

# I.5 Biochemical and Molecular Aspects of Haploid Embryogenesis

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## 1 Introduction

Microspore-derived embryo (MDE) cultures provide a unique system to study fundamental aspects of plant tissue culture and embryo development. In this culture system microspores and immature pollen grains (referred to here for simplicity as microspores) are induced to form haploid embryos by exposing them to a stress treatment, such as increased temperature, starvation or exogenous growth regulators. Under the appropriate culture conditions these embryos undergo numerous rounds of cell division and differentiate to form the sequential embryonic stages that are typical for zygotic embryos of that species. The developmental pathway from microspore to MDE often differs between plant species; however, it is likely that common processes underlie the switch in developmental pathways. As summarized by Yeung (1995), developmental concepts such as competence, induction and determination can be used to describe and understand *in vitro* embryo formation from haploid microspores. Likewise, the developmental processes that characterize zygotic embryogenesis, i.e. morphogenesis and differentiation, where the embryo divides and forms the basic tissue and organ systems of the adult plant (Laux and Jürgens 1997), and maturation, where storage products accumulate and the seed becomes desiccation-tolerant (Wobus and Weber 1999), can also take place during MDE development.

MDE culture has a number of advantages over other *in vitro* embryo systems or in planta models as an experimental system for studying concepts related to embryo formation and differentiation. Zygotic embryos develop within the confines of the maternal tissues and are therefore difficult or tedious to isolate, especially at the zygote and pro-embryo stages. The difficulty in isolating young zygotic embryos is reflected by the small number of publications in which differential screening methods have been used to identify early zygotic embryo-expressed genes (Heck et al. 1995; Li and Thomas 1998). In contrast to zygotic embryos, MDEs are usually produced in large numbers in liquid cultures and can be isolated at different stages of development spanning few-celled to mature embryos. MDEs growing in liquid cultures are more amenable than excised zygotic embryos as an experimental

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system to study the effect of cell perturbation or exogenously applied substances, such as growth regulators, on embryo development (Liu et al. 1993; Fischer and Neuhaus 1996; Hays et al. 2000, 2002). Somatic embryo cultures also provide a valuable in vitro experimental system for studying aspects of totipotency and embryo development; however, most somatic embryo cultures require long induction periods, develop through an intermediate callus phase and contain both embryogenic and non-embryogenic cell clusters (Zimmerman 1993; Mordhorst et al. 1997). These characteristics can make it difficult to study the stages of competence, induction and determination that occur during the initiation of somatic embryo formation. It is not surprising then that MDE cultures, especially those in which embryos develop at a high frequency from single cells, have been used as an experimental system to study the molecular, biochemical and cellular aspects of plant cell totipotency and embryo development (Touraev et al. 1996; Goralski et al. 1999; Custers et al. 2001; Yeung 2002).

This chapter describes the application of MDE cultures as an experimental system to understand two fundamental aspects of plant development: the formation of embryogenic cells and the early differentiation of the embryo. We will focus on two aspects of the molecular and biochemical research being carried out in this area, namely the identification of genes that are preferentially expressed during early MDE development, and the role of extracellular signalling molecules in MDE development. Recent developments in this area will be highlighted using published data, as well as unpublished results from experiments in our group using the *Brassica napus* MDE culture system. Where possible, we have tried to place these developments in the broader context of somatic and zygotic embryo development.

## 2 Gene Identification Strategies for Early MDE Development

A number of gene expression studies have been performed over the years using MDE cultures of different monocot and dicot species. Some of these studies have examined expression patterns of specific genes and proteins known to be involved in zygotic embryogenesis or thought to play a specific role in MDE development (Crouch 1982; Boutilier et al. 1994; Cordewener et al. 1995; Perry et al. 1999; Smykal and Pechan 2000; de Faria Maraschin et al. 2003a,b). Other studies have been directed toward analysis of differential gene expression or protein profiles at a single time point, or a developmental time course spanning multiple stages. Some of the considerations involved in setting up a differential screen, as well as the outcomes of a number of specific screens that have been performed in our laboratory as well as in other groups, are described below.

Differential gene analysis strategies using MDE cultures have been largely targeted toward understanding the molecular processes underlying two key

stages of MDE development: (1) the developmental switch, where the developmental fate of the immature pollen grains is altered by a stress treatment so that embryogenic rather than gametophytic cells are formed; and (2) the differentiation stage, where embryogenic cells continue to divide and sequentially establish the tissue and organ patterns that characterise the developing embryo. There are a number of difficulties involved in setting up good molecular screens to identify genes that are differentially expressed during the switch from microspore to MDE development. Ideally, a good molecular screen would allow one to isolate and compare cells from the same donor plants that have been exposed to the same induction treatment, but which differ only in their response to this treatment, e.g. embryogenic or non-embryogenic. In addition, the screen should take place at the earliest time point at which embryogenic cell formation has been established. However, practical problems relating to the asynchronous response of individual microspores to the induction treatment and in separating responsive from non-responsive cells at a very early stage make it difficult to obtain homogeneous and specific cell populations for screening. The ability to enrich at a very early stage for embryogenic versus non-embryogenic cell types from the same culture requires good cellular or molecular markers. A number of candidate marker genes that could be used to predict embryogenic cell formation are now available. These marker genes are expressed during early zygotic or in vitro embryo development, and in some cases have been shown to predict embryogenic cell formation in somatic embryo cultures (Boutilier et al. 1994, 2002; Lotan et al. 1998; Stone et al. 2001; Chesnokov et al. 2002; Zhang et al. 2002). Transformation of donor plants with non-destructive promoter:reporter constructs could be used to identify and isolate viable cells in MDE cultures. Interestingly, the promoter of a gene that was isolated in a differential screen in *B. napus* MDE cultures, p22A1, proved to be a very useful marker for the first embryogenic cell division when fused to the GFP reporter gene (Fukuoka et al. 2003). Flow sorting has also been used in *B. napus* to sort embryogenic and non-embryogenic cells at a very early stage of development (Schulze and Pauls 1998, 2002).

Gene expression analysis of differentiating MDEs is relatively straightforward as compared to screens aimed at identifying genes involved in the switch from microspore to haploid embryo development. Morphologically, embryo cell clusters are first recognisable as bona fide embryos either by the presence of a protoderm layer at the globular stage or by the presence of a suspensor attached to the embryo proper at the proglobular stage. Post-globular stages of embryo development are recognisable by their overall morphology, such as, for example, the characteristic heart, torpedo and cotyledon stages of dicot embryos. Embryos corresponding to specific developmental stages can be purified by sieving through nylon mesh of different pore sizes or, at relatively young stages, by density gradient centrifugation. Proglobular stages of MDE embryo development are more difficult to identify solely on morphology, as unlike zygote embryos of certain species, which

divide in a regular and recognisable pattern, in vitro cultured embryos tend to divide irregularly (Yeung et al. 1996). Recently, Custers et al. (2001) have succeeded in developing *B. napus* MDE cultures with a high frequency of suspensor-bearing embryos. These embryos undergo the same ordered divisions as their zygotic counterparts, making it possible to morphologically identify differentiating embryos as early as the two-celled embryo stage (J.B.M. Custers, pers. comm.). The presence of a long suspensor on these MDEs also makes it possible to separate them by sieving from non-embryogenic cells in the same culture.

## 2.1 Early Embryo-Expressed Genes in Monocot MDE Cultures

Differential gene expression analysis of monocot MDE cultures has been performed in barley, wheat and maize. Vrinten et al. (1999) identified three genes whose expression was strongly upregulated in 3-day-old barley MDE cultures as compared to untreated microspores. The identified cDNAs encode a non-specific lipid transfer protein (ECLTP), a glutathione S transferase (ECGST) and an unknown protein (ECA1). The *ECA1* gene likely encodes an arabinogalactan-like protein (AGP). AGPs are a class of secreted proteins that are thought to play a role in plant development (Kreuger and van Holst 1996; see below). Detailed expression analysis suggests that of the three genes that were identified, only *ECA1* is a specific marker for embryogenic cell formation in barley, as only *ECA1* expression levels were correlated with the embryo forming capacity of the culture.

Reynolds and colleagues took a similar approach to identify MDE-expressed genes in wheat anther cultures (Reynolds and Crawford 1996; Reynolds 2000), except that in this screen cDNA libraries were constructed from advanced-stage 'pollen embryoids'. Embryo-expressed clones were identified by screening the library with mature pollen and pollen embryoids. Subsequent expression analysis of individual genes was used to identify markers for the different stages of MDE development; however, the identity of only one of these clones, *EcMt*, was determined. *EcMt* encodes a cysteine-labelled metallothionein, and its expression is specifically induced in anther cultures starting as early as 6 h after embryo induction (Reynolds and Crawford 1996; Reynolds 2000). *EcMt* expression was reported to be a marker for MDE development; however, *EcMt* is induced by the plant hormone abscisic acid (ABA), both in MDE cultures and other tissues (Reynolds and Crawford 1996; Reynolds 2000), making it unclear whether *EcMt* expression actually marks the formation of embryogenic cells or simply the accumulation of ABA that accompanies the establishment of embryogenic cell formation in wheat. Analysis of ABA levels and *EcMt* expression in embryogenic and non-embryogenic cells from the same culture should answer this question.

## 2.2 Early Embryo-Expressed Genes in *B. napus* MDE Cultures

The majority of screens for early embryo-expressed genes have been performed using the *B. napus* MDE embryo culture system. The popularity of this system is largely due to the ease and efficiency of MDE embryo culture and the availability of good non-embryogenic controls (Custers et al. 2001; Yeung 2002). Another benefit of using *B. napus* as a model plant is that its genes are very similar at the nucleotide level to those of *Arabidopsis thaliana* (*Arabidopsis*; Brunel et al. 1999). This close similarity is also advantageous in that the molecular and functional genomics tools available for *Arabidopsis* research, including an efficient and effective transformation system, a fully sequenced genome, full genome microarrays, and a wide range of characterised mutants and mutant populations, can provide an initial platform for determining the identity and function of *B. napus* MDE-expressed genes (Hall et al. 2002).

### 2.2.1 mRNA Differential Display PCR Identifies a Small Secreted Signalling Peptide

Research on MDE development in our laboratory is mainly focused on the identification and functional characterisation of *B. napus* genes and proteins that are differentially regulated during the switch from microspore to haploid embryo development and during the early differentiation of the embryo. One of the first molecular screens we performed used mRNA differential display PCR to identify genes expressed during the switch from microspore to MDE development, and the transition from globular to heart-shaped embryos (Custers et al. 2001). MDE cultures were analysed at consecutive developmental stages beginning 8 h after the initiation of the embryo culture (developmental switch) through to the torpedo stage of embryo development (establishment of basic organ patterns). A large number of early embryo-expressed genes were identified that belong to a wide range of protein function categories and that show diverse temporal expression patterns (Custers et al. 2001). One of the genes that was isolated in the screen, *DD3-12*, encodes CLE19, a small 74 amino acid secreted protein that belongs to the CLAVATA3/ESR (CLE)-like family of proteins (Cock and McCormick 2001). CLE proteins are characterised by their small size (average size 8 kDa), an N-terminal secretion peptide, and a conserved C-terminal KRXVPXGPNPLHN motif, which does not show similarity to other known functional motifs. Only a few CLE genes have been characterised in detail. One well-characterised CLE gene is *CLV3*. *CLV3* is expressed in the stem cells of the shoot meristem and acts in a negative signalling loop with *WUSCHEL* (*WUS*) to maintain the stem cell population of the meristem (Laux 2003).

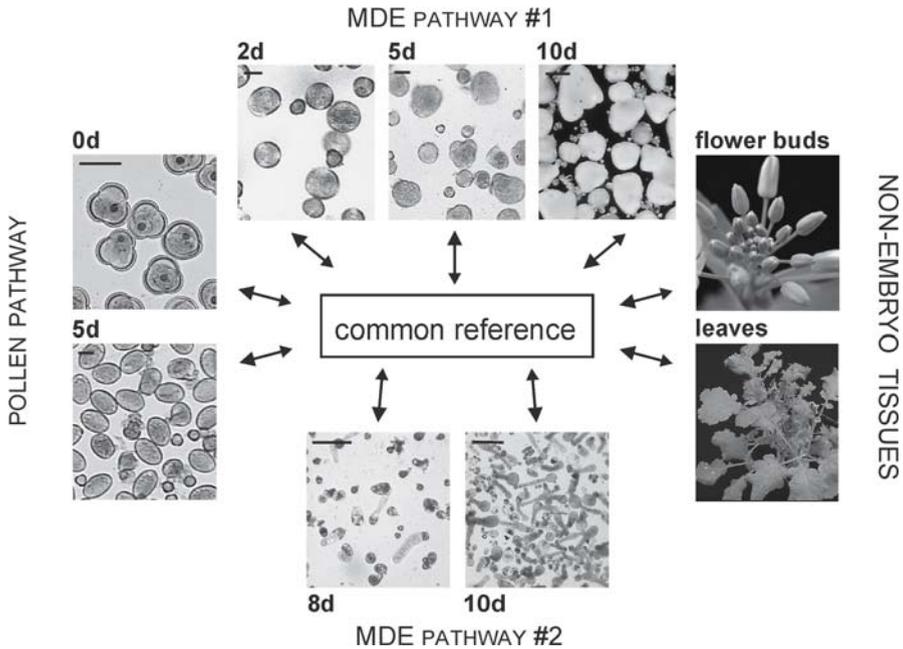
Our initial differential display-PCR analysis indicated that CLE19 is expressed at the globular to heart stage of development. This expression pat-

tern was later confirmed by RNA gel blot and CLE19 promoter:reporter gene analyses in *Arabidopsis* and *B. napus*. In zygotic embryos, CLE19 expression marks the transition from the globular to heart stage of development: CLE19 is first expressed in the cotyledon primordia of triangular stage embryos and becomes gradually restricted to the L1 layer at the tip of the cotyledons and then to the base of the cotyledons surrounding the shoot apical meristem (Fiers et al. 2004). Functional analysis of CLE19 is underway; however, our preliminary results suggest that CLE19 may also function in a CLV3-WUS-like signalling loop.

### 2.2.2 Microarray Analysis of Early MDE Development

We have recently initiated microarray-based expression profiling experiments in *B. napus* MDE cultures to obtain a broader perspective on the molecular pathways that accompany the switch from pollen development to embryo formation in vitro, and to classify temporal patterns of gene expression during early embryo development. DNA microarray analysis is a high throughput technology that allows for the simultaneous and comparative analysis of expression profiles for thousands of genes (Aharoni and Vorst 2002). To date, two cDNA microarray analysis studies have examined changes in gene expression during early embryo development, both of which made use of somatic embryo cultures (Thibaud-Nissen et al. 2003; van Zyl et al. 2003). The cDNAs on these microarrays were derived from random EST collections. The use of such non-targeted arrays can be limiting in that genes preferentially involved in embryo development processes are generally not well represented in EST sequencing programs, and are therefore underrepresented on the array. This indeed appears to be the case, as in both studies a large number of genes were spotted on the arrays, but very few differentially expressed genes were detected. The lack of similar studies using *Arabidopsis* full genome arrays and very young zygotic embryos highlights the difficulty associated with isolating few-celled embryos from seeds.

To identify expression profiles associated with early MDE development, we developed a dedicated microarray carrying cDNAs for genes expressed at three major points of MDE development: (1) 1-day heat-stressed microspore cultures in which a fraction of the microspores have undergone their first sporophytic cell division; (2) 4-day heat-stressed microspore cultures containing a mixture of pre-globular embryos that have started to emerge from the microspore exine wall, developing pollen, and non-embryogenic cells; and (3) purified 10-day globular and heart stage MDEs. A PCR-based technique, suppression subtraction hybridisation (Diatchenko et al. 1996), was also applied to the 4-day and 10-day mRNA samples to normalise the mRNA abundance in these samples and to enrich for genes that are either up- or downregulated relative to non-embryogenic samples. We used the expression profiles of the approximately 1,600 unique arrayed cDNAs to characterise the



**Fig. 1.** Developmental stages and experimental design used to identify MDE-expressed genes on a *B. napus* cDNA microarray. The microarray contains cDNAs from *B. napus* MDE libraries spanning the first embryonic cell division to the heart stage of development. The array was hybridised with nine samples corresponding to four major developmental groups: (1) MDEs at 2, 5 and 10 days after start of culture (MDE pathway #1); (2) suspensor-bearing MDEs at 8 and 10 days after start of culture (MDE pathway #2). Note that the developmental stage of the embryo proper in 8- and 10-day suspensor embryo cultures is approximately the same as that in embryos without suspensors from 2- and 5-day cultures, respectively; (3) developing microspores at start of culture (0d) and 5 days later (pollen pathway); and (4) non-embryo tissues. Each of the nine samples was hybridised to the microarray together with a common reference. The common reference sample was created by combining equal amounts of RNA from the embryo- and microspore culture samples. Scale bar is 20 μm in the pollen pathway 0d and 5d samples and MDE pathway #1 2d and 5d samples, and 100 μm in the MDE pathway #1 10d sample and MDE pathway #2 8d and 10d samples

development of three types of cultures: embryogenic cultures (three time points), embryogenic cultures containing suspensor-bearing MDEs (two time points) and control pollen cultures (two time points). An overview of the experimental setup and the developmental stages studied is shown in Fig. 1.

Our preliminary analysis of the microarray data focused on the identification of genes that are upregulated in embryogenic cultures relative to pollen cultures. Approximately 200 embryo upregulated genes were identified and further subdivided into categories based on their temporal expression patterns and/or preferential expression in suspensor-bearing or non suspensor-bearing MDEs. Five gene expression categories were identified including: (1) genes expressed in 2-day embryogenic cultures (11 genes); (2) genes

expressed in 5-day embryogenic cultures (30 genes); (3) genes expressed in 10-day globular-heart stage embryos (38 genes); (4) genes with upregulated expression in suspensor-bearing embryos (14 genes); and (5) genes expressed during all stages of MDE development (115 genes). Most of the genes we identified have not been assigned a function, nor have they been annotated as being embryo-expressed ESTs. Of the annotated genes we identified, the majority fell into functional classes related to transcription, chromatin remodelling, protein degradation and signal transduction. Very few metabolic genes were identified.

This data set of early MDE-expressed cDNAs provides a rich source of potential targets for further expression and functional analysis. Detailed analysis of a number of these early embryo-expressed genes is in progress and should generate new insight into the factors controlling embryo development in MDE cultures and seeds. Conceivably, a subset of these genes could be further developed to serve as molecular markers for different stages of MDE culture, and may even provide new molecular tools for the improvement of different aspects of MDE culture.

### 2.2.3 *Subtractive Hybridisation Uncovers a Key Regulator of Embryo Development*

In a parallel approach to the differential display-PCR screen, we used *B. napus* MDE cultures to identify genes expressed during the 8- to 32-cell stage of embryo development (Boutilier et al. 2002). In our hands, these proglobular-staged embryos are typically found in microspore cultures subjected to a 4-day heat-stress treatment. However, cultures at this time point also contain a large proportion of non-embryogenic cells that are either following the pollen development pathway, are arrested in development or are non-viable. We therefore made use of a heat-stressed non-embryogenic sample to subtract these non-embryogenic mRNAs from our embryogenic mRNA population (Pechan et al. 1991).

Using this subtractive screen, we identified five cDNA sequences corresponding to four unique genes that were originally named BNM for *Brassica napus* microspore derived-embryo (Table 1). Two of the cDNAs, *BNM2A* and *BNM2B*, encode BURP domain proteins (Hattori et al. 1998); one of the cDNAs, *BNM4*, encodes the orthologue of the *Arabidopsis* inward rectifying K<sup>+</sup> channel protein, AtKAT1 (Sentenac et al. 1992); one of the cDNAs encodes BNM5, an unknown protein; and the last cDNA, *BNM3* or *BABY BOOM* (*BBM*), encodes a protein with similarity to the AP2/EREBP domain transcription factor family. AP2/EREBP transcription factors are characterised by the AP2/ERF domain, a DNA binding domain that was first identified in the APETALA2 (AP2) and ETHYLENE RESPONSE ELEMENT (ERE) BINDING PROTEINS (EREBP; Jofuku et al. 1994; Ohme Takagi and Shinshi 1995). AP2/ERF proteins are plant specific and mediate diverse developmental and

stress-related responses (Riechmann and Meyerowitz 1998). The BBM transcription factor plays a central role in plant embryo development and is described in more detail below.

*BBM* expression pattern and function have been examined in *B. napus* and *Arabidopsis* (Boutilier et al. 2002). *BBM* is expressed throughout the major stages of embryo development in both seeds and MDEs, but is upregulated during early embryo development. Messenger RNA in situ hybridisation to developing MDEs and seeds showed that *BBM* is expressed throughout the developing embryo and transiently in the young endosperm. *BBM* expression does not appear to be seed specific as mRNAs are detected at low level in roots and in other organs. Analysis of gain-of-function mutants shows that *BBM* plays a key role in embryo development. Ectopic overexpression of *BBM* under control of the UBIQUITIN (*UBI*) and 35S promoters induces somatic embryogenesis and the formation of cotyledon-like structures from the vegetative tissues of young seedlings and occasionally from the leaves of mature plants. Both the *UBI:BBM* and the *35S:BBM* transgenic plants also show a low penetrance of other pleiotropic phenotypes that, together with the induction of somatic embryos, point to a general role for *BBM* in promoting cell division and morphogenesis. This capacity for promoting cell growth and differentiation was confirmed by an experiment in which *UBI:BBM* and wild-type *Arabidopsis* explants were placed on minimal medium or on medium containing growth regulators that are normally used to induce regeneration. In both cases the *UBI:BBM* explants outperformed the wild-type explants, showing enhanced and accelerated regeneration under the standard hormone-induced regeneration protocol, and the ability to completely regenerate into plantlets in the absence of added growth regulators (Boutilier et al. 2002).

#### 2.2.4 Transcriptional Regulation of Embryogenesis

Recently, a number of genes have been identified that show gain- or loss-of-function phenotypes similar to those seen in *BBM* gain-of-function plants. These genes include the *LEAFY COTYLEDON (LEC)* genes, *LEC1* and *LEC2* (Lotan et al. 1998; Stone et al. 2001), *WUS* (Zuo et al. 2002) and *PICKLE* (Ogas et al. 1997). The *LEC1* and *LEC2* genes both encode seed-expressed transcription factors: the *LEC1* protein shows similarity to the HAP3 subunit of eukaryotic NF-Y/CCAAT box-binding factors (Lotan et al. 1998), while *LEC2* contains a B3 domain, a plant-specific DNA binding domain (Stone et al. 2001). Both *LEC1* and *LEC2* were originally identified as loss-of-function mutants showing defects during the differentiation and maturation stages of embryo development (Meinke et al. 1994; West et al. 1994). The *lec* loss-of-function phenotypes (desiccation intolerance, failure to accumulate maturation-specific mRNAs, and cotyledons with post-germination leaf characteristics) suggest that wild-type *LEC* genes are required to specify embryo

cell fate and maintain embryo-specific processes. Further support for this idea was obtained by gain-of-function studies in which *LEC1* and *LEC2* were ectopically expressed in *Arabidopsis* under control of the 35S promoter (Lotan et al. 1998; Stone et al. 2001). Both 35S:*LEC1* and 35S:*LEC2* transgenics spontaneously produce somatic embryos on seedlings, although somatic embryo formation in the 35S:*LEC1* plants is considerably weaker than in 35S:*LEC2* transgenics.

A second gene shown to induce spontaneous embryo development is *WUS*. *WUS* encodes a homeobox transcription factor that together with the *CLV1*, *CLV2* and *CLV3* genes is involved in the specification of stem cell identity in the shoot apical meristem (Laux et al. 1996; Schoof et al. 2000). Ectopic expression of *WUS* together with *STM* promotes ectopic organogenesis (Galloy et al. 2002), a phenotype that was confirmed by the discovery of *WUS* in a gain-of-function mutant screen for cytokinin-independent regeneration (Zuo et al. 2002). Zuo et al. (2002) showed that ectopic *WUS* expression induces cell proliferation and somatic embryo formation in the absence of added growth regulators. Using steroid-inducible *WUS* expression it was also shown that *Arabidopsis* seedlings retain the capacity to respond to the *WUS* signal and form somatic embryos up to 7 days after germination (Zuo et al. 2002). We obtained similar results using steroid-inducible BBM activity. The results obtained with *WUS* and BBM are in contrast to those obtained with steroid-inducible LEC transgenics, where somatic embryos are only formed when seeds are plated directly on medium containing the steroid inducer (Zuo et al. 2002). Thus specific factors present in a narrow developmental window are required for LEC-mediated somatic embryo induction, whereas *WUS*- and BBM-mediated somatic embryo formation relies on factors that are more broadly expressed in juvenile plants.

The *PICKLE* gene also plays a central role in the induction of embryo development; however, unlike *LEC*, *WUS* and *BBM*, somatic embryos are formed in a *pk1* loss-of-function background. *PKL* was identified in a genetic screen for mutants showing altered root development (Ogas et al. 1997), and later as a genetic enhancer of the *crabs claw* mutant phenotype (Eshed et al. 1999). *Pkl* mutants fail to repress embryonic programs after germination with the result that *pk1* plants develop greenish pickle-shaped roots that express embryonic characteristics and form somatic embryos when excised and placed on minimal tissue culture medium. *PKL*, like *WUS*, is expressed throughout the life cycle of the plant. These genes seem to play a broader role than *BBM* and *LEC* in promoting meristematic growth during plant development.

What can these observations tell us about embryo formation in MDE cultures? Successful MDE culture depends on many factors including the species or genotype under study, pretreatments used to optimise the competence of the microspores to respond to the induction treatment, the developmental stage used as starting material, the stress treatment used to induce embryogenesis, and the media composition and culture conditions (Ferrie et al. 1995;

Maluszynski et al. 2003). Many species and genotypes are recalcitrant to MDE formation, and even in genotypes that respond well to microspore culture only a fraction of the microspores will develop into embryos. Recalcitrance for MDE formation can occur at many steps in the process and may have many origins. For example, recalcitrance for MDE formation may arise from a lack of competence to respond to the induction signal, or the inability to sustain embryogenic cell formation or initiate embryo differentiation. Ectopic expression of wild-type LEC1/2, BBM or WUS proteins or expression of dominant negative forms of PKL under control of a microspore-specific promoter (Custers et al. 1997) could be used to induce meristematic or embryogenic cell formation in recalcitrant genotypes, or to increase the number of MDEs in responsive genotypes. However, the question remains as to whether microspores initially need to be competent to respond to these embryo-inducing signals, or whether expression of these signals is sufficient to both induce embryogenic competence and sustain embryo formation. Clearly a better understanding of how competence for embryogenesis is established in microspores and how embryogenesis-promoting genes function at a mechanistic level is an essential part of any strategy aimed at improving MDE formation in both recalcitrant and responsive genotypes.

### **3 Extracellular Signalling Molecules in MDE Development**

Medium conditioned by rapidly growing embryo cultures or non-embryogenic nurse cells has been shown to stimulate the formation of embryogenic cells and embryos in both gametophytic and somatic-based culture systems (von Arnold et al. 2002). The effect of conditioned growth medium on embryogenesis has been best studied in carrot and conifer somatic embryo cultures, where it has been shown that certain secreted proteins and oligosaccharides can act as signalling molecules to promote cell proliferation and the growth of embryogenic cells. These signalling molecules include specific endochitinases (de Jong et al. 1992), arabinogalactan proteins (AGPs; Egertsdotter et al. 1993; Kreuger and van Holst 1993, 1995; Egertsdotter and von Arnold 1995; Toonen et al. 1997; van Hengel et al. 2001) and endogenous or bacterially produced lipophilic chitin oligosaccharides (de Jong et al. 1993; Egertsdotter and von Arnold 1998; Dyachok et al. 2000, 2002). The biological function and mode of action of chitinases, AGPs and lipophilic chitin oligosaccharides during somatic embryogenesis is not known; however, the ability of all three types of molecules to substitute for each other in promoting embryogenic cell formation is likely due to the ability of endochitinases to produce signalling molecules through AGP cleavage or lipophilic chitin oligosaccharide formation (Dyachok et al. 2002; van Hengel et al. 2002). How AGPs and lipophilic chitin oligosaccharides themselves promote cell division is also not known, but several studies suggest that they may function by inhibiting programmed cell death (McCabe et al. 1997; Dyachok et al. 2002).

Secreted proteins and oligosaccharides are also likely to stimulate the growth and development of MDE cultures, although direct evidence for this is just beginning to emerge (Zheng et al. 2002). Ovary co-culture and ovary conditioned medium (OVCM) have been used for many years to improve the efficiency of MDE formation in wheat and barley, while conditioned medium from wheat and barley MDE cultures has likewise been used to promote the growth of isolated zygotes in monocot species (Köhler and Wenzel 1985; Ziauddin et al. 1990; Bruins et al. 1996; Hu and Kasha 1997; Li and Devaux 2001). Recent attempts to identify the embryo-promoting molecules in MDE conditioned medium and to understand the role of endosperm-like nurse cells in MDE cultures have shed some light on putative roles for secreted molecules in promoting embryo development.

### 3.1 Ovary Conditioned Media Promotes MDE Development

A number of studies have demonstrated that co-cultivation of isolated barley and monocot microspores with live ovaries increases embryo yield and quality (Köhler and Wenzel 1985; Ziauddin et al. 1990; Bruins et al. 1996; Hu and Kasha 1997; Li and Devaux 2001). Recently, Zheng et al. (2002) showed that medium preconditioned by live ovaries was able to replace direct ovary co-culture in promoting MDE in responsive wheat genotypes. Microspores from responsive genotypes cultured with a single dose of ovary conditioned medium (OVCM) showed accelerated MDE formation as compared to ovary co-culture, most likely due to the early stimulation of cell division. A positive effect of ovary co-culture on cell division was observed earlier in barley-isolated microspore cultures (Li and Devaux 2001). In wheat, the greatest effect on cell division and embryo formation was obtained by OVCM that had been conditioned by ovaries for 7 days (Zheng et al. 2002). However, the final embryo yield was even greater when microspores were cultured continuously with live ovaries or with a combination of live ovaries and OVCM, suggesting that a sustained source of embryo-promoting factors increased embryo yields. In contrast to responsive genotypes, co-culture of microspores from recalcitrant genotypes with live ovaries had no effect on MDE formation. However, the addition of OVCM alone or together with live ovaries at an early stage of microspore culture dramatically enhanced MDE formation to levels observed in responsive genotypes. OVCM appears to stimulate microspore division within a narrow developmental window, as OVCM added at a later stage of development could not induce microspore division in recalcitrant genotypes. Together, these observations led Zheng et al. (2002) to suggest that microspores from responsive genotypes are initially able to secrete sufficient amounts of cell division promoting nursing factors, whereas recalcitrant genotypes lack this ability. These factors can be supplied to recalcitrant microspores by OVCM, but are released too slowly by live ovaries to be effective within the narrow developmental window in which they are needed.

Based on this hypothesis it should be possible to induce MDE in recalcitrant microspores by co-cultivating them with responsive microspores or MDEs.

While it is clear that ovaries have a nursing effect on embryogenesis, the nature of the conditioning factor is not known. Köhler and Wenzel (1985) examined the stimulatory effect of conditioned ovary medium on callus induction in shed pollen cultures of barley microspores and concluded that an auxin-like growth regulator could be the conditioning agent. Phenylacetic acid (PAA), a naturally occurring growth regulator with auxin-like activity, has been suggested to be the growth stimulating factor in OVCM (Ziauddin et al. 1990). However, in barley ovary co-culture appears to be more effective in promoting MDE development than PAA, suggesting that additional components contribute to the growth stimulating effect of OVCM (Li and Devaux 2001).

### 3.2 Signalling Molecules Secreted by Barley MDE Cultures

Conditioned media from barley MDE cultures has been shown to have a stimulating effect on the *in vitro* growth of zygotes excised from developing maize and barley seeds (Holm et al. 1994; Paire et al. 2003). As a first step toward identifying the specific components in MDE conditioned medium that contribute to the beneficial effect on embryo development, Paire et al. (2003) assessed conditioned medium from MDE cultures collected at different time points (4, 7, 11, 14, 18, 21 and 28 days) for its ability to stimulate embryo development in maize zygotes. When zygotes were cultured continuously on conditioned medium from a single time point, only conditioned medium from 18- and 21-day cultures supported embryo development. Conditioned medium from earlier stages of MDE development stimulated cell divisions, leading to small clusters of cells rather than embryos. MDE cultures at 18 and 21 days contain numerous, clearly formed MDEs that had been released from the exine wall of the microspore. The specific conditioned medium component responsible for stimulating zygotic embryogenesis was not identified in this study; however, preliminary evidence suggests that it is protein based. Firstly, the active component in conditioned medium is heat labile and protease sensitive. Secondly, the timing of appearance of significant amounts of secreted proteins in the conditioned medium correlates with the embryo-promoting capacity of the conditioned medium, and, further, non-embryogenic cultures that do not support growth of zygotic embryos do not secrete detectable amounts of protein. Finally, the addition of a greater than 50-kDa purified protein fraction to cultured zygotes was sufficient to stimulate embryo formation. The authors suggest that the active protein could be AGPs, since AGPs are detected in the conditioned medium at 18 days, and AGPs are of a size range that is compatible with the isolated protein fraction. Although there is not enough evidence at this moment to state which components in the barley MDE conditioned medium promote embryo

development, further biochemical analysis should lead to the rapid identification of these factors.

### 3.3 Secreted Peptides in *B. napus* MDE Cultures

*B. napus* is considered a good model system to study MDE development, in part due to the high efficiency of embryo production. However, embryo yield in *B. napus* MDE cultures can vary from 0 up to 10%, possibly due to variability in the condition of the donor plants, the differences in developmental stages of the microspores used as starting material and toxic factors released by dead or dying microspores (Pechan and Smykal 2001).

We are using a proteomics-based approach to study the biochemical differences in the conditioned medium of high-yielding embryogenic cultures and non-responsive cultures. We are focusing our analysis on small proteins, as a number of small proteins, and even peptides, have been shown to play a role in plant cell proliferation and meristem growth (Takayama and Sakagami 2002). One example of a growth stimulating peptide is phytosulfokine. In *Asparagus* cell culture, mesophyll cell division only occurs when the cells are cultured at sufficiently high density. A sulfated pentapeptide (phytosulfokine- $\alpha$ ) and a sulfated tetra-peptide (phytosulfokine- $\beta$ ) released by *Asparagus* cells cultured at high density were shown to stimulate mesophyll cell division in low-density cell cultures (Matsubayashi et al. 1997). Phytosulfokines were also identified as the compounds that contribute to the growth stimulating effect of conditioned medium from carrot somatic embryo cultures (Hanai et al. 2000).

We examined the differences in small protein profiles present in the conditioned medium from high-yield MDE cultures (HEC) in which 2% of microspores developed into embryos, and non-responsive MDE cultures (NRC) that did not produce any embryos. Both cultures were examined at about 10 days after culture initiation. HEC cultures contained a mix of developing embryos at the globular to torpedo stages, together with some arrested but viable microspores, as well as dead microspores. NRC cultures contained only viable-arrested and dead microspores. An isolation protocol was established in which the culture medium was acidified and protein fractions were isolated based on the differences in hydrophobicity and charge. This separation yielded 60 fractions, of which around 40 fractions showed detectable signals at 214 nm (mainly for proteins and peptides) and 254 nm (mainly for metabolites). Clear differences between the HEC and NRC medium could be detected using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS; Fig. 2). Two large non-protein peaks (metabolites) were specific for the HEC medium.

From the 60 fractions isolated we were able to identify 12 proteins in the NRC conditioned medium and 16 proteins in the HEC conditioned medium. Only 2 of these 28 proteins were found in the conditioned medium from both cultures. Fractions representing the most dramatic differences between HEC

and NRC conditioned medium were subjected to trypsin digestion and de novo sequencing, from which six small proteins from different fractions of the HEC and NRC media were identified (Table 1). All of the small proteins listed in Table 1 are encoded by genes with predicted secretion signal peptides, which is consistent with their presence in the culture medium. Two of these small proteins are homologous to Bp4 and BAN54, which are known to be pollen-specific (Albani et al. 1990; Kim 1997). The *Bp4* gene was present on our *B. napus* MDE microarray (see Sect. 2.2.2) and our expression analysis also supports a late pollen-specific rather than embryo expression pattern for this gene. The presence of these proteins in the medium from 10-day MDE cultures, which no longer contain any viable pollen, suggests that these proteins are derived from developing pollen grains from earlier stages of culture, and thus are very stable. Fraction 12 contained a protein that was abundantly present in HEC but absent in the NRC medium. This protein corresponds to an EST sequence identified in *B. napus* flower buds (GenBank acc. CD838723). Mass spectrometry data suggested that there is a potential internal disulfide bond connecting two cysteine groups within the peptide. The protein encoded by the cDNA carries a putative signal sequence, suggesting that it is an extracellular protein. No clear homologue of this protein could be found in the *Arabidopsis* genome.

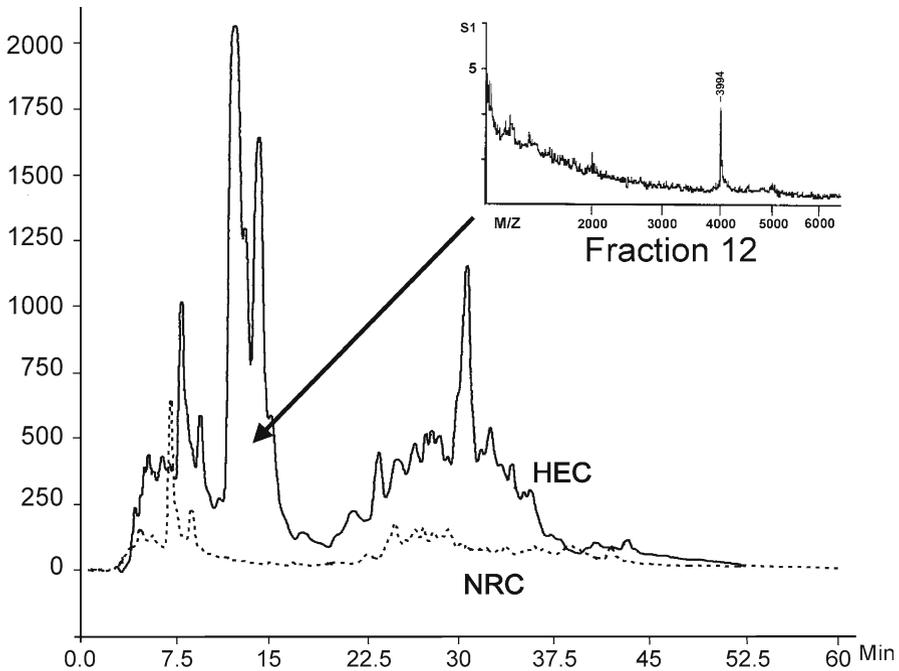
**Table 1.** Tryptic peptides identified by mass spectrometry in the media of high embryogenic cultures (HEC) and non-responsive cultures (NRC). Localisation of all proteins found was extracellular, based on sequence analysis

Mass (Da)	Identified sequences	Percentage of identity <sup>a</sup>	Protein description
(Da)			
1,105	VECDACKPK	80	Unknown floral gene, <i>B. rapa</i>
1,377	GCKVECDACKPK	77	Same as above
1,505	LPNSNWCCNTTPR	100	Same as above
1,503	LPNSNWC:CNTPR <sup>b</sup>	100	Same as above
1,535	LPNSNWC <sup>X</sup> C <sup>X</sup> NTTPR <sup>c</sup>	100	Same as above
1,237	TYPYKLPLDK	Unknown	
1,253	PYKLPLDK	Unknown	
1,336	YCXDEQQLPVNK	73	BAN54, <i>B. rapa</i>
1,392	LMDEQQLPVNK	73	Same as above
1,426	YXDEQQLPVNK	64	Same as above
1,520	LFSCDEQQLPVNK	62	Same as above
1,292	IPITGSYCLPTK	100	Bp4-like, <i>B. oleracea</i>
1,382	IPITGSYXLPTK	92	Same as above
2,606	CIGYLTQNGPLPR	100	LTP, <i>B. napus</i>
1,451	TRTNLNNMAR	80	Same as above

<sup>a</sup> Sequence similarity with known proteins in GenBank

<sup>b</sup> C:C, Internal SS bridge

<sup>c</sup> C<sup>X</sup>, Extra group(s) on cysteine; X, unknown



**Fig. 2.** Analysis of proteins present in the conditioned media of 10-day-old non-responsive (NRC) and high-yield (HEC) *Brassica napus* MDE cultures by high performance liquid chromatography. *Inset* shows the MALDI-TOF-MS profile of fraction 12 from HEC conditioned medium. The 4-kDa protein in this fraction shows similarity to an unknown protein from *B. napus*

In summary, a proteomics approach has been used as an efficient and sensitive way to analyse secreted proteins in *B. napus* culture media. Our future work will focus on the identification of small proteins present at earlier stages of high-yielding embryo and non-responsive cultures. Additional assays will be needed to elucidate the functions of these small proteins, and to determine if any of these proteins are responsible for the increased or decreased embryogenic capacity observed in different microspore cultures.

### 3.4 A Role for Endosperm-Like Nurse Cells in Monocot MDE Cultures

One interesting observation regarding growth stimulating molecules that are secreted into conditioned medium of embryo cultures is that non-embryogenic cells rather than embryogenic cells may actually be the source of these signalling molecules. This appears to be the case for the carrot EP3 protein. EP3 is a member of the class IV family of endochitinases, and was initially identified based on its ability to rescue embryo formation in the temperature-sensitive carrot cell line ts11 (de Jong et al. 1992). Localisation of

*EP3* mRNAs in carrot somatic embryo cultures and seeds showed that the *EP3* genes are not expressed in developing embryos, but rather in non-embryogenic cell types in culture and in integument and endosperm tissues that surround the developing embryo in planta (van Hengel et al. 1998). This observation led to the suggestion that EP3-like chitinases are part of a nursing system that functions in embryo-surrounding cells during zygotic embryo development, and that this nursing system is mimicked by non-embryogenic suspension cells during somatic embryogenesis (van Hengel et al. 1998).

Indirect support for the role of non-embryogenic nurse cells in promoting MDE development arose during a screen for genes that are specifically expressed during early maize MDE development (Magnard et al. 2000). Two genes encoding small secreted proteins, *ZmAE1* and *ZmAE3*, were identified as being expressed in 5-day-old MDEs. However, subsequent expression analysis in developing seeds showed that both *ZmAE1* and *ZmAE3* mRNAs are localised to the endosperm rather than to the zygotic embryo (Opsahl-Ferstad et al. 1997). In seeds, *ZmAE1* is initially expressed in the endosperm in the so-called embryo-surrounding region (Opsahl-Ferstad et al. 1997) just as endosperm cellularisation begins. *ZmAE1* expression persists in this region as the endosperm develops, and later extends to a region of the endosperm adjacent to pedicel, the basal endosperm transfer layer. This layer is thought to be involved in nutrient transfer from the seed coat to the endosperm (Hueros et al. 1999). *ZmAE3* is also expressed in the embryo-surrounding region of the endosperm, but is restricted to a few cells adjacent to the adaxial side of embryo. These expression results were surprising, and suggested that maize MDEs may actually possess endosperm-like characteristics, and, by extension, that these non-embryogenic cells may play a nursing role by promoting the growth of MDEs. Ultrastructural examination of developing MDEs from the same stage as those used to isolate *ZmAE1* and *ZmAE3* showed that MDEs contain two distinct domains of different sizes and cellular characteristics (Magnard et al. 2000; Testillano et al. 2002). The larger domain shows cellular features that are characteristic of early endosperm cells, such as partially coenocytic organisation, synchronised cell division, a central vacuole and incomplete or 'free-growing' cell walls, whereas the smaller domain is embryo-like in that it is cellularised and consists of small proliferating or meristematic-like cells. If maize MDEs do indeed contain separate embryo- and endosperm-like regions then *ZmAE1* and *ZmAE3* mRNAs should be specifically localised to the endosperm-like region of the MDE. However, this is not the case as both *ZmAE* genes are expressed in both compartments of the MDE (Magnard et al. 2000). As suggested by Magnard et al. (2000), expression of the *ZmAE* genes in both compartments could indicate that embryo-endosperm gene expression is deregulated in vitro. Alternatively, the smaller domain may not be embryo-like, but rather homologous to the densely cytoplasmic, cellularised region of the endosperm that normally surrounds the early embryo. If both domains of the androgenic struc-

tures are indeed endosperm-like, then this would imply that MDEs first go through an endosperm-like phase and then later develop into embryos. This discrepancy could be resolved by examining spatial and temporal distribution of a large number of embryo- and endosperm-expressed genes in the androgenic structures.

Although there is no direct evidence for a role of endosperm-like nurse cells in promoting MDE development in maize, the similarities between these cells and the putative non-embryogenic nurse cells in carrot seeds and somatic embryo cultures are striking and deserve further attention. In this respect, laser or genetic ablation experiments in maize MDE cultures and other embryo culture systems could be used to demonstrate a direct role for these non-embryogenic nurse cells in promoting of embryo development *in vitro*.

The identification and further characterisation of the secreted molecules in MDE cultures will also answer some important questions about the role of secreted growth promoting factors in embryo development. For example, are the compounds found in MDE conditioned medium also found in developing seeds, and, if so, are these molecules secreted by the embryo or by the non-embryogenic cells and tissues that surround the embryo in the seed and in MDE cultures?

## 4 Conclusions and Perspectives

The use of MDE culture as a model system to study the molecular control of embryogenesis is resulting in an ever-expanding collection of early embryo-expressed genes. Such expression-based studies could be further refined and enhanced by the use of marker genes for the early detection and enrichment of specific cell types, and through the use of full genome microarrays. Isolation of the promoters for these early embryo-expressed genes and characterisation of transgenic lines carrying promoter:reporter constructs can be used to develop a collection of early embryo-expressed promoters. Such promoter sets are currently underrepresented in the databases, but if developed would provide valuable tools for tissue and stage-specific embryo expression both *in vitro* and in developing seeds. These genes and reporter constructs would also be valuable as markers to identify embryogenic cells in mutant screens and for mutant characterisation.

Functional analysis of this collection of MDE-expressed genes is clearly needed to understand the role of these genes during microspore-derived and zygotic embryo development. Functional analysis of MDE-expressed genes can be carried out in the homologous species using standard gain- and loss-of-function transgenic approaches; however, this is an enormous task, as most species used for MDE culture are less amenable to high throughput analysis. In this respect, it would be more efficient to identify early embryo-

expressed genes using model species for MDE culture, and then to analyse the function of the orthologous genes in a heterologous species such as *Arabidopsis*, where tools are available for high-throughput functional genomics. More targeted approaches such as mutant screens and genetic genomics (Janzen and Nap 2001), although laborious, offer a more direct route to identifying genotype-based differences in embryogenic cell formation in MDE cultures of certain model species.

Another area where MDE culture is proving to be an extremely useful tool is in the area of cell–cell communication and the identification of factors in conditioned medium that promote cell division and differentiation. New developments in the high-throughput and ultra-sensitive detection of proteins and metabolites will facilitate further elucidation of the extracellular signals controlling growth and differentiation during embryogenesis.

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