Chapter 1
Developmental Biology of Somatic Embryogenesis

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1.1 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 (Garces 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 (Müller and Sheen 2007; Tan et al. 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 has been 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 (Williams and Maheswaran 1986; Dijak et al. 1986). As indirect SE is more commonly employed, this form is emphasised, 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.

1.2 Basic Requirements for In Vitro SE

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. 1.1 needs to be understood, which will serve as the basis for considering SE in this chapter.

Fig. 1.1 The main conceptual components to consider for an understanding of the mechanism of somatic embryogenesis
1.3 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.

1.3.1 Genotype

It is apparent from many studies, and a serious problem for biotechnology, that only some cultivars within a species 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. However, the identity of the genes residing at these loci is not clear. 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 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 et al. 2005) disrupts SE. Recent studies show the involvement of small interfering RNAs in the condensation of chromatin (Henderson and Jacobsen 2007; Bäurle et al. 2007). The DNA methylation patterns are heritable and can be maintained across mitosis (Henderson and Jacobsen 2007).

In *M. truncatula*, all highly embryogenic genotypes, including Jemalong 2HA (Nolan et al. 1989; Rose et al. 1999), R108 (Hoffman et al. 1997) and M9-10a (Araújo et al. 2004), were obtained after a cycle of tissue culture. These results suggest the regeneration process selects for somatic cells with competence for SE, a heritable trait. Moreover, it suggests the regeneration process may 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 could 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 below in this chapter (Henderson et al. 2004).

### 1.3.2 Explant Cells

When callus forms from an explant before the appearance of somatic embryos, a question arises as to which cells the embryos derive. Historically, this was 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) that 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 (2004) 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, provide support for two types of progenitor cells for SE. Callus that derives from leaf explants is permeated by vascular tissue. Most SEs develop from the parenchyma-like callus cells near the epidermis (Fig. 1.2), 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

Fig. 1.2 (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 of the leaf away from the medium, 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 μm