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# Microspore culture in wheat *(Triticum aestivum)* — doubled haploid production via induced embryogenesis

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## Abstract

The inherent potential to produce plants from microspores or immature pollen exists naturally in many plant species. Some genotypes in hexaploid wheat *(Triticum aestivum* L.) also exhibit the trait for androgenesis. Under most circumstances, however, an artificial manipulation, in the form of physical, physiological and/or chemical treatment, need to be employed to switch microspores from gametophytic development to a sporophytic pathway. Induced embryogenic microspores, characterized by unique morphological features, undergo organized cell divisions and differentiation that lead to a direct formation of embryoids. Embryoids 'germinate' to give rise to haploid or doubled haploid plants. The switch from terminal differentiation of pollen grain formation to sporophytic development of embryoid production involves a treatment that halts gametogenesis and initiates sporogenesis showing predictable cellular and molecular events. In principle, the inductive treatments may act to release microspores from cell cycle control that ensures mature pollen formation hence overcome a developmental block to embryogenesis. Isolated microspore culture, genetic analyses, and studies of cellular and molecular mechanisms related to microspore embryogenesis have yielded useful information for both understanding androgenesis and improving the efficiency of doubled haploid production. The precise mechanisms for microspore embryogenesis, however, must await more research.

*Abbreviations:* ABA – abscisic acid; DH – doubled haploid; HSP – heat shock protein; OVCM – ovary- conditioned medium; PGR – plant growth regulator; PPB – preprophase band

## Introduction

Angiosperms exhibit life cycles of alternating generations between a haploid gametophyte and a diploid sporophyte. In the gametophyte phase, microspore mother cells in anther locules undergo meiosis and mitosis that result in the formation of male gametophytes or pollen grains. Prompted by certain environmental cues, immature pollen (microspores) can be switched from gametophytic to sporophytic development, leading to the formation of 'pseudoembryos' commonly known as embryoids (Reynolds, 1997; Touraev et al., 2001; Zheng et al., 2001). Embryoids 'germinate' to produce either haploid or doubled haploid plants (DHs). This phenomenon, an excellent example of plant cell totipotency, is defined as androgenesis or microspore embryogenesis, two terms that are often used interchangeably. The production of DHs through androgenesis is a proven method to obtain homozygous individuals in a single step. Thus, the method is useful in crop improvement, genetic manipulation, and in many areas of basic research related to plant developmental biology. The attainment of homozygosity in one generation helps reduce the numerous cycles of inbreeding necessary in conventional pure line breeding systems. With an efficient plant regeneration system,

gametic cells are also preferred targets for genetic transformation and transgenic studies. DHs are frequently used in plant genome mapping. The direct access to single-cells and the formation of true emhryoids makes the microspore culture an ideal system for studies of embryogenesis and other aspects of plant developmental biology (Vicente et al., 1991; Reynolds, 1997; Touraev et al., 2001).

Although the first success in regeneration of wheat *(Triticum aestivun)* plants through anther culture was achieved in the early 1970s (Research Group 303, 1972; Chu et al., 1973; Ouyang et al., 1973; Picard and De Buyser, 1973; Craig, 1974), progress was slow. The early culture methods primarily relied upon the natural occurrence of embryogenic microspores within certain genotypes, hence were inefficient, yielding only a fraction of the plants easily obtained today. In addition, these early systems mainly used agar-solidified media, and rather high levels of PGRs for callus induction. As a result, plants were mostly obtained through differentiation and regeneration of calli. The ratios of green vs. albino plants often were disappointingly low, and the high levels of PGRs induced chromosome aberrations and undesirable somatic variations as well. Once liquid media were employed in culture, the yields of calli/embryoids were markedly improved and the regenerated plants were more stable due to lowered PGR levels (Chuang et al., 1978; Ouyang et al.. 1978). These liquid media included an extract from boiled potatoes. While not readily definable, the media worked well and were effectively used to study and identify other factors that affect the responses of wheat anthers to *in vitro* culture manipulations.

As with other small grain cereals, DR production in wheat has advanced rapidly in recent years through isolated microspore cultures (Hu et al., 1995; Touraev et al., l996b, 2001; Hu and Kasha, 1997; Konzak et al., 1999; Zheng et al., 2001, 2002b; Liu et al., 2002). The increase in efficiency was made possible through the direct access to and artificial manipulation of individual microspores and subsequent success in plant production. This review provides a historical overview on wheat androgenesis and summarizes some recent progress in wheat microspore culture systems as well as our current understanding of the genetic, cellular, biochemical and molecular events associated with microspore embryo-genesis. Reviews on other aspects of wheat anther/microspore culture can be found elsewhere (Kasha et al., 1990; Dunwell, 1992; Jähne and Lörz, 1995; Cordewener et al., 1995a; Reynolds, 1997; Touraev et al., 2001). Although wheat microspore embryogenesis is the focal topic, reference to some aspects of anther/microspore cultures in other species is inevitable due to the similarity in fundamental mechanisms of androgenesis.

## Culture of microspores via induced embryogenesis

The first success in culturing microspores free of the surrounding anther tissue was reported by Nitsch and Norreel (1973) in *Datura innoxia.* This and other early systems, however, are not strictly isolated microspore culture as a pre-culture period for anthers or flower parts is employed prior to the release of microspores. Following the pre-culture period, microspores are either freed mechanically through homogenization (Lichter, 1982) or shed from anthers into liquid medium (Ziauddin et al., 1990). The mechanical isolation and subsequent culture of microspores for plant regeneration in wheat is a more recent event (Mejza et al., 1993; Gustafson et al., 1995). In spite of low efficiencies, these two groups reported first success in culturing wheat microspores isolated from mechanical blending. Since then, the culture systems for isolated wheat microspores have been improved to the practical level. Not only have the culture efficiencies been drastically improved, but also the germplasm range on which microspore culture can be employed successfully has been largely expanded. A reasonable number of DHs can be obtained from genotypes previously described as recalcitrant (Konzak et al., 1999; Zheng et al., 2001; Liu et al., 2002). In these systems, microspores isolated from numerous genotypes are capable of producing adequate DHs for use in wheat breeding. The successes can be attributed to a better understanding of androgenesis, more effective manipulation of microspores for embryogenesis, and optimization of induction culture for embryoid production (Table 1).

### *Growth and harvest of donor plants*

It is widely known that growth conditions influence the androgenic response by affecting the vigor and quality of donor plants (Kasha et al., 1990; Jähne and Lörz, 1995). The main factors include light intensity and quality, nutrition, photoperiod, and temperature.

*Table I.* Highlights of isolated wheat microspore culture processes

| Stage | Manipulation | Key events/parameters | End product |
|---|---|---|---|
| I | *Pretreatment* (Induction of embryogenic potential) | High (33 °C)/low (4 °C) temperature, starvation (sugar, N), and chemical, alone or in combination | Viable microspores (some characterized by 'fibrillar' cytoplasm or 'star-like' structure) |
| II | *Isolation and purification* | Mechanical blending (speed and length); filtration and density centrifugation of 0.3 M mannitol over 0.58 M maltose | Homogeneous population of embryogenic microspores |
| Ill | *Induction culture* (embryoid development) | 9% maltose; PGR balance (2,4-D, PAA, kinetin); ovaries or OVCM; temperature 26–28 °C; osmolarity 300–320 mOsmole $kg^{-1}$ $H_2O$ | Low rate of embryogenic abortion: large number of mature embryoids |
| IV | *Plant recovery* (embryoid germination) | 190–2 semi-solid medium; 0.088 M sucrose; PGR-free; light supplement | Production of green plants |

In wheat, lower temperatures (12–18 °C) have positive effects on culture response (Simmonds, 1989; Ziauddin et al., 1992). However, donor plants grown in a greenhouse with reasonable control of temperature and light intensity should be satisfactory (Zheng et al., 2001). Environmental stresses including diseases and insects should be avoided. Although growth chambers with well-regulated growing conditions are helpful in reducing the fluctuations in microspore cultures, they are not essential for the production of doubled haploid. Microspore donor plants may be raised along with breeding material in the same greenhouse. In general, any standard conditions for growing wheat in a greenhouse are acceptable provided that healthy plants can be obtained (Zheng et al., 2001). For winter wheats, it is essential to complete the vernalization to ensure normal plant growth and optimal culture response.

One of the critical steps in wheat microspore culture is the sampling of donor plants at the appropriate developmental stage. The best time for harvesting wheat spikes is when the majority of microspores are at the mid- to late-uninucleate stage. During this period, microspores are most susceptible to androgenic induction treatment. For a given genotype, plants exhibit very similar morphological measure if grown under similar conditions. Such morphological feature-developmental stage correlation can easily be established for each genotype via microscopic examination of microspores in acetocarmine stain or distilled water at various plant stages (Zheng and Konzak, 1999).

*Pretreatment — transition to sporophytic development*

Although a number of procedures or systems have been used in wheat anther/microspore cultures, almost all employ some type of stress treatment to trigger androgenesis. In other words, proper stress treatment to induce the embryogenic potential is a pre-requisite for success. Microspores having inherent potential for embryogenesis exist naturally in anthers of some plant species (Guha and Maheshwari, 1964; Chuang et al., 1978; Heberle-Bors, 1985; Raghavan, 1986; Ziauddin et al., 1990; Zhou et al., 1991). The frequencies of such natural occurrence (androgenesis without any deliberate stress treatment), however, are usually very low. Even with such 'natural' occurrence, one could argue that the trauma introduced in excising anthers from their host plants represents a physical stress that might have acted as a switch signal for androgenesis. The efficiency of a system relying upon natural phenomena is unacceptably low. Thus an efficient production of DHs requires the artificial manipulation of microspores and subsequent success in the recovery of large number of green plants (Touraev et al., 1996a, b, 2001; Hu and Kasha, 1997; Konzak et al., 1999; Zheng et al., 2001; Liu et al., 2002).

The first of the two critical steps in artificial manipulation involves treatment to switch microspores from naturally determined pollen formation to an alternative development that leads to embryogenesis (Touraev et al., 1996a, 2001; Reynolds, 1997; Zheng et

al., 2001). Various pretreatments can be applied either *in vivo* or *in vitro* to trigger microspore embryogenesis. These include physical, physiological and chemical means in the form of cold or heat shock, sugar starvation and chemical treatment of excised spikes, anthers or microspores. Nitrogen starvation, short days and low temperature treatment of donor plants are also found to increase embryoid yields in anther cultures. Regardless of the type and timing of application, these treatments may act as signal or stimulus to trigger the switch of the developmental program in microspores (Reynolds, 1997; Indrianto et al., 1999; Konzak et al., 1999). Other treatments including water stress, anaerobic conditions, radiation, and inducer chemicals also act as external stimuli to trigger or enhance microspore embryogenesis (Imamura and Harada, 1980, 1981; Kyo and Harada, 1986; Touraev et al., 1997; Konzak et al., 1999). These factors may act alone or in combination in order to achieve an optimal conversion of microspores to embryogenic cells. The requirement of stress for the induction of embryogenesis appears to be universal in plants and animals (Zimmerman and Cohill, 1991). Animal cloning such as the birth of 'Dolly' the sheep employs a stress treatment as at least one of the crucial factors in initiating embryogenic cell division.

In wheat (as in some other species), low temperature (1–5 °C) pretreatment is beneficial for anther culture response. When tillers or spikes are given a cold-shock at ~4 °C for several days prior to normal culture procedures, anthers have significantly higher response than the control (Lazar et al., 1985; Amstrong et al., 1987; Henry and de Buyser, 1989). Both higher callus yield and an increased frequency of spontaneous chromosome doubling among the regenerants are obtained with cold pretreatment. The speculation is that cold-shock pretreatment might have a dual function:

– interrupting normal gametophytic development and
– the nursing effects of anther tissue on microspores.

Others, however, have found no positive or even adverse effects of cold-shock pretreatment (Marsolais et al., 1984; Ouyang, 1986; Li et al., 1988). In barley, pretreatment of spikes for 4 weeks in the dark at 4 °C prior to anther / microspore culture is a common practice (Kasha et al., 1992; Ziauddin et al., 1992). However, the mechanisms by which low temperatures act on anthers or microspores are not well understood. Some suggest that low temperature slows down metabolism of microspores hence arrests cell cycle in preparation for the resumption of mitosis once anthers /microspores are cultured in normal temperatures (25–28 °C). Others speculate that the starvation effects under low temperature may be a primary factor for anther culture response (Kasha et al., 1990; Ziauddin et al., 1992). Compared to pretreatment of excised anthers in 0.3 M mannitol (sugar starvation), cold pretreatment prior to the microspore isolation is less effective for embryogenic induction (Roberts-Oehlschlager and Dunwell, 1990; Olsen, 1991; Ziauddin et al., 1992; Hoekstra et al., 1993; Mejza et al., 1993; Hu et al., 1995). In essence, both low temperature stress and other physiological responses associated with low temperature could be responsible for enhancing anther culture responses.

High temperature, alone or in combination with starvation, seemed to trigger or at least enhance microspore embryogenesis (Baillie et al., 1992; Custers et al., 1994; Touraev et al., 1996a, b). High temperature (32–34 °C) pretreatment applied to excised wheat anthers increases callus yields (Hu, 1986; Li et al., 1988). High temperature (–32 °C), coupled with starvation, dramatically increases embryoid yields from isolated tobacco microspores (Touraev et al., 1996a). Up to a stunning 75% of the initial population of microspores were triggered to divide following a 6-day starvation treatment at 33 °C. When the 33 °C pretreatment was applied to wheat anthers, much higher yield of embryoids were obtained from isolated microspores although the regeneration and green plant frequencies were still low (Touraev et al., 1996b).

More recently, heat- (cold-) shock and starvation coupled with inducer chemical(s) has further increased the efficiency of DH production in wheat through isolated microspore cultures. A frequently used inducer chemical formulation contains no sugar, 0.01% (w/v) 2-hydroxynicotinc acid (2-HNA), $10^{-6}$ M benzamino purine (BAP) and $10^{-6}$ to $10^{-5}$ M 2,4-D (Liu et al., 2002). The two unique and efficient systems – 'flask' and 'fresh microspore' systems are capable of inducing 20–50% of microspores in anthers of some wheat genotypes from gametophytic into sporophytic development (Zheng et al., 2001, 2002b; Liu et al., 2002). In the 'flask' system, tillers with leaves removed are allowed to stand upright in a flask (hence the name) containing inducer chemical formulation. Thus, the base of all tillers was in direct contact with the inducer solution. After the neck of the flask is wrapped around with a thin-walled plastic bag, the flask is incubated at a desired temperature for a period of time (Liu et al., 2002). Transpiration facilitates movement of the chemical through the vascular system

into the anthers and microspores. The 'fresh microspore' system provides direct contact between the inducer chemical formulation and microspores. Microspores are first isolated from spikes sampled afresh (thus the name 'fresh microspore'), and immediately mixed with a solution containing the inducer chemicals and osmoticum (Zheng et al., 2001, 2002b). During the pretreatment phase, microspores swell and their cytoplasm, vacuole and nucleus undergoes structural and positional reorganization. Thus, the reprogrammed microspores typically have eight or more small vacuoles forming a circle in the cytosol. The nucleus moves to the center of the cell and is surrounded by condensed cytoplasm pocket that also forms radiant strands and passes in-between small vacuoles, forming a 'star-like' or fibrillar structure (Touraev et al., 1996b; Liu et al., 2002; Zheng et al., 2002b).

Type and duration of pretreatment was very important in blocking gametophytic development and in inducing embryogenic development. The three pretreatment factors, temperature shock, starvation and inducer chemicals, seemed to act in synergy in embryogenic induction. Using only heat shock and starvation produces lower embryoids and higher albino frequencies (Touraev et al., 1996b). Once the embryogenic potential is fully induced by optimal stress treatment, microspores need to be isolated or cultured immediately in a nutrient-rich medium. Both insufficient and over-extended pre-treatment is detrimental for microspore embryogenesis. The goal for pretreatment is not only to maximize the number of embryogenic microspores, but also to achieve the best quality of competent microspores from which embryoids can mature and produce green plants.

*Isolation methods — releasing and purifying microspores*

At least four different approaches exist for isolating microspores: shedding, magnetic-bar stirring, macera-tion, and blending (see Jähne and Lörz, *1995* for a more extensive review). Shedding is a technique first developed by Sunderland and Roberts (1977) in which cultured tobacco anthers shed their microspores into a liquid medium. These microspores were then collected and cultured for callus development and plant regeneration. The shedding technique was later adopted in wheat (Wei, 1982; Datta and Wenzel, 1987). More recently, a 6–7 day pretreatment in 0.3 M mannitol plus macronutrients was recommended for shedding micro-spores or for a step preceding mechanical isolation of microspores (Kasha et al., 1990; Ru et al., 1995). Magnetic-bar stirring is a derivative of shedding in that a stirring force is added to help release the microspores still enclosed within the anther wall. In effect, magnetic-bar stirring serves to increase microspore yields from the natural shedding (Cho and Zapata, 1990). The shedding and stirring procedures, however, are not effective means in wheat microspore cultures due to the low yields of microspores and plants subsequently recovered.

The first mechanical isolation of microspores was established in *Brassica napus* (Lichter, 1982), in which anthers were pressed through a mesh filter by using a glass or Teflon rod. This technique, termed maceration, was adapted to other species including wheat (Tuvesson and Öhlund, 1993). The most recent technique-isolation by blending was initially developed by Olsen (1991) working in barley and was first adapted to wheat by Mejza et al. (1993). Since then, the blending method has become the method of choice for wheat microspore isolation. Compared to the maceration, the blending technique employs sharp blades at moderate velocity and hence is able to quickly cut anthers in pieces and release microspores. Comparing all four methods of isolating microspores including blending, stirring, macerating, and shedding, isolation by blending gives the highest initial microspore viability of 75% (Gustafson et al., 1995). In the 'flask' system mentioned earlier, isolation by blending is carried out as soon as the pretreatment is completed. Any delay or storage of tillers following the pretreatment leads to rapid decreases in microspore viability, irrespective of temperature (4–23 °C) employed for storage (Zheng et al., 2002b). Micro-spores released through blending usually have less damage, higher embryogenic capacity and reproducibility than those obtained through maceration. However, blending isolation can also damage the microspores, especially when the blending speed and length is not optimized. The blending parameters vary somewhat with the number of florets in the blender-cup, toughness of the tissue surrounding anthers and the relative volume of liquid medium vs. solid tissues.

Although the damage to microspores may not always be visible, the consequence is almost certain – no embryoid development. One needs to know when to make adjustments necessary to achieve the highest microspore yield with the least damage. With optimal conditions, wheat microspore cultures via blending isolation are far superior to anther culture due to a number of reasons. First, blending saves labor and reduces cost as a result of its capability of obtaining large number of microspores without the need of excising anthers. Second, the possibility of running multiple isolations for different crosses or progenies in a single day makes its use

practical in wheat breeding. And finally, the large number of microspores, embryoids and green plants obtained from these microspores enhances the reliability of experimental and statistical analyses in studies of androgenesis.

To achieve best results, microspores in culture need to be relatively pure. Once released through blending, embryogenic microspores can be separated from nonviable cells, dead tissues and cell debris through size- differential filtration and / or density centrifugation (Mejza et al., 1993; Gustafson et al., 1995; Touraev et al., 1996b; Konzak et al., 1999). Although effective for purification, gradient centrifugation with Percoll is too expensive to be practical. A more economic but equally effective procedure involves mesh filter filtration followed by a density centrifugation of 0.3 M mannitol over 0.58 M maltose (Mejza et al., 1993; Zheng et al., 2001; Liu et al., 2002). Immediately following the blending, the slurry is poured into mesh filter cups of 100 and 38-µm pore size. Microspores trapped in the 38-µm mesh filter cup are rinsed off with 0.3 M mannitol and laid over on 0.58 M maltose. When centrifuged at low speed (l00–l50×g), viable embryogenic microspores form a band in the interface of 0.3 M mannitol and 0.58 M maltose, while other cells or tissues form a pellet (Mejza et al., 1993; Konzak et al., 1999; Liu et al., 2002). The purification is based upon the presence of small vacuoles in embryogenic microspores and the characteristic density associated with these vacuoles. The purified microspores are collected and mixed with culture medium for embryoid development. Impurities or contaminants of non-viable cells or tissue debris tend to inhibit embryogenesis hence reduce the number of embryoids.

*Induction culture – elaboration of embryo genic program*

Once the embryogenic potential is triggered, it is vital to provide microspores with an adequate environment to carry out embryogenesis. Favorable factors during this phase may include both optimized physical conditions such as temperature and osmolarity, proper nutrients in the medium including carbohydrates, PGRs, minerals, vitamins, and an adequate density, etc. (Mejza et al., 1993; Tuvesson and Ohlund, 1993; Gustafson et al., 1995; Touraev et al., 1997; Zheng et al., 2001). The optimal temperature for embryoid development from wheat microspores is 26–28 °C. The working range of medium osmolarity is 300–320 mOsmole $kg^{-1}$ $H_2O$, an osmolarity equivalent of 0.3 M mannitol or 0.25 M maltose. Although various microspore densities ranging from $2\times10^4$ to $2\times10^5$ / ml had been reported effective for embryoid induction (Ziauddin et al., 1990; Olsen, 1991; Hoekstra et al., 1993; Gustafson et al., 1995), a high density is not necessary for success. In fact, a density of $7–8\times10^3$ / ml is quite effective for embryoid development (Zheng et al., 2002b). The effective density ranges from $5\times10^3$ to $2\times10^4$/ ml. Relatively low but adequate microspore density eases the competition for nutrients, oxygen, and space for cell divisions and embryoid formation, hence improves both the number and quality of embryoids. The co-culture of microspores with ovaries and/or ovary-conditioned medium (OVCM) makes it possible to employ a lower density (Zheng et al., 2002a). In addition, microspores of high purity in culture also contribute to the success of using lower microspore densities.

Medium composition has long been known to affect the success of anther/microspore cultures. Although variations exist for mineral nutrients and vitamins, wheat microspores respond quite favorably to various recipes, as long as a proper nutrient balance is maintained. The basal medium is derived either from N6 (Chu, 1978) or from modified MS (Hu et al., 1995) although many other variations may also be effective. The carbohydrate of choice for wheat microspore cultures has been maltose following the discovery of its positive effects on barley and wheat anther/microspore cultures (Hunter, 1988; Orshinsky et al., 1990; Mejza et al., 1993). In fact, medium consisting of sucrose exhibits toxic effects on isolated wheat microspores partly due to a greater fluctuation in osmolarity than medium with the same level of maltose. A substantial rise in osmolarity is probably due to the hydrolysis of sucrose in the medium. Inhibition on androgenesis by sucrose also occurs in anther culture (Zhou et al., 1991). Media with maltose as sole carbon source showed negligible changes in osmolarity over time, and resulted in the production of embryoids of superior quality that yielded higher ‘germination’ rate, and higher green vs. albino ratio than those from media with sucrose (Zheng, data unpublished). The positive effects of maltose on wheat microspore culture might also be due to a starvation effect on microspores early in the culture as a slow hydrolysis of maltose provides insufficient amount of glucose.

Balance of exogenous hormones in the culture medium is crucial for both the yield and quality of embryoids. Although there is no general agreement on the optimal PGR compositions, the consensus is to use the lowest possible concentrations of PGRs to produce embryoids (Ru and Kasha, 1997; Zheng et al., 2001; Liu et al.,

2002). Higher PGR levels in induction culture are generally detrimental to plant production as cell division and differentiation shift towards calli rather than embryoids (Zheng and Konzak, 1999). Auxin and cytokinin are the two types of PGRs most widely used in media for anther/microspore cultures. The inclusion of PAA is beneficial for wheat microspore culture (Hu et al., 1995). A combination of 2,4-D, kinetin and PAA in wheat microspore culture is optimal for embryoid development (Zheng et al., 2001). With an optimal PGR combination, many dividing microspores evolve into true embryoids that have very high 'germination' rates. In addition, inclusion of PGR such as ABA (Hu et al., 1995), 2,4-D and BAP (Liu et al., 2002) in the pretreatment phase is also beneficial for embryogenic induction. It is, however, a nearly impossible task to have a generalized PGR combination that works for all. Optimal PGR combination is affected by the variation in genotypes, quality of donor plants, pretreatment regime and physiological status of microspores in the culture. It is vital to maintain a threshold level of PGRs in the culture that leads to both high quality and large number of embryoids.

Another important aspect for induction culture is the co-cultivation of isolated microspores with live ovaries or OVCM (Bruins et al., 1996; Puolimatka et al., 1996; Hu and Kasha, 1997; Devaux and Li, 2001; Zheng et al.. 2001, 2002a; Liu et al., 2002). When ovaries are added to wheat microspore cultures, the number of pre-embryoids, embryoids and regenerated plants increase significantly (Ru and Kasha, 1997; Zheng et al., 2002a). Without ovaries, the frequency of multi-cellular structure is reduced (Liu et al., 2002). Most microspores in culture without ovaries stop dividing beyond 14 days hence fail to break out the exine, leading to aborted embryogenic development (Zheng et al., 2002a). More importantly, the inclusion of OVCM is essential for microspore embryogenesis of some less responsive or recalcitrant genotypes. Without OVCM, no embryoids can be obtained from some recalcitrant genotypes, although cell divisions or multi-cellular structures are observed. OVCM apparently provides essential substance(s) to maintain embryogenesis already triggered during pretreatment (Zheng et al., 2002a). The presence of OVCM helps microspores to realize their embryogenic potential. Although the functional component(s) in OVCM is (are) yet to be discovered, the positive effect appears to result from its ability to accelerate and maintain microspore divisions (Puolimatka et al., 1996; Ru and Kasha, 1997; Zheng et al., 2002a). In addition to the inclusion of ovaries and OVCM, other aspects of culture conditions may also need to be adjusted for the least responsive genotypes (Lazar et al., 1990).

While in a balanced embryoid development medium, embryogenic microspores follow a well-organized and predictable pattern of cell divisions and differentiations that lead to formation of embryoids. The first cell divisions normally occur in 14–20 h, and well-defined multicellular structures form within 1 week. Pre-embryoids begin to emerge out of the microspore wall in 10–14 days. Mature embryoids (≥2 mm) can be harvested after 28 days (Zheng et al., 2001; Liu et al., 2002).

*'Germination' of embryo ids – production of doubled haploid plants*

Once mature embryoids are obtained, plant regeneration is a relatively simple matter compared to the other steps of microspore culture. The handling of plantlets is also standard for those who have ordinary skills in this field. Embryoids reaching the size of approximately 2 mm in diameter are usually transferred to a PGR free, semi-solid medium with a reduced carbohydrate (0.088 M sucrose). A widely used embryoid 'germination' medium for wheat anther/microspore culture is 190–2 (Zhuang and Xu, 1983). Upon the transfer, these embryoids are incubated under light at a room temperature. Green shoots begin to emerge and increase in size in 5–7 days. After 10–14 days on the 'germination' medium, green plantlets are transplanted to soil and are raised in a greenhouse, much like plants grown from seeds (Zheng et al., 2001; Liu et al., 2002). It is important to note that the transfer of embryoids to 'germination' medium is not a once-for-all step because embryoidmaturation in induction culture is a process lasting about 30 days from the transfer of the first batch of embryoids. To minimize the decline in 'germination' rate of embryoids maturing later in the induction culture, ovaries and induction medium are conveniently refreshed following the first transfer of embryoids to 'germination' medium (Liu et al., 2002; Zheng et al., 2002b).

If plants appear to be haploid by checking the root tip or the size of stomata, colchicine or caffeine can be applied to induce chromosome doubling. The standard colchicine treatment is a 3 h immersion of root tips of haploid plantlets in 2 g $l^{-1}$ colchicine solution followed by thorough rinses with running water (Metz et al., 1988). Although colchicine treatment is still the most

effective way for doubling the chromosome, the highly carcinogenic nature limits its application. Treatment with caffeine is less effective and produces fewer seeds than colchicine (Thomas et al., 1997). Considering that caffeine is much less toxic, its use should be sought as an alternative for chromosome doubling. Further, a transient use of coichicine (100 mg $1^{-1}$ colchicine for 5 d) in induction culture may also be used to increase the *in vitro* doubling frequencies (Redha et al., 1998). The use of colchicine, at or before the first mitotic division, also induces the symmetrical division of microspores, considered as an important event for embryoid development (Redha et al., 1998, Hu and Kasha, 1999). However, the use of colchicine or caffeine may be unnecessary when a large number of green plants can be obtained with even moderate spontaneous doubling frequencies (Liu et al., 2002; Zheng et al., 2002b).

*Summary*

In short, the most effective wheat microspore culture systems typically rely upon four key steps: In the first, the microspores are switched from their naturally programmed pathway for gametophytic development to sporophytic development by a physical/physiological stress (e.g. high or low temperature, starvation) coupled with a chemical formulation. In the second step, the embryogenic microspores featured with unique morphology are purified through size differential filtration and density centrifugation. In the third step. optimal culture conditions are provided, which include adequate nutrition, co-culture with ovaries / OVCM and a favorable physical environment to help miciospores elaborate their embryogenic program leading to embryoid production. Finally, embryoids ‘germinate’ on solid, hormone-free media to form haploid/DH plants. It is now possible to produce thousands of green plants from microspores isolated from a single wheat spike (Hu and Kasha, 1997; Konzak et al., 1999; Zheng et al., 2001; Liu et al., 2002).

## Genetic control of microspore embryogenesis

It has long been recognized that both environmental and genetic factors contribute significantly to the androgenic responses in wheat. The influence of environmental factors has been extensively reviewed elsewhere (Dunwell, 1985; Kasha et al., 1990; Pickering and Devaux, 1992) and will not be discussed here. All three components in wheat anther culture, embryoid induction, total plant regeneration and green / albino plant ratio have been determined to be independently inherited traits (Agache et al., 1988). The inheritable nature of androgenic traits provides the basis for introducing these traits into non-responsive genotypes. In most cases, the genetic component of culturability is attributed to additive gene effects (Deaton et al., 1987; Szakacs et al., 1988), although epistatic and dominant effects have also been observed (Agache et al., 1988). The dominant and additive gene effects provide opportunity to improve androgenic response through cross breeding and recurrent selection.

In addition, significant interactions exist between nuclear genes and cytoplasm type for all three components of the androgenic response (Ekiz and Konzak, 1991a, b). Some types of cytoplasm have strong positive or negative effects on anther culturability while others have minor effects. Among the 18 pairs of reciprocal crosses studied in common wheat, nine showed reciprocal differences for green plant regeneration, four showed significant differences for callus induction, and three were different for overall plant regeneration (Ekiz and Konzak, 1991 b). These results suggest that the three components of androgenesis are expressed differently in the same nuclear and cytoplasmic background. The influence of cytoplasm in androgenic response makes the choice of paternal vs. maternal parent in crosses an important step. For germplasm with unknown androgenic response in particular, it is better to make reciprocal crosses before comparing the androgenic responses between progenies and their parents.

The location of nuclear genes has not been ascertained although some effort has been made to place gene(s) on specific chromosomes or identify markers linked to genes involved in the androgenic response (Zhang and Li, 1984; Agache et al., 1989; Devaux et al., 1990; Ben Amer et al., 1995). Using genetic analyses of mono-somic lines, several chromosomes are found to be involved in embryoid production (Agache et al., 1989). Chromosomes 2A and 2D bear major genes for androgenesis whereas chromosomes 2B, 4A, 5A and 5B carry minor genes acting to inhibit embryoid production (Zhang and Li, 1984). A comparative study on the genetic basis of androgenic response finds that genes on CS chromosome 1D and chromosome 5BL increase the embryoid frequency (Agache et al., 1989). Gene(s) in-volved in regeneration ability locate(s) on chromosome 1RS, acquired through a translocation from rye (R) genome. A gene increasing albino frequency is on CS-5B chromosome (Agache et al., 1989). Obviously, due to a reduced viability of both plants and microspores associated with them, results from monosomic studies

should be interpreted with caution. Furthermore, a significant portion of uncontrollable and environmentally induced variations makes genetic analyses of anther/microspore culture all the more difficult.

Quantitative and complex gene actions are involved in the determination of androgenic responses although some believe that major genes are mainly responsible for the inheritance of androgenesis. Additive effects are identified in both callus induction and plantlet regeneration from anther culture in a diallel population produced from five inbred spring wheat cultivars of different anther culturability (Lazar et al., 1984). Three quantitative trait loci for tissue culture response were mapped on chromosome 2B of hexaploid wheat *(Triticum aestivum* L.) by Ben Amer et al. (1997). However, the callus induction and plantlet regeneration seem to be two independent, heritable traits (Holme et al., 1999). The gene loci for these two traits are not yet mapped to specific chromosomes. A comparative study in anther cultures using hexaploid wheat (AABBDD) and tetraploid wheat (AABB) found that the D genome carries genes that contribute to higher efficiency in both embryoid yields and green plant frequencies (Ghaemi and Sarrafi, 1994). Overall, tetraploid durum wheat exhibits much lower androgenic responsiveness than does hexaploid common wheat. Results from microspore cultures using durum and common wheat appear to be in agreement with those from anther culture (Zheng et al., unpublished data). In general, durum wheat shows much lower response to culture manipulation, with fewer embryogenic microspores induced, much higher rate of embryogenic development abortion, and higher albino frequencies than common wheat. In addition to nuclear genes, cytoplasmic factors in durum wheat also affect culture responses (Ghaemi et al., 1993). As with hexaploid wheat, favorable combinations of nucleus and cytoplasm can somewhat improve androgenesis in tetraploid wheat. Nevertheless, low frequencies of embryoid development and the high rate of albino plants remain to be major obstacles for DH production in tetraploid wheat. It is yet to be identified to which extent the genes in D genome act independently or contribute through their interactions with genes in A and B genomes in determining androgenic responses.

More recent studies with hexaploid wheat reveal significant genetic variability and relatively high heritability for androgenic traits. Among 49 winter wheat cultivars/lines tested, heritability for embryoid yield, total plant regeneration and green plant percentage are relatively high (0.80–0.88). In addition, both general and specific combining abilities are estimated to be highly significant (Moieni and Sarrafi, 1995). Fortunately, albino plant regeneration has the lowest heritability value (0.68) when compared with other traits, making it possible to reduce albinos by controlling the culture conditions or other environmental factors. In a study using 43 cultivars and six Fl crosses representing different market classes of spring wheat, androgenic responses are strongly affected by the genotype of the donor plants (Masojc et al., 1993). Cultivars with hard grain (either red or white) generally showed higher response than soft wheats in both anther culture and isolated microspore culture (Liu et al., 2002; Zheng et al., 2002b). These results again suggest that androgenic traits are heritable and variable. It is possible to convert recalcitrant yet elite germplasms into responsive genotypes through cross breeding. The feature of independent inheritance of various culture components, i.e. embryoid induction, total plant regeneration and the ratio of green to albino plants, makes it possible to create new lines with superior combination of all androgenic traits.

Marker analyses on androgenic response are largely carried out in crop species other than wheat. The information, however, should be valuable for future marker analysis in wheat. An RFLP analysis in maize identifies six regions on chromosomes 1 and 2, and two regions each on chromosomes 3, 6 and 8 that are linked to microspore embryogenesis (Wan et al., 1992). Further RFLP marker analyses on $F_2$ generation of these crosses locate a total of six regions on chromosomes 1, 3, 5, 7 and 8 that are associated with androgenesis (Beaumont et al., 1995). A more extensive mapping study on androgenic response was carried out using a total of 98 $S_1$ families (Cowen et al., 1992). Two major recessive genes on chromosomes 3 and 9, which exhibit epistasis, and two minor genes on chromosomes 1 and 10 are involved in microspore embryogenesis. Although the precise location and the nature of these genes is still unknown, such mapping effort will certainly help lead to the eventual cloning and characterization of genes that control microspore embryogenesis. With the highly efficient systems of isolating and culturing single-cell microspores in wheat, it should be feasible to conduct marker analyses aiming at identifying genes that are critical for androgenesis. Once candidate genes (markers) are identified, they can be used to aid in biochemical, molecular and developmental investigations of the underlining mechanisms of microspore embryogenesis (Reynolds, 1997; Touraev, 2001). The favorable genes (alleles) can also be introduced into less responsive or recalcitrant genotypes through genetic transformation. Together, these genetic studies will likely provide useful information in at least one of two ways: to find genes that are related to androgenesis and to increase the efficiency of DH production by transferring these genes to recalcitrant genotypes. Before we gain a better precision on manipulating gene(s) responsible for androgenesis, however, finding ways to improve the culture system is obviously a more effective

approach to overcome or alleviate genotype problems.

## Cellular mechanisms

Cytological observations and analyses of cellular mechanism in androgenesis were first conducted using anther cultures. Sunderland and Dunwell (1977) presented the first extensive discussion on the pathways in microspore embryogenesis. Embryoids arise in one of three main pathways although in some species (e.g. *Nicotiana tabacum, Hordeum vulgare)* only two occur. The first embryogenic division in pathway A and B are asymmetric, leading to the formation of a typical bicellular pollen grain. In pathway A, the normally quiescent vegetative cell divides while the generative cell degenerates. By contrast in pathway B, typically the generative cell divides and the vegetative cell degenerates. The third pathway (pathway C) exhibits symmetric first division. Subsequent divisions of both vegetative and generative cell lead to formation of embryoid/callus. These pathways are defined on the basis of cellular origin of the calli/embryoids. Additional variations under the three main pathways also occur (Sunderland and Dunwell, 1977). Although very informative, their discussion was not directed specifically at androgenesis of any particular species. No supporting cytological evidence was presented for their proposed pathways.

In wheat, all three pathways, symmetrical and asymmetrical cell divisions within microspores are said to occur during androgenesis (Ouyang et al., 1973; Reynolds, 1993; Touraev et al., l996b, 2001; Ru and Kasha, 1999; Zheng et al., 2002b). However, some early studies failed to present convincing evidence to support the claims for various pathways, largely due to the limit on the access to cytological studies offered by cultured anthers (Ouyang et al., 1973; Ruang, 1986). Two pathways of androgenesis are later documented for embryogenic microspores in anther culture (Reynolds, 1993). The first is pathway A, by which vegetative cell undergoes repeated divisions to form the embryoid. In the second, the first division is symmetric and both nuclei divide (pathway C) to form embryoid (Reynolds, 1993). One major drawback using anther culture for cellular analyses is apparent: the early stages of embryogenesis occur within the locule of the anther. Hence, it is difficult to access the inductive phase and early cellular events.

A great deal of progress has been made recently in our understanding of microspore embryogenesis at the cellular level. Much of the recent advance in our understanding relies upon the success in obtaining large numbers of microspores responsive to induction, and the ability to identify these cells at an early stage of their androgenic development. To date, the cellular origin of embryoids in wheat androgenesis has been well documented at the level of light and electron microscopy (Reynolds, 1993, 1997; Hu and Kasha, 1999; Indrianto et al., 2001; Zheng et al., 2001; Liu et al., 2002). Following a stress pretreatment of anthers at 33 °C for several days, the embryogenic microspores showed a ‘star-like’ structure and produced embryoids following repeated symmetric divisions (Touraev et al., l996b). In a separate cytological study comparing the effects of various pretreatments, mostly symmetric first divisions were observed during mannitol pretreatment while only asymmetric first divisions were seen during or after cold pretreatment (Hu and Kasha, 1999; Kasha et al., 2001). Apparently, the type and duration of pretreatment affect the symmetry of the initial nuclear division hence the pathway of microspore embryogenesis. Following the symmetrical division, the two nuclei typically fuse and form a diploid nucleus, which undergoes rapid nuclear and cell divisions to form multicellular structures in induction medium (Hu and Kasha, 1999; Kasha et al., 2001). The fusion of two nuclei has profound practical significance as it leads to the production of doubled haploid embryoids and fertile plantlets. Although a variety of pretreatment methods have been used to induce embryoid or callus formation from anthers / microspores, only the three (A, B and C) pathways for androgenesis are believed to occur in wheat (Reynolds, 1993; Hu and Kasha, 1999; Indrianto et al., 2001; Kasha et al., 2001).

More recently, three types of microspores showing different morphological features are identified following pretreatment of anthers with heat (Indrianto et al., 2001). Type-1 microspores, morphologically indistinguishable from normal late uninucleate microspores, have a large central vacuole and a nucleus embedded in a thin layer of cytoplasm opposite to the germination pore. Type-2 microspores have a vacuole fragmented by cytoplasmic strands with the nucleus positioned close to the microspore wall. Type-3 microspores exhibit a centralized nucleus surrounded by star-like cytoplasmic strands radiating towards cell wall. More interestingly, the authors conclude that these three types of microspores are not distinct classes of responding and non-responding microspores as previously perceived but rather represent different stages in a continuous process. The sequential cellular events associated with microspore embryogenesis are documented to a great extent (Indrianto et al., 2001). Cell divisions by type-1 microspores in most cases fail to produce embryoids.

Type-3 microspores have the highest rate of conversion into embryoids while type-2 microspores are intermediate. A portion of type-1 and type-2 microspores is able to evolve into type-3 microspores during the first few days in induction culture. The sequence of events in development of type-2 and type-3 microspores is similar to that of type-1 from 4d onwards: disappearance of the vacuole, enrichment of the microspore lumen with cytoplasm, and appearance and accumulation of granules often at the side of the germ pore (Indrianto et al., 2001). The emergence of multicellular structures always occurs in the region of the microspore wall opposite to where granules accumulated, at days 5–8 of incubation for type-2 and type-3 microspores, and at 16 days for type-1 microspores. For the first time, the full developmental sequence of events from individual microspores to embryoids is documented in isolated wheat microspore culture (Indrianto et al., 2001).

The type of microspores at culture initiation also affects the pace of emergence of multicellular structure from the microspore wall, the quality of embryoids, and spontaneous doubling of chromosomes in induction cultures. Embryoids evolved from type-3 microspores mature faster and exhibit highest values in total and green plant regeneration compared to those from type-1 and type-2 (Indrianto et al., 2001). Although a fraction of type-1 microspores could evolve into type-2 and type-3, most of the cell divisions from type-l microspores lead to the formation of calli rather than embryoids, resulting in lower frequencies of plant regeneration. From a practical standpoint, one should attempt to achieve a large percentage of microspores with typical morphological features of type-3 through optimal pretreatment. Microspores with 'star-like' structure (Touraev et al., 1996b) or fibrillar cytoplasmic structure (Zheng et al., 2001) are the equivalent of type-3 characterized by Indrianto et al. (2001). They are the true embryogenic microspores induced by a defined pretreatment. Embryoids from these embryogenic microspores also exhibit a high rate of spontaneous chromosome doubling among the regenerated plantlets (Hu and Kasha, 1999; Kasha et al., 2001). Taken together, a high efficiency in doubled haploid production requires an optimal pretreatment to obtain both a large number and high quality of embryogenic microspores. One could argue, of course, that genetic background sets a cap to which the culture response can be artificially manipulated. We can, however, at least maximize the degree of success in microspore culture by using the best pretreatment until we find ways to overcome such genetic limits.

Microtubules and their related cellular structures are clearly involved in the determination of symmetrical vs. asymmetrical divisions (Zhao et al., 1996; Simmonds and Keller, 1999). A change in microtubule organization, more specifically the appearance of a preprophase band (PPB) of microtubules in heat-treated microspores is linked to division symmetry (Simmonds and Keller, 1999). In a normal cell division cycle, a PPB is a band of cortical microtubules that appears transiently prior to prophase and disappears before metaphase. Two types of PPB are typically observed, continuous and discontinuous. Most heat-treated micro-spores exhibit discontinuous PPB. The continuous PPB seems to be pre-requisite for symmetric division that later leads to embryoid formation. Colchicine, an inhibitor for the polymerization of microtubule (hence prevents the formation of spindle fibers), is effective in triggering microspore embryogenesis (Zhao et al., 1996). Considering the natural role of spindle fibers (made up of microtubules) in the cell cycle, it is reasonable to suggest that stress (e.g. heat) may act upon microtubules and contribute to the transformation of microspores into embryogenesis. However, the precise role of microtubules and the mechanisms by which they act inside microspores are yet to be revealed.

The developmental origin of embryogenic microspores was once a controversial issue due to the 'P-grain' hypothesis (Heberle-Bors, 1985). The hypothesis states that a morphologically distinct type of immature pollen grains (P-grains) forms *in vivo* determines the androgenic potential. *In vitro* culture simply provided a means to express the embryogenic potential of these P-grains. This hypothesis was further expanded to explain the basis of androgenesis of wheat microspores. Pollen embryogenesis in wheat is limited only to cultivars possessing P-grains, the frequencies of which determine the levels of embryogenic induction (Picard et al., 1990). However, subsequent observations and cytological analyses in wheat androgenesis lend no supporting evidence for the presence of P-grains (Reynolds, 1993; Hu and Kasha, 1999; Liu et al., 2002). Studies of isolated microspore cultures reveal that embryogenic microspores are induced by defined stress treatment prior to or during the *in vitro* culture of microspores in the induction medium (Reynolds, 1993; Touraev et al., 1996b; Hu and Kasha, 1999; Zheng et al., 2001; Liu et al., 2002). Thus, the P-grain hypothesis needs to be modified.

Once the embryogenic microspores are obtained, subsequent cellular events follow a predictable pattern.

Both symmetric and asymmetric first cell division was observed for embryogenic microspores, depending upon the type of pretreatment used. Using cold pretreatment in wheat, the 1st division is usually asymmetric and the induction frequency is very similar to that following mannitol pretreatment where it is a symmetric 1st division (Hu and Kasha, 1999). In the nutrient medium, embryogenic microspores undergo rapid cell divisions to form embryoids within 4 weeks (Zheng et al., 2001; Liu et al., 2002). Mature embryoids, having a well-developed scutellum, coleoptile, coleorhiza and epiplast, are morphologically indistinguishable from zygotic embryos of wheat. It is worth noting that even among embryogenic microspores, some would abort the development and fail to evolve into embryoids. Nevertheless, the fact that thousands of embryoids can be harvested in the culture of microspores from one spike of many genotypes should make it a realistic approach for routine DH production in wheat breeding and genetic transformation. The defined and predictable morphology of embryogenic microspores can also be used as a convenient criterion for rapid screening of wheat genotypes for culture response and for evaluating the efficiency of given pretreatments in embryogenic induction.

**Molecular and biochemical mechanisms**

Studies of androgenesis have implied a signal transduction pathway in the conversion of gametophytic microspores into embryogenic cells. However, the biochemical or molecular basis for the developmental transition of microspores into embryoids has not been established for any plant species. This is in part due to the long-standing emphasis on the modification of culture parameters aimed at increasing the culture efficiency and the number of species capable of producing haploids/doubled haploid. In principle, three important events seem to take place during the pretreatment or inductive phase of microspore embryogenesis:

–the inhibition of gametophytic development,
–the commitment of microspore to the embryogenic process, and
–the complete elaboration of embryogenic program (Reynolds, 1997; Touraev et al., 1997; Hu and Kasha, 1999; Zheng et al., 2001; Liu et al., 2002).

One of the main areas of research interest is how microspores become committed to the embryogenic process. The environmental stress model is widely held among scientists (Dunwell, 1992; Touraev et al., l996a; Reynolds, 1997). The application of stress to microspores (anthers, spikes) halts gametophytic development by arresting the expression of gamete-specific genes. A new set of genes is initiated in potentially embryogenic microspores, leading to the first sporophytic divisions (Reynolds and Kitto, 1992; Reynolds, 1997). A great deal is known on how to make microspores in many plant species commit to sporophytic development. However, not much information is available about the commitment process itself. There is a lack of understanding especially at the biochemical and molecular level (Reynolds, 1997; Zheng et al., 2002a, b). Based upon the cellular observations on embryogenic microspores and the whole process of embryoid development, androgenesis in wheat is likely to occur in three related yet distinct stages. They are: the induction phase, the pre-embryoid (multicellular) stage, and the embryoid phase. The first phase ends with the attainment of embryogenic potential by microspores. The second stage begins with the first embryogenic division and ends prior to the rupture of microspore wall, which is the beginning point for the embryoid stage. Conditions must be optimized for at least the first two stages to achieve a high yield of embryoids because embryogenic development is more likely to abort prior to the emergence out of microspore wall (Reynolds, 1997; Ru and Kasha, 1999; Indrianto et al., 2001; Zheng et al., 2002a).

Several studies have been aimed at identifying specific molecular changes during the induction of embryogenesis from microspores (Kyo and Harada, 1990; Pechan et al., 1991; Reynolds and Kitto, 1992; Zarsky et al., 1995; Cordewener et al., 1995b). Phosphoproteins are found in the initial period of tobacco pollen embryogenesis (Kyo and Harada, 1990). These phosphoproteins are not detectable after the beginning of cell division in the culture medium. The transient appearance of these proteins seems to suggest a possible role in the onset of pollen embryogenesis. The heat treatment to microspores for a few hours is accompanied by synthesis of a number of heat-shock proteins (hsps) including members of hsp68 and hsp70 families in *Brassicas* (Cordewener et al., 1995b). The fact that certain members of the 70-kDa family are closely linked to a number of proteins in cell-cycle regulation and the cytoskeleton further affirms the possible role of hsp70 during the inductive phase of microspore embryogenesis. The hsp70s could act either directly via

recruiting DnaB helicase to the origin of DNA replication (prelude for new DNA synthesis) or indirectly by allowing the transfer of transcription factors to the nucleus, triggering specific gene expression (Cordewener et al., 1995b). The synthesis of a small heat shock protein is also activated by starvation during the induction of pollen embryogenesis (Zarsky et al., 1995). The differences in pretreatment procedures and developmental stages of microspores amenable to embryogenic induction do not allow direct comparisons of results from studying different species. However, I believe that there is much in common among plant species in their basic strategy of responding to stimuli and the subsequent cellular and molecular events leading to embryogenesis. Obviously one could also argue that these heat shock proteins may be required for the microspores to survive the stress treatment, and the activation of hsp genes might simply be a normal response to stresses. Nevertheless, it is possible that heat shock proteins are directly associated with the induction of microspore embryogenesis since many heat shock genes, in both animals and plants, are developmentally regulated during early embryo-genesis *in vivo* (Zimmerman and Cohill, 1991).

Embryogenic induction of wheat microspores by pretreatment seems to involve a differential repressing of gametophytic genes and activating of sporophytic genes. A few embryoid-specific genes that were temporally expressed during microspore embryogenesis have been identified in wheat, with some expressed earlier than others (Reynolds and Kitto, 1992). These genes are thought to be associated with the major morphological and physiological activities related to the differentiation of embryoids. The first cDNA library using microspore-derived embryoids of common wheat was prepared and screened with cDNA probes prepared from embryogenic microspores at various stages of development (Reynolds and Kitto, 1992). An early cysteine-labeled metallothionein (EcMt) transcript was identified (Reynolds and Crawford, 1996). EcMt transcript is embryoid-specific that expresses only in embryogenic microspores, embryoids, and developing zygotic embryos. The authors propose the use of EcMt gene as a molecular marker for wheat microspore embryogenesis. Other cloned mRNAs are also developmentally regulated and seem to be linked with the earliest stages of microspore embryogenesis (Reynolds and Kitto, 1992). Using a cDNA library prepared to *Brassica* microspore embryoids, Boutilier et al. (1994) found that the expression of napin (a seed storage protein) was closely related to the induction of microspore embryogenesis. Napin might be used as a molecular marker for early events of *Brassica* microspore embryogenesis. In both cases, the genes are embryoid-specific, as their expression was not detected in mature pollen or any somatic tissues other than in the zygotic embryo.

The mechanisms by which the embryoid-specific or developmentally regulated genes act on microspore embryogenesis are not well understood. Pretreatment in the form of stress, starvation or chemical disrupts the gametophytic programs, while simultaneously or in turn switching on genes crucial for sporophytic development. The altered synthesis and accumulation of mRNA and proteins in microspores leads to the first sporophytic cell divisions. In addition to the synthesis of hsps, protein phosphorylation and DNA replication had also been associated with the early stages of the formation of embryogenic microspores (Kyo and Harada, 1990; Pechan et al., 1991; Reynolds and Kitto, 1992; Touraev et al., 1997). The early cysteine-labled metallothionein genes mentioned above are expressed strongly in microspore embryoids but with no detectable presence of mRNA in vegetative tissues or developing pollen. The metallothioneins are small, cysteine-rich proteins having a high affinity in binding metal ions hence its synthesis is regulated by metal ion concentration. Due to a direct and positive correlation between the presence of EcMt mRNA and the appearance of ABA throughout the microspore embryogenesis in wheat, microspore embryogenesis might be mediated at least in part through ABA modulation of the EcMt gene (Reynolds and Crawford, 1996). Fluridone, an ABA synthesis inhibitor, suppressed both ABA accumulation and the EcMt transcription hence the embryogenic competency of microspores. However, the addition of exogenous ABA to fluridone-treated cultures reversed the inhibitory effects on both EcMt expression and embryogenesis. Stresses seem to trigger the synthesis of ABA, which alters the pattern of gene expression in competent microspores so that the transition from gametogenesis to embryogenesis occurs during the first haploid mitosis (Reynolds and Crawford, 1996). A more recent study showed that $Ca^{2+}$ movement across membranes was required for pollen embryogenesis (Reynolds, 2000). The reduction of external $Ca^{2+}$ to cultured wheat microspores inhibited androgenic potential and suppressed the expression of the EcMt transcript while no corresponding reduction of endogenous ABA was observed. Although a possible modulator of microspore embryogenesis in wheat, ABA alone cannot maintain sporophytic differentiation *in vitro.* Due to the lack of evidence linking metallothioneins directly to wheat

microspore embryogenesis, this gene has not been characterized as a direct result or cause of embryogenic induction. Nevertheless, the presence of metallothionein transcripts was considered to be a molecular marker for wheat microspore embryogenesis (Reynolds and Crawford, 1996; Reynolds, 2000).

Although studies in recent years regarding the molecular mechanisms controlling the microspore embryogenesis provide some useful information, we are still far from a complete understanding of this phenomenon. The importance of studying this process is obvious, both for the application of DHs in crop improvement and the understanding of developmental events. Once more genes or markers are identified, it may be possible to unravel the signal transduction pathway linking the external signals to the gene expression and the developmental response of the microspores. The knowledge is not only vital for theoretical understanding, but also may present opportunities for increasing doubling frequencies without the need to use known carcinogens in the cultures.

## Conclusion

In recent years, great progress in the development of wheat microspore culture has been made. Major factors affecting the efficiency of microspore culture include genotype, donor plant physiology, microspore developmental stage, pretreatment conditions, purity of microspores in culture, physical and chemical conditions for induction culture and plant regeneration. First, androgenic traits are genetic and heritable hence genotype difference in culture response is a norm rather than exception. Second, healthy donor plants are important for success. Although there is no generalization for raising donor plants, any stresses during growing would lead to deviation of culture response. Third, microspores must be at mid- to late-uninucleate stage when subjected to a pretreatment. Fourth, an optimized stress treatment is needed to convert gametophytic microspores into embryogenic cells. Combinations of multiple factors (e.g. temperature, starvation and inducer chemical) have additive effects on embryogenic induction of wheat microspores. Fifth, microspore isolation by blending spikes and collection of relatively pure microspores via density centrifugation has really enhanced the culture efficiency. Finally, an optimized osmolarity (300 mOsmol $kg^{-1}$ $H_20$) and temperature (27–28 °C), an addition of live ovaries or OVCM to a balanced medium enhance direct production of large number of embryoids, capable of germinating and forming hapbid/doubled haploid plants.

While we expect further optimization of the culture protocol, the basic research on mechanisms governing microspore embryogenesis has just begun to draw on the culture system already established. At least two effective approaches may be used to identify develop-mental markers for androgenesis of wheat microspores. The first employs gene products synthesized during embryogenesis *in vivo* as probes for developing microspore embryoids. The second approach uses comparative studies on gene expression during gametogenesis and microspore embryogenesis. A better understanding of the genetic, cellular, biochemical and molecular basis associated with wheat microspore embryogenesis will further improve the technology for DR production and expand the germplasm basis upon which DH can be integrated in routine wheat breeding. The recent advances in technology have already made isolated microspore culture far superior to anther culture systems in several ways. First, with a large number of embryogenic microspores comes a large number of DH. Second, the single-celled microspores provide direct access to manipulate the induction process, hence the opportunity for maximizing the culture efficiency. And finally, the reality of collecting and culturing homogenous popu-lations of embryogenic microspores provides an ideal system analysis of signal transduction pathway and the developmental events associated with *in vitro* embryogenesis.

However, establishing a culture system universal to all wheat genotypes remains elusive due to the genetic differences in their responses to artificial manipulations. Genotype variation and differential responses of genotypes to changes in growth and culture conditions make it difficult to devise growth and culture procedures suitable for all genotypes. In addition, some genotypes are inherently more tolerant to stress treatment hence exhibit lower frequencies of embryogenic microspores following the pretreatment. Among genotypes conducive to pretreatment, some show much higher 'abortion' rates during embryogenic development than others. Although the differential response to embryogenic induction by wheat microspores is genetically based, termination of embryogenic development is probably caused by the imbalance of components in the culture medium. The success achieved by adding OVCM to culture medium of previously recalcitrant microspores is a good example. Thus, until we fully understand the process of microspore embryogenesis, the culture medium may need to be modified and tailored to specific genotypes. Future research should be directed at understanding the genetic control, and the molecular and biochemical mechanisms governing the entire process of microspore embryogenesis, probably through comparative analyses

between responsive vs. recalcitrant genotypes. Nevertheless, the systems developed thus far are efficient for most wheat genotypes and can be incorporated into practical breeding.

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