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# Functional genomics of microspore embryogenesis

**Julia Hosp · Simone de Faria Maraschin · Alisher Touraev · Kim Boutilier**

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**Abstract** Isolated plant microspores, when stressed and cultured in vitro, can be diverted from their normal gametophytic pathway towards sporophytic development, with the formation of haploid embryos and ultimately doubled-haploid plants. This process is called androgenesis or microspore embryogenesis, and is widely used in plant breeding programmes to generate homozygous lines for breeding purposes. Protocols for the induction of microspore embryogenesis and the subsequent regeneration of doubled haploid (DH) plants have been successfully developed for more than 200 species. These practical advances stand in stark contrast to our knowledge of the underlying molecular genetic mechanism controlling this process. The majority of information regarding the genetic and molecular control of the developmental switch from gametophytic to sporophytic development has been garnered from four intensely studied (crop) plants comprising two dicotyledonous species, rapeseed (*Brassica napus*) and tobacco (*Nicotiana tabacum*), and two monocotyledonous species, wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). In these species the efficiency of microspore embryogenesis is very high and reproducible, making them suitable models for molecular studies. In the past, molecular studies on microspore embryogenesis have focussed mainly on the identification of genes that are differentially expressed during this developmental transition and/or early in embryo development, and have identified a number of genes whose expression marks or predicts the developmental fate of stressed microspores. More recently, functional genomics approaches have been used to obtain a broad overview of the molecular processes that take place during the establishment of microspore embryogenesis. In this review we summarise accumulated molecular data obtained in rapeseed, tobacco, wheat and barley on embryogenic induction of microspores and define common aspects involved in the androgenic switch.

**Keywords** Microspore embryogenesis · Doubled haploids · Functional genomics · Microarray · Subtractive hybridisation

J. Hosp · A. Touraev (✉)
Max F. Perutz Laboratories, University Departments at the Vienna Biocenter, Department of Genetics, Vienna University, Dr. Bohrgasse 9, A-1030 Vienna, Austria
e-mail: Alisher.Touraev@univie.ac.at

S. de Faria Maraschin
De Ruiter Seeds, P.O. Box 1050, Bergschenhoek 2660, The Netherlands

K. Boutilier
Plant Research International, P.O. Box 16, Wageningen 6700 AA, The Netherlands

**Abbreviation**

EST Expressed Sequence Tag
SSH Suppression Subtractive Hybridisation

## Introduction

Microspore embryogenesis of flowering plants is a fascinating system that is being used as a tool to facilitate plant breeding and, in more pure research, studies on embryogenesis. A central issue is cell totipotency. Microspores can be diverted from their gametophytic pathway to a totipotent, de-differentiated intermediate stage and further, towards embryogenic development after exposure to a variety of stress treatments such as temperature, carbohydrate or nitrogen starvation, chemical, hormone or irradiation (Touraev et al. 1997, 2001; Zoriniants et al. 2005). These embryogenic cells develop through the same series of morphological stages as diploid zygotic (seed) embryos and have gene expression and biochemical profiles that are similar to those of zygotic embryos (Ilic-Grubor et al. 1998; Yeung et al. 1996; Boutilier et al. 2002; Fiers et al. 2004). This similarity in developmental pathways, combined with the ability to obtain large and homogenous populations of purified microspores and microspore-derived embryos at diverse stages has made microspore culture a valuable tool in studying a range of processes related to plant embryogenesis, including lipid and hormone biosynthesis and signalling, and stress responses (Weselake 2000; Hays et al. 2001; Rays et al. 2000).

The mechanisms underlying the stress-induced switch from gametogenesis to embryogenesis are largely unknown, however a large body of cell biological data accumulated over the years in different species indicates that a number of common cellular processes take place during the transition from microspore to embryogenic cell formation. Stressed microspores that enter the embryogenic pathway exhibit changes in their overall morphology that suggest a transition towards a more de-differentiated state. This transition is characterised by cellular enlargement, along with a typical ''star-like'' vacuolisation and cytoskeletal rearrangements that result in the migration of the nucleus towards a more central position. Stressed microspores also show a decrease in the number of starch and lipid granules and ribosomes. It has become widely accepted that the degradation of cellular components and proteins is a prerequisite for developmental re-programming in many organisms. This re-programming is mediated on the one hand by the 26S proteasome and on the other hand through autophagy and thus lysosomal recycling (Maraschin et al. 2005c and references therein). During the exposure to embryo-inducing stress treatments, tobacco and rapeseed microspores undergo a transient cell cycle arrest (Zarsky et al. 1992; Binarova et al. 1993). In tobacco, this cell cycle arrest is relieved when the stressed microspores are transferred to non-stress conditions in a culture medium that supports sustained cell divisions and enables embryo formation. When transferred into a ''maturation'' medium, in which non-stressed microspores develop into fertile pollen grains, these same stressed microspores still retain their irreversible commitment to embryo formation (Touraev et al. 1996a).

Molecular studies aimed at unravelling the changes that occur during the induction of microspore embryogenesis have largely focussed on the identification of genes that are differentially expressed during this developmental transition and/or early embryogenic development (Vrinten et al. 1999, Reynolds and Kitto 1992; Custers et al. 2001; Boutilier et al. 2002). These studies have identified a number of genes whose expression marks or predicts the developmental fate of stressed microspores. More recently, concerted efforts have taken place to obtain a broad overview of the molecular processes that occur during the establishment of microspore embryogenesis. Although *Arabidopsis thaliana* has been the model of choice for most plant molecular-genetic studies, microspore embryogenesis in *Arabidopsis* has not yet been attained despite much (unpublished) effort. As a result, the majority of information regarding the genetic and molecular control of the developmental switch from gametophytic to sporophytic development has been garnered from four well studied plants comprising two dicotyledonous species, rapeseed (*Brassica napus*) and tobacco (*Nicotiana tabacum*), and two monocotyledonous

species, wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). For rapeseed, tobacco and barley the major factor contributing to their popularity is the very efficient and reproducible production of microspore embryos (Jähne and Lörz 1995; Touraev et al. 1997); up to 70% of the cultured microspores can be induced to divide initially, and embryos usually develop by direct embryogenesis i.e. without a callus phase (Indrianto et al. 2001). In the case of wheat research on DH production has been driven by the value of homozygous lines to cultivar production.

## Transcriptomics as a strategy for gene discovery in microspore embryogenesis

Recently, a number of microarray-based studies of the *Arabidopsis* pollen transcriptome have been published, giving a detailed view on the transcriptional peculiarities of these specialised cells (Honys and Twell 2003; Becker et al. 2003; Lee and Lee 2003). Since microspore embryogenesis is not established in *Arabidopsis*, no large-scale studies on microspore embryogenesis using *Arabidopsis* whole-genome based microarrays have been carried out. Therefore, differential screening techniques such as differential display, subtraction hybridisation and dedicated cDNA arrays have been the methods of choice to identify genes and analysing gene expression profiles associated with microspore totipotency in species other than *Arabidopsis*, including tobacco, rapeseed, wheat and barley. These differential screens have been used to investigate genes that are (1) involved in the switch between the two pathways and thus in de-differentiation and (2) govern differentiation in the course of embryogenesis (Boutilier et al. 2005). Finding marker genes involved in the developmental switch is of major interest to both the pure and applied research communities. Such candidate genes, whose expression ideally would coincide with the earliest step of de-differentiation and the commitment for embryogenic development, could be of help in overcoming the limitations in regard to microspore embryogenesis in recalcitrant species, and at the same time could reveal and be diagnostic of the earliest steps in the course of genetic regulation of microspore embryogenesis.

## Macroarray studies yield a wealth of information on the induction of barley microspore embryogenesis

Different types of stress treatments are able to trigger the switch of barley microspore development from the gametophytic pathway towards the sporophytic route, including temperature-, osmotic- and starvation-stress (Jähne et al. 1994). Despite the fact that many stress treatments lead to the re-programming of microspores into embryos, a combination of starvation and osmotic stress (mannitol treatment) has been the method of choice in studies aimed at identifying genes involved in the induction of barley microspore embryogenesis. Following mannitol stress treatment, up to 50% of the enlarged microspores are switched towards the sporophytic pathway, thus providing excellent starting material for differential screening approaches (Maraschin et al. 2005a).

The first genes identified as being differentially expressed during the initiation process of barley microspore embryogenesis encode a lipid transfer protein (ECLTP), a glutathione-S-transferase (ECGST) and an unknown protein (ECA1) that possibly encodes an arabinogalactan-like protein. *ECLTP*, *ECGST* and *ECA1* were identified via differential screening of a cDNA library prepared from 3 day old mannitol-induced microspore cultures. Further expression analysis revealed that only the increase in expression of *ECA1* is specific for embryogenic microspores (Vrinten et al. 1999).

Maraschin and co-workers (2005b) have employed microarrays containing ESTs isolated from early stages of barley zygotic embryogenesis for screening mRNA populations derived from: (1) uni-nucleate microspores on the verge of the first pollen mitosis, (2) bi-cellular pollen, and (3) mannitol-stressed, embryogenic microspores. A total of 96 ESTs were found to be differentially expressed (up- or down-regulated) between these distinct developmental stages, with expression fold changes ranging from 2 to 52 times. Principle component analysis (PCA) of normalised gene expression data was used to identify gene expression programmes associated with each developmental stage. The uni-nucleate microspore stage

was characterised by the induction of ESTs from functional classes involved in mitosis and lipid biosynthesis, while bi-cellular pollen was marked by the induction of ESTs that were associated with carbohydrate and energy metabolism. In contrast, embryogenic microspores showed a very distinct gene expression profile as compared to developing pollen. Based on the PCA model, the ESTs that were either specifically down-regulated in bi-cellular pollen or up-regulated in embryogenic microspores were considered to be involved in the microspore embryogenesis process. These ESTs belong to distinct functional groups, mainly involved in protein degradation (ubiquitin-conjugating enzyme, 20S proteasome subunit alpha-5 and alpha-2, 26S protease regulatory subunit-8, cysteine protease, aspartic protease and FtsH metalloprotease), starch and sugar hydrolysis (maltase, cell wall invertase), stress responses (GST, catalase), inhibition of programmed cell death (Bax-inhibitor 1), metabolism (alcohol dehydrogenase 3; ADH 3) and cell signalling (GTPase). The authors have studied further the expression of the most dynamic ESTs associated with mannitol-stressed microspores upon optimal and sub-optimal stress treatments to induce microspore embryogenesis. Using this approach, it was demonstrated that ESTs involved in stress responses and cell signalling were induced irrespective of the stress treatment, while the level of induction of *ADH3* and of proteolytic genes were positively associated with the embryogenic potential of microspores. *ADH3* expression during microspore embryogenesis, and thus the catalysis of aldehyde or ketone into alcohol, points to a switch from oxidation to fermentation, and represents an indication that metabolic changes are a part of the de-differentiation phase of microspore embryogenesis. Further research needs to be done to understand the role of metabolic changes and proteolysis in the regulation of stress-induced androgenesis. Proteolysis is an issue that is attracting increasing attention as an important regulatory mechanism in cell differentiation and cell cycle progression in plant cells (Hellman and Estelle 2004), and plays a role in many aspects of plant development including somatic and zygotic embryogenesis, germination, tissue re-modelling, and programmed cell death (Beers et al. 2004). It will be a challenge to determine whether the induction of ESTs coding for proteolytic enzymes is reflected in the degradation of pollen-specific proteins and in the re-entry of the pollen nucleus into the cell cycle.

## Differential screens point to a complex genetic regulation of events during wheat microspore embryogenesis

The combination of carbohydrate starvation and heat stress efficiently induces microspore embryogenesis in isolated wheat microspores (Touraev et al. 1996b). A number of genes expressed in wheat anther cultures have been isolated that are differentially expressed between late stage microspore-derived embryos and mature pollen (Reynolds and Crawford 1996; Reynolds 2000). One of these genes encodes a cysteine-labelled metallothionein, EcMt, whose expression is restricted to anther cultures 6 h after embryo induction. In mammals, metallothioneins are involved in defence mechanisms associated with toxicity and carcinogenicity (Theocharis et al. 2003). They are unusually rich in cysteine residues that coordinate multiple zinc and copper atoms under physiological conditions, although the functional significance of these residues is yet to be determined. EcMt was suggested to be a marker for microspore embryogenesis, although it was also found to be induced after ABA treatment in diverse tissues.

Subtractive cloning is a powerful technique for isolating genes expressed in one cell population but not in another (Sagerström et al. 1997). Suppression Subtractive Hybridisation (SSH; Diatchenko et al. 1996) between freshly isolated and stressed wheat microspores has been carried out (Tashpulatov et al., unpublished). From 900 screened clones, 200 were found to be differentially expressed between the examined stages, and 65 unique sequences could be identified. About 30% of the sequences are related to genes with unknown function, 36% did not show significant homologies in database searches, but 34% could be assigned to functional categories based on sequence similarities. The majority of these clones encode proteins with a role in metabolism, along with the transcription and translation of RNA

and proteins. Several transposon-related genes were identified, as were other sequences encoding homologues of proteins involved in signal transduction, replication and cytoskeletal re-organisation. These results suggest profound physiological changes involving stress signal transduction and adaptations in metabolism and transcription, which take place during the switch from pollen to embryogenic cell development.

## Gene discovery using rapeseed microspore-derived embryo cultures: from subtractive hybridisation to cDNA microarrays

To date, the majority of gene expression studies on microspore embryogenesis have been carried out in rapeseed. The popularity of rapeseed is in part due to the exceptional speed, efficiency and synchronicity of microspore embryogenesis, and in part due to the similarity of rapeseed at the phylogenetic and genome sequence level to the model plant *Arabidopsis*. Microspore embryogenesis in rapeseed is induced by growing donor plants at low temperatures, usually a 10°C/5°C day/night cycle, followed by isolated microspore culture at approximately 32°C. After 2 days of heat stress approximately 15–25% of the isolated microspores usually exhibit embryogenic divisions, and after 10 days in culture approximately 2–5% of the original microspore population have developed to the globular-heart stage of embryo development. Identification and functional annotation of differentially-expressed rapeseed genes is facilitated by the similarity at the genome level between *B. napus* and *Arabidopsis* (Brunel et al. 1999). The *Arabidopsis* orthologues of rapeseed genes are for the most part easy to identify, and the function of these genes can be determined rapidly using the wealth of insertional mutagenesis lines available for *Arabidopsis* or through heterologous or homologous expression studies in *Arabidopsis*.

Gene expression studies in rapeseed microspore embryogenesis have developed in parallel with the major technological advances in differential gene expression analysis, starting with subtractive hybridisation and ending more recently with microarray analysis. One of the first differential gene expression screens on rapeseed microspore embryogenesis was performed using subtractive hybridisation (Boutilier et al. 2002 and references therein). This screen was established to identify genes that were up-regulated in 4 day heat-stressed embryogenic microspore cultures as compared to 4 day heat-stressed non-embryogenic microspore cultures. Five cDNAs, called *BNM* (B*rassica* n*apus* m*icrospore-derived embryo*) were identified in this screen. Two of the cDNAs encode BURP domain proteins (BNM2A and BNM2B) a new class of plant proteins with a modular structure and a conserved C-terminal motif with unknown functions (Hattori et al. 1998), one of the cDNAs (*BNM3*) encodes an AP2/EREBP domain transcription factor that was later renamed BABY BOOM (BBM), one cDNA (*BNM4*) encodes the *Arabidopsis* orthologue of the AKT1 $K^+$ channel protein (Sentenac et al. 1992), and the fifth cDNA corresponds to a sequence for which no open reading frame has yet been assigned (BNM5).

Bioinformatics analysis of the *BNM2* gene and functional analysis of the *BBM* gene support a role for these two proteins in plant embryogenesis. In rapeseed, *BNM2* is expressed throughout microspore embryogenesis and zygotic embryogenesis, and also in flower buds (K. Boutilier, unpublished data). *BNM2* expression during microspore and zygotic embryos peaks during the initial period of storage product accumulation and in zygotic embryos, declines as the seeds mature and become desiccation tolerant. The *Arabidopsis* orthologue of the *BNM2* is the most similar of all the *Arabidopsis* BURP domain genes to the field bean (*Vicia faba*) *Unkown Seed Storage Protein* gene (Bassüner et al. 1988). The function of USP has not been determined, however as shown by Chesnokov et al. (2002), USP is an early marker for *Nicotiana plumbaginofolia* somatic embryogenesis and can be used in this system to sort embryogenic from non-embryogenic cells.

The BBM gene is preferentially expressed during early zygotic and microspore embryogenesis, and at lower levels in other non-seed organs, including roots and flower buds. BBM overexpression using constitutive promoters led to the ectopic induction of somatic embryos and cotyledon-like structures from young seedling tissues

of *Arabidopsis* and rapeseed, and elevated the regeneration capacities of *Arabidopsis* explants. Both these observations point to the importance of BBM in promoting cell division and morphogenesis. The question that remains to be answered is whether BBM plays a critical role in the induction of microspore embryogenesis per se or whether it simply acts downstream of the induction trigger to maintain an embryogenic state in committed cells.

The well-established technique of subtractive hybridisation was followed by the differential display PCR technique (Liang and Pardee 1992), which was used by Custers et al. (2001) to isolate genes involved in the developmental re-programming of heat-stressed rapeseed microspores and in the early stages of ME development. A number of genes were isolated in this screen, among them a gene encoding a CLAVATA3/ESR (CLE) family member, CLE19. *CLE19* encodes a small secreted protein and is expressed in aerial parts of the plant at the periphery of the incipient meristem, where organs will eventually form, and in roots (Fiers et al. 2004). In microspore and zygotic embryogenesis CLE19 expression is confined to the developmental window between the globular and heart stages of embryo development. CLE19 does not seem to have a role in initiating or maintaining embryogenesis per se, but rather promotes cell differentiation and/or inhibits meristem formation in a range of plant organs (Fiers et al. 2004). Other CLE proteins, including CLAVATA3 (CLV3) and CLE40, play similar roles in the regulation of stem cell populations (Hobe et al. 2003). The study by Fiers et al. (2004) highlights the utility of the microspore embryogenesis system as a functional genomics tool to isolate genes that are expressed during the largely inaccessible stages of early zygotic embryogenesis.

The most recent addition to the molecular studies on microspore embryogenesis makes use of cDNA microarray and 2-dimensional gel electrophoresis (2-DGE) proteomics technologies (Boutilier et al., manuscript in preparation). A dedicated cDNA array was made using cDNAs expressed in one- to ten-day-old microspore cultures. Suppression Subtractive Hybridisation (Diatchenko et al. 1996) was also used to enrich for differentially expressed genes at the pre-globular and globular stages of development. Expression profiles of approximately 1,600 arrayed cDNAs were examined using samples corresponding to (1) pollen cultures, (2) microspore-derived cultures containing embryos from the few-celled to globular-heart stages of development, (3) suspensor-bearing microspore-derived embryo cultures (Custers et al. 2001). The same samples were used to identify global and specific changes that occur at the protein expression level.

PCA of gene and protein expression profiles from standard microspore embryo cultures showed that the proteome and transcriptome predict the same progression of developmental events during microspore-derived embryo development, that is, a gradual transition from pollen-dominated expression profiles in young cultures to embryo-dominated expression profiles in older cultures. Standard microspore-derived cultures less than 5 day old (pre-globular embryo stage) are dominated by abundant pollen-expressed genes that are expressed in the non-embryogenic gametophytic cells that develop in the same culture. These gametophytic cells usually die off *en masse* around day 4–5 of standard microspore embryo culture, at which point their contribution to the total gene and protein expression profiles of a culture is reduced. This early over dominance by pollen-expressed genes and proteins made it difficult to identify expression profiles associated with the stress-induced switch from pollen development to the first few embryogenic cell divisions. However, suspensor-bearing microspore-derived embryo cultures, which develop much more slowly than standard cultures and behave morphologically like true zygotic embryos (Custers et al. 2001), proved to be a valuable resource for identifying early embryo-expressed genes and proteins. Suspensor-bearing microspore embryos first develop via a uni-seriate filament, the apical cell of which divides after about 7–8 days to form the first cell of the embryo-proper. The percentage of pollen-like cells in the suspensor-bearing embryo cultures significantly drops around this time and at 10 days (pre-globular stage embryos) no viable pollen cells are detected. The gene and protein expression

profiles associated with these cultures are accordingly more embryo-like than pollen like. Thus in this study, the 5 day standard microspore embryo cultures and both suspensor-bearing embryo cultures were used to identify genes and proteins that are up-regulated within the first few embryogenic cell divisions in culture. A collection of genes up-regulated during the first few cell divisions was identified, however the general molecular functions of the proteins encoded by these genes as well as the biological processes in which they function did not differ significantly from those of genes expressed at the pre-globular and globular-heart stage of microspore-derived embryo development. This observation suggests that suspensor-bearing embryos comprising only a few embryo-proper cells, may be temporally too far removed from the heat-stress induction phase to allow identification of specific genes associated with the initial switch in developmental pathways from gametophyte to sporophyte development.

## SSH and metabolomics highlight a succession of events that take place during tobacco microspore embryogenesis

Tobacco, like rapeseed, has also been a popular model for understanding the molecular basis of microspore embryogenesis (Touraev et al. 1997). Sucrose and nitrogen starvation applied to isolated young bi-cellular pollen of tobacco induces the formation of embryogenic pollen grains, which after transfer to a simple sucrose- and nitrogen-containing medium, divide repeatedly and produce large numbers of embryos (Garrido et al. 1995; Kyo and Harada 1986). A combination of starvation and heat stress can induce embryogenesis in nearly all viable tobacco microspores (Touraev et al. 1996a), while the same microspores develop into mature fertile pollen under non-stress conditions in a relatively rich medium (Benito Moreno et al. 1988; Touraev and Heberle-Bors 1999).

Kyo et al. (2002) isolated genes encoding embryogenic pollen-abundant phosphoproteins (NtEP) in tobacco and showed that their selective transcription takes place in dedifferentiating pollen in a cell-cycle-independent manner. The same group isolated 16 distinct cDNAs after a differential screen (Kyo et al. 2003) and arranged them into two classes. The 13 cDNAs in class I showed an expression pattern that was restricted to de-differentiating microspores, while the remaining class II cDNAs were expressed in both de-differentiating microspores and actively dividing cell populations, such as pollen mother cells, BY-2 cells and early microspore-derived embryos. The class I genes comprise various sequences, among them the earlier characterised phosphoproteins NtEP, stress- and ABA-responsive genes, a Myb transcription factor, glucanase, chitinase and some unknown genes. The class I genes were all expressed in S-phase enriched cell populations, prior to one of the A-type cyclin genes that has been shown to regulate the S-phase of the cell cycle. The class II genes were represented by plant homologs coding for histones and for a mini-chromosome-maintaining protein, suggesting that microspore embryogenesis is accompanied by re-entry into the cell cycle.

SSH was used to enrich the low abundance RNA products of differentially expressed genes during tobacco microspore embryogenesis (Touraev et al., in preparation). cDNAs from starved tobacco microspore cultures were subtracted against cDNA from untreated microspores. A number of distinct sequences were identified that were up-regulated in stressed, embryogenic microspores. Further analyses of selected clones by multiple tissue Northern and real time RT-PCR confirmed their up-regulation in stressed microspores and haploid embryos compared to untreated microspores. Sequence analyses indicated the involvement of these genes in metabolism, chromosome remodelling, transcription and translation, while one third of sequences did not show a significant match to any known genes or published ESTs. The genes isolated were termed NtSM genes (N*icotiana tabacum* Stressed Microspores).

Unlike previous studies, in which proteomics and/or transcriptomics-based analyses were performed, Touraev et al. (in preparation) also used GC/MS to characterise the metabolic profile changes that take place in stressed, embryogenic microspores in relation to non-stressed microspores. In total 70 compounds could be identified in the investigated samples, partly displaying

significant changes in metabolite levels. Interpretations of the observed results suggest that embryogenic microspores do not merely react to the stress treatment but also undergo more profound developmental changes.

## Functional genomics to predict the mechanism of microspore embryogenesis

An overall picture of the complex regulation of the developmental switch from the gametophytic to sporophytic pathway during microspore embryogenesis has still not emerged despite the large amount of data obtained in the different model species. However, circumstantial evidence suggests that some common aspects govern the re-programming of microspores toward embryos. Cell cycle arrest has already been shown, at both the cellular and molecular level, to be a relevant prerequisite for the adaptation of microspores to adverse conditions (Zarsky et al. 1992; Binarova et al. 1993). According to evidence in yeast (Wysocki and Kron 2004), it could be postulated that cell cycle arrest may lead to an early stage of an autophagic response that enables the ''fittest'' microspores to de-differentiate and develop as embryos.

Chromosome re-modelling might be a further requirement in an early stage of adaptation based on reports of a number of genes involved in chromosome re-organisation isolated in diverse species upon differential screens as described above. However, there has been consistent molecular proof that chromosome remodelling accounts for re-programming, although hypomethylating drugs have been shown to enhance microspore embryogenesis to a certain extent (Li et al. 2001). Along with chromosomal alterations, restructuring of the cytoskeleton (enlargement of the cell, nuclear migration and altered vacuolisation) might be involved in the morphological changes from young pollen to embryogenic cells, namely. Changes in the transcriptional and translational machinery are likely to occur and are clearly supported by isolated sequences that are homologs to genes of such functional classes.

At some stage in androgenic development, proteins are assumed to be recycled and selectively destroyed in favour of synthesis of new proteins that are able to better serve the novel conditions of embryogenic induction. A number of protease and ubiquitin-interacting genes isolated in independent studies supports this assumption. Evidence for targeted protein degradation in starved somatic cells, and thus involvement of vacuolar autophagy has already been demonstrated (Aubert et al. 1996). Morphological and biochemical studies were performed during the transition from multi-cellular structures to globular barley embryos that give sufficient evidence for the importance of programmed cell death at late stages of microspore embryo development. Programmed cell death was demonstrated by chromatin condensation and DNA degradation, a process that preceded cell detachment and cytoplasm dismantling. This morphotype of programmed cell death was accompanied by an increase in the activity of caspase-3-like proteases (Maraschin et al. 2005d).

A master gene capable of initiating embryogenic development from microspores has not yet been identified. However, it seems to be unlikely that only one gene might have the potential to induce a large number of profound cellular adaptations and changes. Assumedly, it might prove more likely that a concerted series of events takes place during the switch from pollen to haploid embryo development. Such a switch may initiate stress-induced metabolic alterations, lead to higher order changes in chromatin structure and gene expression, and end with the activation of cell-cycle and regulatory genes.

Looking at the independent studies presented in this review, some drawbacks resulting from the current approaches have to be noted, namely that apart from the *GST* and *ADH* genes, none of the same genes have been identified in different species. This lack of unity may arise from the complexity of the process of microspore embryogenesis, the different stages of microspore embryogenesis under study and the limited number of genes analysed rather than from key differences in the regulation of microspore embryogenesis. Furthermore, since diverse stress treatments lead to androgenesis in different species, the possibility that different signalling pathways and respective genes might be involved in stress responses and thereby re-programming,

helps to explain the discrepancy in gene expression revealed by studies in the different model species.

In this respect, establishment of a microspore embryogenesis system in *Arabidopsis* would open up new and exciting research possibilities. Firstly, it would provide a single unified system from which to study different aspects of the microspore embryogenesis process. Secondly, researchers could make use of the large amount of functional genomics tools, including full genome microarrays for gene identification and mutant populations for functional analysis, to identify and characterise both marker genes and causal induction genes for microspore embryogenesis. Finally, Arabidopsis' short life cycle and sequenced genome could serve as a basis for QTL and mutagenesis-based mapping studies, aimed at identifying the major and minor genetic components regulating microspore embryogenesis in plants.

## Conclusions

Microspore embryogenesis research still is lacking a common model species or at least common experimental approaches that would facilitate a better fundamental understanding of the process and lead to innovation in application. Despite these limitations, new functional genomics data is helping to forge a link between the different species and systems under study. In the light of the rate at which plant genomes are being sequenced and the feasibility of novel high-throughput approaches such as microarray techniques, research in the next 10 years promises to shed even more light on the intriguing aspects of microspore embryogenesis. Likewise, a number of promising microspore embryogenesis marker genes and embryo-induction genes have already been identified. Further characterisation of the expression patterns of the marker genes during microspore embryogenesis using non-destructive imaging techniques, as well as directed expression of embryo-induction genes in responsive and non-responsive microspore embryo cultures is feasible in most of the model species under study and should therefore provide important insight into the process of microspore embryogenesis within a reasonably short time frame. Together these detailed and broader studies will provide insight into how microspore embryogenesis is controlled in different species and under different culture conditions and will ensure the development of new techniques for doubled haploid induction in recalcitrant organisms.

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