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The Developmental Biology of Somatic Embryogenesis

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Abstract

Somatic embryogenesis (SE) is a remarkable developmental process enabling nonzygotic plant cells to form embryos and ultimately, fertile plants. It is an expression of totipotency. This chapter initially considers the genotypic component and the progenitor stem cells where SE is induced to form the initial asymmetric division of the somatic embryogenesis program. These cells are part of a stem cell niche dependent on the surrounding cells. Recent evidence is discussed that before the SE pathway can be initiated a GA-modulated pathway that represses inappropriate embryogenesis needs to be derepressed. The current understanding of how stress and hormones can induce the activation of specific SE genes is examined. Important stress components are reactive oxygen species and the signalling of stress-related hormones. The action of the key developmental hormone auxin, and also cytokinin, in relation to developmental genes is considered and, based on current understanding, a model is presented for the mechanism of SE. While there are many SE applications in contemporary biotechnology, understanding the reprogramming process associated with SE remains an important question for developmental biology.

1 Introduction

Somatic embryogenesis (SE) is a remarkable developmental process enabling nonzygotic plant cells, including haploid cells, to form embryos and ultimately, fertile plants. An expression of totipotency, this asexual process involves dedifferentiation of a nonzygotic cell and subsequent redifferentiation (reprogramming), resulting in the production of all cells characteristic of the mature plant. Although primarily considered in the context of *in vitro* SE from cultured tissues or cells, SE is also a naturally occurring means of asexual reproduction in some species. For example, somatic embryos form on the succulent leaves of *Kalanchoë* (Garcês et al. 2007) and apomixis, a clonal reproductive process where embryos derive from cells in the ovule wall producing seed genotypically identical to the parent, occurs in a number of evolutionarily divergent plant species (Koltunow and Grossniklaus 2003). This discussion focuses on *in vitro* SE, but an understanding of this process is applicable to all forms of SE.

SE was first demonstrated 50 years ago by Steward et al. (1958) and Reinert (1958) in carrot. More recently, the emphasis has been on inducing SE in an ever-increasing number of species and cultivars to facilitate genetic transformation. The molecular mechanisms of SE remain poorly understood with mechanistic studies focused primarily on the hormonology of this developmental process. The classic paradigm is that auxin is initially required to induce SE, while its subsequent withdrawal, or reduction in concentration, drives embryo development (Halperin 1966; Dudits et al. 1991). In many species, cytokinin in addition to auxin is required for SE (Nolan and Rose 1998). These are not the only hormones to consider, but usually the critical ones. Understanding of how auxin and cytokinin signalling regulates transcription has advanced in recent times (Tan et al. 2007; Müller and Sheen 2007). Application of this understanding to SE, however, is limited and requires an understanding of the interaction of these hormonal signalling pathways with key developmental genes. Increasingly, stress is recognised as having a critical role in the induction of SE (Touraev et al. 1997; Fehér et al. 2003; Nolan et al. 2006). Thus, hormones and stress collectively induce cell dedifferentiation and initiate an embryogenic program in plants with a responsive genotype (Fehér et al. 2003; Ikeda-Iwai et al. 2003; Rose and Nolan 2006).

SE *in vitro* takes two forms, indirect and direct, referring respectively to the presence or absence of a phase of callus development (Dijak et al. 1986; Williams and Maheswaran 1986). As indirect SE is more commonly employed, this form is emphasized, and direct SE is also discussed, given that there is likely a continuum in terms of the amount of dedifferentiation and cell proliferation required before somatic embryos are induced.

The ability to produce embryos from nonzygotic cells and to regenerate whole plants is one of the fundamental questions of contemporary biology (Vogel 2005). This chapter summarises current understanding of the cell and molecular biology of SE and discusses the implications SE has to biotechnology.

2 The Basic Requirements for *In Vitro* Somatic Embryogenesis

Formation of somatic embryos in a given species typically entails culturing an explant (of appropriate tissue type and genotype) in a basal medium with a suitable hormone regime (Rose 2004). Importantly, excision and culture of the tissue introduces a stress component. A number of studies indicate that stress itself acts as an inducer of SE (Kamada et al. 1993; Touraev et al. 1997; Ikeda-Iwai et al. 2003) and, indeed, is the first event experienced by cells on excision of the explant from the plant tissue or the isolation of a cell before initiating the culture process. The basal medium can modify the amount of SE in a given explant (Ammirato 1983) and is, of course, essential for SE, but is usually not a key regulator. In considering the mechanism of SE, Fig.1 needs to be understood, which will serve as the basis for considering SE in this chapter.

3 The Explant and Stem Cell Biology

It may be possible in some circumstances for a somatic embryo to derive from more than a single cell (Williams and Maheswaran 1986). However, it is known from the tracking studies of de Vries and co-workers that an embryo can develop from a single totipotent somatic cell; both in the classic carrot cell

suspension system (Schmidt et al. 1997) as well as in other species where somatic embryos are derived from leaf explants (Somleva et al. 2000). These single-cell progenitors of somatic embryos are totipotent stem cells as opposed to the pluripotent cells of plant meristems. Given the developmental programming required for embryo formation and its vascular independence from surrounding cells or callus (Haccius 1978), it seems likely that development from totipotent stem cells is the norm for SE. What is far from clear is which genotype and specific cell type enable SE.

3.1 Genotype

It is apparent from many studies, and a serious problem for biotechnology, that within a species only some cultivars are regenerable by SE (Bingham et al. 1975; Ammirato 1983; Vasil 1988). Work by Bingham et al. (1975) in *Medicago sativa* demonstrated the possibility to breed for SE, with an ensuing study by Kielly and Bowley (1992) suggesting that a SE-competent phenotype is dominant and that two genetic loci are involved. The identity of the genes residing at these loci, however, remains unknown. While genotypic influences may have a genetic basis, another line of enquiry suggests a role for epigenetic phenomena, in particular, the pattern of chromatin condensation. Investigations in plant epigenetics reveal heterochromatin to be marked by methylation of cytosine and histone H3 (Bender 2004). Data supporting epigenetic control of SE are not extensive, but provide some support for the control of SE by methylation pattern. Thus, disruption of DNA methylation pattern with 5-azacytidine in *M. truncatula* (Santos and Fevereiro 2002) and carrot (Yamamoto 2005) disrupts SE. Recent studies show the involvement of small interfering RNAs in the condensation of chromatin (Bäurle et al. 2007, Henderson and Jacobsen 2007). Importantly, DNA methylation patterns are heritable and can be maintained across mitosis (Henderson and Jacobsen 2007).

Interestingly, in *M. truncatula* all highly embryogenic genotypes, Jemalong 2HA (Nolan et al. 1989; Rose et al. 1999), R108 (Hoffman et al. 1997) and M9-10a (Araújo et al. 2004), were derived after a cycle of tissue culture. This fact suggests the regeneration process selects for somatic cells with competence for SE, a heritable trait. Moreover, it suggests the regeneration process might provide a consistent selection for an epigenetic pattern in the cultured somatic cells, as a cycle of tissue culture is always enough to enhance regenerability (Nolan et al. 1989; Hoffman et al. 1997).

In plant development, genes promoting embryogenesis ought to be repressed as nonzygotic cells develop in the plant body. There is evidence that SE can be induced in the *Arabidopsis pickle* mutant (Ogas et al. 1997) by derepressing the embryogenesis pathway. *PICKLE* acts to repress embryonic traits (Henderson et al. 2004) and encodes a CHD3 chromatin-remodelling factor (Ogas et al. 1999). It is possible that activation of the expression of embryogenesis-promoting genes, repressed during development, enables SE. The repression of embryogenesis appears to involve gibberellic acid (GA) metabolism, discussed later in this chapter (Henderson et al. 2004).

3.2 Explant Cells

When callus forms from an explant before the appearance of somatic embryos, a question exists as to from which cells the embryos derive. Historically, this has been investigated and discussed in relation to carrot cell suspension cultures (Halperin 1966). In establishing cell suspension cultures, explants have been taken from various tissues including hypocotyls (Guzzo et al. 1994; Schmidt et al. 1997), where cell division is initiated in provascular cells that form embryogenic callus that is transferred into liquid cell suspension culture. In the original work on SE by Steward et. al (1958, 1964), explants were obtained from the phloem of the storage root of carrot and then SE induced in liquid culture. What is possibly the case here in the carrot studies is the presence in these tissues of procambial-like cells (involved in the differentiation of vascular tissue) which readily reprogram to a totipotent state. Once the carrot cells are

in liquid culture in the presence of auxin, proembryogenic masses (PEMs) form. Further embryonic development does not then occur until removal of auxin. Tracking cells in a newly initiated cell suspension culture via expression of a p*SERK-LUC* fusion reveals that most somatic embryos develop from single cells or small cell clusters originally derived from provascular cells (Schmidt et al. 1997). In established cultures, somatic embryos derive from cells in PEMs.

As plant biotechnology progressed, regeneration via SE was developed for many species using agar-solidified medium. Histological studies often focused on demonstrating that regeneration was via SE rather than organogenesis (*e.g.* Sharma and Millam 2004). In the Sharma and Millam study, meristematic zones from embryogenically induced internode segments were noted and interpreted as PEMs. Our unpublished data with *M. truncatula*, using both mesophyll protoplasts and leaf explants, provides support for two types of progenitor cells for SE. Callus that derives from leaf explants is permeated by vascular tissue. Some SEs develop from the parenchyma-like callus cells near the epidermis (Fig. 2) while others are from the provascular or procambial tissue, mirroring that observed in carrot hypocotyls. There is good supporting evidence for embryos forming at the periphery of callus in a diversity of species such as guinea grass (Lu and Vasil 1985), pea (Loiseau et al. 1998), chickpea (Sagare et al. 1995) and potato (Sharma and Millam 2004). The other common feature with embryogenic callus in a number of studies is the link to vascular tissue (Lu and Vasil 1985, Schwendiman et al. 1988; Schmidt et al. 1997; Somleva et al. 2000) which goes back to the early studies of Steward et al. (1958). Procambial cells are pluripotent stem cells characterised by their capacity to differentiate into cells of the vasculature. This was emphasised in studies of adventitious root formation in *M. truncatula*, where it was clear that cells proliferating from the procambial-like cells of the veins of leaf explants could differentiate into root meristems in response to auxin (Rose et al. 2006). Kwaaitaal and de Vries (2007) subsequently showed that *SERK1* (which marks cells that form somatic embryos – see later) expressed in procambium cells and immature vascular cells (also observed in *M. truncatula*), responds to auxin.

The *M. truncatula* protoplast system (Rose and Nolan 1995) where SEs derive from mesophyll cells clearly illustrates that mature plant cells can dedifferentiate and ultimately form SEs without any initial vascular tissue being present (Mantiri et al. 2008). Direct SE from mesophyll protoplasts of *M.*

sativa has also been demonstrated (Dijak et al. 1986). These data are brought together in a conceptual manner in Fig. 2.

4 The Earliest Event in Embryogenesis – Asymmetric Cell Division

During zygotic embryogenesis, the zygote divides asymmetrically to produce an apical cell, which forms the majority of the embryo, and a basal cell, which forms the suspensor (Scheres and Benfey 1999). The apical cell is smaller and densely cytoplasmic and the basal cell larger and vacuolated (Jenik and Barton 2005). While the basal cell gives rise to the suspensor and tethers the embryo to the ovule, the suspensor cell adjacent to the apical cell (the hypophysis) generates the quiescent centre of the root pole, as well as the root cap and its associated stem cells. The remainder of the root meristem traces back to the apical cell (Jenik and Barton 2005). Therefore, the first cell divisions of the zygote delineate the polarity that determines the root and shoot apical meristems. Tight regulation of each embryonic cell division produces cellular patterning resulting, ultimately, in a heart stage embryo. Cell division patterning appears to be controlled by auxin fluxes (Friml et al. 2003; Kepinski and Leyser 2003), *WOX* transcription factors (Haecker et al. 2004), cell wall components (Souter and Lindsey 2000; Ingram 2004) and a number of other genes (Chandler et al. 2007).

One protein linked directly to apical-basal pattern formation in the *Arabidopsis* embryo is GNOM, an ADP-ribosylation factor guanine-nucleotide exchange factor (ARF-GEF) required for correct localisation of, among other proteins, the PIN1 auxin transporter (Geldner et al. 2003). Mutations in *GNOM* can produce seedlings that lack basic polarity and possess unusual shapes (Mayer et al. 1993). This patterning can be related to alteration in the first cell division in the *gnom* mutant where two nearly equal-size cells are produced, rather than the distinctive asymmetric division (Mayer et al. 1993).

4.1 The Cell Wall in Establishment of Polarity, Division Asymmetry and Cell Fate

One fundamental set of questions that exists in plant biology is how plant cell identity is determined, how cells decide whether to divide or expand, and whether they commit to a specific developmental fate. In the context of SE, specifically, how does a nonzygotic cell become committed to an embryogenic pathway and develop the intricate patterning present in an embryo? Numerous studies, both classical and recent, reveal that plant cell fate can be respecified according to its position relative to other cells (Ingram 2004). In other words, the fate of a cell depends more on its neighbours than its mother cell (Majewska-Sawka and Nothnagel 2000). This implies that cells continually communicate with one another during plant development. Since a rigid cell wall encompasses the plant cell, communication between the cell interior and the environment occurs via the cell wall (Sato 1998). Indeed, increasing evidence eludes to the fundamental role that cell wall components (both protein and saccharide) have in regulating cell division patterns, cell shape, intercellular attachment, wound/defence-related signalling, programmed cell death (PCD), polarity establishment and determination of cell fate. Arabinogalactan proteins (AGPs), hydroxyproline-rich glycoproteins with complex carbohydrate side-chains comprising approximately 90% of the molecules mass, are believed to be of particular importance in the abovementioned processes (Majewska-Sawka and Nothnagel 2000).

The role of cell wall components in establishing axial-basal polarity (required for division asymmetry) is perhaps best illustrated by early development in *Fucus* zygotes. Here, deposition of sulphated polysaccharides at the base of the cell defines the basal-axial axis leading to the development of thallus (apical) and rhizoid (basal) cell after the first asymmetric division. Interestingly, remnant cell wall in protoplasts isolated from these cells is sufficient to direct the fate of the cells in culture (Souter and Lindsey 2000). Similarly, establishment of the axial-basal polarity in zygotes of higher plants may involve differential distribution of cell wall components. For example, during *Brassica* embryogenesis, the JIM8 antibody, which recognises a specific AGP epitope, only localises to cells fated to become the suspensor (Pennell et al. 1991; Souter and Lindsey 2000). Thus, polarity can be established before the first asymmetric division through differential localisation of AGPs.

Recently, a hydroxyproline-rich glycoprotein, RSH, has been shown to be essential for normal embryo patterning, with influences observed at the first asymmetric division of the zygote (Hall and

Cannon 2002). In rapidly dividing cells of the embryo, RSH localises to the cell plate-cell wall junction, suggesting a role in positioning of the cell plate at cytokinesis. Mutation of *RSH* leads to morphologically defective embryos, with irregularly sized and shaped cells. Similarly, the cellulose synthase - like gene, *AtCSLA7*, which encodes a processive β -glycosyltransferase is critical for normal embryogenesis causing homozygous *AtCSLA7* mutants to have slow developing embryos with abnormal cell patterning that arrest at the globular stage (Goubet et al. 2003). Interestingly, mutation in *GNOM* produces a phenotype most similar to *RSH* and *AtCSLA7* mutants, suggesting that RSH and AtCSLA7 are cargo in GNOM-associated vesicles. Whether the role for GNOM in embryogenesis reflects secretion of cell wall components or localisation of auxin transporters is unclear (Ingram 2004).

4.2 Division Asymmetry in the Initiation of SE

Somatic embryos have a similar developmental pattern to zygotic embryos, passing through globular, heart, torpedo and cotyledonary stages (Rose 2004). However, somatic cells that form embryos do so in various different environments and tissues, notably without endosperm (Williams and Maheswaran 1986). Thus, are the signalling environment and the division asymmetry necessary for zygotic embryogenesis also required for SE? Evidence for the existence of asymmetric divisions comes from a substantive study involving cell suspension cultures of carrot (McCabe et al. 1997). In these cultures, JIM8⁺ (state B) cells undergo asymmetric division giving rise to a JIM8⁺ (state F) cell and a JIM8⁻ (state C) cell. State F cells eventually undergo PCD, while state C cells are competent for SE providing that conditioned media factors from JIM8⁺ cultures (presumed to be AGPs) are present. Soluble signals from JIM8⁺ cells that direct state C cells along a SE pathway may be akin to endosperm-derived signals believed to direct development of the zygotic embryo (Ingram, 2004), suggesting possible commonalities in the signalling environments of zygotic and SE.

In *M. sativa* where leaf protoplasts may undergo direct SE, asymmetric cell divisions result in SE, whereas symmetrically dividing cells form callus (Dudits et al. 1991). Asymmetric cell divisions of elongated cells were also observed in the provascular tissue of carrot explants in response to auxin

(Guzzo et al. 1994). These asymmetrically divided cells give rise to PEMs, from which embryos subsequently develop in the absence of auxin (presumably following a further asymmetric division). Morphological asymmetry, however, was not observed in *Cichorium* (Blervacq et al. 1995). The evidence for the involvement of asymmetric divisions in SE is best in the case of isolated single cells or protoplasts. The evidence in tissue explants or calli derived from protoplasts, however, is more difficult to obtain. The current authors have observed early asymmetric divisions in somatic embryo formation in calli from isolated protoplasts. In many cases of SE it is probably hard to obtain the clear-cut asymmetric division observed in the zygote, a possible explanation for why many somatic embryos do not progress to plants. Overall, however, there is support for asymmetrical divisions being an integral component of SE (Fehér et al. 2003).

4.3 Asymmetric Division and the Suspensor in SE

One of the difficulties in interpreting early development of somatic embryos is that of the suspensor. Are the asymmetric cell divisions of state B cells analogous to the first division of the zygote? Certainly, the JIM8 labelling pattern of divided state B cells is analogous to that observed after the first embryonic division in Brassica (seeSection 4.1), and so potentially, the cell undergoing PCD after the asymmetric division represents an aberrant suspensor. In the cell tracking study of Schmidt et al. (1997), which used carrot cell suspension cultures expressing a *SERK*-reporter, a suspensor attached to elongated cells in the culture can be seen in one tracking series but not in another. Because of the unusual developmental context of SE, suspensor development is rather variable, ranging from a large group of cells to absent (Williams and Maheswaran 1986). Thus, the hypophysis-root pole relationship is unlikely to be the same in both zygotic and SE.

5 The Stress Component in the Initiation of Somatic Embryogenesis

Stress has been proposed for some time as being an important component of SE (Dudits et al. 1991). The first signal perceived by cells destined to become somatic embryos relates to the wounding that accompanies excision of the tissue or isolation of the cells. In *M. truncatula*, there are many stress-related proteins associated with SE (Imin et al. 2004). A number of these proteins are differentially expressed between regenerators and non-regenerators (Imin et al. 2005).

5.1 Reactive Oxygen Species

One of the earliest responses to wounding, and indeed many other abiotic and biotic stresses, is increased production of reactive oxygen species (ROS) at the site of stress, the so-called ‘oxidative burst’ (Orozco-Cardenas and Ryan 1999). ROS, including superoxide and hydroxyl radicals and hydrogen peroxide (H_2O_2) are produced as byproducts of oxygen metabolism (occurring in organelles, the cytosol, plasma membrane and apoplast) and can cause serious oxidative damage to the cell. However, it is increasingly recognized that ROS are important signalling molecules involved in plant development and environmental perception (Noctor 2006). Furthermore, ROS, particularly H_2O_2 , function in lignification and oxidative cross-linking of the cell wall, control of cell growth, destruction (oxidation) of microbial pathogens and PCD/hypersensitive response (Dangl and Jones 2001; Gechev and Hille 2005).

Thus, ROS are produced by cultured explants in response to wounding. Indeed, staining explants with 3,3-diamidinobenzidine (DAB) reveals continual production of H_2O_2 in callus tissue during culture, although importantly, not in somatic embryos that form on the callus (N.A. Saeed, M.B. Sheahan and R.J. Rose, unpublished data). Moreover, culturing explants in the presence of diphenyleneiodonium (DPI), an inhibitor of flavoprotein-dependent ROS production, prevents the re-initiation of cell division in *M. truncatula*, *Arabidopsis* and tobacco leaf explants, indicating a critical role for ROS in explant regeneration (N.A. Saeed, M.B. Sheahan and R.J. Rose, unpublished data). In addition, it is known that oxidative stress causes plants cells to acquire a less differentiated state (Pasternak et al. 2002) and that oxidative stress enhances auxin-dependent cell cycle reactivation (Pasternak et al. 2005). Although ROS initially promote dedifferentiation and re-initiation of cell division, an inability to control levels of ROS

may be a causal factor in recalcitrance of some species to undergo SE. Plants possess an arsenal of small-molecule antioxidants (ascorbate, glutathione, and tocopherol) and antioxidant enzymes (including superoxide dismutases, peroxidases and catalase) and in cultured tobacco protoplasts, up-regulation of the activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase follows the oxidative burst, causing reduced forms of glutathione and ascorbate to predominate. Significantly, this change does not occur in non-totipotent cells and appears necessary for the expression of totipotency (Papadakis et al. 2001).

The nature of downstream events involved in wound-induced ROS signalling remain unclear. However, they may involve direct interaction between ROS and redox sensitive transcription factors such as NPR1 (Mou et al. 2003), MAPK signalling cascades (Kovtun et al. 2000) or modification of thiol residues on proteins such as protein tyrosine phosphatases or glutathione peroxidases (Gupta and Luan 2003, Miao et al. 2006). ROS signalling may also be sensed via the level of small-molecule antioxidants such as glutathione. Indeed, progression from the G₁ to S phases of the cell cycle requires adequate levels of reduced glutathione which may influence, in turn, the expression or activity of A-type cyclins or the cyclin dependent kinase inhibitor, CKI, respectively (Vernoux et al. 2000). Undoubtedly, manifestation of stress signalling involves cross-talk between hormonal and ROS signalling pathways and there exists substantial evidence linking auxin, abscisic acid, ethylene, jasmonic and salicylic acid signalling pathways with ROS signalling (Desikan et al. 2005).

Thus, ROS signalling appears critical to cellular reprogramming and re-initiation of the cell cycle. However, ROS also appear to be important for subsequent development of somatic embryos. Hence, H₂O₂ stimulates SE in *Lycium barbarum* (Cui et al. 1999; Kairong et al. 2002), glutathione redox state influences meristem development in *Picea glauca* SE (Stasolla et al. 2004; Belmonte et al. 2005; Belmonte and Stasolla 2007), while regions of somatic embryo formation are marked by high expression levels of an H₂O₂-producing oxalate oxidase in wheat (Caliskan et al. 2004). The effects of ROS on somatic embryo maturation may be mediated by its effects on cell walls that prevent cell expansion and cell-cell separation, thus promoting an ‘embryogenically competent’ microenvironment (Caliskan et al. 2004; Belmonte and Stasolla 2007).

5.2 Stress-Related Hormone Signalling

In our recent study in *M. truncatula* (Mantiri et al. 2008), where SE is induced by auxin and cytokinin, it was shown that the stress-related hormone, ethylene, is synthesised within the first 24 h of initiating culture. Moreover, inhibitors of ethylene perception or biosynthesis prevent SE and expression of the ethylene response transcription factor (ERF), *MtSERF1*, a member of the AP2/ERF superfamily essential for SE (Mantiri et al. 2008). Knockdown of *MtSERF1* expression by RNAi results in almost total suppression of SE. The expression of *MtSERF1* is not only dependent on ethylene, but also auxin and cytokinin. Furthermore, the *MtSERF1* promoter contains ethylene and auxin response elements and cytokinin-responsive ARR motifs. These data start to provide a molecular basis for the integration of stress and auxin/cytokinin-dependent signalling in SE.

Another stress-related hormone, abscisic acid (ABA), in certain systems can initiate SE (Nishiwaki et al. 2000). ABA can also promote SE induced by auxin and cytokinin in *M. truncatula* (Nolan and Rose 1998), so taken together with the ROS and the MtSERF1 data, these may be a signalling web that is likely to be similar, but subtly different, to other areas of plant development (Beveridge et al. 2007).

Existing evidence provides strong support for the involvement of stress in SE. As discussed in the next section, auxin is the key player in the SE paradigm and indeed 2,4-D, perhaps the most commonly used auxin, has been suggested to promote SE because of its herbicidal properties (Pasternak et al. 2002). Other auxins, however, can substitute for 2,4-D. So, while 2,4-D may add to the stress response, it is more likely to be acting to derepress genes in line with current evidence for auxin action (Rose and Nolan 2006).

6 Hormones and the Initiation of Somatic Embryogenesis

Classically, auxin has been considered the key player in the induction of SE and the formation of somatic embryos. As understanding of the mode of auxin action and the regulation of its transport increases (Friml et al. 2003; Tan et al. 2007) so too does evidence that auxin is the central player in regulation of plant (and somatic embryo) growth and development (Beveridge et al. 2007). The early work of Halperin showed that high concentrations of 2,4-D could produce PEMs from cultured petioles (in a medium containing reduced nitrogen) and somatic embryos were produced when transferred to liquid medium with no or low auxin. It was essential that the auxin concentration be lowered for SE to occur. Though the carrot system has served as the model for SE, there were always some difficulties in studying the activation events in SE, given the proembryogenic nature of the suspension culture (Dudits et al. 1991).

The advent of model plants such as *Arabidopsis* and *M. truncatula* (Rose and Nolan 2006) has, to a degree, lessened the role of the carrot system in SE studies. Nevertheless, it provides an important perspective to SE (Mordhorst et al. 1997) and the work with the *SERK1* gene in carrot ushered in a more concerted effort into the molecular biology of SE. With a luciferase reporter gene for cell tracking, it was shown that SERK is expressed in single cells that develop into somatic embryos and in carrot acted as a marker of cells competent for SE (Schmidt et al. 1997). *SERK1*, when over-expressed in *Arabidopsis*, is able to stimulate SE (Hecht et al. 2001). In *M. truncatula* the *MtSERK1* gene is rapidly induced by auxin in cultured leaf explants and its expression is associated with both SE (SE requires auxin plus cytokinin in *M. truncatula*) and root morphogenesis. The extensive data now available on SERK (Rose and Nolan 2006) is consistent with an important role for *SERK1* in SE competency, although it appears to have a broader role in differentiation as well (Nolan et al. 2003; Kwaaitaal and de Vries 2007).

Investigations into the homeobox gene, *WUSCHEL* (*WUS*), have also served to link SE to hormonal activation of specific developmental genes. *WUS* encodes a transcription factor that regulates the stem cell population in the shoot meristem. Over-expression in *Arabidopsis* formed embryo-like structures and it was suggested that *WUS* acted as both a meristem and embryo organiser (Zuo et al. 2002). Other work by Gallois et al. (2004) has shown in *Arabidopsis* that ectopic expression of *WUS* in roots could induce shoot organogenesis (low auxin) or SE (high auxin). An important consequence of this work is the concept that genes directing stem cell identity can reprogram somatic cells, with the direction

of developmental change dependent on additional cues. Unpublished data of the authors in *M. truncatula* shows rapid induction of *WUS* expression. These data have been interpreted to suggest that genes involved with maintenance of pluripotent stem cells in meristems may have a role in inducing totipotent stem cells in culture. This has been included in the model shown in Fig. 3.

7 Induction of SE by Over-Expression of Leafy Cotyledon Transcription Factors and their Relationship to SE induction and Repression – the GA Connection

A framework is emerging of how stress and hormones can induce the activation of specific genes that can form stem cells capable of initiating the embryogenic pathway. Several transcription factors can induce SE independent of hormones, raising a number of questions in relation to the mechanism of SE. A connection between these different transcription factors is emerging in some cases, for example, the leafy cotyledon (*LEC*) group of transcription factors, required for SE in Arabidopsis, have an interesting connection to GA and ABA. *LEC2* when over-expressed induces both SE and the MADS transcription factor AGL15 (Braybrook et al. 2006). Ectopic expression of AGL15 promotes somatic embryo formation in Arabidopsis (Harding et al. 2003). Further evidence indicates that AGL15 acts by targeting the GA2-oxidase, AtGA2ox6, which catabolises biologically active GA. This decrease in biologically active GA causes increased SE, while addition of GA₃ causes a decrease in SE (Wang et al. 2004). A possible explanation for this effect lies with the *pickle* (*pkl*) mutant of Arabidopsis. The *pkl* mutant, so named because of its abnormal root phenotype, when cultured in the absence of plant hormones generates callus-like growth and forms somatic embryos on roots (Ogas et al. 1997). Wild-type roots do not produce callus or somatic embryos when cultured under the same conditions as the *pkl* roots. The surprising observation is that GA suppresses the *pkl* phenotype. The *PKL* gene has been cloned (Ogas et al. 1999) and encodes a CHD3 protein linked to chromatin remodelling (component of a histone deacetylase complex). The *PKL* gene also appears to be necessary for the repression of *LEC1* (Ogas et al. 1999; Rider et al. 2003).

Thus, AGL15 and PKL investigations reveal a GA-modulated pathway that represses embryonic identity. It is essential to prevent inappropriate embryo formation as germination and subsequent plant development occurs. This repression is happening in parallel to the GA activation of postembryonic development in Arabidopsis. In principle, this is similar to the situation shown by Haerizadeh et al. (2006), who reported the repression of germ cell-specific genes in somatic cells. Results of GA-related studies imply that it is necessary to ensure GA-repression is derepressed before the SE induction pathway can be initiated.

8 ABA, Stress and GA

As discussed above, ABA can stimulate or induce SE in some systems (Nolan and Rose 1998; Nishiwaki et al. 2000) and as ABA and GA act antagonistically in germination (Henderson et al. 2004) it might be that the ABA effect on SE works through antagonising the GA effect. However, Henderson et al. (2004) found that ABA only had a small effect on the penetrance of the *pkl* phenotype. The authors postulated that PKL might regulate a subset of GA-dependent responses. Gazzarrini et al. (2004) have shown FUSA, a LEC transcription factor required for SE, is up-regulated by ABA and down-regulated by GA. Overall, there is some coherency in these data which emphasise the need to have an SE repression system. The stress-related hormone ABA may well exert part of its action by contributing to overriding this repression.

9 Soluble Signals and Cell -Cell Interactions that Promote SE in Suspension Cultures

One observation frequently made in cultured cells, not discussed thus far, is the dependence of SE on cell density. Many compounds, whose concentration can vary with cell density, are released into the medium and affect SE. For example, carrot SE does not proceed at high density due to the release and build-up of 4-hydroxyphenol (Kobayashi et al. 2000). Conversely, conditioned medium factors such as

phytosulfokine peptides can stimulate cell division (although not SE directly) in cultures of suboptimal density (Matsubayashi and Sakagami 1996).

9.1 Secreted Proteins that Influence SE

Some proteins affecting SE progression, such as trypsin inhibitor, are produced by both embryogenic and non-embryogenic carrot cultures, but only secreted by embryogenic cultures, thereby preventing degradation of proteins necessary for progression through SE (Quiroz-Figueroa et al. 2006). The majority of protein secreted into the medium, however, is glycoprotein (Quiroz-Figueroa et al. 2006), most notably AGPs, believed to have important roles in cell-cell interaction and signalling. The importance of AGPs in cell fate determination and in regulating passage through SE is highlighted by tight temporal and spatial regulation of AGP expression in the plant. In carrot cell cultures, a characteristic group of AGPs are secreted into the medium, the composition of which changes with time (Quiroz-Figueroa et al. 2006). These AGPs likely have a structural role. However, that AGP effects (enhancement or reduction in frequency of SE) exerted at nanomolar concentrations also suggests a signalling role. Interestingly, AGPs can contain GlcNAc and Glc residues, sensitive to cleavage by chitinases (van Hengel et al. 2001). AGPs treated with chitinase produce oligosaccharides, which are more active than AGPs in promoting SE (reviewed in Quiroz-Figueroa et al. 2006). Indeed, addition of EP3 chitinase to temperature-sensitive *ts11* carrot cells is capable of rescuing the defective SE phenotype (Mordhorst et al. 1997). Thus, chitinase-modified AGPs are extracellular matrix molecules able to control plant cell fate (van Hengel et al. 2001). It has been suggested that these signals are indicative of cell-cell communication and hence the importance of these molecules.

9.2 AGP Signalling in SE: Mechanisms and Interactions between Signalling Pathways

Experiments in several species indicate that chitinases tend not to be expressed in cells of the embryo proper, but rather surrounding cells, providing evidence for the importance of intercellular

communication in SE and is reminiscent of the endosperm-embryo relationship in zygotic embryogenesis (Wiweger et al. 2003). In *Picea abies* cultures, removal of plant growth regulators up-regulates expression of *Chia4-Pa* chitinase. Chia4-Pa stimulates PCD and promotes the PEM to somatic embryo transition (Wiweger et al. 2003). The presence of JIM13-reactive AGPs, a marker for cells undergoing PCD in maize and zinnia xylem transdifferentiation, on PEMs in *P. abies* provides further evidence to suggest that AGP signalling invokes PCD (Filonova et al. 2000). Thus, AGP signalling likely interacts with an integrated proliferation-PCD control system to regulate SE (Filonova et al. 2000, Majewska-Sawka and Nothnagel 2000).

While soluble signals are undoubtedly important in suspension cultures, it does not necessarily mean that SE from tissue explants depends on the same signals, although clearly the cells that form somatic embryos are part of a stem cell niche dependent on the surrounding cells. It is becoming clear that cell wall related signalling cascades interact with hormonal and stress-signalling pathways invoked during SE. It is of interest in this regard to note that auxin deprivation (often used to promote PEM to somatic embryo transition) in tobacco BY2 suspension cultures results in a 2-fold increase in AGP secretion and a 4-fold increase in JIM13 reactivity (Winicur et al. 1998). Perturbation of cell walls can signal stress responses. For example, aggregation of AGPs with Yariv reagent produces an expression profile most similar to wounding, leads to down-regulation of GA signalling and up-regulation of defence-related genes, while inhibition of cellulose synthesis results in increased production of ethylene and jasmonates (Ellis et al. 2002; Guan and Nothnagel 2004; Mashiguchi et al. 2008). Interestingly, AGPs are cross-linked by H₂O₂ and wounding *in planta*, providing credibility to the hypothesis that stress, hormonal and cell wall signalling interact in SE (Kjellbom et al. 1997). In *Medicago* ethylene-dependent transcription factors are required for SE induced by auxin plus cytokinin.

9.3 Cell-Cell Interaction and Relevance to SE in Suspension Cultures

In carrot and other systems, researchers have noted the loss of embryogenic competence typically results in a reduction in the size of cell clusters that would normally form somatic embryos (Satoh 1998).

Indeed, close observations reveal tighter contact between cells of embryogenic callus than between cells of non-embryogenic callus. Thus, loss of embryogenic competence is associated with a loosening of intercellular attachment, suggesting close attachment is necessary for SE in these culture systems (Satoh 1998). Moreover, in *P. abies*, PEMs readily develop in somatic embryos, whereas single cells (densely cytoplasmic or vacuolated), made by fractionation of crude suspensions, do not (Filonova et al. 2000). This fact demonstrates the obligate requirement for cell-cell interaction in some SE systems. Changes in cell-cell interaction over the course of SE are reflected in, and likely caused by, changes in cell wall composition. For example, pectin comprises a substantially larger proportion of the cell wall in somatic embryos than it does in plantlets regenerated from these embryos (Yeo et al. 1998). Furthermore, hemicelluloses display a transient increase in 5- and 3,5-linked arabinose during embryogenesis (Yeo et al. 1998). Notably, embryogenic cultures have significantly higher levels of neutral sugars like arabinose and xylose than do non-embryogenic cultures (Satoh 1998; Yeo et al. 1998). Neutral sugars are believed to play an important role in intercellular attachment and might act by bridging hemicellulose and pectin molecules (Satoh 1998). One gene product identified as critical for intercellular attachment is C-ESE1 from carrot (Takahata et al. 2004). The *C-ESE1* gene is strongly up-regulated after depletion of 2,4-D from the medium and expressed specifically in the primordial cells of somatic embryos. Co-suppression of *C-ESE1* expression leads to small cell clusters with weak intercellular attachment, thus delaying SE (Takahata et al. 2004). Observation of control and *C-ESE1* co-suppressed cells by scanning electron microscopy reveals smooth cell surfaces of *C-ESE1* co-suppressed cells, which also release large amounts of cell wall polysaccharide into the medium. Thus, C-ESE1 appears to play a role in polysaccharide deposition, in turn important for cell-cell attachment and SE. Notably, control of cell-cell interaction also appears important for SE progression in explant based systems, such as that of coconut (Verdeil et al. 2001).

10 The Development Program after SE induction

This discussion has focused on what is known about the biology of SE induction and the different threads of evidence from a range of culture systems. The tacit assumption is that once SE is induced there is an asymmetric division, albeit with the development fate of the cell that would contribute to the root pole and the suspensor in the zygotic embryo having a more variable history. This latter point is likely one reason for the variable nature of SE. However, once SE initiates, the developmental program is thought to be similar to zygotic embryogenesis, being a primary reason for the adoption of SE as a useful experimental system to understand zygotic embryogenesis. The defining feature of SE, set out in an important review by Zimmerman (1993), is the reprogramming of a somatic cell. Zimmerman's review emphasised that the ability to produce large quantities of embryos at different developmental stages was a distinct advantage in embryogenesis investigations. Zimmerman's validation of SE relied on gene expression data available at that time. Developments since 1993 have revealed the power of genomics, mutants and model organisms, particularly *Arabidopsis* (Fehér et al. 2003), but also *Medicago* (Rose and Nolan 2006). As pointed out by Fehér et al. (2003), research on the *Arabidopsis* embryogenesis mutants have provided insights into SE. However, there have been the discoveries of *SERK* and *SERF* genes from SE studies that are also expressed in zygotic embryogenesis. The reality now is that our understanding of embryogenesis benefits from comparisons between zygotic and SE. While there are likely some gene expression changes that reflect the different environments under which the embryos develop, the accumulated evidence suggests overwhelmingly similar developmental programs (Zimmerman 1993; Dodeman et al. 1997; Mantiri et al. 2008).

11 Concluding Remarks and a Model Based on Studies in *Medicago truncatula*

A model which provides a framework for our understanding and future work is shown in Fig 3. While it is based on experience with *M. truncatula* it draws conceptually from *Arabidopsis* and the wider literature discussed in this chapter. It is known that ROS are rapidly induced when tissue is excised and placed into culture and that ethylene is essential for SE. It is feasible that both ROS and auxin contribute to the induction of ethylene biosynthesis genes. ROS has been reported to promote auxin-induced

ethylene production in mungbean hypocotyls (Song et al. 2007). It is also suggested that WUS is an important early player requiring cytokinins (our unpublished data). WUS, which has been postulated to be an embryo organiser (Zuo et al. 2002), is likely contributing to the production of stem cells similarly to as it does in the shoot meristem *in planta*. There is some evidence, as discussed earlier, that auxin-dependent *SERK* expression is indicative of cells primed for SE and commencing to differentiate. *MtSERF1* expression is initiated after *SERK* expression. MtSERF1 is essential for SE. Based on physiological experiments and putative promoter binding sites, there is evidence that *MtSERF1* expression requires cytokinin, auxin, ethylene and WUS (Mantiri et al. 2008). MtSERF1 may interconnect the stress and hormone signalling pathways. Based on the Arabidopsis studies discussed, it is assumed that the SE pathway is derepressed, involving an AGL15 dependent pathway, and the genes we have discussed are able to initiate the SE pathway.

12 SE and Biotechnology

The immense body of work on SE (more than 400 papers in the past 10 years) has arisen largely in response to the modern era of biotechnology and molecular biology. SE facilitates transformation for many species and, unless the floral dip procedures used in Arabidopsis or other *in planta* procedures can be developed for other species, SE remains a key component of many transformation protocols (organogenesis is the other regeneration pathway). The SE transformation protocols require the gene to be inserted into the somatic cell followed by regeneration via SE. The gene insertion is mostly by *Agrobacterium*, but can involve other methods such as microprojectiles.

The cloning of desirable genotypes using SE can be an advantage in high value plants such as in forestry, where it is a practicable propagation procedure (Sutton 2002), or in horticulture (Bunn et al. 2007), or with crops like potato (Seabrook and Douglass 2001). Greater knowledge may enable SE to be applied more efficiently and more broadly to encompass more species. Embryo production from nonzygotic cells in anther and isolated microspore culture in the production of doubled haploids has become important in breeding programs (Hosp et al. 2007). As pointed out in the introduction, an

understanding of SE will also facilitate an understanding of apomixis. Illuminating how apomixis is induced remains a goal of both fundamental and applied plant research. The ability to produce seed with identical genotype to the parent would provide new plant breeding strategies, particularly with hybrid crops. SE falls under the umbrella of regeneration biology and an understanding of this reprogramming process remains an important biological question (Vogel 2005). Elucidating the mechanisms of SE will contribute to a fuller understanding of plant development.

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Figure Legends

Fig. 1. The main conceptual components to consider for an understanding of the mechanism of somatic embryogenesis.

Fig. 2. Totipotent cells are produced from procambium or differentiated cells such as mesophyll cells and initiate embryogenesis, or may divide a number of times to form a PEM before initiating embryogenesis. (A) Totipotent cells are produced from procambium or differentiated cells, such as mesophyll cells, and initiate embryogenesis, or may divide a number of times to form a PEM before initiating embryogenesis. (B) Section through a *Medicago truncatula* leaf explant initiating somatic embryogenesis. The globular stage somatic embryos have been initiated near the surface on the adaxial side of the leaf quite separate from the vascular tissue and arise from dedifferentiated mesophyll cells. M, dedifferentiating mesophyll cells; VT, vascular tissue; arrowheads, somatic embryos at different stages of development. Bar = 100 μ m.

Fig.3. A model for the induction of somatic embryogenesis and the development of the heart-stage embryo from *Medicago truncatula* mesophyll cells, drawing on research from the authors' laboratory and the literature discussed in this review.

Fig. 1.

Fig. 2

Fig. 3