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Abstract

Plants are sessile organisms and, as such, have evolved a remarkable developmental plasticity allowing them to cope with the adverse effects of numerous biotic and abiotic factors from the environment. One of the most intriguing examples of this plasticity is somatic embryogenesis during which somatic cells dedifferentiate into cells that are capable to form embryos. The latter are morphologically similar to zygotic embryos and can regenerate whole plants. The transition of somatic cells into embryogenic ones is the most intriguing and the part of somatic embryogenesis least understood. To better understand the mechanisms of somatic embryogenesis, comparative proteomic approaches have been used, and in recent years, hundreds of proteins have been identified in embryogenic and nonembryogenic cultures, in somatic and zygotic embryos, and during distinct stages of somatic embryogenesis in both angiosperms and gymnosperms.

This review provides a comprehensive analysis of current advances in the proteomics of SE, focusing on the presence and putative function of differentially expressed proteins involved in processes such as stress response, protein synthesis and processing, cell proliferation, signal transduction and energy metabolism, and their potential use as embryogenic markers.

Keywords

Differentially expressed proteins • Embryogenic and non-embryogenic callus • Proteomics • PR protein • Heat shock protein • Actin • Annexin • Signal transduction • Storage protein • Secretome • Programmed cell death • Up-regulation of genes • Somatic embryogenesis

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5.1 Introduction

Somatic embryogenesis (SE) represents a unique phenomenon in the plant kingdom. This developmental pathway is one of the most striking examples of the plant cell developmental plasticity (Fehér 2008; Fehér 2014). It includes a series of characteristic events such as dedifferentiation of somatic cells, activation of cell division, and reprogramming of their metabolism and gene expression patterns. The transition of somatic cells into embryogenic ones is the most intriguing and the part of somatic embryogenesis least understood (Fehér 2005; Karami et al. 2009; Elhiti et al. 2013). It is still not known why and how somatic cells regain totipotency and embryonic cell fate giving rise to somatic embryos that are morphologically similar to zygotic embryos (Fehér 2014). More than two decades ago, SE was proposed to be a developmental stress response (Dudits et al. 1991), and now it is widely accepted that stress and hormones play a crucial role in collectively inducing cell dedifferentiation and initiation of embryogenic program in plants with responsive genotype (Fehér et al. 2003; Ikeda-Iwai et al. 2003; Rose and Nolan 2006). Still, the underlying mechanisms are hardly known justifying the listing of the question “How does a single somatic cell become a whole plant?” among the current 125 most important scientific questions (Vogel 2005).

Efforts have been made to elucidate the patterns of gene expression that may play a critical role in the process of SE and especially in the somatic-to-embryogenic developmental transition. Several gene classes associated with SE including SERK, LEAFY COTYLEDON, BABY BOOM, WUSCHEL, and PICKLE have been identified (reviewed by Karami et al. 2009; Yang and Zhang 2010; Fehér 2014). However, the lack of a clear correlation between mRNA and protein abundance due to the variation in mRNA stability, translatability, and protein stability sets some limits on mRNA expression profiling. Furthermore, protein structure, activity, and function can be altered and regulated by subcellular localization, interaction with other molecules, and posttranslational modifications that would not be detected by mRNA analysis (Rose et al. 2004). Consequently, there is a growing rec-

ognition that this approach should be complemented with profiling methods of the final gene products or proteins themselves.

Proteomics is a powerful approach aimed at systematic studies of protein structure, function, interaction, and dynamics. Protein changes occurring as a response to developmental and environmental states, abundances of proteins, posttranslational modifications, and protein isoforms can also be investigated (Valledor and Jorrián 2011). Within the last decade, improvements in the high-resolution two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) technique have allowed the identification of proteins linked to SE competence and development in diverse plant species such as *Manihot esculenta*, *Catharanthus roseus*, *Phoenix dactylifera*, *Cyclamen persicum* (reviewed by Aslam et al. 2013), *Quercus suber* (Gomez-Garay et al. 2013), *Vitis vinifera* (Marsoni et al. 2008; Zhang et al. 2009), *Vigna unguiculata* (Nogueira et al. 2007), *Acca sellowiana* (Cangahuala-Inocente et al. 2009), *Citrus sinensis* (Pan et al. 2009), *Coffea arabica* (Tonietto et al. 2012), *Crocus sativus* (Sharifi et al. 2012), *Araucaria angustifolia* (Jo et al. 2014), *Elaeis guineensis* (Silva Rde et al. 2014), *Medicago truncatula* (de Jong et al. 2007; Almeida et al. 2012), *Zea mays* (Sun et al. 2013; Varhaníková et al. 2014), and *Pinus pinaster* (Morel et al. 2014). These reports included studies on protein expression changes during the transitional state from somatic to embryogenic cells, identified differentially expressed proteins in nonembryogenic and embryogenic calli and in somatic and zygotic embryos, compared specific proteins that accumulate during different stages of somatic embryogenesis, and discovered putative protein markers for somatic embryogenesis.

5.2 Proteomics of Somatic Embryogenesis: A Ten-Year Survey (2005–2015)

Transcriptomics and proteomics approaches have been applied to investigate global gene expression during somatic embryo formation in several plant species, including angiosperms and gymnosperms (see Table 5.1). SE can be divided into

Table 5.1 Overview of plant proteomics studies of somatic embryogenesis (2005–2015)

Plant species	Common name	Source	Proteomics techniques	References
<i>Acca sellowiana</i>	Feijoa	Somatic embryo development	2-DE/MALDI-TOF MS	Cangahuala-Inocente et al. (2009)
<i>Acca sellowiana</i> (<i>O. Berg.</i>) Burret	Feijoa	Somatic embryo regeneration	2D DIGE/Q-TOF MS/MS	Fraga et al. (2013)
<i>Araucaria angustifolia</i> (<i>Bert.</i>) <i>O. Ktze</i>	Brazilian pine	Embryogenic vs. nonembryogenic cell line	2-DE/MALDI-TOF/TOF MS/MS	Jo et al. (2014)
<i>Carica papaya L.</i>	Papaya	Somatic embryo development	2-DE/MALDI-TOF/TOF MS/MS	Vale Ede et al. (2014)
<i>Citrus sinensis Osbeck</i>	Valencia sweet orange	Somatic embryo development	2-DE/MALDI-TOF MS	Pan et al. (2009)
<i>Coffea arabica</i>	Coffee	Somatic embryo development	2-DE/MALDI-TOF/TOF MS/MS	Tonietto et al. (2012)
<i>Crocus sativus L.</i>	Saffron	Embryogenic vs. nonembryogenic calli	2-DE/MALDI-TOF/TOF MS/MS	Sharifi et al. (2012)
<i>Cyclamen persicum</i>	The Persian cyclamen	Embryogenic vs. nonembryogenic cell lines	2D DIGE/ MALDI-TOF MS	Lyngved et al. (2008)
<i>Cyclamen persicum</i>	The Persian cyclamen	Somatic vs. zygotic embryos	2-DE/nano-LC-MS/MS	Winkelmann et al. (2006)
<i>Cyclamen persicum</i>	The Persian cyclamen	Somatic vs. zygotic embryos	2-DE/LC-MS/MS	Rode et al. (2011)
<i>Cyclamen persicum</i>	The Persian cyclamen	Somatic embryo development and maturation	2-DE/LC-MS/MS	Rode et al. (2012)
<i>Cyclamen persicum</i>	The Persian cyclamen	Somatic embryo development and nonembryogenic callus	2-DE/MALDI-TOF/TOF MS/MS	Bian et al. (2010)
<i>Cyphomandra betacea</i>	Tamarillo	Embryogenic vs. nonembryogenic calli	2-DE/LC-MS/MS	Correia et al. (2012)
<i>Elaeis guineensis Jacq.</i>	Oil palm	Somatic embryo development	2-DE/MALDI-TOF/TOF MS/MS	Silva Rde et al. (2014)
<i>Eruca sativa Mill</i>	Rucola	Embryogenic vs. nonembryogenic calli	1-DE/MALDI-TOF MS	Chen et al. (2012)
<i>Gossypium hirsutum L.</i>	Cotton	Somatic embryo development	iTRAQ/LC-ESI-MS/MS	Ge et al. (2015)
<i>Larix principis-rupprechtii Mayr</i>	Prince Rupprecht's larch	Embryogenic vs. nonembryogenic calli	iTRAQ/LC-MS/MS	Zhao et al. (2015)
<i>Larix × eurolepis</i>	Hybrid larch	Somatic vs. zygotic embryo maturation	1-DE/LC-MS/MS	Teyssier et al. (2014)
			2-DE/LC-MS/MS	
<i>Manihot esculenta Crantz</i>	Cassava	Secondary somatic embryogenesis	2-DE/MALDI-TOF/TOF MS/MS	Baba et al. (2008)
<i>Manihot esculenta Crantz</i>	Cassava	Somatic embryos, plantlets, and roots	1-DE/LC-ESI-MS/MS	Li et al. (2010)
<i>Medicago truncatula</i>	Barrel clover	Embryogenic (2HA) vs. nonembryogenic cell lines (Jemalong)	2-DE/MALDI-TOF MS/MS	Imin et al. (2005)
<i>Medicago truncatula</i>	Barrel clover	Embryogenic (M9-10A) vs. nonembryogenic (M9) cell lines	2-DE/MALDI-TOF/TOF MS/MS	Almeida et al. (2012)
<i>Persea americana</i>	Avocado	Embryogenic vs. nonembryogenic cell lines	2-D DIGE/MALDI-TOF/TOF MS/MS	Guzmán-García et al. (2013)
<i>Phoenix dactylifera L.</i>	Date palm	Somatic vs. zygotic embryos	2-DE/MALDI-TOF/TOF MS/MS	Sghaier-Hammami et al. (2009)

(continued)

Table 5.1 (continued)

Plant species	Common name	Source	Proteomics techniques	References
<i>Picea balfouriana</i>	Balfour spruce	Somatic embryo development	iTRAQ/LC-MS/MS	Li et al. (2015)
<i>Picea glauca</i>	White spruce	Somatic embryo maturation	2-DE/LC-MS/MS	Lippert et al. (2005)
<i>Pinus pinaster</i> Ait.	Maritime pine	Somatic vs. zygotic embryo maturation	1-DE/LC-MS/MS	Morel et al. (2014)
			2-DE/LC-MS/MS	
<i>Quercus suber</i>	Cork oak	Somatic embryo development	2-D DIGE/MALDI-TOF/TOF MS/MS	Gomez-Garay et al. (2013)
<i>Theobroma cacao</i> L.	Cacao	Somatic vs. zygotic embryos	2-DE/LC-MS/MS	Noah et al. (2013)
<i>Vanilla planifolia</i> Andrews	Vanilla orchid	Callus regeneration	2-DE/MALDI-TOF/TOF MS/MS	Tan et al. (2013)
<i>Vigna unguiculata</i>	Cowpea	Embryogenic cell suspensions	2-DE/MALDI-TOF/TOF MS/MS	Nogueira et al. (2007)
<i>Vitis vinifera</i> cv. <i>Thompson seedless</i>	Grape vine	Embryogenic vs. nonembryogenic calli	2-DE/LC-ESI-MS/MS	Marsoni et al. (2008)
<i>Vitis vinifera</i> L. cv. <i>Cabernet sauvignon</i>	Grape vine	Embryogenic vs. nonembryogenic calli	2-DE/MALDI-TOF/TOF MS/MS	Zhang et al. (2009)
<i>Zea mays</i> L. <i>H99 inbred cell line</i>	Maize	Embryogenic vs. nonembryogenic calli	2-DE/MALDI-TOF MS	Sun et al. (2013)
<i>Zea mays</i> L. <i>A19 inbred cell line</i>	Maize	Embryogenic vs. nonembryogenic calli	2-DE/Q-TOF MS/MS	Varhaniková et al. (2014)

two phases: induction and expression. During the induction phase, somatic cells are undifferentiated, acquire embryogenic competence, and proliferate as embryogenic formations. Cellular reprogramming takes place which is associated with the expression of specific genes. In the expression phase, the embryogenic cells differentiate to form somatic embryos and after that plants. Somatic embryo development encompasses key stages of ZE: the heart and torpedo stages in the case of dicotyledonous species; the globular, scutellar, and coleoptilar stages in the case of monocotyledonous species; and early and late embryogenesis in the case of gymnosperm species (Rocha and Dornelas 2013). Proteomic studies related to SE have focused mainly on (1) early stages of embryogenesis, when the embryogenic competence is acquired; (2) comparison of embryogenic and nonembryogenic calli; and (3) comparison of zygotic and somatic embryos and have revealed a plethora of differentially expressed proteins.

However, the results obtained from these studies are not entirely identical, perhaps due to the different plant species, organs, methods, and con-

ditions used. And still, numerous proteins involved in fundamental cellular processes of SE were found to be identical in the different plant species. The biological significance of key differentially expressed proteins and their associated cellular roles is discussed in this section, stressing on the perspective of their SE or in vitro morphogenesis relevance. At a functional level, proteins identified in this study were derived from a broad variety of cellular processes, which emphasize the range of changes associated with embryo development. Suggested protein markers for SE offer the possibility of determining the embryogenic potential of plant cells in culture long before any morphological changes have taken place and of gaining further information on the molecular basis of induction and differentiation of plant cells (Tchorbadjieva 2005).

The proteins were classified into the following functional categories based on their primary biological process: (1) stress response; (2) protein synthesis, processing, and fate; (3) cell proliferation, cell wall biogenesis, and cell elongation; (4) transcription and signal transduction; (5) metabolism and energy state; and (6) storage proteins.

5.2.1 Proteins Involved in Stress/Defense Response

5.2.1.1 Proteins Involved in Oxidative Stress Response

Recent proteomic studies have strongly emphasized the role of stress proteins during SE (Takáč et al. 2011).

The oxidative stress in plant tissue culture is well documented (Zavattieri et al. 2010). A burst in ROS is indispensable in modulating cell division and the reprogramming of cell metabolism as an adaptation response to stress. The initiation of embryogenic cultures (EC) often involves wounding of anthers, leaf explants, hypocotyls, etc. and placing them on callus induction medium. The latter contains the synthetic auxin 2,4-D, known by its herbicide effect triggering considerable oxidative stress in plant cells. ROS has also been implicated as a second messenger during auxin and stress-induced embryogenesis (Maraschin et al. 2005). Uncontrolled production of ROS could severely damage cellular proteins and membranes. Therefore, plant cells regulate ROS levels through sophisticated mechanisms such as scavenging with antioxidant defense proteins. Peroxidases, catalases, and superoxide dismutases (SOD) function as key players in the modulation of ROS levels and ROS-mediated stress signaling (Apel and Hirt 2004). Thus, it is not surprising that enzymes involved in detoxification have been detected in all studies regarding the early stages of somatic embryogenesis.

Ascorbate peroxidase (APX) plays a critical role in oxidative stress response through reactive oxygen species (ROS) scavenging. APX isozymes differ with respect to structure, substrate specificity, and tissue distribution, and their expression is highly responsive to environmental conditions and stress. A differential accumulation of APX has been reported in *Vitis vinifera* embryogenic (EC) and embryogenic calli (NEC) – two acidic isoforms were detected in EC only, while two basic isoforms were specific to NEC (Marsoni et al. 2008; Zhang et al. 2009). The proteomic profiles of the embryogenic phases of *Elaeis guineensis* revealed an APX present in all stages of somatic embryo develop-

ment and a peroxidase present in primary callus and a putative secretory peroxidase unique to proembryogenic callus (Silva Rde et al. 2014). APX was found more abundant in the EC of two inbred lines of *Zea mays* L. – A19 (Varhaníková et al. 2014) and H99 (Sun et al. 2013) – as well as in callus of saffron (Sharifi et al. 2012). This might suggest that maintaining low levels of hydrogen peroxide is important for cell reprogramming towards SE. An increased APX was detected in mature embryos of cork oak as compared to proliferating embryos, with a slight decrease in intermediate stages as compared to the initial stage (Gomez-Garay et al. 2013).

In *Cyclamen persicum*, catalase was upregulated in embryogenic suspensions (ES) (Lyngved et al. 2008). Rode et al. (2012) found high levels of catalases of which the more acidic forms were specific for callus and early stages of SE. In contrast, the more basic forms had a higher abundance in the late embryos. These catalases are therefore candidates for stage-specific isoforms, probably affected by different phosphorylation levels. In mature embryos of oak, catalase and APX with diminished levels in proliferating embryos and increased abundance in the mature ones were detected (Gomez-Garay et al. 2013).

Glutathione-S-transferase (GST) proteins are involved in several processes, including protection from oxidative stress and detoxification of xenobiotics (Dixon et al. 2002). Winkelmann et al. (2006) found that GST is accumulated in embryogenic tissue and somatic embryos and may also have a possible role in detoxifying excessive amounts of auxin (Fehér et al. 2003). In *Citrus*, three different isoforms of GST with differential expression were detected – one was upregulated in late stages of SE, while the other two were only transiently downregulated in the first weeks after callus induction (Pan et al. 2009). Several examples have been reported in which members of the GST gene family were upregulated during auxin-induced somatic embryogenesis (Imin et al. 2005; Lyngved et al. 2008; Guzmán-García et al. 2013). Consistently, GST accumulation has been reported in somatic embryos of *Cyclamen persicum* (Winkelmann et al. 2006) and *Vitis vinifera* (Marsoni et al.

2008). A differential expression of GST was observed in *Medicago truncatula* – it consistently increased in both embryogenic and nonembryogenic lines from day 0 to 14 d reflecting the explant response to in vitro stress. When the first embryos started to differentiate, it dropped to its initial level in the embryogenic line M9-10a, suggesting that the M9-10a explants were able to cope with imposed oxidative stress (Almeida et al. 2012). Similar results were obtained in *Citrus sinensis* (Pan et al. 2009).

Thus, it can be concluded that oxidative stress may stimulate cell differentiation to promote somatic embryo formation, whereas other antioxidative proteins may serve to protect cells from toxicity caused by long-term in vitro culturing.

Superoxide dismutase (SOD) converts superoxide radicals to hydrogen peroxide, thus providing the first line of defense against oxidative stress. Mn-SOD is mainly localized in the mitochondrial matrix and the peroxisomes. SOD accumulation has been reported to be involved in triggering SE and is thought to be required for embryo germination. In *Cyclamen persicum*, SODs were abundant in both somatic and zygotic embryos (Winkelmann et al. 2006), but in *Quercus*, a significantly higher abundance of this enzyme was detected in somatic versus zygotic embryos (Gomez-Garay et al. 2013). A Mn-SOD was upregulated to much higher levels in EC than in NEC of *Vitis* (Zhang et al. 2009) and was over-expressed in cork oak (Gomez-Garay et al. 2013) and cotton cotyledonary and mature stages of somatic embryos (Ge et al. 2015).

During the scavenging of ROS, scavenger recovery proteins such as thioredoxin and glutaredoxin are necessary to regenerate scavengers. Imin et al. (2005) identified a thioredoxin H (Trx) which appeared early in explant cultures of the embryogenic cell line 2HA and became undetectable at later stages of cell proliferation suggesting that Trx H plays a significant role during early stages of commitment from the vegetative stage to a pathway of cellular differentiation and proliferation. The Trx H group of proteins is involved in a broad scope of biological functions, acting as cofactors, transcription regulators, protein-binding regulators, protein folding cata-

lysts, growth factors, and antioxidants. In cotton cotyledonary embryos, three scavenger recovery proteins, namely, glutaredoxin, glutaredoxin-related proteins, and thiol–disulfide isomerase/thioredoxins, showed various abundance profiles, indicating that these proteins might regenerate scavengers for response to ROS in cotton embryo growth (Ge et al. 2015). In oak torpedo somatic embryos, Trx showed increased level upon maturation (Gomez-Garay et al. 2013). Peroxiredoxin (1-Cys Prx) showed considerably elevated levels of expression in later stages of SE development in the highly embryogenic cell lines 2HA (Imin et al. 2005) and M9-10a (Almeida et al. 2012) of *Medicago sativa*. Another peroxiredoxin – 2-Cys Prx – was highly expressed in *Quercus* torpedo somatic embryos (Gomez-Garay et al. 2013) and somatic embryos of *Cyclamen* (Rode et al. 2011). Peroxiredoxins are thiol-dependent antioxidants containing one (1-Cys) or two (2-Cys) conserved Cys residues that protect lipids, enzymes, and DNA against reactive oxygen species (Imin et al. 2005). A high abundance of these enzymes suggests the occurrence of stress within the somatic embryos tissue beyond that due to embryogenesis.

Cyclophilins constitute a subgroup of a large family of proteins called immunophilins. Most cyclophilins display peptidylprolyl isomerase (PPIase) enzymatic activity and active role in protein folding which render them molecular chaperones. Several of these members have also been directly linked to multiple stresses (Kumari et al. 2012). A higher expression of cyclophilin in the early stages of embryogenesis and response to various stresses has been reported as well as its involvement in the control of ROS (Ruan et al. 2011). The upregulation of cyclophilin in the globular and cotyledonary phases of *Coffea arabica* (Noah et al. 2013) may be related to the control of ROS levels. It is possible that a higher accumulation of ROS occurs in the globular stage and may account for the higher expression of cyclophilin to control ROS levels in the cell.

These results show that a dynamic expression of oxidative stress-related proteins occurs during SE. Somatic embryogenic lines show higher levels of GST, APX, and SOD proteins. Assuming

that the generation of a significant amount of stress and ROS is a prerequisite to induce SE, it is possible that SE lines may have a better ability of controlling oxidative stress by regulation of the ROS-scavenging system and hence maintaining ROS homeostasis during somatic embryo growth and maturation. In this way, ROS can act as signaling molecules playing an important role during auxin-induced SE. As a whole, this sustains the hypothesis that SE is a cellular stress-adaptive response to in vitro culture conditions (Dudits et al. 1991; Pasternak et al. 2002).

5.2.1.2 Pathogenesis-Related Proteins

Another group of proteins involved in stress/defense response is the pathogenesis-related proteins (PR). PR proteins have been observed mostly during early stages of embryogenesis. A chitinase and β -1,3-glucanase considerably increased during the formation of proembryogenic masses (PEMs) in oil palm (Silva Rde et al. 2014). An increased expression of endochitinase was observed during the induction phase of SE in the highly embryogenic M9-10A of *M. truncatula* (Almeida et al. 2012). Chitinase IV was significantly more expressed in EC of *Larix principis* (Zhao et al. 2015). One of the most abundant proteins in embryogenic suspension cultures of cowpea was identified as chitinase from the PR-4 family of PR proteins (Nogueira et al. 2007). During SE in *Picea glauca*, the transcript of a β -1,3-glucanase gene was highly abundant in embryogenic tissues and gradually decreased during the induction of somatic embryos with the lowest abundance occurring during the globular embryo stage (Dong and Dunstan 1997). Five types of β -1,3-glucanase were detected at the globular stage in *M. truncatula* embryogenic cultures (Imin et al. 2004).

Chitinases catalyze the hydrolytic cleavage of the β -1,4-glycoside bond between N-acetylglucosamine residues, mainly present in chitin. β -1,3-glucanases are enzymes that catalyze the hydrolysis of the fungal cell wall polymer β -1,3-glucan. Chitinase induction is often coordinated with the expression of specific β -1,3-glucanases and other PR proteins in response to pathogen attack, as well as abiotic and biotic

stresses. Despite their typical involvement in the defense response of plants, chitinases and glucanases are also expressed in healthy plants in an organ-specific and developmentally regulated pattern, suggesting a nondefensive role in plant development as well (Kasprzewska 2003). The accumulation of chitinases at a much higher level in the medium of embryo cultures than in nonembryogenic lines suggested that chitinase could be related to embryogenic competence for SE. A frequent occurrence of chitinases in embryogenic tissues has been associated with enzymatic activity on arabinogalactan proteins that enable the control or maintenance of embryogenic cell fate (van Hengel et al. 2001).

In both embryogenic and nonembryogenic cell lines of *Medicago truncatula*, two of the most abundant proteins were an abscisic acid (ABA)-responsive protein with homology to the pathogenesis-related protein PR10-1 and PR10-1 itself. Interestingly, they changed little throughout the 8 weeks of culture, suggesting a general role for ABA-responsive proteins and PR10 proteins in cell maintenance or cell defense (Imin et al. 2005). In EC of cowpea, a highly abundant protein was identified as PR10 (Nogueira et al. 2007). As some classes of PR-10 proteins are inducible by auxin, it is possible that this protein mediates the cell responses to growth regulators in cowpea. Also, PR10 proteins were found to be upregulated in NEC (Marsoni et al. 2008; Cangahuala-Inocente et al. 2009; Zhang et al. 2009) and downregulated in EC of *Vitis* (Zhang et al. 2009) and *Zea mays* inbred line H99 (Sun et al. 2013). PR10 proteins belong to an intracellular defense-related protein family and show homology to ribonucleases. In addition, PR10 genes are highly expressed in response to biotic and abiotic stresses (van Loon et al. 2006). The downregulation of PR10 in EC appears correlated with a better ability of controlling oxidative stress in EC cells. Thaumatin (a PR5 protein) was identified with maximum abundance at initial embryo stage of *Quercus suber* (Gomez-Garay et al. 2013). Thaumatin is subject to complex expression profiles regulated by environmental factors and developmental stages (Liu et al. 2010).

PR protein synthesis during *in vitro* cultures may be associated with the adaptation of plant cells to new environmental conditions, resulting in the activation of defense mechanisms that are likely not directly related to a specific morphogenic pathway such as SE. One hypothesis is that plant cells may activate signaling pathways that trigger cellular events in response to environmental stress, leading to the formation of embryonal structures (Van Loon et al. 2006). These findings indicate that PR-type proteins can have a developmental role and, through their enzymatic activities, may generate signal molecules that could act as endogenous elicitors in morphogenesis. Such elicitors could also play a role in activating other types of defensive responses.

5.2.2 Protein Synthesis, Processing, Folding, and Fate

5.2.2.1 Heat Shock Proteins (HSPs)

SE is a complete cell reprogramming process, and the initial stage of dedifferentiation of somatic cells to embryo-like structure needs global change in gene expression and protein complement. It requires the assembly and stabilization of newly synthesized proteins, as well as the modification and removal of peptides (Fehér et al. 2003).

The consideration of SE as a specific form of the stress response is supported by experimental findings that show the involvement of the heat shock systems in this developmental reprogramming. Many heat shock proteins (HSPs) are molecular chaperones, which are formed in response to stress and also are developmentally regulated. They assist in the correct folding of nascent and misfolded polypeptides by preventing their aggregation. Some of them are responsible for assembly translocation and degradation. Their function may be of increased importance under stress conditions, where misfolding of polypeptides occurs more commonly (Wang et al. 2004). Although HSPs are referred to as stress-responsive proteins, however, many of them are expressed in the absence of stress, during normal cell growth aiding in protein folding

and subcellular sorting. HSPs are involved in SE even without being triggered by external signals.

Members of the HSP family HSP60 and HSP70 have been reported to be expressed at higher levels in EC than in NEC (Correia et al. 2012; Guzmán-García et al. 2013; Teyssier et al. 2014; Vale Ede et al. 2014; Zhao et al. 2015). Two different studies performed in *V. vinifera* reported the use of HSP70 as a possible marker for the embryogenic capacity based on their higher levels in embryogenic callus than in non-embryogenic callus (Marsoni et al. 2008; Zhang et al. 2009).

The ER luminal binding protein (BiP), a member of the HSP70 family, was overexpressed in EC of *Larix principis* (Zhao et al. 2015) and *Carica papaya* (Vale Ede et al. 2014). BiP localizes to the endoplasmic reticulum (ER). It acts in the translocation of proteins through the ER and assists in the proper folding and maturation of newly synthesized proteins entering the organelle (Vale Ede et al. 2014). HSP70 and luminal binding proteins have also been reported to be more abundant during the first stages of the development of SE in *M. truncatula* (Imin et al. 2005) and *Crocus sativus* (Sharifi et al. 2012). The expression level of these proteins was the highest at 5 weeks after the induction of SE from leaf explants, coinciding with the generation of calli. However, the chaperones showed a decrease in the 8-week cultures, when somatic embryos appeared. Similar results were reported in *P. glauca* (Lippert et al. 2005). In oil palm, an HSP90 was identified in all stages of SE with a higher intensity in callus after 14 days on induction medium. HSP90 seems an interesting candidate to be further investigated regarding its ability to induce embryogenic competence in oil palm (Silva Rde et al. 2014).

An ATP-binding protein is another HSP that showed increased abundance in the initial proliferation embryo stage (PSE) of cork oak. This protein controls protein folding during cell reorganization from somatic plant cells to the embryogenic pathway (Gomez-Garay et al. 2013).

This may imply that a higher level of expression of the chaperones is required for the maintenance of cells during early culture, playing

a protective function in response to the stress conditions that characterize in vitro growth.

Different HSP levels were found to be stage specific during embryogenesis in *Cyclamen*. High levels of HSP20 and HSP70 were representative for differentiated embryos marking late stages of embryogenesis, while increased abundances of HSP60 and HSP101 were typical for earlier stages and callus. These proteins help newly synthesized proteins to fold and minimize protein aggregation upon stresses imposed during initiation of SE from somatic cells (Rode et al. 2012).

Besides at early stages of SE, HSPs were more abundant at later stages of embryo development, highly increasing during somatic embryo maturation of cork oak (Gomez-Garay et al. 2013), hybrid larch (Teyssier et al. 2014), and somatic and zygotic embryos of *C. persicum* (Winkelmann et al. 2006). Probably their high expression is necessary to prepare the embryos for desiccation, consistent with the high level of proteins synthesized at this stage. In coffee SE, an HSP70 was more abundant in the cotyledonary phase when compared to the torpedo stage and was not observed in the globular stage (Tonietto et al. 2012).

Small heat shock proteins (sHSPs) of 15–30 kDa have been reported in high abundance during the late stages of SE. Small HSPs are not only crucial components of the plant heat shock response but also play important roles in adaptation to various other stresses and development (Waters 2011). One sHSP was much more expressed in EC than in NEC of *Vitis* (Zhang et al. 2009). An sHSP was found to accumulate during maturation of oak somatic embryos (Gomez-Garay et al. 2013). Small HSPs have been also found to be upregulated from early to mature stages of SE development in *P. glauca* (Lippert et al. 2005) and *P. abies* (Businge et al. 2013). In *Pinus pinaster*, two sHSPs were found to be overexpressed in cotyledonary somatic embryos (two isoforms of HSP 18.2), and one in cotyledonary zygotic embryos (class II HSP 17.6) together with HSP60 and HSP70 has been overexpressed in both types of embryos (Morel et al. 2014). In *Phoenix dactylifera*, stress-related proteins of the HSP family (17.6 and 70 kDa)

were detected in zygotic embryos only. These proteins are especially abundant at the late stages of embryo maturation (Sghaier-Hammami et al. 2009). The presence of sHSP 17.6 in zygotic embryos only makes it a potential marker of zygotic embryo maturity in *Pinus pinaster* (Morel et al. 2014) and *Phoenix dactylifera*. HSP 18.2 and HSP 17.6 belong to a small HSP family (HSP 20) which has been detected in differentiated embryos in *Cyclamen* (Rode et al. 2012), but their function is not yet known.

The data suggest that the increased chaperone proteins may play a fundamental role in SE possibly by alleviating stresses associated with global reprogramming during somatic to embryogenic transition. In addition, HSP proteins all contribute to the preparation of the embryo for subsequent desiccation and germination phases.

5.2.2.2 Protein Processing and Fate

Cellular reprogramming ultimately requires the synthesis, protein folding, and posttranslational modification of newly synthesized proteins, as well as the removal of proteins that are no longer needed. The ubiquitin/26S proteasome pathway is a major factor in controlled proteolysis. The ubiquitin–proteasome pathway can be regulated at the level of ubiquitination or the level of proteasome activity (Glickman and Ciechanover 2002). In plants, the ubiquitin–proteasome system can control nearly every aspect of growth and development, such as the cell cycle, embryogenesis, defense, environmental responses, and hormone signaling (Vierstra 2009).

Consistently, 26S proteasome regulatory particle triple-A ATPase subunits α and β were found to be upregulated in EC of *C. persicum* (Lyngved et al. 2008), *V. vinifera* (Zhang et al. 2009), *C. sativus* (Sharifi et al. 2012), and *Z. mays* (Sun et al. 2013). High expression levels of the proteasome subunits in EC imply the possible role of proteasome machinery in callus establishment through removal of explant-associated proteins that are no longer needed. Subunits of the 26S proteasome and ubiquitin were highly abundant throughout SE in *C. persicum* and *C. sativus*, but especially noticeable in the late torpedo stages (Sharifi et al. 2012; Rode et al. 2012). It is well

known that proteasome activity is closely aligned with cell proliferation processes. Rapid cell division marking the early stages of SE is accompanied by an increase in proteasome subunits. Thus, high levels of enzymes involved in the 26S proteasome-dependent proteolysis pathway seem to be important for the switch from callus to globular embryo as well as from globular to torpedo-shaped embryo in these species. A decrease in proteasome subunit abundance was observed during cotyledonary embryo development in cork oak (Gomez-Garay et al. 2013) and cotton (Ge et al. 2015). In cotyledonary embryos, the need for saving energy slows down protein synthesis, whereas reduction in protein degradation also facilitates nutrient storage for germination and plantlet regeneration (Ge et al. 2015). Also, proteasome activity decreases when cells are stimulated to differentiate as is the case. In a previous study on early somatic embryo development in *Picea glauca*, it has been reported that three proteasome proteins were simultaneously downregulated as the culture medium containing abscisic acid stimulates embryo maturation and concomitant differentiation, thereby reducing cell division (Lippert et al. 2005). Hence, the hypothesis that a decrease or absence of proteasome subunits might be an appropriate marker for tracking proper embryonic development has been widely accepted.

To summarize, controlled proteolysis executed by the 26S proteasome–ubiquitin system is needed in order for the somatic embryogenesis to succeed.

Proteomic studies of SE revealed the presence of proteinases and proteinase inhibitors in several plant species (Nogueira et al. 2007; Marsoni et al. 2008; Sharifi et al. 2012; Noah et al. 2013). A leucine aminopeptidase was found to be unique to the PEG treatment of embryogenic cultures of *Carica papaya* improving somatic embryo production thus being indicative of better control of embryonic development under this treatment (Vale Ede et al. 2014). An exceptional high accumulation of trypsin inhibitors and aspartic proteinase was observed in somatic and zygotic embryos of *Theobroma cacao* L., respectively (Noah et al. 2013). Regarding the crucial role of aspartic proteinase, which is responsible for the

initial breakdown of proteins during germination, its relatively early accumulation in zygotic embryo in the torpedo stage might be an indication of the onset of maturation. Recently, Guilloteau et al. (2005) found trypsin inhibitor to be part of an active aspartic proteinase complex in cacao seeds. They suggested this trypsin inhibitor subunit to protect storage proteins from precocious hydrolysis that might result from the high aspartic proteinase content in cacao seeds.

Besides their well-known function to degrade damaged, misfolded, and harmful nonfunctional proteins, proteases indeed play key roles in the maturation of cell wall proteins and the generation of active peptides (van der Hoorn 2008). The endogenous cysteine proteinase inhibitors – the cystatins – form a tight, reversible complex with cysteine proteases, thus exerting a fine control of their activity during embryogenesis, organogenesis, programmed cell death, and tolerance to abiotic and biotic stresses (Benchabane et al. 2010; Turk et al. 2012). Thus, the precise control of the proteolytic processes is of utmost importance for the correct plant growth and development (Novinec and Lenarčič 2013).

5.2.2.3 Protein Synthesis

Protein metabolism is a key factor in somatic embryogenesis induction. In line with this, one of the largest functional groups found in ECs in *Cyphomandra betacea* was that of proteins involved in protein biosynthesis, namely, ribosomal proteins. In addition, pentatricopeptide repeat-containing proteins, known by their role in protein–protein interactions related to a large variety of functions (chaperoning, transcription, etc.), were exclusively found in EC (Correia et al. 2012). Accordingly, three protein synthesis-related proteins [histone H2B.2; 40S ribosomal protein, protein disulfide isomerase (PDI)], were detected in EC of maize which is in agreement with the high number of proteins synthesized during the formation of calli (Sun et al. 2013). Ten differentially expressed ribosomal proteins were downregulated in cotyledonary embryos, indicating that biosynthesis of proteins was decreased in cotyledonary embryos (Ge et al. 2015).

To summarize, a global change in protein metabolism takes place during the somatic-to-

embryogenic cell transition. This is reflected in the overexpression of molecular chaperones such as HSPs and cyclophilins, proteasome subunits to process unnecessary proteins, a duo of proteinases and their inhibitors to fine-tune developmental processes, as well as proteins involved in intense protein synthesis.

5.2.3 Cell Proliferation and Cell Wall Metabolism

The induction of SE includes dedifferentiation in somatic plant cells and establishment of embryogenic competence. These processes require reactivation of the cell division in somatic plant cells. Embryogenesis is accompanied by a morphogenesis process during which the embryo differentiates through several distinct stages – globular, heart-shaped, torpedo, and cotyledon stages. The morphogenesis is based on coordinated cell elongation and division (Pan et al. 2009).

Tubulin and actin are ubiquitous components of the eukaryotic cell. Actin microfilaments and tubulin microtubules comprise the cytoskeleton. Alpha- and β -tubulins are assembled coordinately, forming microtubules in response to various intracellular and extracellular signals and participating in many different functions in eukaryotic cells. Tubulins are known to be associated with cell division and cell elongation and played a significant role in the separation of the organelles and daughter chromosomes (mitosis) (Pan et al. 2009).

Although tubulin is considered a housekeeping protein and has been widely used as a constitutive standard in gene expression, its upregulated differential expression was observed during SE until the globular stage in *C. sinensis* (Pan et al. 2009), but gradually downregulated upon maturation in white spruce (Lippert et al. 2005) and cork oak (Gomez-Garay et al. 2013). Also, some tubulins exhibited higher expression in the EC compared to the NEC (Zhang et al. 2009; Sun et al. 2013; Zhao et al. 2015).

In plants, the actin cytoskeleton plays significant roles in the definition of cell polarity and orientation of cell division, cell elongation, cell

wall development, transport processes, positioning of membrane receptors, and in PCD. A study on the role of actin isoforms in SE in Norway spruce demonstrated that actin isoforms were expressed predominantly in suspensor cells (Schwarzerova et al. 2010).

Actin is also a common reference gene in gene quantitative expression and was recently found to be involved in programmed cell death (PCD) and cell defense mechanisms against biotic and abiotic stress. Depolymerization of the actin cytoskeleton acts as a downstream regulatory factor in the plant stress adaptation networks and participates in triggering the execution of PCD (Malerba et al. 2008). In accordance, different actin forms were found in embryonic masses of cassava (Baba et al. 2008) and *C. persicum* (Lyngved et al. 2008). Two actin isoforms were observed at higher levels in EC of *Larix principis* (Zhao et al. 2015).

Proliferating cell nuclear antigen (PCNA) is an evolutionarily well-conserved protein found in all eukaryotic species. It is able to interact with multiple partners in several metabolic pathways such as DNA repair, translation DNA synthesis, DNA methylation, chromatin remodeling, and cell growth and apoptosis, suggesting a function in the regulation of the cell cycle (Maga and Hubscher 2003). In accordance, the involvement of PCNA in the SE process was first shown in *Vitis vinifera* (Marsoni et al. 2008) and Zhao et al. (2015) detected an elevated level of PCNA in EC of *Larix principis*.

Annexins have been identified in cotyledons of cassava somatic embryos undergoing secondary SE (Baba et al. 2008). Annexins occurred at more than double the abundance in EC of *Larix principis* (Zhao et al. 2015) and were found highly expressed during SE of oil palm (Silva Rde et al. 2014) and in globular and cotyledonary embryos of coffee (Tonietto et al. 2012). Annexins are multifunctional proteins expressed throughout the life cycle that appear capable of linking Ca^{2+} , redox reactions, and lipid signaling to coordinate development in response to the biotic and abiotic environment. Strong evidence also indicates that annexins are involved in cell division during the cell cycle (Laohavisit and Davies 2011).

Accepting the fact that the initiation of somatic embryogenesis is closely linked to hormone-induced cell divisions, its molecular characterization can also be based on genes with cell cycle-dependent expression (Dudits et al. 1995). One protein that is involved in cell growth and cell division through microtubule stabilization, the translationally controlled tumor protein homolog (TCTP), was unique to ES (Lyngved et al. 2008). This protein has previously been identified in embryogenic cell suspensions of cowpea (Nogueira et al. 2007).

The plant cell wall is a highly dynamic entity whose structure and composition changes dramatically during plant growth and cellular differentiation; it serves as the first mediator in cell-to-cell communications and protects cells from biotic and abiotic stresses (Wolf et al. 2012). Cell wall and membrane formation are enhanced during embryogenesis. In line with this, α -1,4-glucan protein synthase, a glycosyltransferase, was upregulated in EC of *Larix principis* (Zhao et al. 2015), and this is consistent with observations in *C. persicum* (Lyngved et al. 2008). In addition, higher abundance of this protein was found in mature somatic embryos during the SE process (Teyssier et al. 2014). The enzyme builds up α -1,4-glucan chains covalently bound to protein and is involved in UDP forming and the synthesis of polysaccharides in the cell wall. A cell wall development protein containing a leucine-rich repeat (LRR) and an extension domain, a LRR family protein/extension family protein which is involved in cell expansion and growth, was identified in embryogenic cultures of *Cyclamen*. The presence of high levels of this protein in both globular embryos and torpedo embryos is consistent with the active embryogenic cell division that occurs during these developmental stages of SE (Bian et al. 2010).

Enzymes involved in cell wall and membrane synthesis were upregulated in embryogenic suspension cultures (ES). Acyl-[acyl-carrier protein] desaturase (unique to ES) takes part in fatty acid biosynthesis and is a key determinant of the overall level of unsaturated fatty acids in the cell (Lyngved et al. 2008). Several proteins involved in cell wall metabolism were found upregulated

(pectin methylesterase, pectate lyase, cinnamyl alcohol dehydrogenase) and others downregulated (pectin acetyltransferase and beta-expansin 1a precursor) in cotyledonary embryos of cotton. The downregulated pectin acetyltransferase and upregulated pectin methylesterases promote cell and organelle elongation in cotyledonary embryos. Overall, cell wall synthesis and the loosening of cross-links are likely to regulate cell expansion in somatic embryos (Ge et al. 2015).

To summarize, higher levels of some proteins involved in the cell cycle, cell wall biogenesis, and expansion were found in EC than in NEC as well as throughout the process of SE in agreement with an active embryogenic cells division, cell wall plasticity, cell growth, and differentiation.

5.2.4 Signal Transduction

Levels of some proteins involved in signaling were found to be higher in ES than in NES in *C. persicum* (Lyngved et al. 2008) and *Larix principis* (Zhao et al. 2015). The 14-3-3-like protein GF14-D, similar to a protein found in embryogenic cell suspensions by Nogueira et al. (2007), belongs to the 14-3-3 proteins, which are highly conserved phosphoserine-/phosphothreonine-binding proteins. 14-3-3 proteins regulate a broad range of target proteins in all eukaryotes through phosphorylation and may allow the growth and development of cells to be coordinated with the metabolic status of the plant as well as environmental stresses (reviewed in Denison et al. 2011). The phosphorylation-dependent binding of 14-3-3 proteins regulate negatively the activity of mitochondrial and chloroplast ATP synthases that may suggest a mechanism for plant cells to adapt to environmental changes such as nutrient supply, and especially exogenous plant growth regulators, during the initiation step of SE. In addition, these proteins participate in the regulation of various biochemical processes during seed development (Zhao et al. 2015). Such proteins were found in embryogenic cultures of *C. papaya* (Vale Ede et al. 2014). Two 14-3-3 proteins were more abundant in proliferating embryos than in their mature counterparts in oak (Gomez-Garay et al. 2013).

Protein phosphatase 2A (PP2A) 65-kDa regulatory subunit has been previously associated with the embryogenesis process by Marsoni et al. (2008). PP2A is a ubiquitous and conserved serine/threonine phosphatase with a multifunctional regulatory activity in plants. The A subunit, in particular, is essential for auxin transport, functioning as a positive regulator of the PP2A holoenzyme, and is involved in differential cell elongation responses. Plant PP2As also participate in biotic and abiotic stress signaling pathways. Recent functional analysis of PP2As revealed that they are key components of stress signal transduction pathways, playing positive and dynamic roles in stress signaling (reviewed in Pais et al. 2009). Dudits et al. (1995) reported a marked increase in phosphorylation of defined proteins in embryogenic cells compared to the nonembryogenic cells. Accordingly, the embryogenic suspensions of *C. persicum* expressed higher levels of PP2A and a 14-3-3-like protein, both of which play important roles in protein phosphorylation (Lyngved et al. 2008). In addition, G proteins and calreticulin have been classified as promising candidates for involvement in signal transduction in *C. persicum* and *Larix principis* embryogenic cultures (Lyngved et al. 2008; Zhao et al. 2015).

The higher abundance of PP2A regulatory subunit and 14-3-3 proteins in embryogenic cultures and at early stages of embryogenesis suggests that they might play a protective role against the stress from in vitro culturing in addition to the stress accompanying the cellular reprogramming.

5.2.5 Proteins Involved in the Metabolism

During SE₂ plant cells undergo reprogramming of their metabolism and energy consumption, especially carbon and nitrogen metabolism (Fehér et al. 2003). The fast growth and high cell division rate of callus and developing somatic embryos require high amounts of energy. This explains why the largest class of differentially expressed proteins in EC and during SE usually are proteins involved in energy metabolism.

Several glycolytic enzymes, as well as proteins involved in energy metabolism most frequently detected in the proteomic studies of different plant species, are discussed below.

The glycolytic enzyme, triosephosphate isomerase, plays a significant role in glycolysis and is essential for effective energy generation. It catalyzes the reversible interconversion of the triosephosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. In comparison with zygotic embryos, higher accumulation of triosephosphate isomerase occurred in somatic embryos in *C. persicum* (Winkelmann et al. 2006) and in EC of *Larix principis* (Zhao et al. 2015) and cowpea (Nogueira et al. 2007). However, in EC of *Vitis* (Marsoni et al. 2008), triosephosphate isomerase was downregulated. A similar result was found in *Cyclamen* torpedo embryos (Bian et al. 2010). Ito et al. (2003) proposed that triosephosphate isomerase was among the key enzymes of the regulation mechanism that slowed down glycolysis and the tricarboxylic acid cycle rate under oxidative stress in order to lower the deleterious production of reactive oxygen species (Marsoni et al. 2008).

The overexpression of transketolase, one enzyme of the pentose pathway, could be correlated with the active proliferation of somatic embryos that need biosynthetic intermediate and NADPH (Marsoni et al. 2008; Lyngved et al. 2008).

Fructokinase (FRK) is a primary enzyme of glycolysis and is involved in starch synthesis. FRK was one of the most abundant proteins in the proteome reference map generated from embryogenic cell cultures in *M. truncatula* (Imin et al. 2005). This enzyme was also detected during the SE of *Citrus sinensis*, with the highest expression level in early stages of embryogenic callus (Pan et al. 2009). This protein has been reported previously as exclusively increased in developed calli compared with corm explant (Sharifi et al. 2012). In oil palm, fructokinase was detected first at the earliest stage of SE induction and was also observed in the next stages of development indicating that starch synthesis occurred during the first 2 weeks of SE induction. The deposition of starch in the cortex of the embryogenic callus has been observed in *C. sativa* and is

mobilized and used as an energy source by the meristematic cells during intense cell division and differentiation (Silva Rde et al. 2014).

Enolase that catalyzes the conversion of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP) in the glycolytic pathway was found highly abundant in somatic embryos of *C. persicum* (Rode et al. 2012), *Eruca sativa* (Chen et al. 2012), and oil palm (Silva Rde et al. 2014). Lippert et al. (2005) detected a high expression of this protein only in the torpedo stage of *Picea glauca* and therefore suggested that enolase could be an interesting candidate to be used as a molecular marker of embryogenesis maturation. Similarly, enolase was also expressed only in the torpedo stage of coffee somatic embryos, indicating that this protein could be used as a molecular marker of the torpedo stage in different plants (Tonietto et al. 2012). A highly abundant enolase in EC was also confirmed by two independent proteomic studies in *Z. mays* (Sun et al. 2013; Varhaníková et al. 2014).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an essential enzyme of glycolysis. GAPDH has been found in higher amounts in somatic embryos compared to zygotic embryos (Winkelmann et al. 2006). Four GAPDH proteins were unique to ES in *Cyclamen* (Lyngved et al. 2008). Nogueira et al. (2007) also identified several proteins as GAPDH in proembryogenic masses of cowpea. GAPDH was highly expressed in the globular phase and showed a reduced expression in the other SE stages of coffee (Tonietto et al. 2012), while GAPDH peaked at oak cotyledonary somatic embryo suggesting that this stage is more demanding in terms of energy and precursors for the synthesis of primary metabolites such as amino acids and fatty acids (Gomez-Garay et al. 2013). The distinct abundance patterns of GAPDH could reflect regulation along the glycolysis pathway at different developmental stages.

Smith and Krikorian (1990) have shown that low pH is essential for maintaining the proembryogenic stage in carrot, while high average intracellular pH favors regeneration. At low pH, the ATPase H⁺ pump is stimulated. Accordingly, vacuolar ATP synthase subunit B2, ATP synthase

subunit beta, and ATPase alpha F1 were highly upregulated in ES compared to NES in *Cyclamen* (Lyngved et al. 2008). The 14-3-3 proteins known as positive regulators of H⁺-ATPase activity (Chen et al. 2006) were upregulated in ES, too. The abundance of ATPase in EC may explain the proembryogenic state of the culture, but may also mean that the embryogenic cells need more energy for their metabolic changes (Lyngved et al. 2008). ATP is a ubiquitous energy source that can also act as a signaling molecule in cellular metabolism. In *Abies alba* Mill., an increase in the levels of adenosine triphosphate (ATP) is associated with the maturation of SEs (Petruša et al. 2009). In addition, mitochondrial activities like ATP catabolism influence plant cell death, which is considered essential for correct SE maturation (Bozhkov et al. 2005). A beta subunit of mitochondrial ATPase in mature ZEs and embryogenic R cell line during the proliferation phase was detected in *A. angustifolia* (Silveira et al. 2008; Jo et al. 2014) defining the mitochondrial ATPase as the marker for selection of EC lines responsive to maturation treatments. In plants (Vianello et al. 2007), the energy status, particularly ATP levels, is crucial to the start of PCD. Thus, it is possible that the proper development of SEs in the R cell line could be linked with an adequate PCD mechanism, which was absent in the nonembryogenic B cell line (Jo et al. 2014). The presence of this protein suggests a high energetic metabolism in ECs of the cell line. However, downregulation of the β subunit of ATP synthase was observed in EC of *Vitis* (Zhang et al. 2009) and *Zea mays* (Sun et al. 2013) possibly indicating considerable damage to this subunit.

The upregulation of S-adenosyl methionine (SAM) synthase has been observed in various conifers at stages from early to late embryogenesis of both somatic and zygotic embryos (Lippert et al. 2005; Balbuena et al. 2009; Jo et al. 2014; Teyssier et al. 2014; Morel et al. 2014). SAM synthase likely contributes to enhanced amino acid metabolism (Teyssier et al. 2014) as well as to alterations in both polyamine content and ethylene biosynthesis (Morel et al. 2014) and might be essential for the transition of PEM to somatic embryos. In *Picea glauca*, SAM synthase has

been characterized as a biochemical marker of early somatic embryo development (Lippert et al. 2005). SAM synthase converts the amino acid methionine into SAM, which is the donor of methyl groups to the DNA methylation system by SAM-dependent methyltransferase. High levels of methylation during plant embryogenesis are associated with chromatin modeling, selective gene expression, and growth of somatic embryos (Morel et al. 2014).

Interestingly, energy metabolism is more active in somatic embryos compared to zygotic embryos, as previously reported by Sghaier-Hammami et al. (2009), Teyssier et al. (2014), Morel et al. (2014), and Noah et al. (2013) in mature somatic embryos from *Phoenix dactylifera* L., *Larix × eurolepis*, *Pinus pinaster*, and *Theobroma cacao* L., respectively. The higher abundance of glycolytic, citrate cycle, and ATP synthesis enzymes in somatic embryos than in zygotic embryos indicates a more active energy metabolism and ATP demand in the former than in the latter and may explain why matured somatic embryos are capable of entering the germination phase without undergoing the desiccation-induced dormancy program observed for zygotic embryos.

SE is a complex developmental process, and the data discussed above indicate that it is extensively based on carbohydrate metabolism ensuring the heavy energy demand required for metabolic processes that occur during cell division and elongation.

5.2.6 Storage Proteins

The differential accumulation of specific storage proteins during different stages of somatic and zygotic embryo development has been reported, and they may be indicators of embryo developmental stages. Proteomic studies reveal that synthesis of storage proteins starts with the globular stage of somatic and zygotic embryos reaching its peak at the maturation stage. 7S globulin has been identified in *Cyclamen* (Winkelmann et al. 2006; Rode et al. 2011) and in *Pinus pinaster* (Morel et al. 2014) zygotic and somatic embryos,

with higher content in zygotic embryos. The accumulation of 11S globulin in the torpedo phase of coffee somatic embryos could be to guarantee the energy necessary during embryo maturation in the cotyledonary phase (Tonietto et al. 2012). In oil palm, glutelin accumulation could be used as a biological marker of an early stage of SE since it was only observed in the first stage of SE (Silva Rde et al. 2014).

Legumin precursor was highly abundant in oak globular and cotyledonary embryos (Gomez-Garay et al. 2013) and has been found upregulated in the mature in vitro derived embryo tissue of *Picea glauca* (Lippert et al. 2005). In some species, the level of this type of reserve proteins has been used to distinguish between zygotic and somatic embryos due to a lower accumulation of reserve proteins in somatic embryos. Three storage proteins, namely, seed storage protein vicilin A and B and 2S albumin storage protein, were upregulated in cotton cotyledonary embryos (Ge et al. 2015). Legumin-like and vicilin-like proteins were highly expressed in both somatic and zygotic cotyledonary embryos of hybrid larch (Teyssier et al. 2014). A glutelin in *Phoenix* (Sghaier-Hammami et al. 2009) and a legumin-like protein in *Pinus pinaster* (Morel et al. 2014) were downregulated in somatic embryos relative to zygotic embryos, supporting the hypothesis of a lower accumulation of reserve proteins in somatic embryos. The putative novel storage proteins in *Cyclamen*, the small enolases (Rode et al. 2011), were present in all developmental stages, but their abundance increased significantly towards later stages of embryogenesis in *Cyclamen* embryos (Rode et al. 2012).

The synthesis of large amounts of storage proteins is regarded as a marker for embryo maturation, and the storage proteins themselves potentially represent excellent markers of embryo quality in gymnosperms as well as in angiosperms. These proteins all contribute to the preparation of the embryo for subsequent desiccation and germination phases. The comparative proteomic studies indicate that the somatic embryo lacks/accumulates fewer reserve proteins compared to the zygotic embryo and there are possibilities of improving the storage protein content

which depends on the SE culture medium as was observed in date palm. The addition of abscisic acid to the culture medium of date palm somatic embryos induced the accumulation of a 22 kDa glutelin enabling the embryos to accumulate the necessary reserves for normal germination (Sghaier et al. 2009). Studies at the proteomic level may help to improve the quality of SE-derived seedlings and to develop new in vitro culture strategies for plant propagation and manipulation.

5.3 Secretome Analysis of SE

Plant secretomics is a newly emerging area of plant proteomics (reviewed by Agrawal et al. 2010; Alexandersson et al. 2013). Agrawal et al. (2010) described it as “the global study of secreted proteins into the ECS by a cell, tissue, organ or organism at any given time and conditions through known and unknown secretory mechanisms involving constitutive and regulated secretory organelles.” The plant cell wall, also called the extracellular matrix (ECM), is an extremely dynamic entity whose structure and composition change dramatically during cellular differentiation; it serves as the first mediator in cell-to-cell communications and protects cells from biotic and abiotic stresses. Extracellular proteins participate in cell morphology, cell division, proliferation, plant defense reactions, responses to stress, and cell-to-cell adhesion (Tian et al. 2009; Rose and Lee 2010). Despite the crucial role played by extracellular proteins in these diverse and significant processes, the extracellular proteome (secretome) has been less well characterized than other subcellular compartments.

SE in cell suspension cultures provides a good model system for investigating early plant development. The culture medium of plant cell cultures may be regarded as a large extension of the intercellular space; soluble secreted molecules that inhabit the apoplast *in planta* accumulate in the medium and exert their effect on cell growth and development. Indeed, conditioned medium harbors a complex array of molecules which play a significant role in SE by their ability to either

promote or inhibit embryo development (for reviews, see Matthys-Rochon 2005; Ruiz-May et al. 2010; Fehér 2014).

We investigated the extracellular proteins in *Dactylis glomerata* L. embryogenic and nonembryogenic suspension cultures searching for potential early embryogenic markers.

Leaf explants from a highly embryogenic *D. glomerata* L. genotype were grown on induction SH30 medium containing the hormone dicamba. After 4 weeks, the younger basal tissue gave rise to callus which upon subculturing on the same medium segregated into embryogenic callus (EC) and nonembryogenic callus (NEC) (Fig. 5.1). Both calluses were morphologically distinct. The EC was more compact and of light yellow color, while the NEC was translucent showing needle-like structures. Somatic embryos from *D. glomerata* L. callus-derived suspension cultures fully developed on a hormone-containing medium only from the embryogenic callus. Two to three days after inoculation of induction medium, competent single cells in embryogenic suspension cultures started to divide intensively and formed microclusters (Fig. 5.1, panel a). Proembryogenic masses (PEMs) containing centers of embryonic growth were formed a week later (Fig. 5.1, panel b), and after 2 weeks, globular embryos began to differentiate from the cell masses (Fig. 5.1, panel c). Single cells from the nonembryogenic suspension culture were divided to form microclusters with blocked further development (Fig. 5.1, panel d).

In an attempt to identify SE-specific extracellular proteins, we analyzed the secretome of microclusters from *D. glomerata* L. embryogenic and nonembryogenic suspension cultures using 2-DE and LC-MS/MS. Fractions of microclusters were collected as described (Tchorbadjieva and Odjakova 2001), and after seven days in culture, the culture medium was used as a source for extracellular proteins. Representative 2-DE gels of extracellular proteins secreted from microclusters of both suspension cultures are shown in Fig. 5.2. Visual inspection of the gels revealed distinct protein patterns. Microclusters from the embryogenic suspension culture secreted approximately 40 proteins as compared to ca. 15 proteins secreted from the microclusters of the nonembryogenic line over

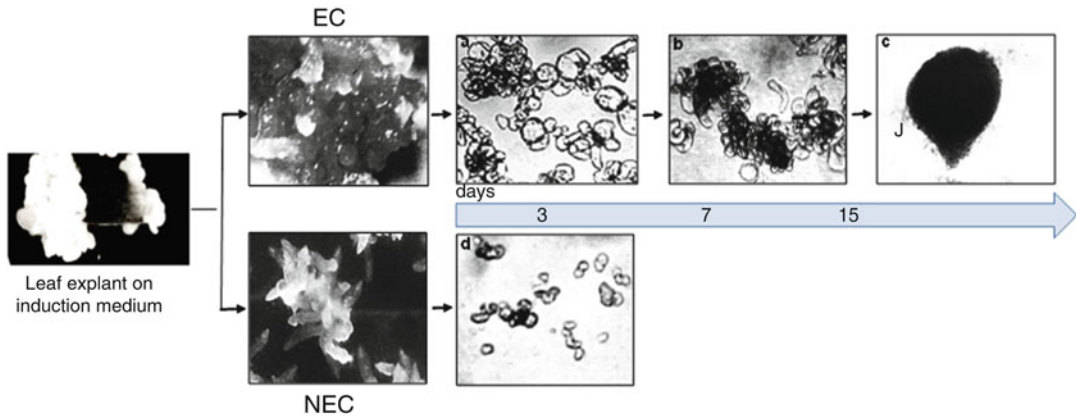


Fig. 5.1 Somatic embryogenesis of *Dactylis glomerata* L. Leaf explants cultured on induction (SH30) medium produce both embryogenic callus (EC) and nonembryogenic callus (NEC). Only EC is able to produce somatic embryos which fully develop in hormone-containing liquid medium before being transferred to a hormone-free

medium for regeneration. Single cells divide to form microclusters (a), microclusters develop to form proembryogenic masses (PEMs) (b), embryos differentiate from PEMs (c). Single cells from NEC form microclusters only whose further development is blocked (d)

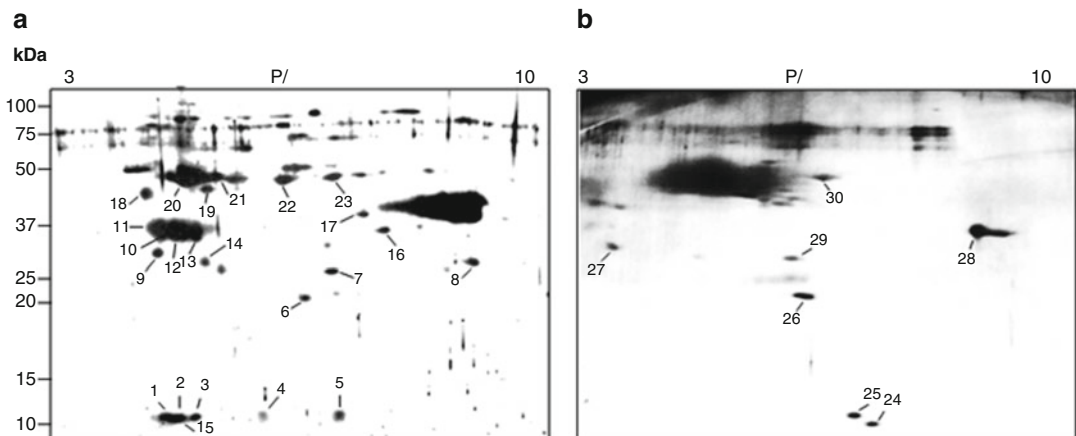


Fig. 5.2 2-DE analysis of proteins secreted into the culture medium of microclusters from *D. glomerata* L. embryogenic (a) and nonembryogenic (b) suspension cultures after 7 days in culture. Proteins were separated in the first dimension on an immobilized linear pH 3–10 gradi-

ent and in the second dimension on a 13 % acrylamide-SDS gel. Protein spots indicated in the gels were selected for protein identification. The positions of pI 3–10 and molecular mass markers are marked. Proteins were visualized by silver staining

a pH range of 3–10 and a size range of 10–100 kDa. In all, 30 differentially expressed proteins were excised, digested in-gel with trypsin, and subjected to LC-MS/MS identification. The proteins were identified by search against nonredundant protein database at the NCBI. Of the 30 candidate spots, only ten proteins (33 %) were successfully identified. This lower percentage of identification is typical of orphan organisms that are almost

absent in public databases. In addition, because of the particular interest towards spots numbered 1, 2, 3, 4, and 5 provoked from previous studies and the unsuccessful attempt to identify them by MS/MS, we used N-terminal sequencing and identified five isoforms of an acidic cysteine proteinase inhibitor – cystatin (Table 5.2). For the remaining spots, a low score or no hits were observed. The spot ID corresponds to protein spots shown in

Table 5.2 Extracellular proteins secreted by microclusters from *D. glomerata* L. embryogenic suspension cultures identified by N-terminal sequencing and LC-MS/MS

Spot no	Protein name	Accession	Species	Theor. pI/MM	Exper. pI/MM	Peptides	Seq. coverage
			<i>N-terminal sequencing</i>				
1	Cystatin Hv-CP18	CAG38129.1	<i>Hordeum vulgare</i>	8.4/10.7	4.3/11.0	1	20
2	Cystatin Hv-CP18	CAG38129.1	<i>Hordeum vulgare</i>	8.4/10.7	4.5/11.0	1	20
3	Cystatin Hv-CP18	CAG38129.1	<i>Hordeum vulgare</i>	8.4/10.7	4.8/11.0	1	20
4	Cystatin Hv-CP18	CAG38129.1	<i>Hordeum vulgare</i>	8.4/10.7	5.2/11.0	1	20
5	Cystatin Hv-CP18	CAG38129.1	<i>Hordeum vulgare</i>	8.4/10.7	6.4/11.0	1	20
			MS/MS analysis				
8	Desiccation-related protein PCC13-62-like	XP_006653362.1	<i>Oryza brachyantha</i>	9.2/28.8	6.0/28.0	2	12
9	Chitinase	BAA22968.1	<i>Chenopodium amaranticolor</i>	8.6/26.1	4.2/32.0	4	16
10	Putative class III chitinase	BAC10165.1	<i>Oryza sativa</i>	9.3/28.7	4.2/34.0	3	14
11	Cathepsin B	CAC83720.1	<i>Hordeum vulgare</i>	6.0/34.9	4.3/36.0	7	22
12	Cysteine endopeptidase precursor	AAD20453.1	<i>Oryza sativa</i>	5.6/37.2	4.6/36.0	6	25
13	Cysteine protease Cp precursor	ABS12459	<i>Citrus sinensis</i>	6.0/36.9	4.8/36.0	7	34
14	Class III peroxidase	ACF08095.1	<i>Triticum aestivum</i>	9.0/31.3	4.8/30.0	2	7
15	Pathogenesis-related protein 4	AAT67050.1	<i>Triticum monococcum</i>	4.5/13.0	4.5/11.2	1	16
20	Barley α -amylase 2	IAVA_A	<i>Hordeum vulgare</i>	5.6/45.0	4.5/45.0	16	41
21	Barley α -amylase 2	IAVA_A	<i>Hordeum vulgare</i>	5.6/45.0	5.1/45.0	16	41

The table shows, spot no, spot number in Fig. 5.2; accession, NCBI accession number; peptides, number of identified tryptic peptides; theor. pI/MM, theoretical pI values and molecular mass; exper. pI/MM, the experimental pI values and molecular mass were determined according to the spot position in the 2-DE gel; seq. coverage, the percentage of sequence coverage of the match

Fig. 5.2. In most cases, the theoretical MMs agreed well with experimental values, still for some proteins (spots 9, 10, 12, 13, and 14) a discrepancy was observed. Additionally, two proteins (spots 20 and 21) matched the same gene sequence but had different pIs. Interestingly, for all spots identified, the experimental pI values were much lower and largely deviated from that of the corresponding theoretical ones. These phenomena are commonly found on 2-DE gels and are invariably due to post-translational modifications or protein degradation.

Studies of SE show that asymmetric cell division and controlled cell expansion are important mechanisms for the generation of embryogenic plant cells indicating a significant role for the plant cell wall in these processes (Fehér et al. 2003). A set of proteins was identified that take part in the remodeling of the cell wall architecture and stress defense, as well as in cell signaling. These included proteins acting on polysaccharides such as endochitinases and α -amylases, proteases and a protease inhibitor, an oxidoreductase, as well as pathogenesis-related proteins whose putative role in SE is discussed below.

In a previous study using a monoclonal antibody, we found an extracellular acidic 48-kDa glycoprotein (gp48) secreted into the medium of microclusters from embryogenic *D. glomerata* L. suspension cultures and proposed its use as an early marker for embryogenic potential (Tchorbadjieva et al. 2005). Further, we identified the gp48 as α -amylase (Fig. 5.2, spot 20), cloned the full-length cDNA of the α -amylase gene (designated *DgAmy1*), and expressed the protein in *E. coli* (Rakleova et al. 2012). *DgAmy1* is a plant α -amylase that belongs to the glycoside hydrolase family 13 (GH13). Considerable differences in the structure of *DgAmy1* and other cereal α -amylases make unlikely its participation in starch degradation. Its transient expression in dividing microclusters during their developmental transition to proembryogenic masses and predominant localization at the regions of cell-to-cell adhesion make it more likely that the secreted *DgAmy1* may act on some unknown carbohydrate in the cell wall liberating signals necessary for the development of microclusters into PEMs or it may locally modify the cell wall and thus

ensure close cell-to-cell contact between the developing embryogenic structures (Rakleova et al. 2012).

Previously, the protein corresponding to spot 9 (Fig. 5.2) was immunologically identified as an acidic endochitinase (Tchorbadjieva and Pantchev 2006). This enzyme was expressed in embryogenic suspension cultures only. Subsequently, using the sequence information from the MS/MS analysis of spots 9 and 10, the full-length cDNAs for both proteins were cloned and showed that one of the proteins (designated *DgChiIV*) is a class IV endochitinase (JN191351) and the other (designated *DgChiIII*) is a class III endochitinase (JN191350) (Tchorbadjieva et al., manuscript in preparation). Typically, chitinases are involved in plant defense, but a nondefensive role of these enzymes in plant development has been suggested as well (Kasprzewska 2003). Plant cells from embryogenic suspension cultures secrete into the medium arabinogalactan proteins (AGPs) believed to have important roles in cell–cell interaction and signaling (Quiroz-Figueroa et al. 2006). Interestingly, AGPs contain GlcNAc and Glc residues, sensitive to cleavage by chitinases, and it has been suggested that chitinase-modified AGPs are extracellular matrix signaling molecules able to control plant cell fate (van Hengel et al. 2001). The presence of *DgChiIV* in embryogenic suspension cultures of *D. glomerata* L. only (Tchorbadjieva and Pantchev 2006) and the secretion of AGPs in the latter (unpublished results) suggest a similar role in the process of SE.

Cell wall proteome and secretome analysis of *Arabidopsis*, *O. sativa*, *N. tabacum*, *M. sativa*, and *Vitis* revealed the presence of unexpected large number of proteases in the cell wall and the culture medium with putative signaling function (Albenne et al. 2013; Krause et al. 2013). In a previous study, we detected an extracellular cysteine proteinase activity band specific for embryogenic suspension cultures of *D. glomerata* L. (Rakleova et al. 2010). Mass spectrometry analysis showed that it contained two cysteine proteinases (Fig. 5.2, spot 11 and spot 13). Both cysteine proteinases were cloned and identified as cathepsin B and cathepsin L (GU067465.1 and GU067466.2,

respectively). In an independent study, using N-terminal sequencing, we identified five isoforms of an acidic cysteine proteinase inhibitor – cystatin. The cystatin (designated *DgECPI*) was also cloned (GU065373.1) and expressed in *E. coli* (manuscript in preparation). It is well known that during SE, cystatins play a fundamental role in providing a suitable microenvironment of the embryogenic cell which is necessary for its further development into an embryo. At the same time, the surrounding somatic cells are prevented to dedifferentiate into competitive embryogenic cells (Rose and Lee 2010; Wolf et al. 2012). A putative role of the cystatin *DgECPI* might be the control of cell proliferation activated by cysteine proteinases or it might take part in the remodeling of the plant cell wall during embryo development. A cysteine proteinase inhibitor and cysteine proteinase were more abundantly expressed in embryogenic suspension cultures of *C. sativus* (Sharifi et al. 2012) and EC of cowpea (Nogueira et al. 2007). The opposite action of a cysteine proteinase (*OsCP*) and a cystatin (*OC-1*) was found to control cell proliferation in rice suspension cultures (Tian et al. 2009). Kusumawati et al. (2008) identified three secreted proteases only in an embryogenic cell line of *Medicago truncatula*. Redifferentiation processes during SE are involved in the general reprogramming of gene expression (chromatin remodeling, transcription machinery), and they require complex changes in the protein pattern and proteolysis.

Thus, a fine control of the protease activity during embryogenesis, organogenesis, programmed cell death, and tolerance to abiotic and biotic stresses mediated by protease inhibitors is of utmost importance (Benchabane et al. 2010; Martínez et al. 2012).

5.4 Concluding Remarks

Within the last 10 years, transcriptomics and proteomics studies have significantly contributed to an improved understanding of plant SE (Elhiti et al. 2013; Rocha and Dornelas 2013; Fehér 2014). Proteomics of SE has allowed the precise identification and quantification of differentially

expressed proteins in embryogenic and nonembryogenic cell lines, in the different stages of SE, and in somatic and zygotic embryos. The results indicate that a complex molecular system is turned on during SE to control processes such as ROS detoxification, energy metabolism, protein synthesis and processing, and cell division among others. An interesting outcome of these studies is the potential use of certain proteins as molecular markers for specific developmental stages or to differentiate embryogenic from nonembryogenic genotypes.

Stress-induced in vitro somatic embryogenesis is an extreme example of plant developmental plasticity (Fehér 2014). The expression of totipotency in cultured somatic cells is part of a general stress-adaptive process that involves a fine regulation of hormone and stress signaling resulting in the restart of cell division and embryogenic competence acquisition. There is probably no single model applicable to all plant species for differentiating into an embryo. However, the observation that embryogenic tissues of different origins and obtained with different hormones display similar protein profiles indicates a general behavior of cellular metabolism that can give valuable insights into the mechanisms of SE (Correia et al. 2012).

The protein expression data complemented with that of transcriptomics and metabolomics will certainly help understand the molecular basis of plant SE and allow the development of more effective in vitro regeneration protocols for many plant species.

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