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Chapter 9

MICROSPORES AND THEIR APPLICATIONS IN BASIC AND APPLIED PLANT SCIENCES

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ABSTRACT

Nowadays, the use of isolated and *in vitro* cultured microspores is not limited for the production of doubled haploids which are important part of modern plant breeding programmes. Microspores became also a powerful tool to investigate important biological processes such as cell totipotency, differentiation, embryogenesis, cell cycle, plant reproduction and development. In the normal pathway microspores develop into mature pollen *in vivo* or when cultured *in vitro* in a rich medium with sugars, however they can be reprogrammed into the totipotent state and further induced to become embryogenic and produce embryos when subjected to various stresses, such as nutrient starvation, heat or cold shock. Microspore cultures are the most efficient method to produce doubled haploids, excellent system for *in vitro* mutagenesis and selection, attractive target for genetic transformation and for gene targeting in plants. This review paper describes the potential applications of plant microspores in plant breeding, genetics, cellular and molecular biology and biotechnology.

Keywords: Microspore, embryogenesis, haploid, plant breeding, genetics.

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INTRODUCTION

Haploid is the general term for plants (sporophytes) that contain the gametic chromosome number (n). The haploid could also be called either monoploid (x) in a diploid sporophytic (2n) species in which only one set of chromosomes exists or polyhaploids in polyploidy species as they have more than one set of chromosomes. The haploid plant obtained from an autotetraploid (4x) with four sets of one genome was originally called a dihaploid (because 2n=2x). When the chromosome number of a haploid is doubled, it is called a doubled haploid (DH). It should be mentioned that a doubled haploid is different from dihaploid. The dihaploid is not homozygous since it represents two chromosome sets selected from four sets in the autotetraploid, whereas the doubled haploid from a monoploid or an allohaploid should be completely homozygous [Kasha and Maluszynski 2003].

The main advantage of doubled haploids in breeding is the reduction of the time required to develop new cultivars [Snape et al. 1986; Touraev et al. 2001; Thomas et al. 2003]. For annual self-pollinated crops it generally takes 10-15 years to produce a cultivar through a conventional plant breeding program such as the pedigree method, which includes selfing and subsequent selection. The time delay is costly and prevents breeders from responding rapidly to end users need. The production of haploids followed by chromosome doubling to produce homozygous lines, from which superior lines are selected, can reduce the time required for cultivar development by 3-4 years. For cross-pollinated heterozygous crops, doubled haploids are a rapid method to produce homozygous pure breeding lines, which can be used in the development of synthetic varieties or hybrids. The production of doubled haploids can improve selection efficiency as the phenotype of the plant is not masked by dominance effects. Traits encoded by recessive genes can be easily identified. A smaller population of doubled haploids is required when screening for desirable recombinants than would be the case for conventional diploid populations [Touraev et al. 2001].Over 200 varieties have been produced by deploying various DH methods [Thomas et al. 2003]. The vast majority of varieties derived from doubled haploidy are in barley (96), followed by rapeseed (47), wheat (20) and the rest [Thomas et al. 2003].

Application of DH systems for induction and selection of mutants has the benefits [reviewed by Szarejko 2003] such as (a) possibility to screen for both recessive and dominant mutants in the first generation after mutagenic treatment; (b) immediate fixation of mutated genotypes, which saves time in the production of pure mutant lines; (c) increased selection efficiency of desired mutants due to the gametic *versus* zygotic segregation ratios (1:1 *vs* 3:1, respectively) and the lack of chimerism; (d) possibility of applying *in vitro* selection methods at the haploid or doubled haploid level.

Doubled haploid populations are ideal for genetic mapping [Forster and Thomas 2003]. DH populations are available for DNA extraction and mapping 1.5 years after the initial cross, i.e. almost as quick as an F_2 or BC_1 population and definitely much faster than a pedigree inbred or single seed descent population. DHs can be re-grown and distributed in seed form so that it is comparatively easy to screen it with a large number of markers. Map construction from a DH population derived from the F_1 of a cross is relatively simple because the expected segregation is that of a backcross [Snape 1976]. DH populations are of benefit in the identification of Quantitative Trait Loci (QTL) as one can grow multi-site replicated trials,

to derive the most meaningful data, three years after the initiation cross [Forster and Thomas 2003].

DH plants can be obtained by several strategies including wide hybridization followed by embryo rescue, parthenogenesis, gynogenesis or microspore embryogenesis (ME) in anther or isolated microspore cultures [Touraev et al. 2001]. Microspore embryogenesis is the most developed and documented approach to produce doubled haploids. ME can be induced either in excised and *in vitro* cultured anthers or in isolated microspore cultures. The induction of ME requires first to reprogram the development of the microspores from the gametophytic (pollen) pathway into the sporophytic (embryo) pathway via applying various stress pretreatments [Shariatpanahi et al. 2006a].

The phenomenon of microspore embryogenesis was first demonstrated by Guha and Maheshwari [1964, 1966], using the Solanaceous plant *Datura innoxia*. These investigators found that when anthers of *D. innoxia* were cultured on a mineral-salt medium containing casein hydrolysate, indole acetic acid, and kinetin, a substantial number of the enclosed pollen grains became embryogenic and within 6 to 7 weeks, developing embryos were observed emerging from the anther. Nowadays researchers have demonstrated that embryogenesis and plant regeneration could be achieved in isolated microspore cultures. The induction of microspore embryogenesis from the culture of isolated microspores was first achieved in *D. innoxia* by Nitsch and Norreel [1973]. In this procedure flower buds were pretreated for 48h at 3°C prior to microspore isolation.

In comparison with other methods, some advantages of microspore embryogenesis are its applicability to all pollen-producing plant species and demonstrated feasibility in a large number of crop species, the good scientific basis of microspore embryogenesis, the high number of pollen grains in most species allowing a large number of doubled haploids to be produced by microbiological techniques, the possibility to double chromosome number during anther or microspore culture, etc. A disadvantage is the formation of albino plants (but only in cereals) and the somaclonal variation as a consequence of suboptimal tissue culture conditions [Touraev et al. 2001].

DEVELOPMENTAL PATHWAYS OF IN VITRO ISOLATED MICROSPORES

Isolated and *in vitro* cultured microspores or immature pollen grains under certain conditions regenerate into embryos and whole plants. This phenomenon is called “totipotency”. Conversion of differentiated plant cells into totipotent cells depends on extraordinary conditions acting upon cells. A triggering factor in the form of stress is necessary to induce embryogenesis in cultured microspores [Nitsch and Norreel 1973, Heberle-Bors 1985, Touraev et al. 1997a]. Another important factor for successful conversion is the stage at which inflorescences, flower buds, anthers or microspores are excised, put into *in vitro* culture or isolated from the anther, respectively. An important issue, however, is to find and choose the right conditions at a certain stage in a given species [Heberle-Bors 1985]. In addition, physiological state and conditions of growth of the donor plants, isolation methods and the culture media are important in optimizing induction efficiency of microspore embryogenesis [Bajaj 1990; Ferrie et al. 1995].

STRESS AS TRIGGER TO INDUCE MICROSPORE EMBRYOGENESIS

Stresses widely used for the induction of microspore embryogenesis are cold, heat, carbon starvation and colchicine [reviewed by Shariatpanahi et al. 2006a]. Heat pre-treatment is usually carried out at 33°C to 37°C for a duration varying from several hours to several days, whereas cold treatment is carried out at 4°C to 10°C from some days to several weeks. Incubation of microspores in media containing non-metabolizable carbon sources, i.e. in mannitol-containing media, is also used with success [Touraev et al. 2001]. In addition, colchicine, a microtubule-depolymerizing agent, is being used as a stress pre-treatment [Zaki and Dickinson 1991, Zhao et al. 1996].

The neglected stresses such as abscisic acid [Imamura and Harada 1980a], feminizing agents [Heberle-Bors 1983], reduced atmospheric pressure [Imamura and Harada 1980b], ethanol [Pechan and Keller 1989], hypertonic shock [Wang et al. 1981], centrifugal treatment [Tanaka 1973] and gamma irradiation [Sangwan and Sangwan 1986] have been tested only in few species. Hardly any recent reports widening the application of these stresses in other species are available. Scarcity of reports may be due to the fact that, in general, widely used stresses, where applicable, are easier to handle and does not require extra labor. However, these stresses may prove effective to induce microspore embryogenesis in recalcitrant species in which the conventional stresses were not successful.

Recently several novel stresses have been reported to induce microspore embryogenesis with success in some species. Incubation of microspores in a high medium pH [Barinova et al. 2004], or in the presence of inducer chemicals [Zheng et al. 2001, Liu et al. 2001], heavy metals [Zonia and Tupy 1995], carrageenan oligosaccharides [Lemonnier-Le Penhuizic et al. 2001] and 2,4-D [Shariatpanahi et al. 2010] are all novel stresses to be tested in detail on other species.

IDENTIFICATION OF EMBRYOGENIC MICROSPORES

A number of early markers for the conversion of microspores towards embryogenesis have been described in the literature. Some of these markers, found in different species, i.e. *Nicotiana tabacum* [Dunwell and Sunderland 1974 a, b, Garrido et al. 1995, Touraev et al. 1996a], *Brassica napus* [Zaki and Dikinson 1990, Telmer et al. 1993], *Datura* [Sangwan and Camefort 1983, 1984] are: (a) fragmentation of the vacuole by formation of cytoplasmic strands from the peri-nuclear to the sub-cortical cytoplasm, (b) movement of the nucleus to the center of the microspore resulting in a central phragmosome, (c) increase in the size of the cell, (d) formation of a new cell wall below the exine, (e) size reduction of the nucleolus, (f) compaction of chromatin, (g) the appearance of a zone of multi-vesiculate bodies, resembling lysosomes, and the degeneration of plastids, (h) tannin-coated tonoplasts, (i) size reduction of the starch grains, (k) no marked structural changes of mitochondria, (l) symmetric division with a planar wall instead of the regular asymmetric cell division in gametophytic microspores [for details see Touraev et al. 2001, Aionesei et al. 2005]. But none of these markers were found to be universal in embryogenic microspores of all species. However, among them, the “star-like” structure of a microspore after stress treatment, seen under the light microscope [Touraev et al. 1996a] and exhibiting a centralized nucleus surrounded by

"star-like" cytoplasmic strands was shown to be sign of embryogenic microspores in a number of different species such as wheat [Touraev et al. 1996b], tobacco [Touraev et al. 1996a], apple [Höfer et al. 1999], and rice [Raina and Irfan 1998]. Furthermore, recent studies using tracking of the entire process of embryogenesis from single selected wheat microspores clarified that microspores with a star-like internal structure and a symmetrical cell division is essential for the initiation of the embryogenic development of isolated microspores [Indrianto et al. 2001]. In other species just the frequency of microspores with a symmetrical division is used as a marker [Telmer et al. 1993] although it has been shown that microspores with two cells of equal size can develop gametophytically [Touraev et al. 1995].

MICROSPORE-DERIVED ALBINO PLANTS

In cereals, the use of microspore-derived doubled haploids in breeding is limited by the occurrence of pigment-deficient (albino) regenerants. These albino plants lack chlorophyll and are unable to carry out photosynthesis. Albino regenerants are frequently found in cereal crop species such as wheat (*Triticum aestivum*, *Triticum durum*), rice (*Oryza sativa*), barley (*Hordeum vulgare*) and forage grasses like *Lolium* or *Festuca*, whereas it is less frequent in maize and not a problem at all in dicot plants [Touraev et al. 2001].

The expression of the albino phenotype in microspore-derived plants is dependent on a variety of factors, including genetic as well as environmental factors. Some of these factors are (a) the lack of genes required for chloroplast differentiation and chlorophyll synthesis [for ref. See Touraev et al. 2001]; (b) plastid DNA deletions as the result of a normal mechanism during pollen development ensuring maternal inheritance of plastids [Day and Ellis 1984]; (c) the absence of the plastid-encoded proteins [Hess et al. 1993; Hofinger et al. 2001]; (d) high temperature (32-34°C)-induced translation deficiency in plastids [Herrman and Feirerabend 1980; Hess et al. 1992].In the absence of evidence for deletions or transcription failure as primary mechanisms, a block in translation has therefore been postulated as the primary cause for albino formation [Hofinger et al. 2001].

It has been shown that manipulations of the microspore culture conditions may solve the problem. In one-step regeneration anther culture, which avoids callus formation, fewer albinos are produced [Liang et al. 1987]. In addition, direct embryogenesis system in isolated microspore culture of wheat in which embryogenic microspores are formed without any apparent stress treatment, enhanced significantly frequency of green plants [Shariapnahi et al. 2006b]. Recently, it was proved that stressful *in vitro* conditions in microspore cultures could make the plants fight their own plastids with antibiotic like compounds resulting in formation of albino [Torp and Andersen 2009].

GENE EXPRESSION DURING INDUCTION OF MICROSPORE EMBRYOGENESIS

Tobacco and rapeseed in which microspore cultures have been developed to their highest efficiency in haploid production [Custers et al. 1994, Touraev and Heberle-Bors 1999] have

been used to study the molecular mechanisms underlying the developmental switch in microspores from gametophytic to the sporophytic mode of development.

Genes specifically expressed during embryogenic induction of microspores have been studied extensively [Touraev et al. 2001, Marashin et al. 2005, Malik and Krochko 2009]. The BABY BOOM (*BBM*) gene is one of many genes identified as being differentially expressed in embryogenic microspores. *BBM* was shown to encode a new member of the AP2/ERF transcription factor family and led to the formation of embryogenic structures on seedlings when over-expressed ectopically in *Arabidopsis* [Boutilier et al. 2002]. However, the role of this gene in microspore embryogenesis is not known.

During heat-induced microspore embryogenesis in *Brassica napus*, both mRNAs and several specific proteins are synthesized *de novo* in response to the stress treatment and some of the genes activated during embryogenic induction have been identified as members of different families of heat-shock genes [Cordewener et al. 1994, 1995]. The question remains as to whether these stress-induced proteins are really involved in the process of microspore embryogenesis or are simply required for the microspores to survive the stress treatment. However, it was shown that much higher levels of a small heat-shock protein (18 kDa) are present in embryogenic tobacco pollen in which no heat shock was applied as compared with mid-bicellular pollen [Zarsky et al. 1995].

In maize, Magnard et al. [2000] showed using differential display that transcripts of two genes (*zmae1* and *zmae3*), normally expressed in maize endosperm, interestingly also accumulated in microspore-derived multicellular pro-embryos after 5 days of culture.

Three phosphoproteins, NtEPb1, -b2 and –b3, were also found to be abundant in embryogenic tobacco microspores [Kyo et al. 2002]. *In vitro* phosphorylation assays in extracts of mid-bicellular and embryogenic tobacco pollen showed quantitative and qualitative changes on protein kinase activities during the starvation treatment [Garrido et al. 1993]. Therefore, protein kinases are likely to be involved in the transduction of the hunger signal, mediating the effects of starvation on gene expression and cell-cycle regulation.

Malik and Krochko [2009] used transcript profiling methods to identify differentially-expressed genes as well as shifts in metabolism during the early stages of microspore embryogenesis. Differentially- regulated gene clusters and 16 genes were identified that could be used as specific markers for microspore embryogenesis.

It can be concluded that the switch of microspores towards embryogenesis is a complex of changes in gene regulation, transcription and metabolism. And, depending on the type of stress applied, different genes may trigger microspores towards sporophytic development.

MICROSPORE EMBRYOGENESIS AND DOULBED HAPLOIDS IN PLANT BREEDING

Modern plant breeding programs depend on the availability of homogenous genetic resources with guaranteed levels of identity. Doubled haploids obtained by ME are the fastest route to homozygosity, in just one generation. They are absolutely pure lines with enormous advantages for line and hybrid breeding, as they speed up breeding programmes [Forster et al. 2007]. Most excitingly, in combination with genetic marker technology, they allow to define the best recombinant profiles early in a plant breeding programs and the most desirable

lines can then be selected for further breeding. However, the technology is not available for all crop species and even genotypes of a given species, irrespective of the method used. For example, *Brassica napus*, var. Topas is highly responsive for ME whereas in many other varieties induction of ME and regeneration are still limiting factors [Swanson 1990]. The same is true for tobacco: *Nicotiana tabacum* varieties are, in general, highly responsive for ME and the large number of doubled haploids are produced routinely whereas *Nicotiana alata* is recalcitrant and ME is still not possible (unpublished data).

MICROSPORE EMBRYOGENESIS AS A TOOL FOR MUTAGENESIS AND MUTANT SELECTION

Microspore cultures *in vitro* can be used efficiently for mutagenesis and selection. Because the microspore is a single haploid cell, any genetic variation induced by the mutagen will be expressed in the entire regenerated plant and progeny, therefore eliminating chimeras [Kott, et al. 1996]. All recessive and dominant traits are readily expressed and are easily selectable in culture. Such traits can be fixed by chromosome doubling to achieve homozygosity. This is unlike diploid cell selection where recessive traits can be masked by a dominant trait in the heterozygote. Since selection can be made at the haploid or doubled haploid state, plants carrying undesirable trait combinations can be readily discarded instead of being carried for generations as in heterozygotes. Apart from the highly regenerative potential of microspores, there are other advantages for their use in mutagenesis. Many uniform cells can be exposed to chemical or physical mutagens in a relatively small space. Appropriate selection pressure can be applied in the culture to select mutants. Mutagenesis was used to develop plants resistant to imidazolinone herbicides in *Brassica napus* cultures by mutagenising microspores with ethylnitrosourea (ENU) [Swanson et al. 1989]. Microspores or microspore-derived haploids are also useful for selecting mutants that accumulate storage products. Mutants with high oleic acid were isolated from mutagen-treated microspores of *Brassica napus* [Kott et al. 1996].

MICROSPORE EMBRYOGENESIS AND DOUBLED HAPLOIDS ARE CORE FOR REVERSE BREEDING

ME and DH plants also play an integral part in reverse breeding (RB), a recently developed technology [Dun et al. 2006]. This technology involves down regulation of meiotic recombination to develop a concept termed reverse breeding. DH are recovered from microspores of plants in which recombination has been suppressed through down regulation of genes like DMC1 and SPO11 via RNAi. Because of the lack of recombination only $(1/2)^5$ normal (euploid) spores are expected to survive until plant regeneration. This means still about 3% of the spores will be normal. The population that will be generated from such RB events is highly unique because it will contain only combination of parental chromosomes without scrambling by recombination. More importantly, chromosome substitution lines are generated in only one step. By back-crossing a substitution line to its most related parent and subsequently inbreeding, Introgression Lines are obtained “per chromosome”.

MICROSPORE EMBRYOGENESIS IN FRUIT TREES

In perennial plants particularly, where conventional breeding is usually time-consuming due to their long reproductive cycle, high degree of heterozygosity and complex reproductive biology, the potential of microspore embryogenesis shows a great advantage in the development of haploids and doubled haploids from heterozygous fruit trees in a single step which is almost impossible through classical breeding methods [reviewed by Germana 2009]. In some fruit trees such as peach, F_1 hybrids have been developed from microspore derived-doubled haploids offering the production of uniform seedling scion cultivars which can be profitable since it is much cheaper to plant non-grafted seedlings than to use grafted plants. In future, haploid and doubled haploid technology in fruit trees will be optimized and widely used as a powerful fruit tree breeding tool [Germana 2009].

OVERCOMING SELF-INCOMPATIBILITY USING IN VITO MATURATION AND GERMINATION OF MICROSPORES

In cross-pollinated plants with homomorphic flowers, self-fertilization can be avoided on genetic/biochemical mechanisms. There are two quite different types of self-incompatibility including Sporophytic self-incompatibility (SSI) and Gametophytic self-incompatibility (GSI). Sporophytic self-incompatibility has been studied intensively in members of the mustard family (*Brassica)*, including turnips, rape, cabbage, broccoli, and cauliflower. In this system, rejection of self pollen is controlled by the diploid genotype of the sporophyte generation. Because the plants cannot fertilize themselves, they tend to be heterozygous. In this system, Pollen will not germinate on the stigma (diploid) of a flower that contains either of two alleles in the sporophyte parent that produced the pollen. In such a cross-pollinated plant, Sporophytic self-incompatibility can be overcome using isolated microspore culture method. Microspores can be isolated *in vitro* and be matured and germinated. *In vitro* germinated pollen grains can be used for *in vivo* pollination of SSI plants. Thus, self-fertilization can be restored [reviewed by Touraev et al. 2001].

MICROSPORE-BASED TRANSFORMATION

The techniques, by which transformation are carried out on microspore or pollen [reviewed by Touraev et al. 2001], can be divided into three, i.e. (I) mature pollen-based transformation in which the DNA either is delivered into pollen before pollination or is applied to stigma before or after pollination (pollen tube pathway); (II) Male Germ Line Transformation (MAGELITR) in which the DNA is transferred biolistically into unicellular microspores in the G1 phase of their cell cycle and cultured *in vitro* to form mature pollen and then this pollen is used for pollination *in vivo* and the resulting seeds are selected for transformants; (III) microspore or immature pollen embryogenesis-based transformation in which the embryogenic microspores or immature pollen grains induced by stress are transformed and divided symmetrically giving rise to embryos and haploid plants under optimal conditions.

Microspore is an attractive target for transformation because it is an easily available and accessible single haploid cell and ME is also available in many important crops [Harwood et al. 1996]. In addition, the transgenes can be studied in both haploid and doubled haploid plants. Although the primary target is the uni-cellular microspore, cells or explants at all stages of ME and regeneration can be used as recipients for gene delivery. Applying haploid cells for gene transfer avoid hemizygosity [Touraev et al. 2001]. In the case when chromosome doubling occurs very early during microspore culture, either spontaneously or induced, homozygosity already exists in the regeneration process [Touraev et al. 2001].

Particle bombardment of barley embryogenic microspores has resulted in transgenic doubled haploids that were homozygous for the transgene [Shim et al. 2009a, b], and similar results were reported in *Brassica napus* [Abdollahi et al. 2007, Cegielska-Taras et al. 2008]. More efficient was the transformation of barley microspore-derived multi-cellular structures using *Agrobacterium tumefaciens* [Kumlehn et al. 2006], demonstrating the feasibility of this technology for other cereals where ME is amenable. In tobacco, particle bombardment has been used to transform embryogenic and non-embryogenic microspores and immature pollen [Aionesei ei al. 2006, Touraev et al. 1997b].

DEVELOPMENT OF AN ENVIRONMENTAL-FRIENDLY F_1 HYBRID BREEDING TECHNOLOGY

Male sterility that facilitates F_1 hybrid seed production and double haploid technology that help to speed up the breeding cycle are two most important technologies in modern breeding [Budar and Pelletier 2001, Ribarits et al. 2009]. A novel technology package has been developed that is able to combine these two valuable technologies and present an environment-friendly breeding system for both seed and non-seed crops [Ribarits et al. 2007, 2009]. Double point mutations in two critical positions were introduced into tobacco cytoplasmic glutamine synthetase (GS1), fused to the microspore-specific NTM19 promoter, and transformed to tobacco. Approximately 50% of pollen in T_0 primary transgenic lines aborted close to the first pollen mitosis stage and homozygous 100% male-sterile T_1 lines were produced via *in vitro* microspore embryogenesis. Male-sterile T_1 progenies are able to set seeds after glutamine spraying and pollinated with wild-type pollen. Thus, microspore-specific inhibition of GS1 in developing pollen allowed the rapid generation of male-sterile inbred lines and the maintenance by fertility restoration.

It widely avoids limitations of currently existing systems and, as a novelty, allows the generation of male-sterile doubled haploids via microspore embryogenesis. Such doubled haploid plants are 100% homozygous and nowadays used by many breeders to produce recombinant inbred lines. This technology allows maintaining the male-sterile lines by three different approaches (by *in vitro* maturation, glutamine sprays and microspore embryogenesis). The use of the microspore-specific NTM19-promoter renders F_1 hybrids fertile due to pollination with segregating wild-type pollen. In any instance, the release of transgenic pollen is precluded in the production field where fertility restoration is not essential. The displayed technology avoids toxic substances or chemicals with potential side effects to induce male sterility or to restore fertility, and employs plant sequences. In addition, we believe that it is a technological advance, feasible in an agricultural setting, and provides a

smart alternative to previously published systems. It is the first example of a molecularly designed breeding technology that combines reversible male sterility and doubled haploid production, and can potentially be applied to virtually any important staple crop. Currently we have established the same technology in important vegetable tomato [unpublished data].

REPROGRAMMED AND EMBRYOGENIC MICROSPORES AS TARGET FOR TARGETED GENE REPLACEMENT

Gene targeting (GT) by homologous recombination (HR) is a genetic tool allowing precise integration of genes at predetermined genome positions as well as the production of specific and predictable changes in the host genome [Reiss 2003]. Homologous recombination (HR) and gene targeting occurs efficiently in many lower eukaryotes such as bacteria and budding yeast and recently became available as a powerful tool also in some higher eukaryotes such as *Drosophila*, mouse, and human somatic cells [Hanson, and Sedivy 1995]. In flowering plants GT technology is still inefficient and has limited reproducibility [Reiss 2003, Tzfira and White 2005, Puchta and Hohn 2005] . The successful gene targeting in rice [Terada et al. 2002] and moss [Schaefer and Zryd 1997] was attributed to the use of competent cells to HR and GT. In the moss (*Physcomitrella*) gene replacement occurs efficiently in the chloronemal cells which are haploids and arrested in the G2 phase of the cell cycle prior to gene transfer [Resch et al. 2009].

The plant male gametophyte as a target for gene targeting has been proposed earlier in the light of success of gene targeting in moss [Puchta 1998]. Higher plant microspores and immature pollen similar to moss are haploid, gametophytic cells which experimentally can be arrested in the G_2 phase of the cell cycle. They may, for these reasons, also offer high HR frequencies and, thus, be the best cell type for gene targeting experiments [Resch et al. 2009].

Recently, immature pollen grains were used for GT to evaluate the potential of higher plant male gametophyte as a target for GT experiments [Resch et al. 2009]. The artificial B18/4 target locus inserted to tobacco genome was used to assess gene targeting in tobacco mid-bicellular pollen. In this system, a neomycin-phosphotransferase (*npt* II) gene which is expressed exclusively in seeds was converted into a constitutive *npt* II gene by insertion of the CaMV 35S promotor between the HMW seeds-specific promotor and a functional restored *npt* II gene at the target locus. The tobacco mid-bi-cellular pollen isolated from the B18/4 target locus plants were transformed biolistically with the repair construct. Southern analysis confirmed an ectopic GT event occurred in one transgenic line via modification of the repair construct by the target locus and subsequent integration elsewhere in the tobacco genome [Resch et al. 2009].

MICROSPORE EMBRYOGENESIS AS A SYSTEM TO STUDY PLANT CELL REPROGRAMMING, TOTIPOTENCY AND EMBRYOGENESIS

One of the most intriguing questions in developmental biology is the reprogramming of a somatic cell with restricted developmental options into a cell, which is able to give rise to an embryo and a reproductively competent organism [Heberle-Bors and De Vries 1997]. This

phenomenon, called "totipotency," has first been proposed by the Austrian botanist Haberlandt [Haberlandt 1922] and experimentally induced in higher plants [Reinert 1959]. Despite a large body of experimental data, obtained in almost 50 years, little is known about the mechanism of cell reprogramming and formation of totipotent cells [Halperin 1995]. Several experimental systems have been developed and used with various levels of success to study this process.

Reprogramming and totipotency have been demonstrated also in male reproductive cells [Touraev et al 1997a, 2001, Guha and Maheshwari 1964]. Isolated and *in vitro* cultured microspores or immature pollen of flowering plants can be induced to undergo a developmental switch by certain physical and chemical stress treatments, such as cold, heat or starvation [Touraev et al 1997a, Shariatpanahi et al. 2006a]. More precisely, microspores convert from their intrinsic gametophytic development through a stage of totipotency towards a sporophytic pathway, resulting in the formation of haploid embryos and plants [Touraev et al. 1997a].

Microspore embryogenesis has several unique properties which make this system ideal to study the molecular and cellular biology of plant cell reprogramming, totipotency and embryogenesis: direct embryogenesis from a single, isolated cell, free of surrounding tissues, large amount of synchronised embryogenic cells, and the possibility to study the formation of the embryo founder cell.

CONCLUSION

Plant microspores, when isolated, stressed and cultured *in vitro*, can be diverted from their normal gametophytic pathway towards embryogenesis, with the formation of haploid embryos and, ultimately, doubled haploid plants. This process, called microspore embryogenesis (ME), is a very attractive system to study the mechanism of plant cell reprogramming, totipotency and embryogenesis. In addition, ME is widely used a) to generate haploids and homozygous doubled haploids, which are core technology for many genetic studies and plant breeding, b) in plant transformation, mutagenesis, gene targeting etc. Doubled haploids obtained by microspore embryogenesis are the fastest route to homozygosity, in just one generation [Touraev et al. 2001; Thomas et al. 2003].

Isolated microspores are unique plant haploid cells as they can develop *in vitro* sprophytically forming embryos via stress treatment or gametophytically producing mature pollen grains. This ability enables microspores to be used in both fundamental and applied science [Touraev et al. 1997a , 2001].

Recently, the *NtDCN1* gene (the *Nicotiana tabacum DCN1* ortholog) has been identified in our group to play a significant role during the transition of microspores into pollen grains *in vivo*, into embryogenic microspores to initiate sporophyte formation *in vitro*, and from the globular stage of both microspore-derived and zygotic embryos to the heart-shaped stage (Hosp et al., submitted)**.** It has been demonstrated that loss of function of *NtDCN1* caused by RNAi blocked these transitions. Over-expression of *NtDCN*1 accelerated the formation of embryogenic microspores by reducing the duration of the stress treatment required to reprogram microspores into sporophytic development. These results clearly indicate that NtDCN1 is involved in phase changes during gametophyte development and embryogenesis

in plants. Biochemical experiments defined that *NtDCN1* binds both ubiquitin and RUB1/NEDD8 and associates with cullin, suggesting that indeed cullin neddylation followed by targeted protein degradation may be required for the above mentioned transitions.

Development of a novel and reversible male sterility system using targeted inactivation of glutamine synthetase presents an environment-friendly breeding system for both seed and non-seed crops [Ribarits et al. 2007, 2009] which is very useful for breeders. We think that in future F_1 hybrids via this technology will be developed in more species.

In the end, it can be concluded that microspore is a remarkable multi-functional haploid cell which can be used for various purposes.

REFERENCES

Abdollahi, MR; Moieni, A; Mousavi, A; Salmanian, AH;Jalali Javaran, M;Majdi, M. *Effect of integrated bombardment and Agrobacterium transformation system on transient GUS expression in hypocotyls of rapeseed (Brassica napus L. cv. PF704) microspore-derived embryos.* Pak J Biol Sci, 2007, 10(18), 3141-3145.

Aionesei, TE; Touraev, A; Heberle-Bors, E. *Title: Pathways to microspore embryogenesis.* In: Palmer CE, Keller WA, Kasha KJ editors. Title: Biotechnology in Agriculture and Forestry, vol.56. Berlin, Heidelberg: Springer-Verlag; 2005; 11-34.

Aionesei, TE; Hosp, J; Voronin, V; Heberle-Bors, E, Touraev, A. *Methotrexate is a new selectable marker for tobacco immature pollen transformation.* Plant Cell Rep, 2006, 25(5), 410-416.

Bajaj, YPS. *Title: In vitro production of haploids and their use in cell genetics and plant breeding.* In: Bajaj YPS, editor. Title: Haploids in Crop Improvement I, Vol 12. New York: Springer Verlag; 1990; 1-44.

Barinova, J; Clement C; Marting, L; Baillieul, F; Soukupova, H; Heberle-Bors, E; Touraev, A. *Regulation of developmental pathways in cultured microspores of tobacco and snapdragon by medium pH.* Planta, 2004, 219, 141-146.

Boutilier, K; Offringa, R; Sharma, VK; Kieft, H; Quellet, T; Zhang, L; Hattori, J; Liu, CM; van Lammern, AA; Miki, BL; Custers, JB; van Lookeren, Campagne, MM. *Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth.* Plant Cell, 2002, 14, 1737-1749.

Budar, F; Pelletier, G. *Male sterility in plants: occurrence, determinism, significance and use.* C R Acad Sci III, 2001, 324, 543-550.

Cegielska-Taras, T; Pniewski, T; Szala, L. *Transformation of microspore-derived embryos of winter oilseed rape (Brassica napus L.) by using Agrobacterium tumefaciens*. J Appl Genet, 2008, 49(4), 343-347.

Cordewener, JHG; Busink, R; Traas, JA; Custers, JBM; Dons, HJM; Van Lookeren Campagne, MM. *Induction of microspore embryogenesis in Brassica napus L. is accompanied by specific changes in protein synthesis*. Planta, 1994, 195, 50-56.

Cordewener, JHG; Hause, G; Görgen, E; Busink, R; Hause, B; Dons, JJM; Van Lammeren, AAM; Van Lookeren Campagne, MM; Pechan, P. *Changes in synthesis and localization of the 70 kDa class of heat shock proteins accompany the induction of embryogenesis in Brassica napus L microspores*. Planta, 1995, 196, 747-755.

Custers, JBM; Cordewener, JHG; Nöllen, Y; Dons, HJM; Van Lookeren Campagne, MM. *Temperature controls both gametophytic and sporophytic development in microspore cultures of Brassica napus*. Plant Cell Rep, 1994, 13, 267-271.

Day, A; Ellis, THN. *Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible basis for maternal inheritance of chloroplasts.* Cell, 1984, 39, 359-368.

Dun, van k; Lelivelt, C; Snoo, de B; Dirks R. *Title: Reverse breeding: a novel plant breeding concept.* In: Title: Proceeding of the international conference on Haploids in higher plants III. Vienna: Vienna; 2006; 29.

Dunwell, JM; Sunderland, N. *Pollen ultrastructure in anther cultures of Nicotiana tabacum I. Early stages of culture.* J Exp Bot, 1974a, 25, 352-361.

Dunwell, JM; Sunderland, N. *Pollen ultrastructure in anther cultures of Nicotiana tabacum II. Changes associated with embryogenesis.* J Exp Bot, 1974b, 25, 363-373.

Ferrie, AMR; Palmer, CE; Keller, WA. Title*: Haploid embryogenesis*. In: Thorpe TA, editor. Title: *In vitro* Embryogenesis in Plants, Vol 20. Dordrecht: Kluwer Academic Publishers; 1995; 309-344.

Forster, BP; Thomas, WTB. Title: *Doubled haploids in genetic mapping and genomics.* In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I editors. Title: Doubled Haploid Production in Crop Plants, A Manual. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2003; 367-390.

Forster, BP; Heberle-Bors, E; Kasha, KJ; Touraev, A. *The resurgence of haploids in higher plants.* Trends Plant Sci, 2007, 12(8), 368-375.

Garrido, D; Eller, N; Heberle-Bors, E; Vicete, O. *De novo transcription of specific mRNAs during the induction of tobacco pollen embryogenesis*. Sex Plant Reprod, 1993, 6, 40-45.

Garrido, D; Vicente, O; Heberle-Bors, E; Isabel Rodriguez-Garcia, M. *Cellular changes during the acquisition of embryogenic potential in isolated pollen grains of Nicotiana tabacum.* Protoplasma, 1995, 186, 220-230.

Germana, MA. *Title: Haploids and doubled haploids in fruit trees.* In: Touraev A, Forster BP, Mohan Jain S editors. Title: Advances in Haploid Production in Higher Plants. United Kingdom: Springer Science+ Business Media B.V.; 2009; 241-263.

Guha, S; Maheshwari, SC. *In vitro production of embryos from anthers of Datura*. Nature, 1964, 204, 497.

Guha, S; Maheshwari, SC. *Cell division and differentiation of embryos in the pollen grains of Datura in vitro.* Nature, 1966, 212, 97-98.

Haberlandt, G. *Über Zellteilungshormone und ihre beziehungen zur wundheilung, befruchtung, parthenogenesis und adventivembryonie*. Biol. Zentbl., 1922, 42, 145.

Halperin, W. Title: *In vitro embryogenesis: some historical issues and unresolved problems.* In: Thorpe T, editor. Title: In vitro embryogenesis in plants. Dordrecht / Boston/ London: Kluwer Acad. Publishers; 1995; 1-16.

Hanson, KD; Sedivy, JM. *Analysis of biological selections for high-efficiency gene targeting.* Mol Cell Biol, 1995, 15(1), 45-51.

Harwood, WA; Chen, DF; Creissen, GP. Title: *Transformation of pollen and microspores*. In: Mohan Jain S, Sopory SK, Veilleux RE, editors. Title: In vitro haploid production in higher plants. Dordrecht, Boston, London: Kluwer Academic Publishers; 1996; 53-71.

Heberle-Bors, E. *Induction of embryogenic pollen grains in situ and subsequent in vitro pollens embryogenesis in Nicotiana tabacum L. by treatments of the pollen donor plants with feminizing agents.* Physiol Plant, 1983, 59, 67-72.

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

Heberle-Bors, E ; De Vries, S. *Le développment embryonnaire des végétaux.* Biofutur, 1997, 172, 38-42.

Herrmann, RG; Feierabend, J. *The presence of DNA in ribosome-deficient plastids of heat-bleached rye leaves.* European Journal of Biochemistry, 1980, 104, 603-609.

Hess, WR; Blank-Huber, M; Fieder, B; Börner, T; Rüdiger, W. *Chlorophyl II synthethase and chloroplast tRNAglu are present in heat-bleached, ribosome-deficient plastids*. J Plant Physiol, 1992, 139, 427-430.

Hess, WR; Prombona, A; Fieder, B; Subramanian, AR; Börner, T. *Chloroplast rps15 and the rpoB/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids: evidence for a functioning non-chloroplast-encoded RNA polymerase*. EMBO J, 1993, 12, 563-571.

Hofingr, BJ; Ankele, E; Gülly, C; Heberle-Bors, E; Pfosser, MF. *Title: The involvement of the plastid genome in albino plant regeneration from microspores in wheat*. In: Bohanec B, editor. Title: Biotechnological Approaches for Utilization of Gametic Cells-COST824. Brussels: European Commission; 2001; 215-228.

Höfer, M; Touraev, A; Heberle-Bors, E. *Induction of embryogenesis from isolated apple microspores*. Plant Cell Rep, 1999, 18, 1012-1017.

Imamura, J; Harada, H. *Effects of abscisic acid and water stress on the embryo and plantlet formation in anther culture of Nicotiana tabacum cv. Samsun*. Z. Pflanzenphysiol, 1980a, 100, 285-289.

Imamura, J; Harada, H. *Stimulatory effect of reduced atmospheric pressure on pollen embryogenesis.* Naturwissenschaften, 1980b, 67, 357-358.

Indrianto, A; Barinova, J; Touraev, A; Heberle-Bors, E. *Tracking individual wheat microspores in vitro: identification of embryogenic microspores and body axis formation in the embryo.* Planta, 2001, 212, 163-174.

Kasha, KJ; Maluszynski, M. *Title: Production of doubled haploids in crop plants; an introduction*. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I editors. Title: Doubled Haploid Production in Crop Plants, A Manual. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2003; 5-14.

Kott, L; Wong, R; Swanson, E; Chen, J. *Title: Mutation and selection for improved oil and meal quality in Brassica napus utilising microspore culture*. In: Mohan Jain S, Sopory SK, Veilleux RE, editors. Title: *In vitro* haploid production in higher plants. Dordrecht, Boston, London: Kluwer Academic Publishers; 1996; 151-167.

Kumlehn, J; Serazetdinova, L; Hensel, G; Becker, D; Loerz, H. *Genetic transformation of barley (Hordeum vulgare L.) via infection of androgenetic pollen cultures with Agrobacterium tumefaciens*. Plant Biotechnol J, 2006, **4**(2), 251-261.

Kyo, M, Yamaji, N; Yuasa, Y; Maeda, T; Fukui, H. *Isolation of cDNA coding for NtEPb1-b3, marker proteins for pollen dedifferentiation in a tobacco pollen culture system*. Plant Sci, 2002, 163, 1055-1062.

Lemonnier-Le Penhuizic, C; Chatelet, C; Kloareg, B; Potin, P. *Carrageenan oligosaccharides enhance stress-induced microspore embryogenesis in Brassica oleracea var italica* . Plant Sci, 2001, 160, 1211-1220.

Liang, GH; Xu, A; Hoang, T. *Direct generation of wheat haploids via anther culture*. Crop Science, 1987, 27, 336-339

Liu, W; Zheng, MY; Konzak, CF. *Improving green plant production via isolated microspore culture in bread wheat (Triticum aestivum L.).* Plant Cell Rep, 2001, 20, 821-824.

Magnard, JL; Le Deunff, E; Domenech, J; Rogowsky, PM; Testillano, PS; Rougier, M; Risueno, MC; Vergne, P; Damas, C. *Genes normally expressed in the endosperm are expressed at early stages of microspore embryogenesis in maize*. Plant Mol Biol, 2000, 44, 559-574.

Malik, MR; Krochko, JE. Title: *Gene expression profiling of microspore embryogenesis in Brassica napus*. In: Touraev A, Forster BP, Mohan Jain S editors. Title: Advances in Haploid Production in Higher Plants. United Kingdom: Springer Science+ Business Media B.V.; 2009; 115-125.

Maraschin, SF; de Priester, W; Spaink, HP; Wang, M. *Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective*. J Exp Bot, 2005, 56, 1711-1726.

Nitsch, C; Norreel, B. *Effet d'un choc thermique sur le pouvoir embryogene du pollen de Datura innoxia cultive dans l'anthere ou isole de l'anthere.* C R Acad Sci, Paris, 1973, 276, 303-306.

Pechan, PM; Keller, WA. *Induction of microspore embryogenesis in Brassica napus L. by gamma irradiation and ethanol stress.* In Vitro Cell Dev B, 1989, 25, 1073-1074.

Puchta, H. *Towards targeted transformation in plants.* Trends in Plant Sci, 1998, 3, 77-78.

Puchta, H; Hohn, B. *Green light for gene targeting in plants.* Proc Natl Acad Sci U S A, 2005, 102(34), 11961-11962.

Raina, SK; Irfan, ST. *High frequency of embryogenesis and plantlet regeneration from isolated microspores of indica rice*. Plant Cell Rep, 1998, 17, 957-962.

Reinert, J. *Über die kontrolle der morphogenese und die von adventivembryonen an gewerbekulturen aus karotten.* Planta, 1959, 53, 318-324.

Reiss, B. *Homologous recombination and gene targeting in plant cells*. Inter Rev Cytol, 2003, 28, 85-139.

Resch, T; Ankele, E; Badur, R; Reiss, B; Heberle-Bors, E; Touraev, A. *Title: Immature pollen as a target for gene targeting.* In: Touraev A, Forster BP, Mohan Jain S editors. Title: Advances in Haploid Production in Higher Plants. United Kingdom: Springer Science+ Business Media B.V.; 2009; 307-317.

Ribarits, A; Mamun, ANK; Li, S; Resch, T; Fiers, M; Heberle-Bors, E; Liu, CM; Touraev, A; *Combination of reversible male sterility and doubled haploid production by targeted inactivation of cytoplasmic glutamine synthetase in developing anthers and pollen*. Plant Biotechnology Journal, 2007, 5, 483-494.

Ribarits, A; Mamun, ANK; Li, S; Resch, T; Fiers, M; Heberle-Bors, E; Liu, CM; Touraev, A. *A novel and reversible male sterility system using targeted inactivation of glutamine synthetase and doubled haploidy*. In: Touraev A, Forster BP, Mohan Jain S editors. Title: Advances in Haploid Production in Higher Plants. United Kingdom: Springer Science+ Business Media B.V.; 2009; 285-294.

Sangwan, RS; Camefort, H; *The tonoplast, a specific marker of embryogenic microspores of Datura cultured in vitro.* Histochemistry, 1983, 78, 473-480.

Sangwan, RS; Camefort, H. *Cold-treatment related structural modifications in the embryogenic anthers of Datura.* Cytologia, 1984, 49, 473-487.

Sangwan, RS; Sangwan, BS. Title : *Effets des rayons gamma sur l´embryogenèse somatique et l´androgenèse chez divers tissue végétaux cultives in vitro*. In: Sangwan RS, editor. Title: Nuclear Techniques and In Vitro Culture for Plant Improvement. Vienna: International Atomic Energy Agency; 1986; 181-185.

Schaefer, DG; Zryd, JP. *Efficient gene targeting in the moss Physcomitrella patens*. Plant J, 1997, 11, 1195-1206.

Shariatpanahi, ME; Bal, U; Heberle-Bors, E; Touraev, A. *Stresses applied for the re-programming of plant microspores towards in vitro embryogenesis*. Physiol Plant, 2006a, 127, 519-534.

Shariatpanahi, ME; Belogradova, K; Hessamvaziri, L; Heberle-Bors, E; Touraev, *A. Efficient embryogenesis and regeneration in freshly isolated and cultured wheat (Triticum aestivum L.) microspores without stress pretreatment*. Plant Cell Rep, 2006b, 25, 1294-1299.

Shariatpanahi, ME; Emamifar, M; Habibzadeh, S; Amiri, R; Nematzadeh, G; Oroojloo, M. *Title: Effect of 2,4-D as an inducer of embryogenesis in microspores of Brassica napus L.* In: Title: Proceeding of the International Conference "Green Plant Breeding Technologies". Austria: Vienna; 2010; 17.

Shim, YS; Pauls, KP; Kasha, KJ. *Transformation of isolated barley (Hordeum vulgare L.) microspores: II. Timing of pretreatment and temperatures relative to results of bombardment.* Genome, 2009a, 52(2), 175-190.

Shim, YS; Pauls, KP; Kasha, KJ. *Transformation of isolated barley (Hordeum vulgare L.) microspores: I. the influence of pretreatments and osmotic treatment on the time of DNA synthesis.* Genome, 2009b, 52(2), 166-174.

Snape, JW. *A theoretical comparison of diploidised haploid and single seed descent populations.* Heredity, 1976, 36, 275-277.

Snape, JW; Simpson, E; Parker, BB. *Title: Criteria for the selection and use of doubled haploid systems in cereal breeding programmes*. In: Horn W, Jensen CJ, Odenbach W, Schieder O editors. Title: Genetic Manipulation in Plant Breeding. New York, Berlin: Walter de Gruiter; 1986; 217-229.

Swanson, ER. Title: *Microspore culture in Brassica*. In: Pollard JW, Walker JM, editors. Title: Methods in molecular biology. Humana Press; 1990.

Swanson, EB, Herrgesell, M; J.Arnoldo, M; Sippell, DW; Wong, RSC. *Microspore mutagenesis and selection. Canola plants with field tolerance to the imidazolinones.* Theor Appl Genet, 1989, 78, 525-530.

Szarejko, I. Title: *Doubled haploid mutant production*. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I editors. Title: Doubled Haploid Production in Crop Plants, A Manual. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2003; 351-361.

Tanaka, M. *The effect of centrifugal treatment on the emergence of plantlets from cultured anther of tobacco* .Japan J Breed, 1973, 23, 171-174.

Telmer, CA; Newcom, W; Simmonds, DH. *Microspore development in Brassica napus and the effect of high temperature on division in vivo and in vitro*. Protoplasma, 1993, 172, 154-165.

Terada, R; Urawa, H; Inagaki, Y; Tsugana, K; Iida, S. *Efficient gene targeting by homologous recombination in rice.* Nat Biotechnol, 2002, 20, 1030-1034.

Thomas, WTB; Forster, BP; Gertsson, B. Title: *Doubled haploids in breeding*. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I editors. Title: Doubled Haploid Production in Crop Plants, A Manual. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2003; 337-349.

Torp, AM; Andersen, SB. Title: *Albinism in microspore culture*. In: Touraev A, Forster BP, Mohan Jain S editors. Title: Advances in Haploid Production in Higher Plants. United Kingdom: Springer Science+ Business Media B.V.; 2009; 155-160.

Touraev, A; Stöger, A; Voronin, V; Heberle-Bors, E. *Maintenance of gametophytic development after symmetrical development in tobacco microspore culture*. Sex Plant Reprod, 1995, 9, 209-215.

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

Touraev, A; Indrianto, A; Wratschko, I; Vicente, O; Heberle-Bors, E. *Efficient microspore embryogenesis in wheat (Triticum aestivum L.) induced by starvation at high temperatures.* Sex Plant Reprod, 1996b, 9, 209-215.

Touraev, A; Vicente, O; Heberle-Bors, E. *Initiation of microspore embryogenesis by stress*. Trends Plant Sci, 1997a, 2, 297–302.

Touraev, A; Stöger, E; voronin, V; Heberle-Bors, E.. *Plant male germ line transformation*. Plant J, 1997b, **12**, 949-956.

Touraev, A; Heberle-Bors, E. Title: *Microspore embryogenesis and in vitro pollen maturation in tobacco.* In: Hall RD, editor. Title: Methods in Molecular Biology, Vol.111: Plant Cell Culture Protocols. Totowa, NJ: Humana Press Inc; 1999; 281-291.

Touraev, A; Pfosser, M; Heberle-Bors, E. *The microspore: a haploid multipurpose cell*. Adv Bot Res, 2001, 35, 53-109.

Tzfira, T; White, C. *Towards targeted mutagenesis and gene replacement in plants.* Trends Biotechnol, 2005, 23(12), 567-569.

Wang, JJ; Hu, DF; Wang, HM; Tang, YL. *Studies on increasing the induction frequency of pollen callus in wheat*. Hereditas (Beiging), 1981, 3, 28-29.

Zaki, MAM; Dickinson, HG. *Structural change during the first divisions of embryos resulting from anther and microspore culture in Brassica napus*. Protoplasma, 1990, 156, 149-162.

Zaki, MAM; Dickinson, HG. *Microspore-derived embryos in Brassica: The significance of division symmetry in pollen mitosis I to embryogenic development*. Sex Plant Rep, 1991, 4, 48-55.

Zarsky, V; Garrido, D; Eller, N; Tupy, J; Vicente, O; Schöfel, 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, 1995, 18, 139-147.

Zhao, JP; Simmonds, DH; Newcomb, W. *Induction of embryogenesis with colchicine instead of heat in microspores of Brassica napus L. cv. Topas*. Planta, 1996, 198, 433-439.

Zheng, MY; Liu, W; Weng, Y; Polle, E; Konzak, CF. *Culture of freshly isolated wheat (Triticum aestivum L.) microspores treated with inducer chemicals*. Plant Cell Rep, 2001, 20, 685-690.

Zonia, LE; Tupy, J. *Lithium treatment of Nicotiana tabacum microspores blocks polar nuclear migration, disrupts the partitioning of membrane-associated Ca+2, and induces symmetrical mitosis.* Sex Plant Reprod, 1995, 8, 152-160.