BIOLOGIA PLANTARUM 43 (4): 481-489, 2000

REVIEW

Pollen embryogenesis - the stress mediated switch from gametophytic to sporophytic development. Current status and future prospects

P. SMÝKAL*

Department of Plant Physiology, Faculty of Science, Charles University, Viničná 5, CZ-12844 Prague, Czech Republic

Abstract

Embryogenesis can be initiated directly from microspores or pollen grains. This is known as androgenesis and refers to the process of redirection of normal pollen development (gametophytic pathway) towards the embryo formation (sporophytic). This review mainly deals with the current knowledge of stress and developmental aspects of induction of androgenesis. The crucial role of stress inductive treatment together with changes in cell polarity are discussed in relation to other relevant biological systems. The intriguing speculations are made on the basis of these comparisons which may point out the direction of future investigations.

Additional key words: cell symmetry, gene expression, heat shock proteins, polarity.

Introduction

The unique phenomenon of *in vitro* pollen embryogenesis (androgenesis) concerns of redirection of normal gametophytic pollen development towards embryo formation pathway. This process can be studied either as reprogramming of seemly determined pollen grains or be used as a convenient model to address the questions of early events of plant embryogenesis. From practical point of view androgenesis resulting in production of homozygous double haploid plants facilitates the rapid isolation of homozygous lines, selection for recessive traits and has become a part of breeding programmes in variety of crops. Androgenesis was initially described by using anther culture (Guha and Maheshwari 1964), later on the more effective systems of isolated microspores and pollen (Nitsch and Norrel 1973) were used. During the last three decades, research in androgenesis has generated a long list of plants in which pollen sporophytic growth was initiated (for review see Raghawan 1997, Ferrie *et al.* 1995, Sopory and Munshi 1996). In *Nicotiana tabacum*, *Brassica napus*, *Hordeum vulgare*, *Oryza sativa*, *Triticum aestivum*, and *Zea mays* the frequency of embryogenic induction is high enough to perform molecular and biochemical characterization of this process.

Factors controlling sporophytic pollen development

Numerous factors were described to influence the induction of pollen embryogenesis. Following parameters have been considered to be important: *1*) genotypic effects of donor plants, *2*) stage of pollen development, *3*) nature of nutrient medium, *4*) temperature used for *in vitro* cultures, *5*) pre-treatment of flower buds or inductive treatment of isolated microspores, *6*) physiological state and conditions of growth of donor plants.

Received 9 December 1999, *accepted* 11 April 2000.

Abbreviations: ABA - abscisic acid; BrdU - bromodeoxyuridine, GC - generative cell, GTPase - guanosine triphosphatase; GUS - glucuronidase, HSP - heat shock protein, PMI - first pollen mitosis; smHSP - small (low molecular mass) heat shock protein, VC - vegetative cell.

Acknowledgement: The work has been supported by Czech Ministry of Education, Grant VS 96145, The Plant Cell and Stress.

*Present address: Department of Plant Genetics, Swiss Federal Institute of Technology, Universitätstrasse 2, CH-8092 Zürich, Switzerland; fax: (+410) 1 6321080, e-mail: psmykal@ipw.biol.ethz.ch

Effects of genotype: Genetic control of different steps of androgenesis has been demonstrated in several species (for review see Powell 1990, Chupeau *et al.* 1998). In all the systems used, the genotype plays a major role in the embryogenic response. Studies performed on *Solanum tuberosum* have indicated that the ability to undergo androgenesis is controlled by more than one gene and that the genes are recessive. Genetic analysis carried out on substitution lines of wheat have shown that the responsiveness is controlled by at least three independent genes on 1D and 5BL chromosome arms and additionally the most responsive genotypes possessed a 1B/1R translocation (Chupeau *et al.* 1998). Analyses of response frequencies in crosses between high and low-yielding genotypes of maize showed that breeding for improved responsiveness is possible (Petolino and Thomson 1987). The ability is conditioned by nuclear genes but at least in wheat a cytoplasm background has an effect (Ferrie *et al.* 1995).

Stage of pollen development: The role of cytological stage of pollen grains at the time of culture initiation is still discussed. Although this stage varies between the species, the developmental window of embryogenic competence lies between the mid-unicellular and the mid-bicellular stage. Non-homogeneity of starting pollen population is in part responsible for this wide developmental window. In *Brassica napus*, the optimal stages for the induction are restricted to the period around the first pollen mitosis, starting from late-uninucleate to early-binucleate pollen grains (Pechan and Keller 1988, Telmer *et al.* 1992). Recently, this narrow window has been questioned by embryogenic induction of mid-bicellular *B. napus* pollen grains (Binarová *et al.* 1997). These authors applied additional short and a more severe heat stress treatment of 41 °C, resulting in the division of already formed vegetative cell. Tobacco pollen grains can be switched towards embryogenesis in different stages according to applied treatment. When a starvation is used, bicellular pollen grains are the best responding, when a heat shock is applied, younger, uninucleate microspores can be used (Touraev *et al.* 1996a,b). Ultrastructural studies together with cytochemical, immunochemical and *in situ* hybridization approaches have shown that the most favourable stage for embryogenic induction in *Capsicum annuum* is when the nucleus shows features of an active transcription (Gonzalez-Melendi *et al.* 1995, Testillano *et al.* 1995).

Medium composition: For a limited number of model species optimalized inductive conditions allow the use of simple media containing only macro- and micronutrient salts, vitamins, myo-inositol and sucrose. In cases of barley, potato and wheat, the sucrose may be replaced by maltose (Scott *et al.* 1995). However, pollen grains of the vast majority of plant species require either an auxin, a cytokinin, or a combination of both in the medium.

The role of anther wall and other sporophytic tissues has been initially over-emphasised. Supplementation of culture media with amino acids, such as serine, glutamine and glutathione which are otherwise released during the degeneration of anther tissues led to improvement (Nitsch 1974). The role of certain secreted arabinogalactan proteins has being recently raised again.

Thermal stress and nutrient starvation: Temperature shock is considered to be the most effective treatment to induce pollen embryogenic development. In several plants, temperature shocks trigger a parthenogenetic activation of the egg leading to the production of haploid plant.

Originally mainly a cold treatment was applied, which was known to alter the division plane of the pollen grains and to result in symmetrical cells. The first reports of favourable effects of low temperature on pollen embryogenesis come from the study of isolated pollen grains of *Datura innoxia* (Nitsch and Norrel 1973), later on in tobacco (Rashid and Reinert 1983). This treatment can be now viewed as a starvation. The cold pre-treatment of excised barley spikes for 2 - 3 weeks at 4 °C results in the same induction (Huang and Sunderland 1982). On the other hand, especially in *Brassica* species, short period of high temperature treatment (30 - 35 °C), before further culture at 25 °C, is required to switch efficiently developmental pathway. Direct demonstration that manipulation in culture temperature controls developmental pathway came from the work of Custers *et al.* (1994). The same *in vitro* culture at 17.5 °C continued towards pollen maturation, whereas at 32.5 °C embryogenesis was initiated. The severity of heat shock treatment can manipulate the stage requirement for effective pollen embryogenesis induction (Binarová *et al.* 1997). Nutrient starvation especially for sugars and nitrogen has been routinely used to induce pollen embryogenesis in tobacco (Kyo and Harada 1986). In tobacco the possibility to replace starvation by heat shock was shown (Touraev *et al.* 1996b, 1997). The unifying concept of stress requirements to trigger pollen embryogenesis comes from the analysis of heat shock proteins expression as the markers of stress.

Growth conditions and physiological status of donor plants: The growth conditions of the plants used for pollen culture have a profound effect on the embryogenic response. The optimum conditions for each species appear to be different. Significant interactions between genotype and environment were detected (for review see Powell 1990). Plants sufficiently supplied with nutrients grown at lower limit of the temperature optimum give the maximal embryogenic potential (Afele *et al.* 1992). Also the age of donor plants was reported to be important for embryogenic potential of pollen grains. The young inflorescences were shown to contain the most responsive pollen grains (for review see Powell 1990, Chupeau *et al.* 1998).

Cytology of pollen embryogenesis

Pathways of androgenesis: The largely used classification derived from Raghawan (1997) scheme considers following possible routes of the pollen derived embryos formation: *1*) repeated divisions of the vegetative cell, *2*) repeated divisions of the generative cell, *3*) division of both vegetative and generative cells, and *4*) symmetrical division of still uninucleate microspore.

Division of the vegetative cell (A pathway): The vegetative cell loses its morphogenic individuality in culture and is partitioned by a series of internal walls until a mass of somatic cells is produced. Cytokinesis is frequently proceeded by nuclear division resulting in multinuclear pro-embryo. The generative cell remains undivided or undergoes few divisions, without contributing to final embryo formation (Sunderland and Wicks 1971). In barley and wheat, this proliferation of vegetative cell results in formation of callus rather than embryo (for review see Raghawan 1997). The fusion of nuclei have been reported and can contribute to the formation of mixoploid callus from single pollen grain.

Division of generative cell or of both generative and vegetative cells (E pathway): The formation of a pollen derived embryo exclusively from generative cell remains the matter of speculations and need to be demonstrated (for review see Raghawan 1997). In *Datura innoxia*, a unique type of pollen embryogenesis involving both the generative and vegetative cells or their nuclei has been described. The cell fusion forms an abnormal cell, where both nuclei divide simultaneously on a common spindle. The fusion occur between one or two haploid vegetative cell nuclei, and a haploid or endopolyploid generative cell nucleus, which in turn leads to occurrence of embryos with non-haploid chromosome numbers (Sunderland *et al.* 1974).

Symmetrical division of microspores (B pathway): Symmetricity of nuclear division upon stress has been implicated to be a major pathway of embryo formation when microspores are taken prior to first pollen mitosis. It has not been firmly determined whether there are any cytological and histochemical differences between two similar-looking cells or nuclei. Since a colchicine treatment has been shown to increase the number of symmetrically dividing embryogenic pollen grains in cultures of *Brassica napus* (Zaki and Dickinson 1991, 1995, Zhao *et al.* 1996), it has been suggested that cytokinetic symmetry is an important factor in deflecting the gametophytic program of the pollen grain toward the embryogenic pathway. The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation has been addressed in microspores from transgenic tobacco plants transformed with promoter of vegetative-cell specific tomato *lat52* gene fused to reporter *GUS* gene. *In vitro* maturation in the presence of high concentrations of colchicine effectively blocked first pollen mitosis, resulting in the formation of uninucleate pollen grains expressing both genes, capable of germination and a pollen tube growth despite of an absence of a generative cell. Lower amounts of colchicine induced symmetric division, producing two similar daughter cells, both expressing *GUS* gene. These results demonstrate that division asymmetry at the first pollen mitosis is essential for correct generative cell differentiation and that activation of vegetative cell specific transcription and functional maturation may be uncoupled from cytokinesis (Eady *et al.* 1995). On the other hand, cultivation of pollen grains containing even two-similarly sized cells under the maturation conditions led to development of mature pollen grains (Touraev *et al.* 1995). This indicates that a symmetry of first pollen mitosis is not essential but the irreversible commitment to embryogenesis. Interesting observations have been made using lithium treatment, which disrupts the partitioning of membrane-associated calcium, blocks a polar nuclear migration and subsequently induces a symmetrical mitosis of tobacco pollen grains (Zonia and Tupý 1995).

Two models explaining the significance of first pollen mitosis for pollen determination and differentiation have been proposed (Eady *et al.* 1995). One simpler model of passive repression supposes that low levels of gametophytically expressed factors, the result of asymmetric division, are present in generative cell. In symmetrically dividing cells or in case of colchicine blocked uninucleate pollen grains, no such repression of vegetative cell specific genes occurs. Another model counts for an existence of an active repressor, blocking transcription in the generative cell, which is again selectively retained in generative cell upon asymmetrical division. More recently the questions of microspore polarity, division symmetry and pollen cell fate have been addressed genetically using *Arabidopsis* gametophytic mutation *gemini pollen* (Park *et al.* 1998). Spatial uncoupling of karyogenesis and cytokinesis suggests that Gemini pollen 1 protein may be required for proper localization of phragmoplast. Cell fate studies suggest that the cell fate is quantitatively related to cell size. This finding has a parallel in multicellular alga *Volvox carten*, where even some genes responsible for cell fate determination have been cloned (for review see Kirk *et al.* 1997). Another pollen mutant of *Arabidopsis, sidecar pollen (scp)* divides prematurely and only one daughter cell retains the ability to divide asymmetrically, which supports the model of polarity determination by asymmetrically localized factors (Chen and McCormick 1996). The inability of *Arabidopsis* microspores to undergo pollen embryo formation do not allow to test such symmetric pollen.

Pollen embryogenesis *in vivo* and anomalous pollen grains: In mature anthers of many plants, anomalous pollen grains were described, based on their smaller size and lower affinity to cytoplasmic stains. The frequency of these pollen grains varies greatly with genotype and environment under which plants are grown. Sometimes they can undergo extra mitosis to form multicellular pollen *in vivo*. The first discovery of an anomalous pollen can be traced back to last century (Němec 1898). Their formation was regarded as a transition from maleness to femaleness, due to a similarity to female embryo sac. Suggestion that pollen embryogenesis *in vitro* is originated from the anomalous pollen formed already *in vivo* have frequently appeared (Sunderland and Wicks 1971). The phenomenon of *in vivo* formed embryoids has been described in several hybrids within the genus *Solanum* (Ramanna 1974) and in *Narcissus* (Koul and Karihaloo 1977). Heberle-Bors (1985) showed that growth conditions influenced the frequency of anomalous pollen and that purified anomalous pollen formed embryos *in vitro*. Nowadays this hypothesis of pre-formed embryogenesis-competent pollen grains is largely abandoned.

Molecular biology of pollen dedifferentiation

DNA, RNA metabolism: The division of the pollen cells in the embryogenic pathway is accompanied by renewed DNA replication. In normal gametophytic pathway the nucleus of the vegetative cell is arrested at G1 stage and never divides *in vivo*. The culture of isolated tobacco pollen grains in a starvation medium before transfer to an enriched medium renewed DNA synthesis in the vegetative cell (Žárský *et al.* 1992). Cell cycle progression during starvation-induced pollen embryogenesis has been thoroughly examined by flow cytometry and radioactive thymidine incorporation (Touraev *et al.* 1996a,b). In tobacco, these experi-ments showed that all viable microspores entered the S-phase during the starvation treatment. Doubling of DNA content occurred during the second day of starvation and at the end of the treatment most micro-spores were arrested in G2. In *Brassica napus* system late-uninucleate microspores at G2 phase entered rapidly within one hour into S phase, as assayed by BrdU incorporation (Binarová *et al.* 1993). These authors also showed arrest of the generative cell and a further proliferation of only vegetative cell when initially binucleate pollen grains were used.

During early steps of the induction of embryogenic development, changes in gene expression occur. This could be linked to three different event going simultaneously: the gametophytic gene expression is repressed, stress gene expression is initiated due to inductive treatments and subsequently sporophytic developmental program is initiated.

Starvation of tobacco pollen grains results in a decrease in RNA content. Beneficial effects of application of actinomycin D, a mRNA synthesis inhibitor, or ABA were demonstrated in case of tobacco pollen grains (Harada *et al.* 1986). These effects have precise temporal window and can be now viewed as blockage of pollen-determining gene expression. After heat shock treatment, the changes in expression pattern occur rapidly (within hours), with prominent detection of corresponding HSP transcripts. *In vitro* translation profiles of mRNAs isolated from embryogenic pollen grains support the results from cytological studies and indicated the appearance of new and disappearance of some mRNAs (Pechan *et al.* 1991, Cordewener *et al.* 1995). Some of the messages apparently code for the heat shock proteins of either low molecular mass family (smHSPs) or 70 kDa HSP (HSP70), but their relevance to embryogenic induction remains to be clarified. Unifying concept of HSPs expression which will be discussed later, is that stress triggers temporal expression of HSPs during the critical period of pollen determination resulting in the block of further gametophytic development.

Protein metabolism: Less information is available on protein metabolism in embryogenic pollen grains than on RNA metabolism. Reduction of a mRNA content results in reduction of a protein content. The most thorough description of protein changes comes from *Brassica napus* microspore system, where the changes in the spatial accumulation of HSP70 members during the thermal shock have been detected (Cordewener *et al.* 1995). Compared to normal pollen grains, the embryogenic ones display an intense and differential labelling of the nuclei with anti-HSP70 antibodies. These observations were supported by results on embryogenic induction of late-binucleate pollen grains of *B. napus*, where staining of vegetative cell nucleoplasm was observed, whereas in non-induced pollen grains only generative nucleus was labelled (Binarová *et al.* 1997). This shift of HSP70 from cytoplasm to nuclei can be either associated with the entrance on new cell cycle and/or associated with cell protection during heat stress.

Identification of specific proteins that are synthesized sufficiently early to have a role in the embryogenic determination, with the use of two-dimensional electrophoretic analysis revealed no qualitative differences. Only slight qualitative and minor quantitative differences were detected using radioactivelly labelled proteins (Cordewener *et al.* 1994, Custers *et al.* 1994). In contrast to these results, using silver stained two-dimensional electrophoretic gels, large differences were observed by Pechan *et al.* (1991). In embryogenic pollen

grains of tobacco, new set of membrane-bound phosphoproteins (Kyo and Harada 1990, Kyo and Ohkawa 1991), soluble and insoluble glycoproteins (Říhová *et al.* 1996) were synthesized. Dramatic decrease of both soluble and insoluble glycoproteins during inductive starvation treatment of tobacco pollen grains was observed (Říhová *et al.* 1996).

Attempts to identify changes in mitosis-specific phosphoproteins during embryogenic induction of *Brassica napus* microspores were not successful (Hause *et al.* 1995). However they detected monoclonal antibody MPM-2 reactive epitopes in the nucleoplasm from G1 to G2 phase and in cytoplasm during mitosis. Taking together these results may suggest that except of dramatic induction of HSPs synthesis and decrease of pollen-specific proteins (which still remains to be demonstrated directly) no abrupt changes occur in the general pattern of protein synthesis.

Gene expression during pollen embryogenesis: Only limited information are available on the gene expression pattern during developmental switch. All up to now detected changes in a gene expression occur late in the development, when the embryogenic fate is already fixed. *Brassica napus* microspore-derived embryos express genes for storage proteins, as in case of zygotic embryos.

There is a good evidence linking the expression of napin gene with the embryogenic induction of isolated microspores of *B. napus*. The corresponding transcript is first being detected after 4 d of inductive treatment, a time when microspores start to divide and form pro-embryos. Older microspores not capable to undergo induction did not express napin mRNA (Boutilier *et al.* 1994). Inductive treatments result in already mentioned expression of heat shock genes. The expression of smHSPs genes has been revealed during the induction of pollen embryogenesis in tobacco (Žárský *et al.* 1995) and rape (Pechan *et al.* unpublished). Three embryoid-specific cDNA clones have been isolated from wheat pollen embryoids cDNA library. Two of these clones are expressed only early in embryogenesis (Reynolds and Kitto 1992). Other embryo specific gene codes for metallothionein-like protein, which expression is restricted to embryogenic microspores, embryoids, and zygotic embryos and is modulated by ABA (Reynolds and Crawford 1996). Recently, several *de novo* induced or up-regulated cDNA clones have been reported in embryogenic microspores of *Brassica napus*. One of the interesting clones is Bnp23, a homologue of mammalian p23 glucocorticoid receptor, which expression is strongly up-regulated during the induction (Cordewener *et al.* 1998) or Bnm3, an embryo expressed apetala 2 domain transcription factor (Boutilier *et al.* 1998). The earliest *de novo* expressed clone is DD3-15, encoding 8 kDa putatively secreted protein, which is detected 24 h after culture initiation. Another DD7-48 clone is homologous to RNA polymerase subunit (RBP5) (van Lookeren Campagne, personal communication). The very recently several cDNAs expressed in the early stages of microspore embryogenesis in barley have been described (Vrinten *et al.* 1999). One of these is highly homologous to glutathione-S-transferase and may be important in protecting cells from oxidative stress during the culture. Other has homology to lipid transfer proteins, which are know to be a marker of the early stages in the carrot somatic embryogenesis (Sterk *et al.* 1991).

Symmetric or asymmetric divisions and role of cytoskeletal changes: The importance of cell asymmetry has long been evident, *e.g.*, the asymmetric localization of fate determinants in the eggs of invertebrates (*Drosophila melanogaster*, *Caenorhabditis elegans*) and vertebrates (*Xenopus laevis*), the asymmetric cell division in budding yeast and caulobacter.

The information obtained from brown algae studies highlighted the importance of the cytoskeleton and the membrane for the proper distribution of molecular determinants. Early indicators of *Fucus* and *Pelvetia* zygotes polarisation include the accumulation of membrane-associated dihydropyridine receptors, that may be functional Ca channels and of cortical F-actin (Shaw and Quatrano 1996a). In addition, the Golgi apparatus becomes polarised and cell wall molecules are secreted asymmetrically. Cell polarization in animal cells and yeast requires reorganization of microfilaments and microtubules which is regulated by a hierarchy of GTPases and protein kinases (Twell and Howden 1998).

Although the identity of cell fate determinants remains unknown in these models, additional experiments have implied a key role for secretion in the polarisation of *Fucus* embryos (Shaw and Quatrano 1996b). The crucial role of cytoskeleton in the localization of mRNAs and proteins cell-fate determinants forming consequently a cytoplasmic gradients is evident from numerous experiments (for review see Nasmyth and Jansen 1997).

All these findings have intriguing implications to the pollen embryogenesis model. The asymmetry of first pollen mitotic division (pollen mitosis I, PMI) is discussed here in a context of the alternation of this asymmetry towards "symmetry". Four different *Arabidopsis* mutants exist, all with defective PMI. *Solo* mutant fails to undergo PMI, resulting in the occurrence of unicellular pollen with a VC phenotype. *Gemini* mutant completes PMI but do so symmetrically, producing daughter cells with VC fates (Park *et al.* 1998). In *sidecar pollen* mutants, PMI is often symmetrical, but one of the daughter cells then undergoes additional a "normal" PMI division producing a VC and GC, resulting in three-celled pollen grain with extra VC (Chen and McCormick 1996). In another mutant *limpet pollen*, an engulfment and inward migration of the generative cell is prevented (Twell and Howden 1998), *duo pollen* mutation prevents the division of the generative cell.

This genetic approach, proved to be efficient in dissection of polarity and asymmetric division in other organisms (Horwitz and Herskowitz 1992, Jan and Jan 1998) and point out the direction which may bring new information of microspore polarity and cell fate determination (Twell and Howden 1998). Unfortunately, current inability to induce pollen embryogenesis in *Arabidopsis,* does not allow to perform the screen for mutations affecting this process.

Importance of cytoskeleton arrangement during the induction steps of microspore embryogenesis was highlighted by colchicine induced embryogenic development in *B. napus* (Zhao *et al.* 1996). Stress treatments such as starvation and temperature shocks also dramatically affect cytoskeleton (Hause *et al.* 1993). The microfilamental system exhibited a loss of polarity in embryogenic cells but disruptive cytochalasin treatment did not influence embryogenesis. Microtubules likely play a major role in newly induced symmetrical division. Most notably the rotation of division plane up to 90 ° was described only at inductive conditions. Reorganization of cytoskeleton leads to nucleus positioning in the centre of the microspore, when uninucleate microspores are used. Movement of the nucleus away from the edge of the cell and subsequent symmetrical division are observed frequently (Telmer *et al.* 1993, 1995, Hause *et al.* 1993, Zaki and Dickinson 1991, 1995). However exclusive involvement of microtubules in nuclear positioning can not be presumed because other cytoskeletal elements may be indirectly affected by microtubule depolymerization. These is supported by investigation of actin microfilament organization (Hause *et al.* 1992, 1993).

In tobacco, low concentration colchicine treatment of microspores also leads to a symmetric first pollen mitosis, but this does not result in embryogenic development (Eady *et al.* 1995, Touraev *et al.* 1995).The vital role of cytoskeleton in cell cycle regulation is supported by data of Alique *et al.* (1994) which proposed a sequestration of cell cycle regulators.

Crucial role of stress in the inductive process: It has been known for a long time, that stress treatment or suboptimal conditions can alter developmental programs with dramatic consequences. The early in the development stress occurs the more profound developmental alternations will be.

Analysis of sublethal heat shock treatment that causes specific developmental anomalies and mimic the effects of known mutations was first pioneered on *Drosophila* embryos (Goldchmidt 1935). These mutation-like nonheritable morphological alternation were termed phenocopies (Mitchell and Lipps 1978). The resemblance of phenocopies to mutants and the correlation with specific stages of gene expression in different tissues suggest that the heat-induced defects are caused by failure to synthesize specific gene products when they are needed during the development.

The novel hypothesis is that heat shock causes developmental defects by increasing the expression or stabilization of a critical target gene (Petersen and Lindquist 1989). Another proposed mechanism is the repression of a critical genes below a threshold level (Petersen and Young 1989). Prevention of phenocopies by thermotolerance-inducing treatments suggests more direct role of HSPs in these events. Also reported failure in mRNA processing and transport during and following heat shock can explain heat-induced developmental defects. Whether these mechanisms play a role in the androgenesis process remains to be tested, however expression analysis of pollen specific genes during the early steps of pollen embryogenesis supported this (Smýkal 1999).

As already mentioned the abiotic stresses play a pivotal role in the induction of pollen embryogenesis. The influence of temperature and nutrition is well documented (Keller and Amstrong 1979, Heberle-Bors 1985, Kyo and Harada 1986, Afele *et al.* 1992). Other most common stress treatments that bring about embryogenesis are cold pre-treatment of anthers or flower buds, period of metabolic starvation (Touraev *et al.* 1996a), ethanol, γ-irradiation (Pechan and Keller 1989) and microtubuli disruptive agents (Zaki and Dickinson 1990, 1991, 1995, Zhao *et al.* 1996). The mechanism how stresses affect pollen differentiation has not yet been firmly established.

As stress response in affected cells is associated with the appearance of heat shock proteins, the connection between stress, HSPs and the induction of androgenesis had become the subject of investigation (Pechan and Keller 1989, Pechan *et al.* 1991, Cordewener *et al.* 1995, Žárský *et al.* 1995, Touraev *et al.* 1996b, 1997, Smýkal 1999). Our work in *B. napus* highlighted several pre-conditions which must be met to effectively trigger embryogenesis. The first is that the *in vitro* temperature of cultivated microspores or young pollen grains must be near their physiological limit of 32 - 35 °C. The second precondition is that the temperature difference between the donor plants and *in vitro* conditions should be greater than 10 - 12 °C. This would explain a low temperature requirement of donor plants. These conditions elicit the stress response and synthesis of HSPs. Our results of colchicine induced sHSPs synthesis and quantitative sHSPs differences between high and low-embryogenic lines support the hypothesis of direct HSPs involvement in induction. However, sHSPs are unlikely to be solely and preferentially associated with induction, as also developmentally advanced pollen grains still synthetize HSP and yet are not capable of embryogenesis. Regardless of their stress regulation, HSP synthesis is also developmentally regulated during the gametogenesis, and somatic and zygotic embryogenesis (Gyorgyey *et al.* 1991, Zimmerman and Cohill 1991, Atkinson *et al.* 1993, DeRocher and Vierling 1994). The more direct evidence would come from the analysis of transgenic plants.

Pollen embryogensis - idea of atavism or totipotency: The search for biological explanation of pollen embryogenesis is probably as old as the discovery of this phenomenon. The most widely used is the explanation of pollen embryogenesis as a classical example of the cell totipotency (Reynolds 1997). Recently Bonet *et al.* (1998) have tried to find an answer to this intriguing question looking at the evolutionary history of higher plant pollen grain. They propose a model of pollen embryogenesis as a result of atavism in the plant kingdom, *e.g.*, that embryogenesis may reflects the morphogenic capability of pollen being an archaic characteristic expressed in its ancestral structures, which nowadays has lost its original function, but remains latent (Bonet *et al.* 1998). The visible display of this atavistic characteristic may only be manifested under certain stress conditions. Authors propose a phylogenic connection to the spores of tree-like pteridophytes, a putative ancestors of nowadays flowering plants. These spores had likely the capability to form multicellular haploid gametophyte, a characteristic preserved in present-day ferns. They conclude that meiospores of the green algae and pollen grains are evolutionary related, which could so explain morphogenic capability of pollen embryogenesis.

References

Afele, J.C., Kannenberg, L.W., Keats, R., Sohota, S., Swanson, E.B.: Increased induction of microspore embryos following manipulation of donor plant environment and culture temperature in corn (*Zea mays* L.). - Plant Cell Tissue Organ Cult. **28**: 87-90, 1992.

Alique, R., Akhavan-Niak, H., Russel, P.: A role for Hsp90 in cell cycle control: Wee1 tyrosine kinase activity requires interaction with Hsp90. - EMBO J. **13**: 6099-6106, 1994.

Atkinson, B.G., Raizada, M., Bouchard, R.A., Frappier, J.R.H., Walden, D.B.: The independent stage-specific expression of the 18 kDa heat shock protein genes during microsporogenesis in *Zea mays*. - Develop. Genet. **14**: 15-26, 1993.

Binarová, P., Hause, G., Cenklova, V., Cordewener, J.H.G., van Lookeren Campagne, M.M.: A short severe heat shock is required to induce embryogenesis in late bicellular pollen of *Brassica napus* L. - Sex. Plant Reprod. **10**: 200-208, 1997.

Binarová, P., Straatman, K., Hause, B., Hause, G., van Lammeren, A.A.M.: Nuclear DNA-synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* L. - Theor. appl. Genet. **87**: 9-16, 1993.

Bonet, F.J., Azbaid, L., Olmedilla, A.: Pollen embryogenesis: atavism or totipotency? - Protoplasma **202**: 115-121, 1998.

Boutilier, K.A., Gines, M.J., DeMoor, J.M., Huang, B., Baczynski, C.L., Iyer, V.N., Miki, B.L.: Expression of the BnNAP subfamily of napin genes coincide with the induction of *Brassica* microspore embryogenesis. - Plant Mol.Biol. **26**: 1711-1723, 1994.

Boutilier, K., Hattori, J., Oullet, T., van Blijderveen, M., van Lookeren Campagne, M.M., Miki, B.: Bnm3, an embryo-expressed AP2 domain transcription factor from *Brassica napus*. - In.: Abstracts of XVth International Congress on Sexual Plant Reproduction, Wageningen 1998.

Chen, Y.C., McCormick, S.: *sidecar pollen*, an *Arabidopsis thaliana* male gametophytic mutant with aberrant cell divisions during pollen development. - Development **122**: 3243-3253, 1996.

Chupeau, Y., Caboche, M., Henry, Y. (ed.): Androgenesis and Haploid Plants. - Springer, Berlin 1998

Cordewener, J., Jansen, H., Hause, G., Fiers, M., van Lookeren Campagne, M.: Bnp23, the plant homologue of mammalian p23, is induced during microspore embryogenesis in *Brassica napus*. - In: Abstracts of XVth International Congress on Sexual Plant Reproduction, Wageningen 1998.

Cordewener, J.H.G., Busink, R., Traas, J.A., Custers, J.B.M., Dons, H.J.M., van Lookeren Campagne, M.M.: Induction of microspore embryogenesis in *Brassica napus* L. is accompanied by specific changes in protein synthesis. - Planta **195**: 50-56, 1994.

Cordewener, J.H.G., Hause, G., Gorgen, E., Bunsik, R., Hause, B., Dons, H.J.M., van Lammeren, A.A.M., van Lookeren Campagne, M.M., Pechan, P.M.: Changes in synthesis and localization of members of the 70-kDa class of heat-shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. - Planta **196**: 747-755, 1995.

Custers, J.B.M., Cordewener, J.H.G., Nollen, Y., Dons, H.J.M., van Lookeren Campagne, M.M.: Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. - Plant Cell Rep. **13**: 267-271, 1994.

DeRocher, A.E., Vierling, E.: Developmental control of small heat shock protein expression during pea seed maturation. Plant J. **5**: 93-102, 1994.

Eady, C., Lindsey, K., Twell, D.: The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. - Plant Cell **7**: 65-74, 1995.

Ferrie, A.M.R., Palmer, C.E., Keller, W.A.: Haploid Embryogenesis. - In: Thorpe, T.A. (ed.): *In Vitro* Embryogenesis in Plants. Pp. 309-344. Kluwer Academic Publishers, Dordrecht 1995.

Golschmidt, R.: Gen und Ausseneigenschaft (Untersuchungen an *Drosophila*). - Z. Indukt. Abstamm. Vererbungsl. **69**: 38-69, 70-131, 1935.

Gonzalez-Melendi, P., Testillano, P.S., Ahmadian, P., Fadon, B., Vicente, O., Risueno, M.C.: *In situ* characterization of the late vacuolate microspore as a convenient stage to induce embryogenesis in *Capsicum*. - Protoplasma **187**: 60-71, 1995.

Guha, S., Maheshwari, S.C.: *In vitro* production of embryos from anthers of *Datura*. - Nature **204**: 497, 1964.

Gyorgyey, J., Gartner, A., Nemeth, K., Magyar, Z., Hirt, H., Heberle-Bors, E., Dudits, D.: Alfalfa heat shock genes are differentially expressed during somatic embryogenesis. - Plant mol. Biol. **16**: 999-1007, 1991.

Harada, H., Kyo, M., Imamura, J.: Induction of embryogenesis and regulation of the developmental pathway in immature pollen of *Nicotiana* species. - Curr. Topics Develop. Biol.

20: 397-408, 1986.

Hause, G., Cordewener, J.H.G., Ehrmanova, M., Hause, B., Binarová, P., van Lookeren Campagne, M.M., van Lammeren, A.A.M.: Cell cycle dependent distribution of phosphorylated proteins in microspores and pollen of *Brassica napus* L., detected by the monoclonal antibody MPM-2. - Protoplasma **187**: 117-126, 1995.

Hause, G., Hause, B., van Lammeren, A.A.M.: Microtubular and actin filament configurations during microspore and pollen development in *Brassica napus* cv. Topas. - Can. J. Bot. **70**: 1369-1376, 1992.

Hause, G., Hause, G., Pechan, P., van Lammeren, A.A.M.: Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. - Cell Biol. int. **17**: 153-168, 1993.

Heberle-Bors, E.: *In vitro* haploid formation from pollen: a critical review. - Theor. appl. Genet. **71**: 361-374, 1985.

Horwitz, R.H., Herskowitz, I.: Mechanisms of asymmetric cell division: Two Bs or not two Bs, that is the question. - Cell **68**: 237-255, 1992.

Huang, B., Sunderland, N.: Temperature-stress pretreatment in barley anther culture. - Ann. Bot. **49**: 77-88, 1982.

Jan, Y.N., Jan, L.Y.: Asymetric cell division. - Nature **392**: 775-778, 1998.

Keller, W.A., Armstrong, K.C.: Stimulation of embryogenesis and haploid production in *Brassica campestris* anther cultures by elevated temperature treatments. - Theor. appl. Genet. **55**: 65-67, 1979.

Kirk, D.L.: The genetic program for germ-soma differentiation in *Volvox*. - Annu. Rev. Genet. **31**: 359-380, 1997.

Koul, A.K., Karihaloo, J.L.: *In vivo* embryoids from anthers of *Narcissus bioflorus* curt. - Euphytica **26**: 97-102, 1977.

Kyo, M., Harada, H.: Control of the developmental pathway of tobacco pollen *in vitro*. - Planta **168**: 427-432, 1986.

Kyo, M., Harada, H.: Specific phosphoproteins in the initial period of tobacco pollen embryogenesis. - Planta **182**: 58-63, 1990.

Kyo, M., Ohkawa, T.: Investigation of subcellular localization of several phosphoproteins in embryogenic pollen grains of tobacco. - J. Plant Physiol. **137**: 525-529, 1991.

Nasmyth, K., Jansen, R.P.: The cytoskeleton in mRNA localization and cell differentiation. - Curr. Opin. Cell Biol. **9**: 396-400, 1997.

Němec, B.: [About the pollen grains of petaloid anthers of *Hyacinthus orientalis* L.] - Rozpr. Čes. Akad. **7**(17): 1-18, 1898. [In Czech.]

Nitsch, C.: Pollen culture – a new technique for mass production of haploid and homozygous plants. - In: Kasha, K.J. (ed.): Haploids in Higher Plants. Advances and Potential. Pp. 123-135. University of Guelph, Guelph 1974.

Nitsch, C., Norrel, B.: Effect d'un choc thermique sur le pouvoir embryogène du pollen de *Datura innoxia* cultivé dans l'anthère ou isolé de l'anthère. - Compt. rend. Acad. Sci. Paris, Sér. D **276**: 303-306, 1973.

Park, S.K., Howden, R., Twell, D.: The *Arabidopsis thaliana* gametophytic mutation gemini pollen1 disrupts microspore polarity, division asymmetry and pollen cell fate. - Development **125**: 3789-3799, 1998.

Pechan, P., Keller, W.A.: Induction of microspore embryogenesis in *Brassica napus* by gamma irradiation and ethanol stress. - *In vitro* cell. develop. Biol. **25**: 1073-1074, 1989.

Pechan, P.M., Bartels, D., Brown, D.C.W., Schell, J.: Messenger-RNA and protein changes associated with induction of *Brassica* microspore embryogenesis. - Planta **184**: 161-165, 1991.

Pechan, P.M., Keller, W.A.: Indentification of potentially embryogenic microspores in *Brassica napus*. - Physiol. Plant. **74**: 377-384, 1988.

Petersen, N.S., Young, P.: Effects of heat shock on protein processing and turnover in developing *Drosophila* wings. - Develop. Genet. **10**: 11-15, 1989.

Petersen, R., Lindquist, S.: Regulation of HSP 70 synthesis by messenger RNA degradation. - Cell Regul. **1**: 135-149, 1989.

Petolino, J.F., Thomson, S.A.: Genetic analysis of anther culture response in maize. - Theor. appl. Genet. **74**: 284-286, 1987.

Powell, W.: Environmental and genetical aspects of pollen embryogenesis. - In: Bajaj, V.P.S. (ed.): Haploids in Crop Improvement I. Biotechnology in Agriculture and Forestry. Vol. 12. Pp. 45-65. Springer-Verlag, Berlin 1990.

Raghawan, V.: Molecular Embryology of Flowering Plants. - Cambridge University Press. Cambridge 1997.

Ramanna, M.S.: The origin and *in vivo* development of embryoids in the anthers of *Solanum* hybrids. - Euphytica **23**: 623-632, 1974.

Rashid, A., Reinert, J.: Factors affecting high-frequency embryo formation in *ab initio* pollen cultures of *Nicotiana*. - Protoplasma **116**: 155-160, 1983.

Reynolds, T.L.: Pollen embryogenesis. - Plant mol. Biol. **33**: 1-10, 1997.

Reynolds, T.L., Crawford, R.L.: Changes in abundance of an anbscisic acid-responsive, early cysteine-labelled metallothionein transcript during pollen embryogenesis in bread wheat (*Triticum aestivum*). - Plant mol. Biol. **32**: 823-829, 1996.

Reynolds, T.L., Kitto, S.L.: Identification of embryoid-abundant genes that are temporally expressed during pollen embryogenesis in wheat anther cultures. - Plant Physiol. **100**: 1744-1750, 1992.

Říhová, L., Čapková, V., Tupý, J.: Changes in glycoprotein patterns associated with male gametophyte development and with induction of pollen embryogenesis in *Nicotiana tabacum*. - J. Plant Physiol. **147**: 573-581, 1996.

Scott, P., Lyne, R.L., ap Rees, T.: Metabolism of maltose and sucrose by microspores isolated from barley (*Hordeum vulgare* L.). - Planta **197**: 435-441, 1995.

Shaw, S.L., Quatrano, R.S.: Polar localization of adihydropyridine receptor on living *Fucus* zygotes. - J. Cell Sci. **109**: 335-342, 1996a.

Shaw, S.L., Quatrano, R.S.: The role of targeted secretion in the establisment of cell polarity and the orientation of the division plane in *Fucus* zygotes. - Development **122**: 2623-2630, 1996b.

Smýkal, P.: Effect of high temperature on gene expression and develomental fate of plant cells. - PhD Thesis, Charles University, Prague 1999.

Sopory, S.K., Munshi, M.: Anther culture. - In: Jain, S.M., Sopory, S.K., Veilleux, R.E., (ed.): *In Vitro* Haploid Production in Higher Plants. Vol. 1. Pp. 145-176. Kluwer Academic Publishers, Dordrecht 1996.

Sterk, P., Booij, H., Schellekens, G.A., van Kammen, A., de Vries, S.C.: Cell-specific expression of the carrot EP2 lipid transfer protein gene. - Plant Cell **3**: 907-921, 1991.

Sunderland, N., Collins, G.B., Dunwell, J.M.: The role of nuclear fusion in pollen embryogenesis of *Datura innoxia*.

Mill. - Planta **117**: 227-241, 1974.

Sunderland, N., Wicks, F.M.: Embryoid formation in pollen grains of *Nicotiana tabacum*. - J. exp. Bot. **22**: 213-226, 1971.

Telmer, C.A., Newcomb, W., Simmons, D.H.: Microspore development in *Brassica napus* and the effect of high temperature on division *in vivo* and *in vitro*. - Protoplasma **172**: 154-165, 1993.

Telmer, C.A., Newcomb, W., Simmonds, D.H.: Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. - Protoplasma **185**: 106-112, 1995.

Telmer, C.A., Simmonds, D.H., Newcomb, W.: Determination of developmental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. - Physiol. Plant. **84**: 417-424, 1992.

Testillano, P.S., Gonzales-Melendi, P., Ahmadian, P., Fadon, B., Risueno, M.C.: The immunolocalization of nuclear antigens during the pollen developmental program and the induction of pollen embryogenesis. - Exp. Cell Res. **221**: 41-54, 1995.

Touraev, A., Ilham, A., Vicente, O., Heberle-Bors, E.: Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. - Plant Cell Rep. **15**: 561-565, 1996a.

Touraev, A., Lezin, F., Heberle-Bors, E., Vicente, O.: Maintenance of gametophytic development after symmetrical division in tobacco microspore culture. - Sex. Plant Reprod. **8**: 70-76, 1995.

Touraev, A., Pfosser, M., Vicente, O., Heberle-Bors, E.: Stress as the major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/pollen embryogenesis. - Planta **200**: 144-152, 1996b.

Touraev, A., Vicente, O., Heberle-Bors, E.: Initiation of microspore embryogenesis by stress. - Trends Plant Sci. **2**: 297-301, 1997.

Twell, D., Howden, R.: Mechanism of asymmetric division and cell fate determination in developing pollen. - In: Chupeau, Y., Caboche, M., Henry, Y. (ed.): Androgenesis and Haploid Plants. Pp. 67-103. Springer, Berlin 1998.

Vrinten, P.I., Nakamuar, T., Kasha, K.J.: Characterization of cDNAs expressed in the early stages of microspore embryogenesis in barley (*Hordeum vulgare*) L. - Plant mol. Biol. **41**: 455-463, 1999.

Zaki, M.A.M., Dickinson, H.: Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. - Protoplasma **156**: 149-162, 1990.

Zaki, M.A.M., Dickinson, H.G.: Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. - Sex. Plant Reprod. **4**: 48-55, 1991.

Zaki, M.A.M., Dickinson, H.G.: Modification of cell development *in vitro*: the effect of colchicine on anther and isolated microspore culture in *Brassica napus*. - Plant Cell Tissue Organ Cult. **40**: 255-270, 1995.

Žárský, V., Garrido, D., Eller, N., Tupý, J., Vicente, O., Schoffl, F., Heberle-Bors, E.: The expression of a small heat shock gene is activated during induction of tobacco pollen embryogenesis by starvation. - Plant Cell Environ. **18**: 139-147, 1995.

Žárský, V., Garrido, D., Říhová, L., Tupý, J., Vicente, O., Heberle-Bors, E.: Derepression of the cell cycle by starvation is involved in the induction of tobacco pollen embryogenesis. - Sex. Plant Reprod. **5**: 189-194, 1992.

Zhao, J.P., Simmonds, D.H., Newcomb, W.: Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L. cv. Topas. - Planta **198**: 433-439, 1996.

Zimmeramn, J.L., Cohill, P.R.: Heat shock and thermotolerance in plant and animal embryogenesis. - New Biol. **3**: 641-650, 1991.

Zonia, L.E., Tupý, J.: Lithium treatment of *Nicotiana tabacum* microspores blocks polar nuclear migration, disrupts the partitioning of membrane-associated Ca, and induces symmetrical mitosis. - Sex. Plant Reprod. **8**: 152-160, 1995.