

The Life and Death Signalling Underlying Cell Fate Determination During Somatic Embryogenesis

Andrei Smertenko and Peter Bozhkov

Abstract Somatic embryogenesis (SE) is a sequence of stereotypical morphological transformations, which results in differentiation of cells into a plant body bypassing the fusion of gametes. As such, it represents a very powerful tool in biotechnology to propagate species with long reproductive cycles or low seed set and the production of genetically modified plants with improved traits. The initiation of SE can be divided into five major stages: (i) perception of extracellular signals or stress stimuli, (ii) transduction of the extracellular signal through the cytoplasm into the nucleus, (iii) induction of gene transcription required for embryogenesis, (iv) reorganisation of cytoplasm and (v) onset of embryonic development. The further embryonic development during SE resembles its zygotic counterpart and begins with the establishment of apical-basal asymmetry. The apical domain, the embryo proper, proliferates and eventually gives rise to the plantlet, while the basal part, the embryo suspensor, becomes a subject of terminal differentiation and gradually degrades via vacuolar programmed cell death (PCD). This PCD is essential for normal development of the apical domain. Some signalling events in the apical and basal domains share homologous components. Here, we describe our current knowledge on the control of life and death processes during SE.

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1 Introduction

SE was described by Williams and Maheswaran (1986) as “the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes”. The lack of gamete fusion in SE implies high genetic homogeneity of all derived in this way plants. SE is a common process in nature (Raghavan 1976; Tisserat, et al. 1979; Vasil and Vasil 1980) and can occur as early in plant development as during embryogenesis. A group of cells in embryos of any developmental stage or even plantlets can give rise to new embryos leading to polyembryony or adventive embryony.

In mature plants of some species (e.g. *Kalanchoe*), dedifferentiation of somatic cells can also lead to embryo formation. Propagation through SE allows formation of multiple genetically identical embryos under favourable conditions avoiding the need to wait for the following reproductive season, and this reproductive acceleration bears vast evolutionary importance.

SE can commonly be initiated in vitro from non-differentiated cells which still possess “embryogenic potential” such as explants from microspores, ovules, embryos and seedlings (Williams and Maheswaran 1986). Cultivation of these explants on media containing an appropriate balance of plant growth regulators (PGRs) can induce proliferation of embryo-forming (embryogenic) callus cultures. The formation of embryos from “dedifferentiated” cells in vitro is called indirect SE (see chapter by Opatrný in this volume for the “dedifferentiation” of callus cells). Embryo development can also be initiated in vitro directly from explant cells without passage through callus formation phase, by a process called direct SE.

The production of physiologically normal and genetically homogenous plants through SE has multiple applications for biotechnology, from propagation of species, which take long time to reach the flowering stage or yield insufficient numbers of seeds, to production of genetically modified cultivars with engineered properties. The translational value drives research into characterisation of molecular pathways controlling SE and refined approaches to increase yield and quality. However, the conditions for SE are species specific and require optimisation throughout all its stages from the induction of SE until embryo maturation and germination. New genetic data from model species including thale cress (*Arabidopsis thaliana*) and Norway spruce (*Picea abies*) have significantly advanced our understanding of the molecular mechanisms regulating embryogenesis and have aided in the improvement of existing technologies and development of approaches for induction of SE in recalcitrant species.

Apart from its industrial importance, SE represents an important set of paradigms to study early signalling and morphogenetic processes in plant embryogenesis. As zygotic embryogenesis takes place deep within maternal tissues and the embryos are minute, the molecular, biochemical and cellular analyses of the developmental process are experimentally difficult. In contrast, SE yields macroscopic amounts of material at specific developmental stages for biochemical

studies. These exposed somatic embryos are also suitable for cell biology and live-cell imaging studies.

2 Biology of SE

While in nature SE often originates from cells still possessing “embryogenic potential” (e.g. polyembryony), the first important step of indirect SE is reprogramming of the cell fate which is accompanied by changes in the gene transcription (see below; see also chapter by [Opatrný](#) in the current volume). The first system of indirect SE was established in *Daucus carota* (Steward et al. 1958; Reinert 1958). The observations made in this system demonstrated that while callus cells were assumed to be totipotent and their ability to produce embryos should not be determined by the tissue of origin, in reality, embryogenic cultures from various species are composed of morphologically distinct cell types. One type, often referred to as proembryogenic masses, is composed of small, actively proliferating cells with dense cytoplasm, large nuclei, small vacuoles, smooth surface and high metabolic activity. These cells are accompanied by non-embryogenic cells, which are bigger, rougher, with larger vacuoles and therefore more translucent.

Somatic embryos can originate from a single cell (unicellular pathway; Haccius 1978) or from a group of two or more associated cells (multicellular pathway; Raghavan 1976). Both pathways appear to be redundant and can act in the same species in case of both direct (Maheswaran and Williams 1985; Trigiano et al. 1989) and indirect (Toonen et al. 1994; Fernandez et al. 2000; Somleva et al. 2000) SE.

2.1 SE Resembles Zygotic Pathway

The morphology of somatic embryo development is remarkably similar to zygotic embryogenesis (Fig. 1; von Arnold et al. 2002). Firstly, zygotic embryogenesis starts from a single cell followed by formation of globular embryo containing a defined number of cells. SE starts from a single cell or a group of cells and attains globular structure containing variable number of cells. How a group of cells initiates embryo formation is not clear, but considering our knowledge about zygotic embryogenesis, an asymmetric distribution of auxin is probably established (de Smet et al. 2010). In some instances, an early step in the initiation of SE communication with the surrounding cells is interrupted by increased cell wall thickness and blockage or dismantling of the plasmodesmata (Button et al. 1974). Inside the isolated single cell or cell cluster, the chain of signalling processes initiates cell divisions in parallel with the establishment of embryo polarity. In many cases, however, the embryo-forming group of cells retain plasmodesmata

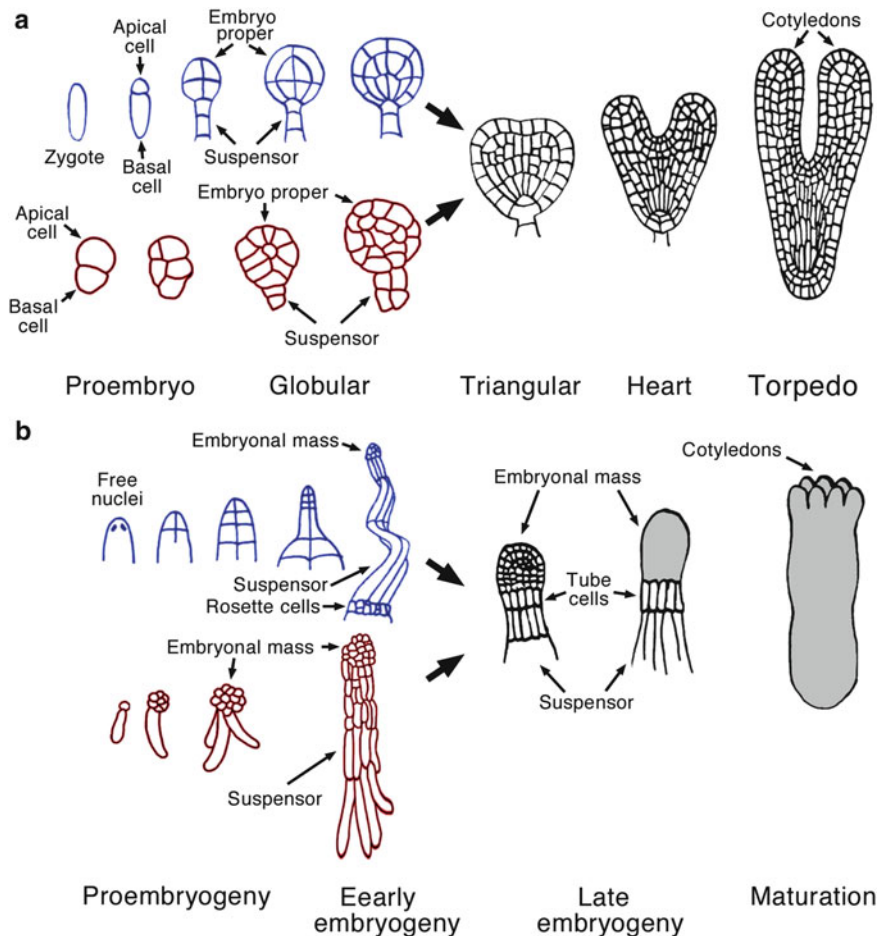


Fig. 1 Comparison of somatic and zygotic embryogenesis pathways in angiosperms (a) and gymnosperms (b)

Stereotypical morphological stages of zygotic and somatic embryogenesis are shown in *blue* and *red*, respectively. The stages of both pathways with common morphology are shown in *black*. The embryo structures are not drawn to scale

In angiosperms, both zygotic (shown for *Arabidopsis*) and somatic (shown for carrot; Street and Withers 1974) embryogenesis start with asymmetric divisions producing cells with different fates. The apical cell proliferates, eventually giving rise to the new plant, while the basal cell forms a terminally differentiated suspensor which disappears by the heart stage

The zygote in gymnosperms contains free nuclei, then undergoes cellularisation and divisions and gradually forms suspensors during the early embryogenic stage. During the somatic pathway, the polar structures composed of proliferating cells on one pole of the embryo and large vacuolated non-proliferating cells on the opposite pole appear already during proembryogeny (Filonova et al. 2000b). The vacuolated cells give rise to the suspensor, which, similarly to the angiosperms, demises during maturation. Zygotic embryos in some genera contain small rosette cells at the base of suspensor, which can divide and form aberrant embryos (rosette polyembryony). Cleavage polyembryony, when the early embryo splits into four or more identical embryos while only a single embryo normally develops to maturity, is a common feature of multiple genera of

contacts with the neighbouring cells and do not display any apparent changes in the cell wall thickness (Williams and Maheswaran 1986).

The somatic embryo development encompasses key stages of zygotic embryogenesis characteristic of the respective taxon: heart and torpedo in case of dicotyledonous species; globular, scutellar and coleoptilar in case of monocotyledonous species; and early and late embryogeny in case of gymnosperm species (Fig. 1; Zimmerman 1993; Singh 1978). This requires establishment of two domains: apical and basal. The apical domain gives rise to shoot apical meristem, cotyledons and hypocotyl, while the basal domain originates root meristem and the root.

The embryo suspensor, a multicellular basally situated organ formed during the first divisions of zygote, plays a pivotal role in the establishment of apical-basal polarity (Zimmerman 1993; Kawashima and Goldberg 2010). The suspensor is a terminally differentiated organ subjected to gradual elimination by vacuolar PCD (Bozhkov et al. 2005a). Cell proliferation and tissue patterning events in the embryo proper must be balanced by terminal cell differentiation and death in the suspensor (see the Suspensor section below).

The mature somatic embryos resemble zygotic embryos, both morphologically and physiologically (Zimmerman 1993; von Arnold et al. 2002; Bozhkov et al. 2005a). Both exhibit apical-basal and radial polarity, possess primary shoot and root meristems and contain typical embryonic organs such as radicle, hypocotyl and cotyledons. The key genes controlling zygotic embryogenesis perform similar roles during SE (Mordhorst et al. 2002). Somatic embryos accumulate similar nutrients required for subsequent germination of the seedling and target them to the same cellular compartments; however, the timing of accumulation and exact proportion between individual types of nutrients can vary (Merkle et al. 1995; Yeung 1995). In addition, somatic embryos may not require desiccation (desiccation is important for SE in *Medicago sativa* and *Picea* spp.) and skip the dormancy period. Instead, they initiate the shoot meristem and seedling growth, eventually producing morphologically normal and fertile plants.

2.2 Induction and Maintenance of SE by External Factors

Comparison of somatic and zygotic embryogenesis shows that the induction and maintenance of embryogenic potential requires exogenous factors, i.e. growth medium or maternal tissues, respectively. The subsequent progression of embryogenesis towards tissue patterning and organ establishment results from the execution of genetic programmes in the cells of globular embryos. This genetic programme results in a stereotypical sequence of morphological changes regulated



Fig. 1 (continued) gymnosperms (e.g. *Pinus*; Fílonova et al. 2002). In SE, cleavage polyembryony was reported even in species which lack this feature during zygotic embryogenesis (e.g. *Picea*). In the latter case, the additional embryos form from the embryonal mass cells. Both zygotic and somatic pathways are shown for *P. abies*

intrinsically by conserved mechanisms with minimal or no impact from surrounding tissues or environment.

Initiation of embryogenic cell cultures is accompanied by the induction of specific sets of genes, primarily those responsible for control of the cell cycle (Zimmerman 1993; Feher et al. 2003; Yang and Zhang 2010). The core cell cycle machinery responsible for driving cell proliferation appears to be conserved between embryogenic cultures and plant meristems (Hirt et al. 1991; De Jong et al. 1993; Emons 1994).

2.2.1 The Role of Hormonal Factors

The gene expression as well as the pattern of cell divisions is regulated by plant growth regulators (PGRs) and composition of the growth medium. Synthetic auxins, in particular 2,4-dichlorophenoxyacetic acid (2,4-D), were originally used for induction and maintenance of embryogenic cell cultures. Other classes of PGRs including cytokinins (Sagare et al. 2000), abscisic acid (ABA; Senger et al. 2001), jasmonates and brassinosteroids (Jimenez 2005) were successfully used for initiation of embryogenic cell lines in different plant species. The variability in embryogenic induction can be explained by the dual role of PGRs. At supra-physiological concentrations, PGRs induce stress responses in addition to their role in signalling, and these stress responses play an essential role in reprogramming gene expression patterns (Feher et al. 2003; Karimi and Saidi 2010). In agreement with this hypothesis, abiotic stresses, such as heat stress, can induce SE even in the absence of added PGRs (Simmonds and Keller 1999; Dubas et al. 2011).

Maintenance of proliferating embryogenic cultures requires a continuous supply of exogenous PGRs auxin and cytokinin. In some instances, for example, in the alfalfa model, only a short (from several minutes and up to several hours) pulse of a relatively high concentration (100 μM) of auxin is sufficient to stimulate embryogenesis (Hirt et al. 1991).

Embryogenic cultures proliferating in the presence of auxin, with or without cytokinin, produce embryos resembling the globular stage of zygotic pathway but composed of variable number of cells. Transition to the next stages is induced by withdrawal of auxin from the medium. This leads to the onset of histogenesis and initiation of tissue and organ patterning. At the same time, the embryo starts to synthesise its own auxin (Michalczuk et al. 1992a, b). While the regulation of auxin distribution in somatic embryos remains unknown, the transition through successive stages of zygotic embryogenesis is accompanied by re-localisation of auxin efflux carrier (PIN proteins) and establishment of auxin gradients through the embryos (Fischer and Neuhaus 1996; Friml et al. 2002, 2004). The exogenously applied auxin interferes with the formation of these gradients and inhibits transition from globular to heart stage. Likewise, application of the polar auxin transport inhibitor 1-N-naphthylphthalamic acid to embryogenic cultures affects cell divisions and interferes with apical-basal patterning of somatic embryos (Fischer and Neuhaus 1996; Larsson et al. 2008).

The maturation of somatic embryos may require additional growth regulators, such as ABA. Although ABA can be produced endogenously by somatic embryos (Hatzopoulos et al 1990), the efficiency of embryo maturation is higher in the presence of exogenous ABA (von Arnold et al. 2002). In zygotic embryos, ABA induces dormancy and upregulates a set of specific genes, known as *Late Embryogenesis Abundant (LEA)*; Dure et al. 1989). Although somatic embryos do not pass through a classical dormancy period, treatment with ABA induces several *LEA* genes including *DC8*, *DC59*, *ECP31* and *ECP40* (Zimmerman 1993), suggesting that ABA induces a semi-dormancy period in somatic embryos, which plays an important role in SE.

2.2.2 Nonhormonal Factors

Apart from hormones, pH and Ca^{2+} are important factors for the induction of SE. The optimal pH of the culture medium lies in the mild acidic range between 4.0 and 5.8 (Von Arnold et al. 2002), while alkaline media inhibit SE (Pasternak et al. 2002), suggesting that initiation of SE requires active ionic transport across membrane.

Calcium as a well-known secondary messenger during plant development and stress responses (Sanders et al. 1999), not surprisingly, also plays a role in SE, especially considering that stress plays an important role in the initiation of the whole process. For example, induction of embryogenesis in carrot cell cultures requires at least 0.2 mM calcium (Overvoorde and Grimes 1994), and increase of calcium concentration from 1 to 10 mM causes a twofold increase in the efficiency of embryogenesis (Jansen et al. 1990). Conversely, sequestering of calcium in the medium using ethylene glycol-bis(aminoethyl ether)- $\text{N,N}'$ -tetraacetic acid (EGTA) inhibits embryogenesis (Anil and Rao 2000). Calcium could even counteract the inhibitory effect of 2,4-D on the initiation of embryogenesis from proembryogenic masses (Jansen et al. 1990).

3 Cell Fate During SE: “Pro-Life” Signalling

Considering the remarkable morphological resemblance of somatic and zygotic embryogenesis, it is assumed that all key regulators of the zygotic pathway discovered in *A. thaliana* and other systems using genetic approaches (De Smet et al. 2010) are equally active in SE. This assumption is supported by numerous experimental studies described below (Table 1). The research on SE is focused on the identification of key regulators that improve the induction and yield of the whole process. While the initiation of embryogenic cultures depends on the developmental stage of the starting plant material and factors in the growth medium (for details, see chapter by Opatrný in this volume) perceived by complementary cellular sensors, the maintenance of the embryogenic potential during subsequent

Table 1 Regulators known to improve the efficiency of somatic embryogenesis

Name	Functions	Species	Overexpression phenotype	References
Agamous-Like 15 (AGL15)	MADS-box TF	<i>A. thaliana</i>	Primary somatic embryogenesis from zygotic embryos and also in shoot apical meristem	Harding et al. 2003
BABY BOOM (BBM1)	AP2/ERF family TF	<i>Glycine max</i> <i>Brassica napus</i> , <i>A. thaliana</i>	Enhanced indirect somatic embryogenesis Direct somatic embryogenesis on seedlings	Thakare et al. 2008 Boutiller et al. 2002; El Ouakfaoui et al. 2010
		<i>Nicotiana tabacum</i>	Sterility if constitutive; spontaneous organogenesis if inducible	Srinivasan et al. 2007
		<i>Capsicum annuum</i>	Indirect somatic embryogenesis in otherwise recalcitrant plants	Heidmann et al. 2011
		<i>Populus tomentosa</i>	Indirect somatic embryogenesis	Deng et al. 2009
EMBRYOMAKER (EMK)	AP2/ERF family TF	<i>A. thaliana</i>	Enhanced direct and indirect somatic embryogenesis	Tsuwamoto et al. 2010
LEAFY COTYLEDON1 (LEC1)	HAP3 domain TF	<i>A. thaliana</i>	Induction of direct somatic embryogenesis	Lotan et al. 1998
LEAFY COTYLEDON2 (LEC2)	B3 domain TF	<i>A. thaliana</i>	Induction of direct somatic embryogenesis without auxin	Stone et al. 2001; Ledwon and Gaj 2011
SHOOTMERISTEMLESS (STM)	Homeobox domain TF	<i>Brassica oleracea</i>	Enhanced efficiently of indirect somatic embryogenesis	Elhiti et al. 2010
WUSCHEL (WUS)	Homeobox domain TF	<i>A. thaliana</i>	Induction of direct somatic embryogenesis without auxin	Zuo et al. 2002
		<i>Coffea canephora</i>	Enhanced efficiently of indirect somatic embryogenesis	Arroyo-Herrera et al. 2008
HBK3	Homeobox domain TF	<i>P. abies</i>	Increased yield of somatic embryogenesis	Belmonte et al. 2007
RKD4	RWP-RK domain TF	<i>A. thaliana</i>	Short-term expression promotes somatic embryogenesis without auxin	Waki et al. 2011
Polycomb Repressive Complex 2 (PRC2)	Trimethylation of histone H3 on K27	<i>A. thaliana</i>	Inhibits embryogenesis, downregulation promotes embryogenesis	Chanvittana et al. 2004

azadC	Inhibitor of DNA methylation	<i>A. thaliana</i>	Upregulation of STM and higher yield of somatic embryos	Elhiti et al. 2010
SERK1	Receptor kinase	<i>A. thaliana</i> <i>Oryza sativa</i>	Enhanced efficiency of indirect somatic embryogenesis Constitutive overexpression boosts the frequency of shoot regeneration in vitro and increased blast resistance in vivo	Hecht et al. 2001 Hu et al. 2005
10 Gibberellin 2-oxidase 6 (GA2ox6)	Gibberellin metabolism	<i>A. thaliana</i>	Constitutive overexpression increases somatic embryo production	Wang et al. 2004

cultivation requires the activity of signalling and genetic pathways, which in turn lead to specific cellular responses such as cytoplasmic remodelling, modification of the division patterns and cell/tissue differentiation. The success of this reprogramming requires a developmental dichotomy: whereas some cells are assigned for survival (“pro-life”), others have to be eliminated by PCD (“pro-death”). In the following sections, the signalling “pro-life” will be considered.

3.1 Cell Wall Components

SE is accompanied by modifications in structure and molecular composition of the cell wall (Emmons 1994; Malinowski and Filipecki 2002). These changes are thought to be important to establish a balance of the mechanic forces required for the maintenance of specific cell shapes and cellular architecture and the determination of the division plane (Malinowski and Filipecki 2002). Apart of controlling the morphogenic events inside the cell, the cell walls are responsible for the communication with neighbouring cells via apoplastic or symplastic flow.

The cells produce a plethora of signalling factors, which upon export into the cell walls can spread through apoplast to surrounding cells and stimulate embryogenesis. In liquid cultures, these factors accumulate in the medium and promote embryogenesis in the whole culture (von Arnold et al. 2002).

Some of these signalling factors isolated from the conditioned medium of the embryogenic cell cultures were able not only to support embryogenesis in low-density embryogenic cultures, which otherwise fail to initiate embryo development (Smith and Sung 1985; de Vries et al. 1988), but could, in addition, induce embryogenic transformation of non-embryogenic cultures (Hari 1980). The outer cell wall surface of embryogenic cells contains specific arabinogalactan proteins (AGPs) and pectins, which are so specific that they can be used as markers of embryogenic cell cultures (Rumyantseva et al. 2003; Konieczny et al. 2005).

3.1.1 Extracellular Proteins

Several cell wall-modifying enzymes are differentially expressed during SE. For example, induction of SE in divergent species is accompanied by upregulation of xyloglucan endotransglycosylases (Malinowski and Filipecki 2002; Thibaud-Nissen et al. 2003; Rensing et al. 2005). This class of enzymes modifies the structure of xyloglucan chains and alters mechanical properties of the cell wall. SE of *Pinus radiata* is accompanied by upregulation of α -D-galactosidase (named SEPR1), which cleaves terminal α -galactosyl moieties of glycolipids and glycoproteins and can thus modify structure and properties of cell wall components (Aquea and Arce-Johnson 2008). Several members of the pectinesterase gene family (SEPR91, SEPR110 and SEPR114) are downregulated during early embryogenesis, but since pectinesterases can cause both stiffening and loosening of the cell

wall, it is difficult to understand the functional context of this downregulation, until the stiffness of the cell wall in embryogenic cells is measured.

The functions of many cell wall proteins in SE remain enigmatic. For example, a glycine-rich protein CEM6 was isolated from a screen for early embryogenesis-abundant genes in carrot cultures (Sato et al. 1995). It is upregulated from pre-globular to early heart stages and contains hydrophobic cell wall localisation signal. A gene encoding a similar glycine-rich protein Atgrp-5 was upregulated during SE in *A. thaliana* and eggplant (Magioli et al. 2001). CEM6 and Atgrp-5 were suggested to play a role in cell wall stiffness-related modifications required for early embryogenesis.

Extracellular glycosylated acidic class IV endochitinase or extracellular protein 3 (EP3) is involved in the transition from globular to heart stage in carrot embryogenic cultures (de Jong et al. 1992). The exact function of endochitinase is unknown; nonetheless, it appears to be a part of a phylogenetically conserved pathway, since endochitinase from sugar beet stimulates SE in the cell cultures of *P. abies* (Egertsdotter and von Arnold 1998). Interestingly, the endochitinase is expressed only in a subset of morphologically distinct cells in the embryogenic cultures located outside the proembryogenic masses and not in the developing somatic embryos themselves. In the seeds, endochitinase is not expressed in the embryos but in the inner integumentary cells of young fruits and in a specific subset of cells located in the middle of the endosperm of mature seeds (van Hengel et al. 1998). These findings suggest that endochitinase is required for the processing of signalling molecules which play a nurturing role during both somatic and zygotic embryogenesis.

The nonspecific lipid transfer proteins (LTP, Sterk et al. 1991) belong to an abundant class of proteins (extracellular protein 2, EP2) found in the conditioned medium of carrot embryogenic lines. The level of LTP expression in cotton cell lines is high before induction of embryogenesis and during the globular stage and then diminishes during post-globular stages (Zeng et al. 2006). The LTPs were implicated in the control of protoderm (the outermost layer of cells above a meristem) differentiation (Thoma et al. 1994; Dodeman et al. 1997). Correspondingly, ectopic overexpression of LTPs under control of a 35S promotor affects establishment of bilateral symmetry of the embryos and disturbs epidermal cell layer morphology (Francois et al. 2008). At the cellular level, LTPs were implicated in the transport of phospholipids or other nonpolar molecules from the endoplasmic reticulum to other cellular compartments (Sterk et al. 1991; Kader 1996; Toonen et al. 1997), so they might be responsible for the stabilisation and transport of signalling molecules through apoplast and symplast.

Germin-like proteins (GLP) belong to one of the most abundant groups of extracellular proteins found in embryogenic lines of *Pinus caribaea* (Domon et al. 1995). Several studies showed upregulation of transcription of GLP-encoding genes in embryogenic lines of other species (Neutelings et al. 1998; Wojtaszek et al. 1998; Çaliskan et al. 2004). In all cases, their expression was limited to embryogenic cells. GLPs are part of the cupin superfamily of ubiquitous plant proteins (Dunwell et al. 2008) with divergent primary sequences,

but conserved tertiary structure. They all are hydrogen peroxide-producing enzymes of two types: oxalate oxidase or superoxide dismutase. Embryogenesis-related GLPs are of the oxalate oxidase type, but their glycosylated form isolated from the conditioned medium possessed no apparent activity. Contrary, a high oxalate oxidase activity of GLPs was detected in the embryogenic lines of wheat (Çalışkan et al. 2004), leading the authors to conclude that active forms of GRPs reside in the cell wall and increase cell wall rigidity by producing hydrogen peroxide, which cross-links glucuronoarabinoxylan polymers. The involvement of GLPs in embryogenesis is further supported by the finding that transcription of an *A. thaliana* cupin At4g36700 is regulated by AGL1, a transcription factor, which controls both zygotic and somatic embryogenesis (Zheng et al. 2009).

3.1.2 Arabinogalactan Proteins

AGPs are a heterogeneous group of molecules composed of a polypeptide, a long chain of branched glycan and a lipid (Fig. 2a; Majewska-Sawka and Nothnagel 2000). Commonly, more than 90 % of the AGP molecule can be constituted by carbohydrates. Only specific types of AGPs can stimulate SE. For example, a fraction of AGPs recognised by the antibody ZUM18 promoted embryogenesis, while the fraction recognised by antibody ZUM15 were inhibitory (Kreuger and van Holst 1995). Removal of AGPs from the culture medium using anti-AGP antibody or AGP-binding synthetic phenyl glycoside (Yariv reagent) blocks SE (McCabe et al. 1997; Butowt et al. 1999; Thompson and Knox 1998; Chapman et al. 2000), while addition of AGPs to old cultures and cultures with low embryogenic potential can promote embryogenesis (Kreuger and van Holst 1995; Egertsdotter and von Arnold 1995). In contrast to the situation with endochitinases, cells producing active AGPs localise within embryos (McCabe et al. 1997).

Although numerous studies have demonstrated a signalling role of AGPs during embryogenesis (Egertsdotter and von Arnold 1995; Kreuger and van Holst 1995; Butowt et al. 1999; Thompson and Knox 1998; Chapman et al. 2000), the molecular mechanisms behind this activity remain poorly understood. One of the possible scenarios involves activity of chitinases. Chitinases can cleave off the glycosyl chains of AGPs (Domon et al. 2000; Passarinho et al. 2001), and the released oligosaccharins can serve as signalling molecules to promote SE (Darvill et al. 1992; Svetek et al. 1999). Chitinase EP3 co-localises with AGPs in developing seeds, providing further evidence that the EP3/AGP signalling module plays a key role in embryogenesis (van Hengel et al. 1998). In agreement with this hypothesis, inactivation of AGPs by Yariv reagent inhibits SE (Chapman et al. 2000). However, treatment of AGPs with endochitinase EP3 produces more active AGPs (Van Hengel et al. 2001). This questions the role of glycosyl residues of AGPs for the induction of SE. Furthermore, a phycocyanin-like domain of AGP from cotton that

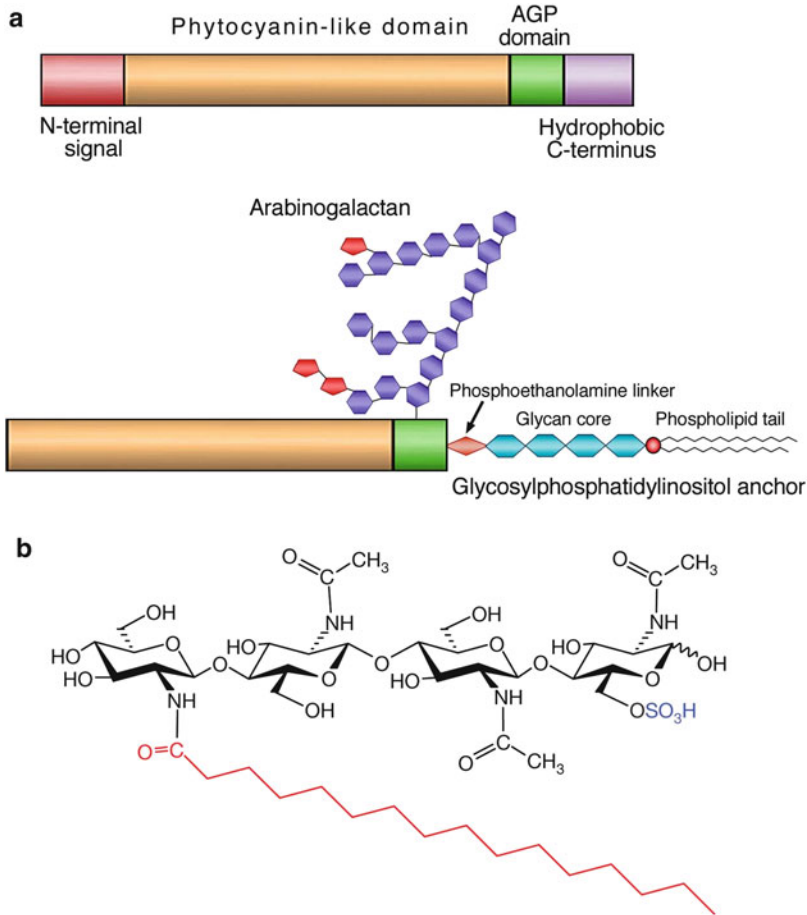


Fig. 2 Structure of the extracellular signalling molecules. **(a)** Hypothetical structure of an arabinogalactan protein (AGP; Poon et al. 2012). The N- and C-terminal domains of the precursor are cleaved, and then the resulting C-terminal region is modified by addition of an arabinogalactan and a glycosylphosphatidylinositol (GPI) anchor which targets AGP molecule to the cell surface. **(b)** Lipochitoooligosaccharide (LCO) responsible for induction of arbuscular mycorrhiza produced by the fungus *Glomus intraradices* (Maillet et al. 2011). The structure of LCOs responsible for the induction of SE is unknown, but likely to resemble the structure given here. The sulphate group shown in blue is optional. The acyl chain can vary (shown in red) and in this particular case can be palmitic (C16:0) or oleic (C18:1 $\Delta 9Z$) fatty acids

lacks glycosylation motifs was alone sufficient to induce SE (Poon et al. 2012). It was so far not possible to assign regulation of SE to the protein or glycan components of AGPs owing to inconsistency of data obtained in different model systems. These inconsistencies may reflect species-specific features of AGP signaling.

3.1.3 Oligosaccharines

A class of oligosaccharines, called lipochitooligosaccharides (LCOs), was originally identified as nodulation (Nod) factors secreted by bacteria of the genus *Rhizobium* to promote plant cell division and formation of nodules for subsequent colonisation by *Rhizobium* (Spaink et al. 1991; Truchet et al. 1991). Nod factors have a uniform backbone made of 3 to 5 chitin (1,4-linked N-acetyl-D-glucosamine) residues with the N-acetyl groups on the terminal nonreducing sugar substituted for an acyl chain (Fig. 2b). The specific activity of LCOs is determined not only by the number of chitin residues and the structure of the acyl chain but also by addition of monosaccharides such as arabinose, mannose and fucose as well as the attachment of sulphate, acetate or carbamoyl groups (Downiw and Walker 1999). Rhizobial Nod factors can stimulate SE up to the globular stage by promoting cell divisions in *D. carota* (De Jong et al. 1993) and *P. abies* cell cultures (Egertsdotter and von Arnold 1998; Dyachok et al. 2000). Embryogenic cell lines produce their own type of LCOs with similar structure to Nod factors. The fraction of conditioned medium enriched with these LCOs can stimulate SE (Dyachok et al. 2002).

3.2 Perception and Transduction of Extracellular Signals

The soluble molecules present in the medium are perceived by the receptor kinases located in the plasma membrane. While the ligands of many receptor kinases are not known, it is plausible that they are produced in the cell walls. The signal can then affect cytoplasmic processes or be transduced into the nucleus where it modulates transcription.

3.2.1 Receptor Kinases

SE receptor-like kinases (SERKs) belong to an evolutionary conserved superfamily of leucine-rich repeat receptor-like kinases (LRR-RLK; Becraft 1998). The first member of this group was identified in maize using degenerate PCR primers to kinases (Walker and Zhang 1990). Later this group was extended by many key regulators of signal transduction during plant development and environmental adaptation, which, apart from SERK, include CLAVATA1 (CLV1), Erecta1, brassinosteroid-insensitive 1 (BRI1) and Crinkly4 (Becraft 1998). All members of this superfamily share an intracellular kinase domain, an extracellular leucine-rich repeats (LRR) domain and several juxtamembrane phosphorylation sites. LRR domains interact mutually resulting in homo- or heterodimerisation (see also chapter by [Robatzek](#), this volume). LRR-RLK can autophosphorylate in

monomeric form and trans-phosphorylate in the oligomerised state modulating in this way interaction of kinases with other proteins and their effectors.

SERKs were originally identified from carrot embryogenic cultures (Schmidt et al. 1997). Their expression levels are elevated in proliferating embryogenic cultures and at the early stages of embryogenesis up to the heart stage (Schmidt et al. 1997; Somleva et al. 2000; Salaj et al. 2008). Like all LRR-RLK, SERKs localise to the plasma membrane (Shah et al. 2001). Similarly, during zygotic embryogenesis in *D. carota*, a SERK was expressed from the eight-celled stage through to the globular stage, while no transcript was detected in unfertilised flowers (Schmidt et al. 1997). The transcription of SERKs is upregulated by auxin (Nolan et al. 2003). The genomes of all angiosperm species examined so far contain several genes encoding SERKs, e.g. five in case of *A. thaliana* and six (excluding three SERK-like homologues) in case of *Medicago truncatula*. Four out of six *M. truncatula* isotypes are upregulated during induction of SE (Nolan et al. 2011), suggesting functional redundancy of individual members of the gene family. Consistently with this conclusion, only quadruple SERK knockout of *A. thaliana* exhibited embryo lethal phenotype (Gou et al. 2012). Ectopic expression of *Arabidopsis* SERK1 isotype promotes formation of somatic embryos (Hecht et al. 2001). Altogether, SERKs are potent signalling molecules for induction and regulation of SE.

There is an intriguing link between SERKs and brassinosteroid signalling. SERK1 was found in a complex with brassinosteroid-insensitive 1 (BRI1) kinase and can trans-phosphorylate BRI1 (Karlova et al. 2006). SERK1 phosphorylation is enhanced in the presence of brassinosteroid, and, in turn, BRI1 can trans-phosphorylate SERK1 (Karlova et al. 2009). So far, no substrates of SERK1 have been identified, but considering that the BRI1 pathway is dependent on SERK activity (Gou et al. 2012) and steroids are required for both somatic (Pullman et al. 2003) and zygotic (Schrack et al. 2004) embryogenesis, it is conceivable that SERKs, at least as one of their functions, can modulate brassinosteroid signalling during embryogenesis. Although they belong to the same phylogenetic group of LRR-RLK, SERK and CLV1 play opposite roles in SE. CLV1 reduces expression of transcription factors promoting embryogenesis and consequently inhibits SE (Elhiti et al. 2010).

3.2.2 Ca²⁺ Effectors

Induction of SE in carrot cell cultures requires at least 0.2 mM calcium (Overvoorde and Grimes 1994), and rising concentration from 1 to 10 mM causes twofold increase in the efficiency of embryogenesis (Jansen et al. 1990). Initiation of embryogenesis from proembryogenic masses of carrot and sandalwood on hormone-free medium is accompanied by increased calcium uptake from the medium, resulting in increased overall intracellular calcium content (Timmers et al. 1996; Anil and Rao 2000). In addition, a short spike of 10- to 16-fold increase of intracellular Ca²⁺ (up to 600–860 nM) was detected by Fura-2 ratiometric

analysis within 10 s after removal of 2,4-D from the medium (Anil and Rao 2000). Interference with the intracellular calcium homeostasis using calcium channel blockers or the ionophore A23187 resulted in complete suppression of embryogenesis (Overvoorde and Grimes 1994; Anil and Rao 2000), similarly to what have been observed when Ca^{2+} in the growth medium was omitted or chelated. This suggests that a specific pattern of intracellular calcium concentration is required to initiate embryogenesis. Consistently, inhibition of calcium signalling by W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide) decreased SE efficiency by 85 % (Anil and Rao 2000). Calcium influx can be translated into physiological responses via several classes of effectors (Galon et al. 2010). So far the role of two classes of calcium effectors during embryogenesis was addressed: calmodulins and calcium-dependent protein kinases (CDPK).

Transcription and protein accumulation of calmodulins can vary between different model systems. In several model systems, the transcription was fairly constant in the course of embryogenesis (Oh et al. 1992; Overvoorde and Grimes 1994), and no changes were detected in calmodulin methylation, which can alter specific activity of calmodulin (Overvoorde and Grimes 1994). In *P. abies*, however, the level of calmodulin was upregulated immediately after initiation of embryo development upon removal of PGRs (van Zyl et al. 2003). The expression of CDPKs and their activity was upregulated in calcium-dependent manner in both proembryogenic masses and somatic embryos (Anil and Rao 2000; Kiselev et al. 2008). Xu and colleagues (1999) used DM-Bodipy-PAA to label calcium changes at the early stages of zygotic embryo development. They reported that the probe preferentially labelled the basal part of the embryo. Therefore, it might be a calcium gradient, but not the overall concentration of calcium effectors, that could mediate the early stages of embryo polarisation and organ patterning.

3.3 Gene Expression

The exogenous signals can be transduced by protein kinase pathways into the nucleus resulting in the switch of gene expression. This switch can be attributed in part to the activity of transcription factors and in part to the epigenetic modifications of chromatin.

3.3.1 Transcription Factors

3.3.1.1 Heme Activator Protein 3 (HAP3) Related

HAP is a multimeric transcriptional activator complex, which recognises the CCAAT box and in plants consists of the three subunits HAP2, HAP3 and HAP5 (Edwards et al. 1998). While in yeast and animals each subunit is encoded by a single gene, in plants, each subunit is encoded by gene families. Two members of

the HAP3 family, LEAFY COTYLEDON1 (LEC1) and LEAFY COTYLEDON1 LIKE (L1L), are key regulators of zygotic embryogenesis (Lotan et al. 1998; Kwong et al. 2003). During the early stages of embryogenesis, LEC1 and L1L specify cotyledon cell identity and maintain the fate of suspensor cells (Meinke 1992; Meinke et al. 1994; West et al. 1994), while during the later stages, they control initiation and/or maintenance of the embryo maturation, but also suppress precocious germination (Parcy et al. 1997; Lotan et al. 1998).

Ectopic overexpression of *LEC1* leads to early growth arrest of the seedling and abnormal plant development, occasionally accompanied by the formation of somatic embryo-like structures (Lotan et al. 1998). Therefore, LEC1 seems to confer embryogenic potential to somatic cells. The gain-of-function mutant of *LEC1*, turnip (*tnp*), exhibits a milder phenotype with ectopic cell divisions and a loss of tissue identity (Casson and Lindsey 2006). Interestingly, the penetrance of the *tnp* phenotype is enhanced by auxin, inhibited by cytokinin, but not affected by ABA and GA. This attributes to LEC1 an important role in the switch of cell identity during auxin-induced dedifferentiation and the proliferation of embryogenic cells. The transcription of *LEC1* is negatively regulated by PICKLE, a chromodomain-helicase-DNA-binding protein 3 (CDH3) chromatin remodelling factor (Ogas et al. 1999). The contribution of LEC1 to SE is far from being clear, but elevated expression levels of *LEC1* are characteristic for embryogenic cell lines and not found in non-embryogenic cell lines of *A. thaliana* (Ledwon and Gaj 2011). Additionally, the conifer *LEC1* homologue (*HAP3A*) is upregulated during SE in *P. abies* and *Pinus sylvestris* (Uddenberg et al. 2011). Contrary, the ectopic expression of *CHAP3A* (*Picea mariana* homologue of LEC1) in embryogenic cell lines did not affect the efficiency of SE (Klimaszewska et al. 2010).

3.3.1.2 B3-Domain Transcription Factors

The B3-domain proteins belong to a plant-specific family of transcription factors with common tertiary structure of seven beta strands and two alpha helices, which binds to the major groove of the DNA double helix but recognises different motifs. This group includes several major regulators of embryogenesis: LEAFY COTYLEDON2 (LEC2), FUSCA3 (FUS3) and ABA INSENSITIVE3 (ABI3) of *A. thaliana* and Viviparous1 (Vp1) of *Zea mays* (McCarty et al. 1991; Luerssen et al. 1998; Stone et al. 2001). LEC2 and FUS3 are important for the early patterning and later embryo maturation stages, while ABI3 is important only for embryo maturation (Stone et al. 2001). Overall, the roles of B3-domain and HAP3-related transcription factors during embryogenesis overlap (Lotan et al. 1998). Consistently, postembryonic ectopic expression of *LEC2* leads to formation of somatic embryos, calli and cotyledon/leaf-like structures (Stone et al. 2001), while downregulation of LEC2, FUS3 and ABI3 significantly inhibits both direct and indirect SE (Gaj et al. 2005).

LEC2 upregulates expression of two transcription factors Agamous-Like 15 (AGL15; see next section) and aux/IAA30 (Braybrook et al. 2006).

Transcription of *AGL15* is upregulated in the embryos, and consequently, overexpression of *AGL15* increases efficiency of induction of embryogenic cell lines in response to exogenous auxin (Harding et al. 2003). *AGL15* in turn upregulates transcription of *aux/IAA30* and *LEC2* (Zheng et al. 2009). *Aux/IAA30* regulates gene transcription in response to auxin (Sato and Yamamoto 2008; see also chapter by Skůpa et al., this volume). Therefore, these three transcription factors form a positive feedback loop essential for activation of an embryogenic programme in somatic cells. In addition, *LEC2* increases transcription of two flavin monooxygenases, *YUC2* and *YUC4*, responsible for the auxin synthesis. Consequently, the overall auxin level in the seedlings ectopically expressing *LEC2* was significantly higher than in the control plants (Stone et al. 2008).

Expression of *FUS3* is regulated by auxin (Gazzarrini et al. 2004). Along with *LEC1* and *LEC2*, *FUS3* is essential for the induction of SE (Gaj et al. 2005). At the same time, *FUS3* upregulates the synthesis of ABA and downregulates synthesis of GA during embryo maturation (Gazzarrini et al. 2004). This alteration of the hormonal balance inhibits cell divisions and delays senescence during leaf differentiation. In this way, *FUS3* couples auxin signalling with the equilibrium of physiological responses to GA and ABA. However, the changes in the hormonal balance regulated by *FUS3* do not apparently reduce embryogenic potential of cells in mature embryos.

Another member of the B3-domain proteins, *VP1/ABI1-LIKE (VAL)*, represses transcription of *LEC1*, *LIL*, *FUS3* and *ABI3* genes, but not of *LEC2*, and knockout of *VAL* mimics ectopic expression phenotypes of *LEC1*, *LIL* and *LEC2* (Suzuki et al. 2007), resulting in embryo-like outgrowth in the region of apical meristem and callus-like formations on the roots.

3.3.1.3 Agamous-Like 15 (*AGL15*)

AGL15 belongs to a divergent family of eukaryotic transcription factors found in phylogenetically distant species from yeast to human and angiosperm plants. All members of this family contain a conserved MADS-box motif within their DNA-binding domain. The name MADS-box derives from the first letters of four originally identified proteins: *MCM1* (minichromosome maintenance-defective1 from *Saccharomyces cerevisiae*), *AG* (*AGAMOUS* from *A. thaliana*), *DEFA* (*DEFICIENS* from *Antirrhinum majus*) and *SRF* (serum response factor from *Homo sapiens*). *AGL15* expression peaks during embryo development starting as early as the globular stage. In agreement with its role, *AGL15* localises to the nuclei of both embryo proper and suspensor. After germination, *AGL15* is transiently expressed in the young shoot apical meristem and floral buds (Heck et al. 1995; Rounsley et al. 1995; Perry et al. 1996; Fernandez et al. 2000; Thakare et al. 2008). The expression of *AGL15* is under tight control of embryo development, and premature termination of embryogenesis in *lec1-2* abolishes expression of *AGL15* (Perry et al. 1996).

Embryogenic cultures exhibit high levels of *AGL15* expression (Thakare et al. 2008). Consistently, ectopic expression of *AGL15* increases the efficiency of both direct and indirect SE (Harding et al. 2003; Thakare et al. 2008), while knockout of *AGL15* reduces the efficiency of SE (Thakare et al. 2008). Importantly, *AGL15* is integrated into different signalling processes during embryogenesis. Firstly, *AGL15* is a component of the SERK complex (Karlova et al. 2006); secondly, its expression is controlled by *LEC2* and is upregulated by auxin (Gazzarrini et al. 2004; Braybrook et al. 2006; Zhu and Perry 2005); thirdly, it promotes transcription of *LEC2*, *FUS3* and *ABI3* (Zheng et al. 2009); fourthly, *AGL15* in cooperation with *LEC2* and *FUS3* reduces the content of active gibberellin and induces expression of gibberellin catabolising GA 2-oxidases *GA2ox6* (Wang et al. 2004) and *GA2ox2* (Zheng et al. 2009). Since it controls the level of active gibberellin, *GA2ox6* is a potent regulator of SE: overexpression of *GA2ox6* increases efficiency of SE by almost sevenfold (Wang et al. 2004).

3.3.1.4 AP2/ERF Domain Proteins

APETALA2/ethylene-responsive factor (AP2/ERF) proteins are a family of plant-specific transcription factors involved in the regulation of a plethora of developmental processes, such as flower meristem identity, flower patterning, lateral root morphogenesis and response to environmental factors, in addition to causing pleiotropic effects on cell size, plant height and fertility (Dietz et al. 2010). The size of the AP2/ERF gene families in phylogenetically divergent species reflects this functional diversity and at the same time correlates with the complexity of morphology and life cycle. For example, the *A. thaliana* genome encodes 147 members, while *Selaginella moellendorffii* and *Chlamydomonas reinhardtii* genomes encode 57 and 14 members, respectively.

Several members of the AP2/ERF family regulate SE. The *M. truncatula* homologue of *A. thaliana* ERF, *MtSERF1*, is an ethylene-inducible gene expressed in zygotic embryos, proliferating embryogenic cell cultures and somatic embryos (Mantiri et al. 2008). The localisation of expression is restricted to the shoot apical meristem region of the heart-stage embryo. Knockdown of *MtSERF1* inhibits SE. Another member, *A. thaliana* *EMBRYOMAKER* (*EMK*), is expressed in early and mature embryos and has a redundant role in maintaining embryonic cell identity (Tsuwamoto et al. 2010). Ectopic expression of *EMK* promotes initiation of somatic embryos from cotyledons.

The best-studied member of the AP2/ERF domain family is BABY BOOM (BBM). Boutilier and co-authors (2002) reported that *BBM* is expressed during all stages of zygotic embryogenesis in *A. thaliana* starting from the globular stage and up to mature seeds. Furthermore, *BBM* was identified as a marker of SE in cell cultures of *Brassica napus* being expressed during all stages of the embryogenesis. Ectopic expression of *BBM* enhances SE and other morphogenic responses. Direct SE, formation of ectopic shoots and calli could be induced in *A. thaliana* and *B. napus* even without any PGRs (Boutilier et al. 2002; El Ouakfaoui et al. 2010).

Overexpression of *BBM* resulted in the induction of indirect SE in tobacco (Srinivasan et al. 2007), poplar *Populus tomentosa* (Deng et al. 2009) and the otherwise recalcitrant *Capsicum annuum* (green pepper; Heidmann et al. 2011) in the presence of PGRs.

In germinating seedlings, *BBM* is expressed in root meristems (Nawy et al. 2005), but constitutive expression of *BBM* does not lead to the formation of neither ectopic root meristems nor roots. This suggests that *BBM* works together with other regulatory elements in the tissue-specific environments. In agreement with this conclusion, *BBM* upregulates TUBBY-LIKE PROTEIN 8 (TLP8), a transcription factor of as yet unknown function (Passarinho et al. 2008). TLP8 in turn is a target of the transcription factor LEAFY (LFY), which is responsible for flower meristem identity and activation of pathways guiding specification of flower organs (William et al. 2004).

3.3.1.5 Homeodomain Transcription Factors

The homeodomain is a highly conserved 60-amino acid long region characteristic for transcription factors found in all eukaryotes. Homeodomain-containing transcription factors regulate different developmental processes including SE. One phylogenetic group of homeodomain containing transcription factors includes *A. thaliana* WUSCHEL (*WUS*) and its fourteen homologues (Mayer et al 1998; Palovaara and Hakman 2008; see also chapter by Opatrný in this volume). *WUS* plays an essential role in keeping cells in a state of proliferation and responsiveness to other developmental cues (Mayer et al 1998; Gallois et al 2002). This activity maintains shoot and flower apical meristems during embryonic and postembryonic development, but does not have any apparent role in the maintenance of the root meristem (Laux et al. 1996). *WUS* is expressed only in a group of cells (organising centres) underlying the shoot and floral apical meristems starting from the 16-celled embryo stage. The lack of *WUS* expression in the meristem cells suggests that it acts in a non-cell autonomous manner (Mayer et al. 1998). Genetic studies revealed that *WUS* is controlled by the LRR-RLK *CLV1* pathway forming a self-regulatory loop in which *CLV1* acts above *WUS*. While *WUS* promotes proliferation of the stem cells, which express members of the *CLV1* pathway, the *CLV1* pathway suppresses *WUS* on the transcriptional level and in this way controls the size of the stem cell niche (Haecker and Laux 2001). The discovery of a *WUS*-binding motif in the promoter of AP2/ERF transcription factor MtSERF1 suggests that *CLV1* controls other transcriptional networks through *WUS* (Mantiri et al. 2008).

The efficiency of callus induction from *wus* and wild-type plants was the same (Mordhorst et al. 2002), indicating redundancy of *WUS*. The subsequent formation of somatic embryos was not assessed by Mordhorst et al. (2002), but in another work, genetic suppression of *WUS* by ectopic expression of *CLV1* significantly reduced the responsiveness to SE stimuli which could be rescued by higher concentrations of 2,4-D (Elhiti et al. 2010). Ectopic inducible expression of *WUS* inhibits seedling development but promotes direct SE from young embryos and

from differentiated tissues in the absence of 2,4-D. The embryos started to form from somatic cells after only several divisions (Zuo et al. 2002). Conversely, knockdown of *WUS* inhibits SE from *B. napus* microspores (Elhiti et al. 2010). The ability of *WUS* to reprogram vegetative tissues towards embryonic development makes it a very useful tool for overcoming difficulties of inducing SE in recalcitrant species. For example, ectopic expression of *A. thaliana WUS* in *Coffea canephora* promoted hormone-induced callus formation and increases the yield of somatic embryos by 400-fold (Arroyo-Herrera et al. 2008).

Angiosperm genomes contain close homologues of *A. thaliana WUS* and large families of WUSCHEL-related homeobox (WOX) proteins. Gymnosperms lack the *WUS* clade, but harbour *WOX* members falling into the conserved clades present in angiosperm species (Palovaara and Hakman 2008). Two members of the *P. abies WOX* gene family, *WOX2* and *WOX8/9*, from independent conserved phylogenetic clades exhibit higher level of transcription during all stages of zygotic embryogenesis, in embryogenic cell lines and during all stages of SE (Palovaara and Hakman 2008; Palovaara et al. 2010). However, the level of *WOX2* was found to be similar in embryogenic and non-embryogenic cell lines (Palovaara and Hakman 2008).

The second group of homeodomain fold transcription factors important for SE are the Knotted1-like homeobox (KNOX) family proteins. This group regulates balance between cell proliferation and cell differentiation during tissue patterning and hence is important for plant development (Hay and Tsantis 2010). The founding member of this group, maize *Knotted1 (kn1; Vollbrecht et al. 1991)*, maintains stem cell niches in the shoot apical meristem by preventing premature differentiation of proliferating cells (Hay and Tsantis 2010), and correspondingly, *kn1* lacks a shoot apical meristem (Vollbrecht et al. 1991). The Arabidopsis KNOX family consists of two clades, KNOXI and KNOXII. KNOXI group consists of four members, *SHOOTMERISTEMLESS (STM)*, *BREVIPEDICELLUS (BP)*, *kn1-like in A. thaliana 2 (KNAT2)* and *KNAT6*. The role of KNOXII group in embryogenesis remains unknown. The phenotype of *stm* resembles *kn1* and *wus* in that it lacks shoot apical meristem (Long et al. 1996). This suggests that *STM* cooperates with *WUS* to maintain the stem cell niche and to regulate the balance between cell proliferation and differentiation, with the difference that *STM* is expressed in all cells of the shoot apical meristem, while *WUS* is expressed only in an organising centre subtending the meristem.

STM expression becomes detectable in the shoot apical meristem at the 32-celled zygotic embryo stage (Long et al. 1996) and persists in shoot apical and floral meristems during postembryonic development. *STM* is also upregulated during SE. The soybean *KNAT2* homologue *SBH* (soybean homeobox-containing gene) is expressed in early somatic embryos reaching maximum transcript level at the cotyledonary stage and decreasing thereafter (Ma et al. 1994). Ectopic expression of *B. napus STM* promotes SE (Elhiti et al. 2010). *P. abies* KNOXI homologues *HBK* (homeobox of KNOX class) are markers and important regulators of SE. *HBK2* is expressed in somatic embryos, but not in a cell line lacking embryogenic potential (Hjortswang et al. 2002). *HBK1* and *HBK3* are upregulated immediately after initiation of embryogenesis on the medium without PGRs.

The expression of *HBK4* becomes apparent during later stages, after degradation of the suspensor, and together with *HBK2*, it plays a role in the establishment of shoot apical meristem (Larsson et al. 2012). Ectopic expression of another member of the *P. abies* KNOXI family, *HBK3*, enhanced the yield of somatic embryogenesis. The shoot apical meristem in these embryos was significantly larger than in wild-type embryos. Accordingly, the downregulation of *HBK3* inhibited embryogenesis (Belmonte et al. 2007).

The KNOXI and WUS pathways are intertwined in the regulation of SE. STM upregulates transcription of *WUS*, and conversely, higher level of *WUS* promotes SE. In addition, STM enhances transcription of genes involved in the hormonal perception (Elhiti et al. 2010), which implies that higher efficiency of SE can be achieved by simultaneous upregulation of both pathways.

3.3.1.6 RKD4

Arabidopsis RKD4 (RWP-RK domain 4) belongs to the RWP-RK group of plant-specific transcription factors conserved in all lineages starting from unicellular algae. The members of this group contain a highly conserved motif RWPxRK (Schäuser et al. 1999) originally isolated as a key regulator of symbiotic root-nodule development. Mutation of RKD4 results in a smaller embryo suspensor, because of suspensor cell identity lost, and defective seed germination (Waki et al. 2011; Jeong et al. 2011). *RKD4* transcription was detected in all cells during early embryogenesis, but starting from the late globular stage, it was confined to the embryo-suspensor cells (Waki et al. 2011). Induction of ectopic expression of *RKD4* for 8 days activates the expression of embryogenesis-related genes, initiates embryogenesis in somatic cells and promotes SE, whereas constitutive ectopic expression of *RKD4* results in continuous proliferation without differentiation (Waki et al. 2011).

3.3.2 Epigenetic Mechanisms

The dedifferentiation of somatic cells and initiation of SE rely on the alteration of gene transcription profile and take up to several weeks. During this time, hormones and stress in addition to changing the gene transcription profile induce epigenetic modifications of the chromatin. This exemplifies notion that cell fate in plants depends on the “genomic memory” as well as positional information. So far, several epigenetic mechanisms have been implicated in the control of SE, including DNA methylation, histone post-translational modifications and microRNA (miRNA) pathways.

3.3.2.1 DNA Methylation

DNA methylation is one of the main heritable genomic modifications that modulate transcriptional activity of specific sequences. This involves covalent attachment of a methyl group to the cytosine base at the CpG, CpHpG and CpHpH sites (where p stands for phosphate and H denotes either A, T or C). The reaction is catalysed by three families of DNA cytosine methyltransferases: chromomethylase (CMT), domains rearranged methyltransferase (DRM) and methyltransferase (MET). In *Arabidopsis*, DRM2 acts as a de novo methylase, and DRM2, MET1 and CMT3 transfer methylation marks to the newly synthesised strand of DNA during replication (Cao and Jacobsen 2002).

Gene silencing regulated by DNA methylation plays a vital role in SE. For example, the promoter region of *LEC1* becomes hypomethylated prior to initiation of SE, whereas methylation levels increase during embryo maturation and subsequent vegetative growth. Hypermethylation of a region within the promoter of *LEC1* using RNA-directed DNA methylation (RdDM) downregulates its transcription. This indicates that transcription of *LEC1* is regulated by methylation of its promoter (Shibukawa et al. 2009). Experiments with modulation of global DNA methylation levels in vivo using inhibitors of methylation generated inconclusive results. Inhibition of methylation in carrot cultures with 5-azacitidine blocked embryogenesis (Yamamoto et al. 2005). In contrast, inhibition of methyltransferase 1 with the drug 5-aza-2'-deoxycytidine (azadC) promoted embryogenesis and increased transcription of the key embryonic regulator *STM* (Elhiti et al. 2010). 5-azacitidine is known to exert a general cytotoxic effect at higher concentrations (Čihák 1974), which could have been the reason for the inhibition of embryogenesis.

3.3.2.2 Histone Post-translational Modifications

Gene expression can also be regulated on the level of chromatin organisation. Chromatin is a complex of DNA and proteins inside eukaryotic nucleus. The basic repeat unit of chromatin is the nucleosome, a 146-base pair unit of DNA wrapped around an octamer protein core containing two copies of each histone protein subunits H2A, H2B, H3 and H4. Nucleosomes are linked together in a fibre of 30 nm thickness by the histone protein H1. This arrangement is important for packaging long DNA molecules into the small volume of the nucleus and at the same time serves as a mechanism of regulation of DNA replication and gene transcription. Transcriptionally inactive chromatin, called heterochromatin, considered to be more packed, while transcriptionally active euchromatin has more relaxed packing. A complex of proteins responsible for the modification of chromatin organisation facilitates the epigenetic regulation of genes (Jarillo et al. 2009). Post-translational modifications of histones, including methylation of lysine and arginine, acetylation or ubiquitination of lysine and phosphorylation of serine

residues, contribute to the establishment and maintenance of chromatin activity states (Costa and Shaw 2007).

Histone acetylation on ϵ -amino groups of lysine residues neutralises the overall positive charge of histones and weakens their interaction with the negatively charged DNA. This relaxes chromatin packaging and promotes the transition into the euchromatin state. Histone acetylation-dependent chromatin remodelling modifies transcription of factors regulating embryogenesis and in this way controls transition from proliferation to embryonic growth. Knockdown of *A. thaliana* *PICKLE*, which encodes subunit CHD3 of histone deacetylase complex, upregulates transcription of *LECI* and delays transition from embryonic to vegetative growth (Ogas et al. 1999). Treatment of seedlings with a gibberellin synthesis inhibitor increased the penetrance of the *PICKLE* phenotype, indicating that its role in suppressing embryonic growth was partially complemented by a redundant gibberellin-dependent pathway. Furthermore, treatment of *P. abies* embryogenic cultures with the inhibitor of histone deacetylase Trichostatin A prior to induction of embryogenesis repressed embryo development and reduced transcription of a key regulator of embryogenesis, the B3-domain transcription factor *PaVPI* (Uddenberg et al. 2011).

Similar to acetylation, histone methylation modifies chromatin packing, but depending on the site, it may either inhibit or stimulate transcription. For example, S-adenosyl-methionine (SAM)-dependent transmethylation can modulate expression of cell cycle regulators (Jones and Wolffe 1999). Several members of the SAM metabolic pathway were upregulated during early stages of hormone-induced cellular dedifferentiation preceding the establishment of embryogenic cell cultures in cotton (Zhu et al. 2008) and during early embryogenesis in *P. abies* (van Zyl et al. 2003).

In addition to the SAM-dependent pathway, histone methylation-dependent silencing is controlled by the highly evolutionary conserved group of Polycomb (PcG) proteins. The Polycomb Repressive Complex 2 (PRC2) is a histone methyltransferase responsible for trimethylation of histone H3 on lysine 27 (Schubert et al. 2006). Plant PRC2 consists of four subunits: the first subunit is encoded by *CLF* (Curly Leaf), *Swn* (Swinger) and *MEA* (Medea); the second subunit by *FIE* (Fertilisation Independent Endosperm); the third subunit by *MSI1* (Multicopy Suppressor of *Ira* 1); and the fourth subunit is encoded by *FIS2* (Fertilisation Independent Seed 2), *EMF2* (Embryonic Flower 2) and *VRN2* (Vernalisation 2). The PRC2 complex is responsible, in particular, for silencing of *STM* (Schubert et al. 2006). The knockout of *FIE* results in callus outgrowth on germinating seedlings (Bouyer et al. 2011). Reduced activity of PRC2 in double homozygous *clf* and *swn* plants results in callus formation and SE even in the absence of PGR (Chanvivattana et al. 2004).

3.3.2.3 MicroRNAs

MicroRNAs (miRNAs) are small, single-stranded, endogenous transcripts capable of forming a stem-loop hairpin structure that can mediate target gene silencing by RNA cleavage or translational inhibition. In this way, miRNAs can act cooperatively with transcription factors to fine-tune gene expression programmes during cell differentiation and proliferation (Willmann and Poething 2007; Willmann et al. 2011). Multiple examples demonstrate the key role of miRNA in the regulation of cell proliferation *in vitro* and SE. Analysis of the miRNA profile in embryogenic callus of rice identified several candidates (e.g. miR397 and miR398) with potential roles in maintaining cells in the meristematic state by mediating gene silencing (Luo et al. 2006). Using Japanese larch as a model system, Zhang and colleagues (2010) identified four distinct miRNA families with different expression in embryogenic and non-embryogenic calli. These miRNAs can target to multifunctional transcription factors involved in regulation of development, ABA signal transduction and response to abiotic stress. miRNAs regulate expression of the transcription factors LEC2 and FUS3 in early embryogenesis of *A. thaliana* (Willmann et al. 2011). A detailed analysis of miRNA profiles during successive stages of indirect SE in sweet orange (Wu et al. 2011) identified miRNA specific for early embryo development, globular embryos, cotyledon-shaped embryos and cell lines lacking embryogenic potential.

3.4 Cytoskeleton

The cytoskeleton is a complex network of cytoplasmic filaments and, in plants, is composed of two principal systems: microtubules and actin filaments. The cytoskeleton determines the composition and properties of the cell wall and interacts with the cell wall components in controlling the distribution of organelles and maintaining cytoplasmic architecture and cell shape. In addition, the cytoskeleton provides tracks for the intracellular transport of organelles and molecules during interphase and cell division. Microtubules are hollow tubes of 25 nm in diameter, composed of α - and β -tubulin heterodimers. During interphase, specific microtubules subtend the plasma membrane forming the “cortical array” controlling the direction of cell expansion, whereas a different set of microtubules tether and position the nucleus (for details, refer to the chapter by Nick in this volume). Actin filaments (F-actin) are 7 nm thick fibers composed of two twisted chains of protein actin. Like microtubules, F-actin can be cortical or endoplasmic.

The role of the cytoskeleton in SE was extensively studied during androgenesis (formation of embryos from cultured microspores) in rapeseed, *B. napus*. Direct androgenesis can be induced from immature pollen (microspores) of the responsive cultivar Topaz by heat stress (Simmonds and Keller 1999; Dubas et al. 2011; see also chapter by Opatrný in this volume). The process starts from a single easily accessible microspore cell containing, depending on the stage of development,

either one or two (vegetative and generative) nuclei (for review, see chapter by Lou et al. in this volume). In case of two nuclei, only the vegetative nucleus responds to the heat treatment. In both cases, the otherwise asymmetrically positioned nucleus moves to the centre of the cell, and subsequently, the microspore divides symmetrically. The central positioning of nucleus is important for the induction of androgenesis in rapeseed, but can vary among species. In case of osmosis-induced androgenesis in barley, the uninucleate microspore must divide asymmetrically to produce generative and vegetative nuclei. Contrary to *B. napus* system, the symmetric division of the microspore in barley does not lead to embryonic development (Maraschin et al. 2005).

The effect of heat stress on the induction of androgenesis in *B. napus* can be mimicked by destabilisation of microtubules using colchicine (25 μ M for 42 h). This treatment caused relocation of the nucleus to the centre of the cell, followed by symmetric division and induction of androgenesis (Zhao et al. 1996). Similarly, depolymerisation of actin by 15-min treatment with high concentration (20 μ M) of cytochalasin D followed by continuous incubation in a medium containing 2 μ M cytochalasin D resulted in the relocation of the nucleus to the central position and induction of androgenesis (Gervais et al. 2000). Thus, induction of the embryogenesis might require a more flexible cytoskeleton. The basal cell (which is generally not surrounded by exine; for details, see the chapter by Lou et al. in this volume) undergoes a series of transverse divisions forming a suspensor-like file of 3–8 cells, while the apical cell (constrained by exine) passes through several rounds of transverse and longitudinal divisions leading to the formation of globular embryo proper (Dubas et al. 2011; Tang et al. 2013).

Induction of SE is accompanied by changes in the expression of the cytoskeletal structural proteins. For instance, an α -tubulin in wheat is upregulated (Singla et al. 2007), while α 1-tubulin in *P. glauca* and β 3-tubulin gene in *P. abies* are downregulated (Lippert et al. 2005; van Zyl et al. 2003). An *A. thaliana* β 2/3-tubulin (At5g62700) is a target of the key transcription factor regulating embryogenesis, AGL15 (Zheng et al. 2009). An F-actin regulator actin-depolymerising protein 9 (ADF9) is a target of BABY BOOM during SE (Passarinho et al. 2008). Actin-depolymerising proteins sever microfilament bundles and facilitate plasticity of F-actin required for the morphogenesis.

4 Cell Fate Regulation During SE: “Pro-Death” Signalling

Plant development requires removal of specific organs and tissues (e.g. leaf and flower senescence) by PCD. In addition, PCD is indispensable for the differentiation of some tissues like xylem or bark. Consistently, numerous studies demonstrate the importance of PCD for both zygotic and somatic embryogenesis highlighting elimination of embryo suspensor as the key PCD process that defines the success of embryogenesis.

4.1 *Functions of the Embryo Suspensor*

The suspensor is a part of the embryo, which does not contribute to the postembryonic phase of plant life. Both the embryo suspensor and embryo proper originate from the zygote following the first division after fertilisation. The apical daughter cell gives rise to the embryo proper, while basal cell develops into suspensor. The embryo proper and suspensor remain interconnected during early stages of embryogenesis.

The suspensor is thought to have co-evolved with the colonisation of land by plants over 450 million years ago (Kawashima and Goldberg 2010) based on the observation of a suspensor-like structure (foot) in bryophytes. In higher plants, the suspensor can have different size and morphology, ranging from only one cell in orchids or a file of seven small cells in *A. thaliana* or *B. napus* to hundreds of cells exceeding the size of the embryo proper in *Phaseolus coccineus* (scarlet runner bean). Somatic embryos form suspensors in many systems (Zimmermann 1993), and like in the case of the zygotic pathway, the suspensor plays an essential role in embryogenesis (Suarez et al. 2004). Three main functions have been attributed to the suspensor: (i) positioning of the embryo in the sack close to endosperm in angiosperms or the megagametophyte in gymnosperms; (ii) conduction of nutrients to the embryo proper using specialised cellular structures responsible for the cell-to-cell transport, including plasmodesmata and cell wall outgrowths (Yeung and Meinke 1993); and (iii) synthesis of the hormones auxin, gibberellic acid, cytokinin and ABA, which are essential for setting up the embryo polarity and regulation of the early stages of embryogenesis (Kawashima and Goldberg 2010).

The suspensor is a terminally differentiated structure that is removed by PCD. The elimination of the suspensor starts at the late globular stage and terminates by the beginning of torpedo stage in angiosperms or during late embryogeny in gymnosperms (Bozhkov et al. 2005a). Therefore, the original cell proliferation programme of the suspensor has to be switched into the self-destruction programme, whereas embryo proper cells continue to express factors facilitating cell proliferation, histogenesis and organ patterning.

Genetic studies highlight the entwining and complex signalling networks, maintaining the balance between embryo formation and suspensor degradation. For example, in *Arabidopsis* mutants *raspberry* and *twin*, suspensor cells are reprogrammed from the cell death pathway to a meristematic fate, resulting in abnormal embryogenesis (Vernon and Meinke 1994; Yadegari et al. 1994; Zhang and Sommerville 1997). Perturbation of suspensor development in mutants of transcription factors (*wox8* and *wox9*), MAP (*yda*, *mpk3*, *mpk6*) and receptor (*ssp*) kinases or the vacuole formation gene *vcl1* leads to embryo lethality. In turn, defective development of the embryo proper in the mutants *rsy2*, *rsy3*, *sus1/dcl1*, *sus*, *twn*, *lec1* and *lil* perturbs suspensor differentiation (Bozhkov et al. 2005a; Kawashima and Goldberg 2010). Inhibition of suspensor differentiation and death during SE has similar prohibitive effect on the progression of embryo development (Suarez et al. 2004; Helmersson et al. 2008).

The balance between survival and development of the embryo proper and death elimination of the suspensor plays a crucial role in zygotic embryo development and has a significant impact on the efficiency of SE. How this balance is established and then maintained is not clear, but intriguingly, the same groups of proteins can play a role in proliferation or cell death, depending on the molecular environment. For example, a member of evolutionary conserved family of the HAP3 transcription factors, LEAFY COTYLEDON1 (LEC1), is essential for specification of cotyledons during early embryogenesis and for the maintenance of suspensor cell fate (Meinke 1992; Meinke et al. 1994; West et al. 1994). Different members of homeodomain containing WUSCHEL transcription factors (WOX) that determine embryo polarity are expressed in the suspensor. WOX2 is specifically expressed in the apical cell, while other members of this family, WOX8 and WOX9, are expressed in the basal cell (Haecker et al. 2004). Understanding the molecular mechanisms of this balance can help to initiate embryogenic cultures and to enhance yield and quality of SE.

4.2 *Vacuolar PCD and SE*

Considering morphological and molecular markers available to date, two most common types of plant cell death have been categorised: vacuolar and necrotic (van Doorn et al. 2011). Vacuolar death is characterised by appearance of double membrane vesicles known as autophagic vacuoles (aka vacuoles, autophagosomes) that engulf portions of the cytoplasm. The cargo of autophagosomes is digested by fusion with lytic vacuoles (equivalent to the lysosomes of animal cells). Gradually, most of the cytoplasm becomes occupied by lytic vacuoles. The process culminates by rupture of the vacuole and apparently uncontrolled degradation of all remaining cellular content. Necrotic cell death is characterised by mitochondrial dysfunction, early rupture of the plasma membrane, protoplast shrinkage and incomplete processing of the cell corpse. This type of cell death typically occurs during plant responses to intracellular pathogens and acute abiotic stress (van Doorn et al. 2011).

Vacuolar PCD is essential for two main processes in plant embryogenesis: (i) establishment of embryo polarity and (ii) differentiation and subsequent elimination of the suspensor. The changes in the cellular organisation accompanying successive stages of vacuolar PCD during SE have been addressed in detail using the *P. abies* model (Fig. 3). This system has the advantage of developing very large multicellular suspensors of several millimetres in length. The suspensor cells undergo stereotypical stages of cell death similar to those reported in other angiosperm and gymnosperm species (Bozhkov et al. 2005a). Thus, there is a gradient of successive PCD stages along the apical-basal axis starting from the proliferating cells of the embryonal masses, initiation of terminal differentiation and commitment to PCD in the embryonal tube cells, execution of PCD in the proximal cells of the suspensor, and empty walled corpses at the distal end (Fig. 3; Smertenko et al. 2003; Bozhkov et al. 2005a).

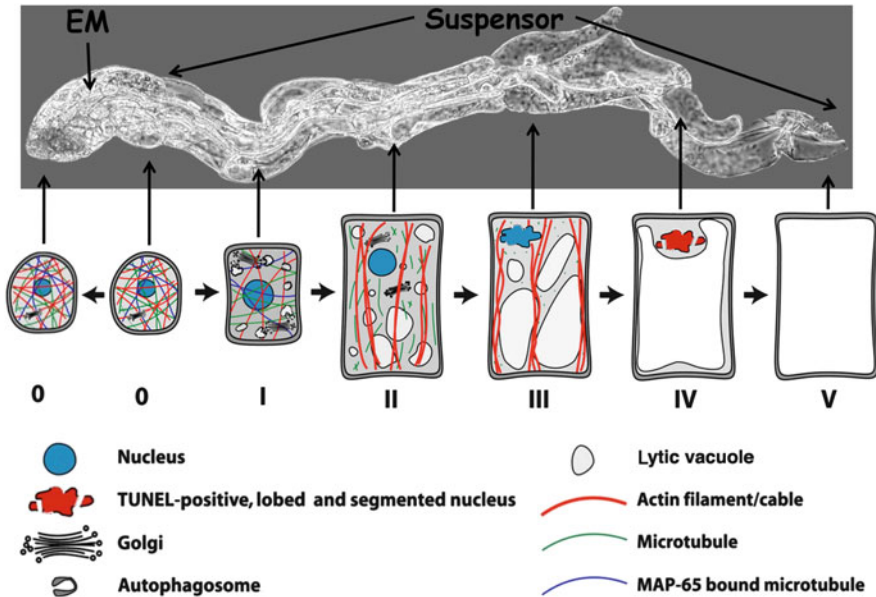


Fig. 3 Programmed cell death during somatic embryogenesis
 A proliferating cell in the embryonal mass (stage 0) can either persist in this stage or switch to differentiation becoming an embryonal tube cell (stage I). The embryonal tube cell at some time point commits to PCD and undergoes a series of stereotypical morphological alterations (stages II to IV), which lead to stepwise clearance of all cellular content by stage V. Terminal differentiation and PCD can be observed simultaneously along the apical-basal axis of the early embryo, with each cell tier of the suspensor featuring one stage

Stage 0 represents meristematic cells of embryonal masses/embryo proper. Cell divisions within embryonal mass provide new cells for growth of both embryo and suspensor. How a meristematic cell initiates terminal differentiation on its path towards a suspensor cell is not known. One scenario proposes that cells in the basal part of the embryonal mass undergo asymmetric division generating daughter cells of unequal structure, molecular composition and fate. Here, the cell fate is determined by both cell composition and positional signalling. This scenario has been shown to occur in other plant systems. For example, during the first division of microspore in *B. napus*, the cell with larger contact surface with exine has a dense cytoplasm and lacks vacuoles, while the cell with the least contact surface possesses distinct vacuoles (Tang et al. 2013). Apart from the vacuoles, the rest of cytoplasmic features including microtubule organisation are indistinguishable in both types of cells (Dubas et al. 2011). The vacuolated basal cell divides to generate the suspensor, while the meristematic or apical cell gives rise to the embryo proper. In case of androgenesis in barley, the early embryo consists of two domains with structurally distinct cells (Maraschin et al. 2005). One domain contains small vacuolated cells descending from generative nuclei and destined for PCD. The larger cells with dense cytoplasm in the second domain originate from the

vegetative cells and form the embryo proper. Alternatively, the cell fate within embryonal mass could be determined by its position relatively to hormonal gradients. There is evidence indicating genetic control of the early cell specification events in the *Arabidopsis* proembryo. The basal cell expresses transcription factor WOX8, while apical cells express a homologue of WOX8, WOX2 (Haecker et al. 2004).

The presence of lytic compartments appears to be the earliest morphological marker of suspensor cell differentiation. While the embryo proper/embryonal mass cells in the embryogenic cultures of *P. abies* contain neither lytic nor protein storage vacuoles, embryonal tube cells can be distinguished by de novo formation of autophagosomes (Filonova et al. 2000a; Fig. 3). In addition, the proliferation ceases, the cell shape changes from spherical to cylindrical, and the cytoskeleton undergoes dramatic reorganisation. The random network of microtubules seen in the meristematic cells becomes transformed into parallel array transversely to the apical-basal axis of the embryo (Fowke et al 1990; Smertenko et al. 2003). The proportion of microtubules bound to microtubule cross-bridging protein MAP65 is diminished in comparison to the embryonal mass cells indicating alterations of microtubule bundling.

All subsequent stages of vacuolar PCD take place in the suspensor, and each stage is typically confined to one tier of cells. Stage 2 is characterised by cell expansion, growing number of autophagosomes, formation of small lytic vacuoles, fragmentation of microtubule network and reduced MAP65 binding to microtubules. The cortical array of microfilaments is substituted by endoplasmic thick longitudinal actin cables (Binarová et al. 1996; Smertenko et al. 2003; Schwarzerová et al. 2010). The transformation of the F-actin network is accompanied by alteration of actin isotypes expression (Schwarzerova et al. 2010). F-actin cables contribute to the regulation of suspensor development by as yet unknown mechanism (Smertenko and Franklin-Tong 2011). Possibly, they regulate spatial distribution of autophagosomes, their fusion and growth of lytic vacuoles. In agreement with this hypothesis, treatment with the actin depolymerisation drugs latrunculin B and cytochalasin D impaired suspensor development and triggered cell death in the embryonal masses (Smertenko et al. 2003). A slight destabilisation of microfilaments by application of low concentrations of latrunculin B promoted cell death in the suspensors and increased the yield of mature embryos consistent with the role of actin in the control of PCD progression (Schwarzerová et al. 2010).

By stage 3, small lytic vacuoles gradually fill most of the cytoplasm and form several large vacuoles. At the same time, anisotropic cell expansion continues, F-actin cables persist, but the microtubule network disintegrates, and only microtubule fragments and tubulin aggregates remain in the cytoplasm. The main morphological feature of this stage is the onset of nuclear dismantling. The nuclear surface forms lobes, which subsequently become detached to produce nuclear segments that eventually are digested by the vacuoles. The nuclear pore complexes disassemble at the sites of nuclear segment dissociation, resulting in chromatin leakage to the cytoplasm.

During stage 4, the large lytic vacuole occupies the whole volume of the cell, F-actin cables disintegrate, the nucleus becomes fragmented, and chromatin aggregates and degrades. Nucleases cleave chromosomal DNA in the easily accessible inter-nucleosomal sites, resulting in accumulation of 50 kbp long chromatin loop-size fragments and 180 bp (and multiples of thereof) nucleosomal fragments (Filonova et al. 2000a). The degradation of the nucleus and the biogenesis of lytic vacuoles are two autonomous processes. Some nuclei become digested before formation of central lytic vacuole is completed (Filonova et al. 2000a). Mitochondria remain morphologically intact at this stage, but their biochemical activity is altered. The activity of alternative oxidase and external NADH dehydrogenase as well as fatty acid-mediated uncoupling increases, while the activity of mitochondrial K^+ channel decreases (Petruzza et al. 2009). The vacuolar PCD culminates in the rupture of the tonoplast and release of the lytic enzymes digesting all remaining cellular components and leaving a hollow walled cell corpse (stage 5; Fig. 3). The dead cells detach from the distal end of the suspensor.

Cell dismantling during vacuolar PCD in the suspensor takes about 5 days from the transition of a cell to the embryonal tube to complete clearance of the cell content. The transition from one stage of vacuolar PCD to the next takes approximately 24 h (a time required to form a new tier of the suspensor cells) with the exception of stage 5, which is apparently a swift process (Filonova et al. 2000b).

4.3 Cell Death Proteases and SE

All metazoan species from worms to humans share three core families of proteins essential for the regulation of PCD: CED-3/caspases, CED4/Apaf-1 and CED-9/Bcl-2 (Aravind et al. 1999, 2001; Koonin and Aravind 2002). On the basis of classification of “domains of death” present in key metazoan proteins, a common ancestral cell death machinery was proposed for all eukaryotic species (Ameisen 2002; Koonin and Aravind 2002). A growing body of bioinformatics and experimental data show a lack of direct homologues to the key animal cell death regulators in plants. However, some of these regulators are replaced by structurally and functionally related proteins.

The caspases belong to a family of cysteine-dependent *aspartate*-specific proteases (Cohen 1997; Thornberry and Lazebnik 1998). Members of this family are continuously expressed as inactive zymogens in almost all cell types. Following activation by an apoptotic signal, caspases cleave core components of the cytoskeleton, nucleus, signal transduction pathway, protein expression machinery, cell cycle regulators and both positive and negative regulators of cell death (Earnshaw et al. 1999). Combination of all proteolytic events compromises the survival pathways and promotes morphological changes characteristic for apoptosis. Since embryogenesis relies on cell death, caspase mutants exhibit abnormal embryo pattern formation and prenatal or perinatal lethality (Baehrecke 2002).

Plants do not have caspases but possess ancestrally related proteases called *metacaspases* (Uren et al. 2000; Tsiatsiani et al. 2011). Unlike caspases, metacaspases prefer substrates with arginine or lysine instead of aspartic acid in P1 position (Vercammen et al. 2004). On the basis of primary sequence and domain analysis, plant metacaspases are subdivided into two types. Type I metacaspases harbour N-terminal prodomain containing a proline-rich and a Zn-finger motifs. Type II metacaspases lack this N-terminal prodomain, but have a linker region between p20 and p10 catalytic subunits.

McII-Pa is the only member of type II metacaspases expressed in the embryo suspensor of *P. abies* (Suarez et al. 2004). McII-Pa is cytoplasmic during the first three stages of PCD, then associates with the nuclear membrane between stages 3 and 4 and finally localises inside the nucleus around condensed chromatin during later stages 4 and 5 (Bozhkov et al. 2005b). Enzymatically active mcII-Pa promotes nuclear lobing, chromatin condensation and DNA fragmentation in vitro and hence was suggested to be responsible for the disassembly of the nuclear envelope and the initiation of chromatin condensation. Knockdown of *mcII-Pa* results in the suppression of vacuolar cell death, inhibition of suspensor formation and failure of embryogenesis (Suarez et al. 2004).

The first natural substrate of mcII-Pa is a multifunctional regulator of gene expression, Tudor staphylococcal nuclease (TSN; Sundström et al. 2009), a member of multimeric complexes responsible for the transcription, mRNA splicing and RNA silencing (Tsiatsiani et al. 2011). TSN is a substrate of caspase-3, indicating that despite different substrate specificity of caspases and metacaspases, their substrates partially overlap. TSN and mcII-Pa are initially localised in the cytoplasm, where mcII-Pa-mediated cleavage of TSN may perturb post-transcriptional RNA processing and promote cell death.

Several other groups of proteases with no structural homology to caspases exhibit caspase-like substrate specificity in cell death-dependent manner (Bozhkov and Lam 2011). The first group is represented by *subtilisin-like serine proteases* (Vartapetian et al. 2011). These proteases recognise a wide range of peptidic substrates containing aspartic acid residue in P1 position, including VEID, VAD, YVAD, IETD, VDVAD and LEHD, but they do not cleave DEVD. On the basis of their substrate specificity, this group was initially named “saspases” for serine *asparagine-specific proteases*. An alternative name for saspases is phytaspases (Vartapetian et al. 2011). Like caspases, saspases or phytaspases prefer particular residues in the positions P2, P3 and P4. Processing of phytaspases includes cleavage of a prodomain, and under normal conditions, the active enzyme is exported to the apoplast space. Induction of PCD triggers import of phytaspases back to the cytoplasm, where they play a key role in the execution of PCD (Chichkova et al. 2010).

Although the precise role of phytaspases/saspases in embryogenesis remains unknown, we have previously reported that VEIDase activity, typical of this group of proteases, can be detected in the embryonal tube and suspensor cells during early embryogeny of *P. abies* and that this activity plays an important role in the suspensor differentiation and somatic embryo development (Bozhkov

et al. 2004). Pharmacological inhibition of VEIDase activity promotes proliferation of meristematic cell clusters, suppresses suspensor differentiation, prevents cell death and consequently abrogates embryogenesis (Bozhkov et al. 2004). Intriguingly, VEIDase activity was shown to localise in autophagosome-like structures in barley endosperm cells during the early stages of developmental PCD (Boren et al. 2006).

The second group of proteases is constituted by *vacuolar processing enzymes* (VPEs) with an YVADase, caspase-1-like, substrate specificity (Hatsugai et al. 2004). VPE exhibits caspase-like catalytic dyad, the catalytic site and the substrate Asp pocket. In addition, it is produced as inactive precursor and undergoes proteolytic processing to become an active enzyme. The activity of VPEs is upregulated in response to tobacco mosaic virus (TMV) infection and the active enzymes localise to the vacuoles, where they regulate maturation of vacuolar proteins during senescence and stress-induced cell death (Hara-Nishimura and Hatsugai 2011). The role of VPEs in SE remains unknown.

The caspase-3-like activity with the cleavage specificity towards DEVD peptide was attributed to proteasome. This activity is principal for both pathogen-induced cell death and vacuolar cell death in xylem (Hatsugai et al. 2009; Han et al. 2012). A significant increase of DEVDase activity accompanied androgenesis in barley (Maraschin et al. 2005), suggesting proteasome could regulate PCD during embryogenesis.

Recently, a novel regulatory module consisting of cathepsin protease NtCP14 and its inhibitor cystatin NtCYS was shown to play a key role in the differentiation and elimination of suspensor in tobacco embryos (Zhao et al. 2013). High expression levels of NtCYS during early stages of embryogenesis suppress NtCP14 activity and allow suspensor to grow, while downregulation of NtCYS expression during later stages leads to higher NtCP14 activity and to the initiation of PCD. These findings demonstrate that several divergent proteolytic events act as part of the same pathway or in parallel pathways to control initiation and execution of PCD in the suspensor.

4.4 Redox Homeostasis

Many different types of stresses, such as heat, heavy metal, dehydration or osmotic stress, are efficient inducers of SE (Simmonds and Keller 1999; Ikeda-Iwai et al. 2003; Dubas et al. 2011). In agreement with this hypothesis, induction and progression of SE is accompanied by upregulation of genes responsible for production of reactive oxygen species (ROS) and for induction of oxidative stress (e.g. germin-like oxalate oxidase and peroxidase; Caliskan et al. 2004; Thibaud-Nissen et al. 2003; Marsoni et al. 2008). Exogenous application of hydrogen peroxide at low concentrations (0.25 mM) can increase the yield of SE (Luo et al. 2001). Therefore, ROS accumulation might tentatively induce transcription of genes required for the embryogenesis.

Accumulation of ROS is a hallmark of oxidative stress-related cell death (De Pinto et al. 2012), and treatment with hydrogen peroxide can trigger cell death responses in different types of cells including embryos (Sundström et al. 2009; De Pinto et al. 2012). It was suggested that production of ROS triggers expression of *glutathione S-transferase (GST)* genes (Nagata et al. 1994; Thibaud-Nissen et al. 2003; Marsoni et al. 2008), which catalyse reversible modification of the active groups of biological molecules and protect them against oxidation during intracellular transport (Cummins et al. 2011; see also chapter by Khan and Hell in the current volume). The spatial profile of *GST* expression in the embryos is not known, but upregulation of *GST* during SE (Joosen et al. 2007) indicates that GSTs protect specific cells in the embryo against premature death and reduction of *GST* expression allows cell death to proceed.

5 Concluding Remarks

SE starts from single cells or a group of cells with similar morphology and transcriptional profile in response to external stimuli either produced by the surrounding tissue *in plant* or added to the tissue culture medium. These stimuli cause genetic reprogramming that leads to the establishment of cell lineages with altered gene transcription pattern, different morphology and developmental fate. The first result of these multidimensional alterations is the formation of morphologically asymmetric (polar) structure. Following the initiation of SE, the process becomes autoregulatory and can sustain successive stages of the embryogenesis pathway without any or with minimal contribution from the external signals. In spite of how complex the subsequent stages may seem, the original establishment and persistence of this polarity from a physiologically homogenous group of cells, including subsequent terminal differentiation and demise of the embryo suspensor, represents a fundamental paradigm of plant developmental biology.

Transcription of specific sets of downstream genes in the divergent cell lineages during early stages of SE is responsible for the specific cellular physiology and morphology. These downstream genes encode mainly components of the cytoskeleton and membranes, proteases, cell cycle regulators and cell wall-modifying enzymes.

Molecules secreted by embryogenic cultures, such as AGPs and oligosaccharines, can efficiently induce embryogenesis (Fig. 4). The extracellular signals are perceived by receptor kinases at the membrane, primarily by SERKs. SERKs can activate divergent signalling pathways by forming complexes with other receptor kinases and, in addition to plant development, can regulate cell death and response to pathogen attack (Chinchilla et al. 2008). The interleukin-1 receptor kinase/Pelle-like kinase is required for division of the basal cell following the first division of zygote and establishment of the suspensor (Bayer et al. 2009). Therefore, distinct classes of receptor kinases are responsible for the induction of PCD in the suspensor and maintaining proliferation status in the embryo proper.

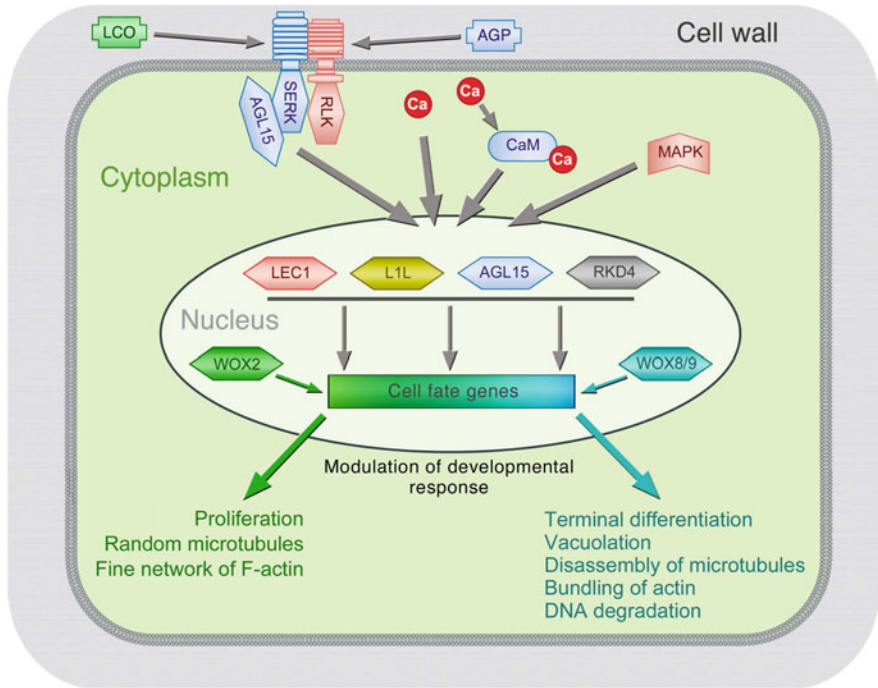


Fig. 4 The key components responsible for the balance of life-death processes during embryogenesis

Proliferation and death of cells are regulated by common key signalling pathways and gene transcription elements. The LCOs and AGPs directly or via interaction with components of the cell walls activate receptor kinases, consequently inducing the rise of intracellular Ca^{2+} concentration, calmodulin activity (CaM), MAP kinases activity and translocation of gene transcription regulatory elements to the nucleus. Some of these elements, like AGL15, are part of the receptor kinase complexes. In the nucleus, transcription factors LEC1, L1L, AGL15 and RKD4 control expression of core cell fate genes essential for both proliferation and terminal differentiation. The switch between the pathways is regulated by specific transcription factors exemplified by WOX2 expressed in the embryo proper and WOX8/9 which is expressed in the suspensor. In addition, cytoplasmic signalling pathways (e.g. depending on positional information) can direct the cell fate

The signal must then reach the nucleus in order to alter transcription patterns. Several pathways could be involved in the signal transduction. First is calcium-dependent pathway involving calmodulin and calmodulin domain kinases. Second is calcium-independent protein phosphorylation mediated by mitogen-activated kinases. Third, transcription factors can directly participate in the signal perception complex. For example, a MADS domain transcription factor, AGL15, is a component of SERK complex (Karlova et al. 2006) and relocates from cytoplasm into the nucleus of cells in embryo suspensor and embryo proper during early stages of embryogenesis (Perry et al. 1996). AGL15 upregulates expression of several transcription factors essential for embryogenesis including LEC2, FUS3 and

ABI3 (Zheng et al. 2009) and has a predicted phosphorylation motif RQVT20 within the conserved MADS domain (<http://phosphat.mpimp-golm.mpg.de/>; Heazlewood et al. 2008). Therefore, phosphorylation by SERK or other kinases could regulate AGL15 activity.

The establishment of apical-basal gradients during zygotic embryogenesis is controlled by WOX homeobox family of transcription factors. WOX8 and WOX9 are expressed in the basal cell, which will give rise to the terminally differentiated suspensor, while WOX2 is expressed in the apical cell destined to form the embryo proper. The RWP-RK transcription factor RKD4 regulates development of both embryo proper and suspensor (Waki et al. 2011; Jeong et al. 2011).

An extensive body of evidence accumulated during more than half a century of studies showcases the requirement of dynamic balance between pro-life and pro-death processes during SE. Understanding this balance is important to induce SE in recalcitrant species and to improve the yields of generated somatic seedlings by the existing biotechnologies. The original “symptomatic” analyses on the “macro-morphological” level have been recently complemented by cytological, biochemical and molecular studies. Owing to the genetically and physiologically diverse models used in this field to date, discrepancies have to be resolved. However, sequencing the genomes of many common SE models in combination with postgenomic technologies and more traditional techniques will help to sharpen the main concept, where the balance of “pro-life” versus “pro-death” signalling towards cell fate determines the induction and efficiency of SE.

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