrose. Details of sex organ formation in this species were described by Rybczyński and Mikula (2011). Following fertilization, early development of the zygotic embryo takes place inside an archegonium. Following rupture of the archegonial envelope by the first leaf (Fig. 6.1a), subsequent embryonic growth is rapid. Within 4–5 weeks, the main organographic regions are determined, and the leaf-root axis is visible (Fig. 6.1b). Subsequently, the first leaf elongates, and the development of the second follows shortly thereafter (Fig. 6.1c, d). The lamina of the first leaf, grown under photoperiod conditions, is entirely different from that of mature adult leaves (Fig. 6.1e, f). The first leaf shows dichotomous venation, whereas the second is more complex and pinnate (Fig. 6.1f). Zygotic embryo-derived sporophytes were readily induced on gametophytes growing on culture medium lacking ammonium nitrate (NH_4NO_3) and with the mineral salt concentration of the medium reduced to 1/8 MS (Fig. 6.1e).

These findings confirm that requirements for nutrients for zygotic embryo initiation in the tree fern *C. delgadii* are low. Indeed, starvation has previously been used for the initiation of fern sporophytes in *Pteris ensiformis* Burm. f., *Adiantum reniforme* var. *sinense* Y. X. Lin., and *Osmunda regalis* L. (Fernández et al. 1999; Wu et al. 2010; Makowski et al. 2016). Parts of very young zygotic embryos and zygotic

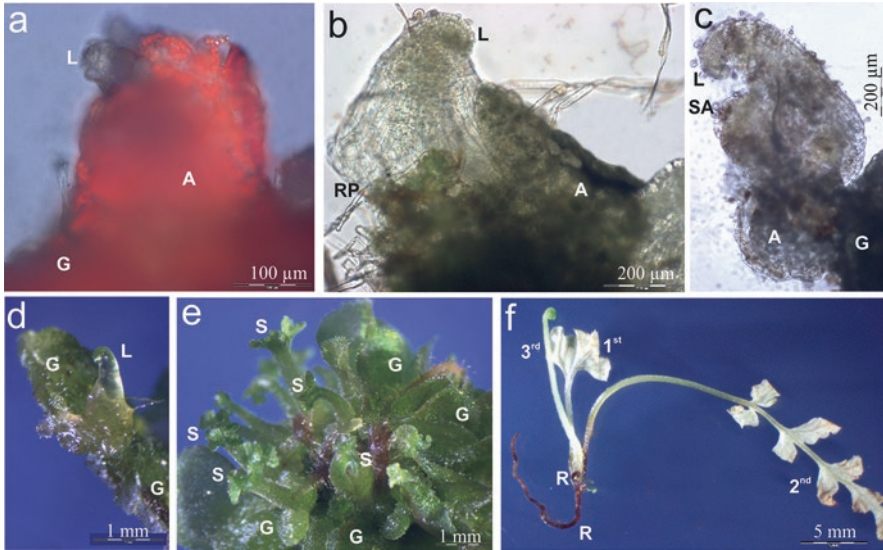


Fig. 6.1 Details of zygotic embryo development in *Cyathea delgadii* maintained in vitro at a 16 h/8 h photoperiod on 1/2 MS medium. (a) Archegonial envelope ruptured by the first emerging leaf of the zygotic embryo; red autofluorescence of chlorophyll was induced by exposure to blue-violet light (BV filter: 400–440 nm). (b) Well-developed zygotic embryo showing first leaf and a root primordium; thus, the leaf-root axis is visible. (c) The first crozier of a zygotic embryo. (d) An elongating leaf of a zygotic embryo. (e) Numerous zygotic embryo-derived sporophytes formed on 1/8 MS medium lacking ammonium nitrate (NH_4NO_3). (f) Zygotic embryo-derived sporophyte with first juvenile leaf, second pinnate leaf, and third young crozier. A archegonium, G gametophyte, L leaf, RP root primordium, R root, S sporophyte, SA shoot apex, 1st first leaf, 2nd second leaf, 3rd third leaf

embryo-derived sporophytes that were allowed to develop under reduced light conditions ($3.5 \mu\text{E m}^{-2} \text{s}^{-1}$) were capable of producing somatic embryos (19% efficient) (Mikuła et al. 2015b). Ultimately, the capacity of *C. delgadii* explants to undergo SE depends on the light conditions that prevail during the development of source plantlets. No embryogenic induction was observed in stipes excised from sporophytes cultured at a light intensity of $50 \mu\text{E m}^{-2} \text{s}^{-1}$ (Mikuła et al. 2015a). Long-term etiolation of somatic embryo-derived sporophytes has proved to be the main factor controlling induction of SE on hormone-free medium. Later studies revealed that the course of SE is dependent on the type of explant used for culture initiation. Stipe explant is important for inducing a single-celled source of somatic embryos (Fig. 6.2a–d) (Mikuła et al. 2015b), whereas an internodal explant is required to induce a multicellular SE pathway (Fig. 6.2e–g). When stipe explants were used for culture initiation, the first cell divisions typically commenced at 10 days of culture (Fig. 6.2a). During the next 4–6 days, the epidermal cells divided several times (Fig. 6.2b). Early (Fig. 6.2c) and late embryonic leaf stages (Fig. 6.2d), with numerous trichomes on the embryo surface, were clearly visible at about 21–28 days following culture initiation. Numerous divided cells were visible on the surface of internodal explants after 7 days of culture. These divisions led to the formation of

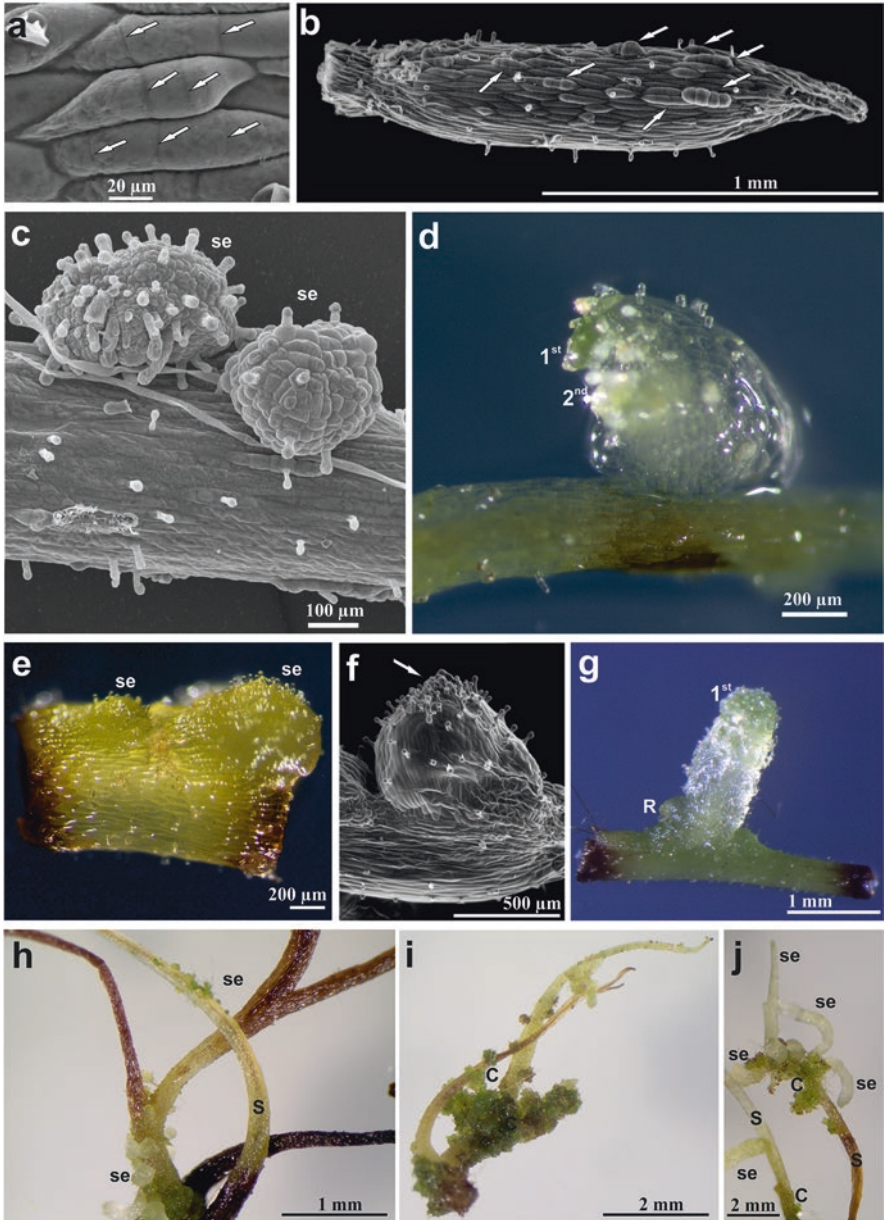


Fig. 6.2 Different methods of inducing somatic embryogenesis in *Cyathea delgadii*. (**a–d**) Direct unicellular pathway method induced on stipe explants. (**a**) First divisions (arrows) of epidermal cells of stipe explant; day 12 of culture. (**b**) Numerous proembryos at linear stage of development; day 14 of culture. (**c**) Somatic embryos at early (day 16 of culture) and (**d**) late embryonic leaf stages (day 21 of culture). (**e–g**) Direct multicellular pathway method induced on internodal explants. (**e**) Emerging nodular proembryos on internodal explant; day 13 of culture.

compact, nodular structures of multicellular origin (Fig. 6.2e), and these subsequently developed very quickly. Differentiation of the leaf primordium was observed at about day 16 of culture (Fig. 6.2f). Elongation of both embryo body and root then occurred (Fig. 6.2g). This method of somatic embryo formation in fern species is shown here for the first time. Sporophyte cultures maintained in darkness without any subculture for more than 5 months became an additional source of somatic embryos (Mikuła et al. 2015b). These embryos were spontaneously formed on various parts of donor plantlets by direct (Fig. 6.2h) or indirect SE (Fig. 6.2i, j). Both processes have been observed to occur simultaneously under the same tissue culture conditions. The course of SE in *C. delgadii*, commencing with spores followed by the culturing of gametophytes and formation of zygotic embryo-derived sporophytes, to the formation of somatic embryos and, ultimately, the derivation of mature plantlets from these, is summarized in Fig. 6.3.

Somatic embryo-derived sporophytes that have developed three leaves are the best source of initial explants. The process of SE from stipe explants of sporophytes (Fig. 6.3; in gray) continue in a cyclic manner, thus providing a continuous supply of embryogenic material. As an experimental system, it has certain advantages compared to those systems of SE described for the majority of spermatophytes. The system is induced (1) on hormone-free medium, (2) from a single cell of the stipe epidermis or from several cells of internodal segments, (3) during a short-time period, and (4) with very high multiplication and replication rate. Additionally, embryogenicity can be maintained for prolonged periods independent of a primary explant source. There are a few examples of species for which SE is driven by growth regulator-free medium (Raemakers et al. 1995). Although progress has undeniably been made in characterizing the underlying mechanisms of SE, thanks to the use of model plants such as *Arabidopsis* and *Daucus carota* (Raghavan 2006), the unique system that occurs in *C. delgadii* is extremely helpful in advancing this research.

6.3 Insight into Early Somatic Embryogenesis Using the *C. delgadii* Model System

The acquisition of embryogenic capacity occurs with greater or lesser ease depending on the plant species and the *in vitro* regeneration system employed. For most, the response of explant cells and the method of somatic embryo formation are



Fig. 6.2 (continued) (f) Further embryo development showing location of differentiation of leaf primordium (arrow) at the apex of the embryonic structure; day 16 of culture. (g) Elongation of embryo body and differentiation of both the first leaf and root of somatic embryo. (h–j) Direct or indirect pathway method in aging cultures of sporophytes. (h) Numerous somatic embryos induced directly from stipe cells of aging sporophyte. (i) Proliferation of embryogenic callus and (j) differentiation of somatic embryos. Images are based on scanning electron (a–c, f) and light (d–e, g–j) microscopy studies. *C* callus, *R* root, *S* sporophyte, *SE* somatic embryo, *1st* first leaf, *2nd* second leaf

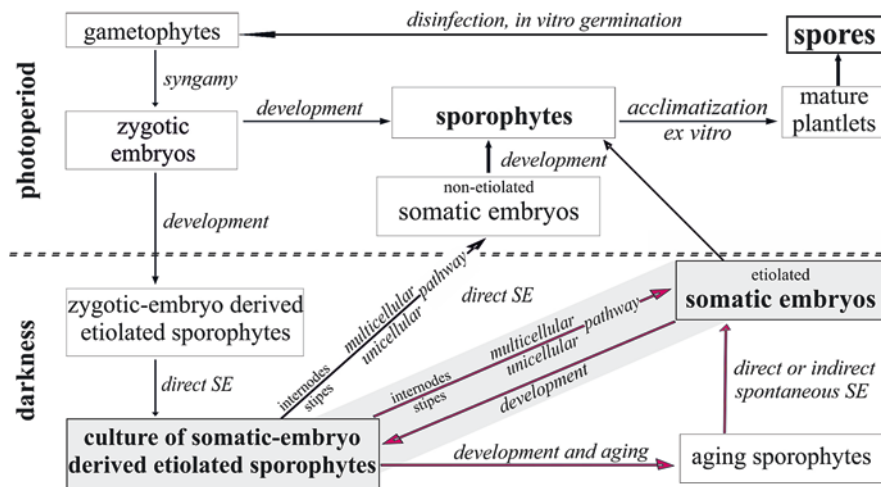


Fig. 6.3 Schematic representation of somatic embryogenesis pathways during micropropagation of *Cyathea delgadii*. Recurrent embryogenesis and continuous production of somatic embryos are shown in gray. This is achieved by maintaining the culture of somatic embryo-derived sporophytes in the dark. The method of somatic embryo formation is dependent on the explant type used for culture initiation. *SE* somatic embryogenesis

modified by the interaction of natural hormones and synthetic plant growth regulators (PGR) (Gaj 2004). Exogenously applied PGRs alter the synthesis, activation, transport, and destruction of endogenous hormones and regulate the morphogenetic response of explant cells (Gaspar et al. 1996). Conversely, SE can be induced in the absence of PGRs by using different stress treatments, such as osmotic stress, high or low temperature, or salinity (Nic-Can et al. 2016). Research on *C. delgadii* showed that the ability of explants to undergo somatic embryo production can also be controlled by the light conditions under which the donor plantlets are grown (Mikula et al. 2015a). This occurs by the regulation of both endogenous hormone contents and hormonal balance (Grzyb et al. 2017). A summary of hormonal changes resulting from the 5-month-long culture of donor plantlets in constant darkness and during early SE is shown in Fig. 6.4.

Etiolation reduces by almost 12-fold the concentration of the phytohormone abscisic acid (ABA), and it is this PGR that is considered to be the main factor involved in inhibiting the explant to undergo SE (Ivanova et al. 1994; Jiménez and Bangerth 2001). It also reduces the ABA/cytokinin (CK) and ABA/ indole-3-acetic acid (IAA) ratios by as much as 107-fold and 29-fold, respectively (Fig. 6.4). In the absence of photosynthesis, there is also a clear reduction in sugar content. However, light per se does not appear to be associated with the acquisition of embryogenic totipotency by cells of etiolated explants since both dark- and light-cultured explants are highly efficient in producing somatic embryos (Mikula et al. 2015a). However, light is nevertheless one of the most important external factors in that it affects both the type of morphogenetic response and also fern sporophyte development. In *C.*

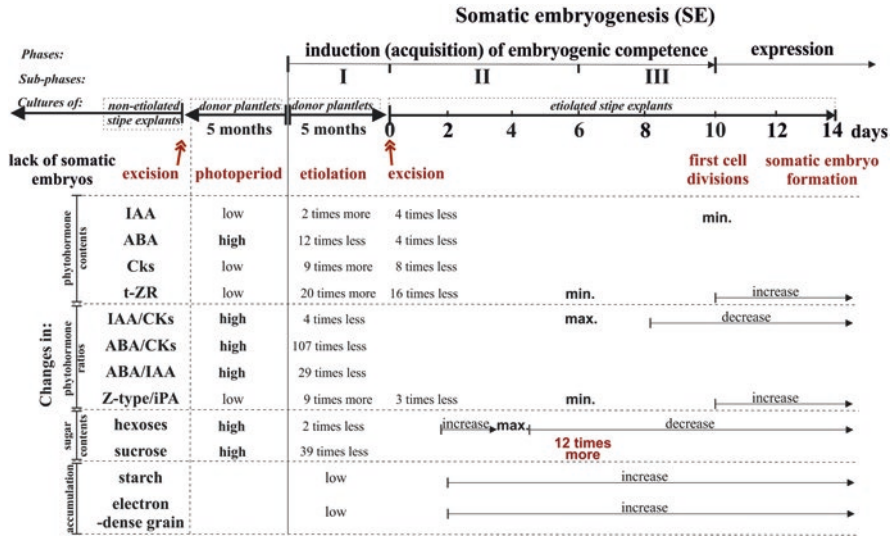


Fig. 6.4 Physiological events leading to the formation of single-cell origin of somatic embryos in the tree fern *Cyathea delgadii*

delgadii, stipe explants cultured under photoperiod conditions are able to form both somatic embryos and gametophytes next to each other (Mikuła et al. 2015a).

Although there has been considerable progress in the identification of genes implicated in SE, our understanding of the mechanisms by which stresses influence the acquisition of embryogenic capacity still remains unclear (Elhiti et al. 2013). In *C. delgadii*, the events that lead to single-cell somatic embryogenesis have been analyzed at the level of hormonal and cytomorphological responses. These results enable one to speculate on how SE started (Table 6.1). It is proposed that the excision of a stipe explant from an etiolated donor plantlet of *C. delgadii* leads to the loss of the resources required for hormone biosynthesis, resulting in a dramatic reduction in the concentration of all phytohormones, yet the hormonal balance remains almost unchanged (Fig. 6.4; Grzyb et al. 2017). The leakage of cytoplasm from mechanically disrupted cells probably results in a fall in turgor pressure (Nonami and Schulze 1989). One of the earliest responses to excision is the production of reactive oxygen species (ROS) that promote redifferentiation (Rose et al. 2010). Studies in cotton have indicated the involvement of ROS homeostasis in the initiation of SE by modifying auxin signaling (Zhou et al. 2016). Alternatively, a shortage of water activates the osmoregulation process (Pelleschi et al. 1997) that functions in accordance with acid growth theory (Wang and Ruan 2013). As a result, fructose and glucose accumulate over the 4 days of *C. delgadii* stipe explant culture (Grzyb et al. 2017). The influx of glucose and fructose into the cells, followed by an almost 12-fold increase in sucrose concentration, may represent a short-term osmotic signal required for the somatic-to-embryogenic transition. Associations between acid invertase activity, sucrose metabolism, and the cell water status, as

Table 6.1 Proposed and described physiological changes preceding the formation of somatic embryos in *Cyathea delgadii*

| Exogenous treatments | Proposed changes | Described changes |
|--|--|--|
| 1. Long-term darkness treatment of donor plantlets | Etiolation | Changes in the content of phytohormones Establishment of a new hormonal balance that is suitable for the induction of SE |
| 2. Explant excision | Loss of endogenous hormone sources Leakage of cytoplasm Reduction in turgor pressure ^a Reactive oxygen species (ROS) generation ^b | Reduction in the content of IAA, ABA, and cytokinins without disturbing the hormonal balance |
| 3. Explant culture | Increase in acid invertase activity ^{c,d} | Accumulation of fructose and glucose |
| | Increase in sucrose phosphate synthase activity ^{c,d} | Accumulation of sucrose |
| | Activation of the osmoregulation process ^{c,d} | Accumulation of starch in amyloplasts Reduction in sucrose content Accumulation of intravacuolar electron-dense grains |

^aNonami and Schulze (1989)

^bRose et al. (2010)

^cWang and Ruan (2013)

^dPelleschi et al. (1997)

shown for carrot suspension cultures (Ikeda et al. 1999), support our hypothesis. Sucrose also appears to be involved in the morphogenetic response of juvenile leaves of the fern *Microgramma vacciniifolia* (Langsd. & Fisch.) Copel. (Hirsch 1976). In the presence of low level of sucrose (1%) in the induction medium, both embryo-like structures and gametophytes were produced. On the higher sucrose levels (3–4%), the development of aposporous gametophytes was inhibited and the sporophytes developed on a large number of excised leaves.

The significance of auxin as the main controlling factor in SE induction was recognized by Fehér (2015). The role of endogenous cytokinins, however, is still poorly understood. Using the *C. delgadii* model system, it has been shown that establishing an equilibrium between endogenous IAA and cytokinin content is essential both for the acquisition of embryogenic competence and the expression phase of SE (Grzyb et al. 2017). The embryogenic process may be completely arrested by disrupting this balance (Grzyb et al. 2018). A thorough examination of the effect of transport and hormone biosynthesis inhibitors on SE in *C. delgadii* showed that the role of phytohormones in somatic embryo induction is a highly complex process. Under the influence of different inhibitor substances (such as the auxin polar transport inhibitor 2, 3, 5-triiodobenzoic acid, the ABA biosynthe-

sis inhibitor fluridone, or ethylene biosynthesis inhibitor salicylic acid), the concentrations of both indole-3-acetic acid (IAA) and cytokinins became strongly modified. Moreover, imbalances in phytohormone levels are responsible for the modification of soluble sugar concentrations, including that of sucrose – the main factor for triggering embryogenesis in *C. delgadii*. A survey of the literature has shown that a synthetic auxin is initially required to induce SE, but that its withdrawal drives embryo formation (Halperin 1966). Similar relationships were also found for changes in phytohormone concentrations in the *C. delgadii* model system. More endogenous auxin than cytokinin was specifically related to the acquisition of embryogenic competence, whereas cytokinins favored SE expression during which frequent cell divisions occur resulting in the formation and appearance of somatic embryos (Grzyb et al. 2017). Of the different cytokinins, the trans-zeatin riboside was clearly associated with this phase of SE (Fig. 6.4). This agrees with results obtained by Centeno et al. (1997), who observed an increase in the level of Z-type cytokinin during the maturation of *Corylus avellana* L. zygotic embryos.

With reference to cytomorphological studies, only two differences were found to occur between initial explants and those cultured on hormone-free 1/2 MS medium for about 16 days (Domžalska et al. 2017). These were a massive accumulation of starch grains in amyloplasts and an accumulation of electron-dense intravacuolar granules, both of which appear to be necessary for the early SE of *C. delgadii*. Increased levels of starch have been reported at the onset of several in vitro development processes, including the induction of SE (Ho and Vasil 1983; Stamp 1987; Barciela and Vieitez 1993; Canhoto et al. 1996; Pinto et al. 2010). By contrast, a dramatic reduction in the amount of starch has been documented at the commencement of SE and organogenesis (Martin et al. 2000; Fortes and Pais 2000; Puigderrajols 2001; Mikuła et al. 2004). Starch is considered a primary source of energy and carbon for cell proliferation during plant morphogenetic events (Martin et al. 2000). Research conducted on *C. delgadii* suggests that the starch accumulation in early SE may play a role in the osmoregulatory response to a sudden increase in endogenous sucrose level. The importance of starch was emphasized by Fortes and Pais (2000) for *Humulus lupulus* var. Nugget. By means of the cyclic process of starch accumulation/mobilization, they demonstrated the potential role of this carbohydrate in the initiation of organs and later in their development. The accumulation of starch in *C. delgadii* stipe explants was reflected in the proteomic analysis. Comparative analysis performed by Domžalska et al. (2017) showed that many proteins were involved in carbohydrate metabolism (33%). The results of this study are in accordance with the data obtained for *Picea glauca*, *Zea mays*, and *Medicago truncatula* (Lippert et al. 2005; Almeida et al. 2012; Varhaníková et al. 2014). Changes in this protein category, represented by enolase and 6-phosphogluconate dehydrogenase, and decarboxylation of 6PGDH, can be directly related to starch accumulation.

6.4 Molecular Aspects of Fern Somatic Embryogenesis

Acquisition of embryogenic capacity and the process of somatic embryo differentiation are a consequence of physiological changes induced by stress treatment (Fehér 2015; Grzyb et al. 2017). It promotes changes in molecular regulation and the proteomic response, especially in proteins that play integral roles in hormone perception and signaling (Elhiti et al. 2013). Evaluation of proteomic changes allows the characterization of different events that occur during SE, and these can be used as markers for monitoring the process (Rosas et al. 2016). In *C. delgadii*, the protein expression patterns associated with early SE were investigated using two-dimensional gel electrophoresis (2-DE) and mass spectrometry (Domžalska et al. 2017). The study revealed the presence of several proteins that are triggered by induction of SE and the embryo-forming process. Of the 114 differentially regulated proteins identified in *C. delgadii* stipe explants at day 16 of culture, it is thought that 16 and 20 are markers of the induction and expression phase of SE, respectively.

Proteins associated with the induction phase play an essential role in the protection of plant cells against oxidative stress (chalcone synthase, chaperone protein ClpB3, HSP70, chaperonin GroEL, MED37), in the interruption of cell communication (RHM1, RGP1, ACC, ACS), and in cellular reprogramming (GST, APX, DAHPS1, DHQ synthase, GDH). These proteins have been found to be associated with the early SE of many seed plants (Moriguchi et al. 1999; Tchorbadjieva 2005; Almeida et al. 2012; Gallego et al. 2014; Heringer et al. 2015; Zhou et al. 2016). Moreover, the activities of two enzymes involved in malate metabolism, i.e., malate dehydrogenase (MDH) and NADP-dependent malic enzyme (NADP-ME), increased more than fourfold. According to Crecelius et al. (2003), malate serves not only as an additional carbon sink but may also function as a vacuolar osmolyte balancing increased concentrations of soluble sugars.

Nine of the proteins characterized for the expression phase of SE in *C. delgadii* are involved in the conversion of sugars and the production of metabolically usable energy. Next, 11 proteins are associated with the high growth rate of embryogenic cells, protein synthesis, and degradation. It is worth noting that enolase is thought to be a molecular marker of late embryogenesis in spermatophytes (Andriotis et al. 2010; Tonietto et al. 2012; Vale et al. 2014), whereas in the tree fern *C. delgadii*, it accumulates during early SE (Domžalska et al. 2017). The study provided a list of early SE-related proteins that are potential biomarker candidates for future investigations of somatic embryogenic development in this species of tree fern.

6.5 Application of Somatic Embryogenesis for the Propagation of Ferns

Ferns (around 12,000 species) are a very ancient group of plants that are distributed worldwide, from Greenland to the Antarctic and from sea level to high altitudes. Of these, tree ferns are the second most diverse group with 600–660 species,

distributed mainly throughout the tropics (Large and Braggins 2004). Most fern species are habitat- and niche- specific because of their sensitivity to wet or cold conditions. In recent years, ferns have become increasingly popular in horticulture. Many fern species are grown for their ornamental value, both for indoor and garden culture (Fernández and Revilla 2003). Ferns are used for food (Liu et al. 2012), medicine (Ho et al. 2011), and phytoremediation (Rathinasabapathi 2011). Tree ferns are also used for the production of handicrafts (Eleutério and Pérez-Salicrup 2006). Edible ferns are some of the most common wild food plants collected by people around the world (Liu et al. 2012). Habitat destruction and fragmentation, deforestation, and overexploitation of fern fronds, stipes, rhizomes/trunks, and scales are the main factors related to the reduction in fern populations. Nearly 70% of fern species are threatened with extinction (Barnicoat et al. 2011).

The conventional methods of fern propagation by spores or vegetative propagules (such as bulbils, aerial growths, stolons, tubers, offsets, stipules, and root buds) are time-consuming procedures that are comparatively slow and inefficient. Furthermore, most tree ferns cannot be multiplied vegetatively, as they do not produce offsets from their trunk or roots (Large and Braggins 2004). Therefore, *in vitro* propagation and collecting systems have been developed for many ferns (Fernández and Revilla 2003; Rybczyński and Mikula 2011; Bharati et al. 2013). These systems are primarily based on the vegetative reproduction of gametophytes from which the formation of sporophytes occurs through sexual (Rybczyński and Mikula 2011; Makowski et al. 2016) or asexual mechanisms (Kuriyama et al. 2004; Camloh and Ambrožič-Dolinšek 2011; Cordle et al. 2011). During *in vitro* culture, the time required for the development of the sporophyte from spore varies from 1 (*Woodwardia virginica*) to 8 months (*Asplenium nidus*) depending on the species of fern and the length of its life cycle (Fernández et al. 1999). Favorable conditions for *in vitro* culture can both increase the number of plants produced and shorten the time required for sexual sporophyte production to occur, as documented for *O. regalis* (Makowski et al. 2016), *Cyathea cooperi*, *C. cunninghamii*, and *Dicksonia antarctica* (Moura et al. 2012). However, this method of sporophyte production can also result in failure, as was shown for *Platyserium andinum* and *P. wandae* (Pérez-García et al. 2010). Other effective *in vitro* propagation methods are based on the culture of homogenized gametophytic (Fernández et al. 1999) and sporophytic tissue (Teng and Teng 1997; Somer et al. 2010; Camloh and Ambrožič-Dolinšek 2011). Homogenization of gametophytes can be considered an excellent method for the propagation of fern species, one that is currently not available for the *in vitro* culture of seed plants. This system is most effective for species with a short life cycle, such as *Woodwardia virginica* and *Dryopteris affinis* sp. *affinis*, but not for *O. regalis* (Fernández et al. 1999). Numerous reports also document the propagation of sporophytes by apogamy, the use of globular green bodies, and direct or indirect shoot organogenesis (Fernández and Revilla 2003; Camloh and Ambrožič-Dolinšek 2011). However, although these systems work successfully for herbaceous ferns, our knowledge of the *in vitro* propagation of tree ferns is still in its infancy. Researchers working on this group of ferns have mainly focused on spore germination and gametophyte development (Huang et al. 2001; Hiendlmayer and Randi 2007; Du et al. 2009; Rechenmacher et al. 2010; Moura et al. 2012; Das et al. 2013;

Vargas and Droste 2014). Progress in the production of tree fern sporophytes was made by Goller and Rybczyński (2007) and Rybczyński and Mikula (2011). In their studies, 12 of 16 tree fern species reached sporophyte stage and were successfully acclimatized to ex vitro conditions (Goller and Rybczyński 2007). Recently, Yu et al. (2017) reported the successful induction of globular green bodies in *Cyathea barometz* (L.) J.Sm. by culturing juvenile sporophytes in the presence of thidiazuron (TDZ) and α -naphthaleneacetic acid (NAA). This is the first species of tree fern for which this rapid regeneration pathway has been described. Another quite efficient system of sporophyte production has also been shown for the tree fern *Cyathea spinulosa* Wall. ex Hook (Shukla and Khare 2013). Here, media supplemented with plant growth regulators promoted indirect shoot production, each having as many as 12.5 shoots per leaf primordium callus.

The excellent multiplication system by direct somatic embryogenesis recently developed for *C. delgadii* (Mikula et al. 2015a, b) contrasts markedly with the low numbers of sporophytes obtained by the methods described above. To date, it is the fastest and most economically efficient system for the in vitro propagation of ferns. Following 2 months of culture, each responding explant measuring 2.5 mm in length produced as many as 42 somatic embryos (Mikula et al. 2015a). The efficiency of this process can be improved upon even further by the spontaneous formation of somatic embryos in aging cultures (Mikula et al. 2015b). This system allows more rapid propagation and may be extended to other fern species, in particular tree ferns. Large-scale propagation of *C. delgadii* plants is one practical application of the process described here, and this may prove beneficial for the conservation of threatened ferns, as well as for the commercial production of ornamental fern species.

6.6 Future Prospects

The tree fern *C. delgadii* is a good experimental model for studying SE at both basic and applied levels. It is still not clear, however, how embryogenic cells differentiate inside explants and what mechanisms control this process. Somatic embryogenesis of *C. delgadii* offers a model system for understanding the physiological and biochemical events that occur during the somatic-to-embryogenic transition. Moreover, the superficial and unicellular origin of somatic embryos makes this process easy to observe in terms of changes to intercellular communication. Symplasmic isolation between neighboring cells is considered an important factor in determining cell fate.

In recent years, substantial progress has been made in the field of SE in seed plants and in various aspects of this process. However, *C. delgadii* is the first fern species for which this process has been described. Consequently, our studies open the door for comparing developmental biology, especially with respect to embryogenesis, in spermatophytes and cryptogamic plants.

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