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Journal of Experimental Botany, Vol. 56, No. 417, pp. 1711–1726, July 2005
doi:10.1093/jxb/eri190 Advance Access publication 31 May, 2005

Journal of Experimental Botany

REVIEW ARTICLE

Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective

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Received 25 November 2004; Accepted 5 April 2005

Abstract

Embryogenesis in plants is a unique process in the sense that it can be initiated from a wide range of cells other than the zygote. Upon stress, microspores or young pollen grains can be switched from their normal pollen development towards an embryogenic pathway, a process called androgenesis. Androgenesis represents an important tool for research in plant genetics and breeding, since androgenic embryos can germinate into completely homozygous, double haploid plants. From a developmental point of view, androgenesis is a rewarding system for understanding the process of embryo formation from single, haploid microspores. Androgenic development can be divided into three main characteristic phases: acquisition of embryogenic potential, initiation of cell divisions, and pattern formation. The aim of this review is to provide an overview of the main cellular and molecular events that characterize these three commitment phases. Molecular approaches such as differential screening and cDNA array have been successfully employed in the characterization of the spatiotemporal changes in gene expression during androgenesis. These results suggest that the activation of key regulators of embryogenesis, such as the *BABY BOOM* transcription factor, is preceded by the stress-induced reprogramming of cellular metabolism. Reprogramming of cellular metabolism includes the repression of gene expression related to starch biosynthesis and the induction of proteolytic genes (e.g. components of the 26S proteasome, metalloprotease, cysteine, and aspartic proteases) and stress-related proteins (e.g. *GST*, *HSP*, *BI-1*, *ADH*). The combination of cell tracking systems with biochemical markers has allowed the key switches in the developmental pathway of microspores to be determined, as well as programmed cell death to be identified as a feature of successful androgenic embryo development. The mechanisms of androgenesis induction and embryo formation are discussed, in relation to other biological systems, in special zygotic and somatic embryogenesis.

Key words: Androgenesis, developmental switch, embryogenesis, embryogenic potential, gene expression, microspore, programmed cell death, stress.

Embryogenesis in higher plants

Embryogenesis has evolved as a successful strategy for the reproduction of higher multicellular organisms. Zygotic embryogenesis in animals and plants starts with the fusion of the haploid female and male gametes, giving rise to a diploid zygote. The zygote possesses the ability to initiate embryogenesis, a developmental programme that leads to the establishment of an embryo with the basic features of the adult body plan. This widely conserved mechanism of reproduction has, however, major differences between animal and plant kingdoms, as embryogenesis in flowering plants starts with two fertilization events. The pollen grain (male gametophyte) is a three-celled structure composed of two generative cells encased within the vegetative cell (McCormick, 1993). During pollination, the vegetative cell serves as a 'powerhouse' to deliver the generative cells to

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the embryo sac (female gametophyte). In the embryo sac, the double fusion of the generative cells with the egg cell and the two nuclei of the central cell give rise to the diploid zygote and the triploid endosperm, respectively (Goldberg *et al*., 1994). Another major difference between animal and plant embryogenesis consists of the ability of plant embryos to develop *in vivo* or *in vitro* from a wide range of cell types other than the zygote (Mordhorst *et al*., 1997). The development of techniques and protocols to produce plant embryos asexually has had a huge technological and economical impact on agricultural systems, and nowadays these biotechnologies represent an integral part in the breeding programmes of agronomically important crops.

Figure 1 provides an overview of the distinct types of cells that can undergo embryogenic development in higher plants. During *in vivo* development, maternal apomixis refers to the asexual formation of a seed from the maternal tissues of the ovule, avoiding the processes of meiosis and fertilization (Koltunow, 1993). Maternal apomictic embryos develop from a somatic cell within the ovule (apospory) or from an unreduced embryo sac derived from the megaspore mother cell (diplospory). In either case, apomictic embryo development is independent of pollination, but in some species this might be required for the initiation of endosperm development and the production of viable seeds (Koltunow *et al*., 1998). Another type of apomictic development has been reported to occur in the gymnosperm *Cupressus dupreziana*, where embryos develop from unreduced pollen grains. This type of apomixis is referred to as paternal apomixis (Pichot *et al*., 2001). Because apomixis offers the possibility of the fixation and indefinite propagation of a desired genotype, there has been a great deal of interest in genetically engineering this ability. Nevertheless, so far it has not been possible to manipulate the apomictic trait for clonal reproduction via seeds (Bicknell and Koltunow, 2004). Clonal propagation is usually achieved via the induction of *in vitro* somatic embryogenesis, a process that is defined as the regeneration of a whole plant from undifferentiated somatic cells in culture. Induction of somatic embryogenesis is usually achieved by a stress and/or hormone treatment of somatic cells. Depending on the donor tissue and the induction treatment conditions, embryos may develop either directly from single cells or indirectly through an intermediary callus phase (Zimmerman, 1993). Additional routes to *in vitro* embryogenesis are defined by the ability of male or female gametophytes to irreversibly switch from their gametophytic pathway towards an embryogenic route. While androgenesis refers to the development of embryos from microspores or immature pollen grains (Touraev *et al*., 1997), gynogenesis refers to the development of embryos from unfertilized ovaries *in vitro* or *in vivo* (Musial *et al*., 2001; Gémes-Jushász *et al*., 2002). By contrast to apomixis and somatic embryogenesis, which lead to clonal propagation of a specific genotype, androgenic and gynogenic plants reflect the product of meiotic segregation. Thus, they have the remarkable characteristic of possessing only one set of chromosomes, and therefore are haploid plants.

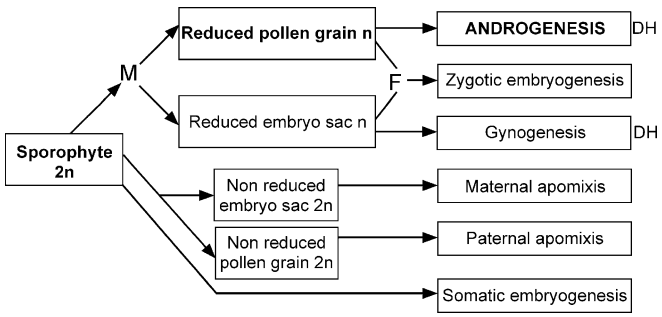

Fig. 1. Overview of the different types of cell structures that can undergo embryogenic development in higher plants. F, fertilization; DH, double haploid; M, mitosis.

Androgenesis as a double haploidization tool for efficient plant breeding

For breeding purposes, the evaluation of diversity in genetic pools and the establishment of homozygous lines are of critical importance. Homozygosity is traditionally achieved by performing time-consuming and labour-intensive backcrosses (Morrison and Evans, 1988). Haploid plants derived from microspores opened a new dimension for the production of homozygous lines due to the large amount of microspores that are produced by a single plant. Due to the colchicine-induced or spontaneous process of chromosome doubling that takes place during the early stages of embryo development, fertile double haploid plants can be easily regenerated within a short period of time (Wang *et al*., 2000). The production of double haploids via androgenesis represents, in this context, a powerful technique both for the production of hybrid seeds and the evaluation of genetic diversity. Though androgenesis is a naturally occurring process in some species, the *in vivo* frequency is very low (Rammana, 1974; Rammana and Hermsen, 1974; Koul and Karihaloo, 1977). Efficient androgenesis is usually induced by the application of a stress treatment to whole plants *in vivo* or tillers, buds, anthers, and isolated microspores *in vitro* (Touraev *et al*., 1997). Since the first description of androgenesis in *in vitro*-cultured anthers of *Datura innoxia* by Guha and Maheshwari (1964), improvement of the conditions for androgenesis induction and microspore culture have resulted in the regeneration of double haploids of many plant species. However, many agronomically important crops are recalcitrant to androgenesis (Wang *et al*., 2000). Further use of this technology is largely hampered by the poor understanding of the mechanisms that render microspore cells embryogenic. *In vitro* embryogenesis systems, here represented by

androgenesis, are excellent model systems to study the developmental aspects of embryogenesis induction and embryo formation from single, haploid microspores. As shown by several experiments, embryogenic development during androgenesis is divided into three main characteristic, overlapping phases: in phase I, acquisition of embryogenic potential by stress involves repression of gametophytic development and leads to the dedifferentiation of the cells; in phase II, cell divisions lead to the formation of multicellular structures (MCSs) contained by the exine wall; in phase III, embryo-like structures (ELS) are released out of the exine wall and pattern formation takes place. A time-line of the three different phases during androgenic development in the model species barley is shown in Fig. 2a. The aim of this review is to provide an overview of the main molecular and cellular events that characterize the different commitment phases of microspores into embryos, and to highlight their similarities and differences with the two most extensively studied model systems, somatic and zygotic embryogenesis. Special emphasis is given to the initial stages of microspore embryogenic potential acquirement and the initiation of cell divisions.

Androgenesis induction: the role of stress

Owing to their high regeneration efficiencies, barley (*Hordeum vulgare* L.), rapeseed (*Brassica napus* L.), tobacco (*Nicotiana* spp.), and wheat (*Triticum aestivum* L.) have been considered model species to study the mechanisms of stress-induced androgenesis (Touraev *et al*., 1997). However, with the recent advances in protocol design, molecular and morphological studies are now possible in other plant species, such as maize (*Zea mays*; Magnard *et al*., 2000) and pepper (*Capsicum annuum* L.; Bárány *et al*., 2001). Lessons learned from these advanced model systems suggest that androgenesis can be efficiently triggered within a relatively wide developmental window. During pollen development, the responsive period for androgenesis is represented by the stages that surround the asymmetric division of the uninucleate microspores, resulting in a polarized pollen grain containing a generative cell embedded in the large vegetative cytoplasm. The vegetative and generative cells differ markedly, as the small condensed generative cell will undergo an additional mitotic division to produce two sperm cells, while the vegetative cell will start an intense programme of accumulation of storage products, namely starch and lipids to drive further pollen maturation (Bedinger, 1992; McCormick, 1993). It is widely accepted that when the vegetative cytoplasm of binucleate pollen starts to accumulate starch, androgenesis can no longer be triggered (Binarova *et al*., 1997; Touraev *et al*., 1997). Another important postulation based on practical experience is that the stress treatment, which is needed to switch efficiently the developmental fate of microspores, varies greatly depending on the plant species and the species genotype. In barley, higher

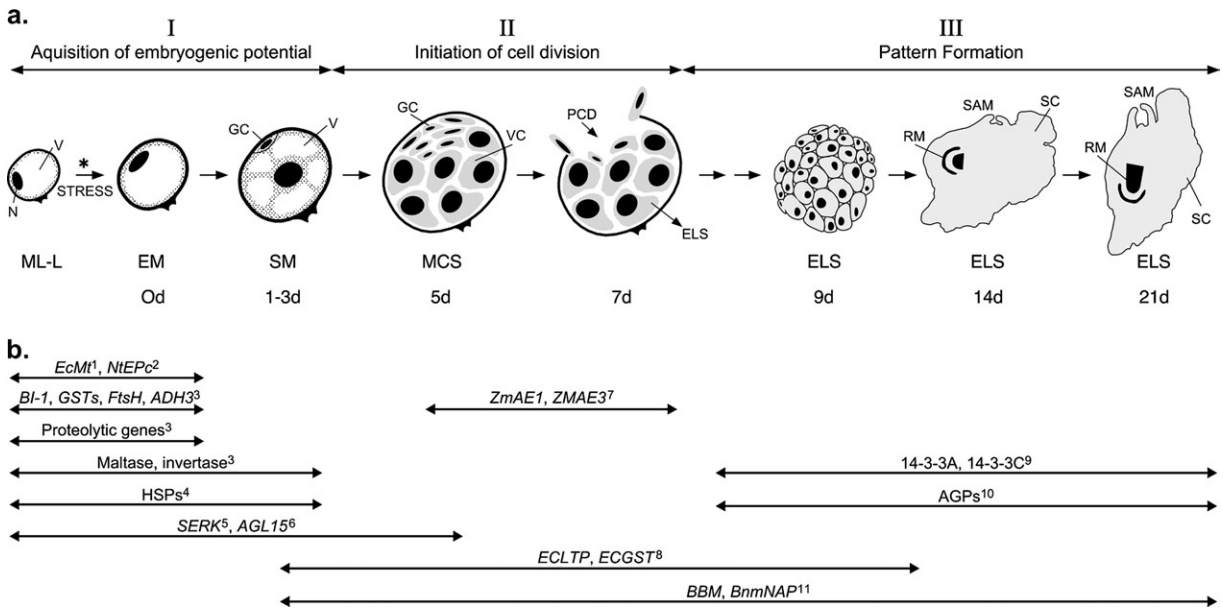

Fig. 2. Cellular and molecular aspects of androgenesis. (a) Time-line of *in vitro* androgenic development in the model species barley illustrating the three different phases of embryogenic development. ELS, Embryo-like structure; EM, enlarged microspore; GC, generative cell; MCS, multicellular structure; ML-L, mid-late to late uninucleate microspore; N, nucleus; PCD, programmed cell death; RM, root meristem; SAM, shoot apical meristem; SC, scutellum; SM, star-like microspore; V, vacuole; VC, vegetative cell. (b) Based on gene expression data collected from barley androgenesis and other androgenic model species, the gene expression programmes associated with each phase are displayed underneath the time-line. [1]Reynolds and Crawford (1996); [2]Kyo *et al*. (2000); [3]Maraschin *et al*. (2005*b*); [4]Pechan *et al*. (1991), Cordewener *et al*. (1994, 1997), Zarsky *et al*. (1995), Smykal and Pechan (2000), Bárány *et al*. (2001); [5]Baudino *et al*. (2001); [6]Perry *et al*. (1999); [7]Magnard *et al*. (2000); [8]Vrienten *et al*. (1999); [9]Maraschin *et al*. (2005*b*); [10]Paire *et al*. (2003), Borderies *et al*. (2004); [11]Boutilier *et al*. (1994, 2002).

regeneration efficiencies are obtained when microspores at the mid-late to late uninucleate stage are subjected to starvation and osmotic stress, which is achieved by incubating anthers in a mannitol solution (Hoekstra *et al*., 1992). In wheat and tobacco, higher induction rates are achieved by a period of starvation in combination with heat shock (Touraev *et al*., 1996*a*, *b*), whereas a heat shock treatment alone is sufficient to induce androgenesis in rapeseed and pepper (Custers *et al*., 1994; Bárány *et al*., 2001). However, other types of stresses applied within the responsive developmental window have been demonstrated to trigger androgenesis at lower rates. They consist of subjecting cells to colchicine (Barnabás *et al*., 1991; Zhao *et al*., 1996; Obert and Barnabás, 2004), nitrogen starvation (Kyo and Harada, 1986), auxin (Reynolds and Kitto, 1992; Hoekstra *et al*., 1996), chemicals, gamma irradiation (Pechan and Keller, 1989; Zheng *et al*., 2001), and cold treatment (Gaillard *et al*., 1991; Kasha *et al*., 2001). Since so many stress factors can trigger the reprogramming of microspores into embryos, it is likely that initiation of androgenesis is induced by converging signalling pathways, although, of course, different stress signals may trigger the same downstream pathways. An analogous situation may be found during the induction of somatic embryogenesis, where the transition of somatic cells to an embryogenic state is regulated by different classes of hormones, namely auxin, cytokinins, and abscisic acid (ABA) (de Vries *et al*., 1988; Filonova *et al*., 2000; Nishiwaki *et al*., 2000), as well as by wounding, osmotic stress, starvation, and heavy metal ions (Ikeda-Iwai *et al*., 2003). During zygotic embryogenesis, however, stress *per se* is not directly involved with zygotic embryogenic competence. The ability of the zygote to initiate embryogenesis appears to be related to an increase in ethylene synthesis and endogenous auxin levels after fertilization (Ribnicky *et al*., 2002; Mòl *et al*., 2004). Interestingly, reactive oxygen species (ROS) are second messengers during auxin- and stress-induced embryogenesis (Nagata *et al*., 1994). Mitogen-activated protein kinase (MAPK) cascades may link auxin signalling to oxidative stress responses and cell cycle regulation (reviewed by Hirt, 2000), and a MAPK has been reported to be activated via stress-related ABA signalling (Knetsch *et al*., 1996). Thus, it is likely that downstream regulatory proteins, such as MAPKs, play an important role in bridging the gap in embryogenesis induction in different types of cells.

Morphological changes associated with embryogenic microspores

Upon mannitol treatment to induce barley androgenesis, microspores enlarge, and this has been correlated with embryogenic potential acquisition during induction of androgenesis in many crop species (Hoekstra *et al*., 1992; Touraev *et al*., 1996*a*, *b*). Embryogenic microspores are characterized by the presence of a large central vacuole, and a clear cytoplasm (Huang, 1986; Hoekstra *et al*., 1992; Maraschin *et al*., 2003*a*). In other embryogenic systems, such as carrot (*Daucus carota* L.) somatic embryogenesis, competent cells are present among a subpopulation of enlarged vacuolated cells (McCabe *et al*., 1997; Schmidt *et al*., 1997), and during zygotic embryogenesis plant egg cells show a rapid increase in volume after fertilization (Mansfield and Briarty, 1991; Mòl *et al*., 1994). However, after the induction of somatic embryogenesis in *Dactylis glomerata* and Norway spruce (*Picea abies* L. Karst), enlarged cells are not competent to become embryos. In these species, it is a subpopulation of small, cytoplasm-rich cells that become embryogenic (Filonova *et al*., 2000; Somleva *et al*., 2000). This indicates that besides cell size, other morphological markers are associated with embryogenic potential. During androgenesis, one of these markers is the degree of cytoplasmic dedifferentiation of enlarged cells. Initiation of cell division from stressed microspores has been correlated with specific ultrastructural changes, including organelle-free regions in the cytoplasm, a significant decrease in the number and size of starch granules and lipid bodies, and an overall decline in the number of ribosomes (Rashid *et al*., 1982; Huang, 1986; Telmer *et al*., 1995; Maraschin *et al*., 2005*a*). Specifically in barley, these cytoplasmic changes are associated with the presence of a thin intine layer, contrasting to the thick intine layer displayed by pollen cells (Maraschin *et al*., 2005*a*). Based on these morphological observations, it has been proposed that stress leads to the dedifferentiation of microspores by the repression of gametophytic development. There are two known pathways in eukaryotic cells that lead to cytoplasmic remodelling: the ubiquitin-26S proteasomal system, which is the major cellular pathway for the degradation of short- and long-lived molecules, and autophagy, which is the primary intracellular mechanism for degrading and recycling organelles via the lysosomes. Though these pathways are developmentally regulated, they are also activated upon stress conditions, e.g. starvation, heat shock, and low temperatures (Levine and Klionsky, 2004). During the initial steps of androgenesis induction in tobacco, cytoplasmic organelles undergo programmed destruction, a process that has been shown to be mediated by the lysosomes (Sunderland and Dunwell, 1974). However, not only autophagy seems to take place in cytoplasm remodelling during the dedifferentiation phase of microspores, as genes coding for enzymes involved in the ubiquitin-26S proteosomal pathway are induced in stressed enlarged barley microspores (Maraschin *et al*., 2005*b*).

Following cytoplasm dedifferentiation, the nucleus migrates towards the centre of the cell, while the large central vacuole is divided into fragments, interspersed by radially oriented cytoplasmic strands. The resulting morphology, often called star-like structure because of its radial polarity,

has been described in several androgenic model systems, including barley, wheat, rapeseed, and tobacco (Zaki and Dickinson, 1991; Touraev *et al*., 1996*a*, *b*; Indrianto *et al*., 2001; Maraschin *et al*., 2005*a*). During pollen development, the peripheral nuclear position is maintained by microtubules and actin filaments (Hause *et al*., 1992). Since the treatment of uninucleate microspores using colchicine or cytochalasin D is sufficient to trigger androgenesis by displacing the microspore nucleus towards the centre of the cell, it has been proposed that cytoskeleton rearrangements are involved in androgenesis induction (Barnabás *et al*., 1991; Zaki and Dickinson, 1991; Zhao *et al*., 1996; Gervais *et al*., 2000; Obert and Barnabás, 2004). One of the proposed models for the role of cytoskeleton rearrangements in androgenesis induction is related to the symmetric divisions that are observed following central positioning of the nucleus (Zaki and Dickinson, 1991). According to Simmonds and Keller (1999), this symmetric division is important in establishing consolidated cell walls via the formation of continuous pre-prophase bands, a crucial step in the formation of a multicellular organism. However, induction of maize androgenesis by colchicine does not lead to symmetric divisions of the microspore nucleus (Barnabás *et al*., 1999). These results indicate that the role of cytoskeleton inhibitors in androgenesis induction is not restricted to the induction of symmetric divisions, but it is likely to involve the induction of radial polarity in the microspores. At the early binucleate stage, after the asymmetric pollen division, androgenesis in rapeseed can be efficiently triggered by a heat shock treatment at 32 °C (Custers *et al*., 1994), and in late binucleate pollen by an extra heat shock treatment at 41 °C (Binarova *et al*., 1997). Interestingly, heat shock leads to cytoskeleton rearrangements and central positioning of the vegetative nucleus (Zhao and Simmonds, 1995; Binarova *et al*., 1997), as do cold (Wallin and Stromberg, 1995; Sopory and Munchi, 1996). Although it is not yet known whether starvation leads to cytoskeleton rearrangements, starvation does lead to the displacement of the nucleus towards the centre of the cell (Touraev *et al*., 1996*a*, *b*; Indrianto *et al*., 2001; Maraschin *et al*., 2005*a*).

Cell tracking studies on barley and wheat revealed that star-like morphology represents the transition from a dedifferentiated state to the initiation of cell division, and therefore corresponds to the first morphological change associated with microspore embryogenic potential (Indrianto *et al*., 2001; Maraschin *et al*., 2005*a*). Further ultrastructural studies of barley star-like structures revealed that the vegetative nucleus migrates to the middle of the structure, while the generative cell remains attached to the intine (Fig. 3). Following the central positioning of the vegetative nucleus, both generative and vegetative cells start to divide (Maraschin *et al*., 2005*a*). In agreement with the hypothesis that central nuclear positioning is related to initiation of cell divisions, a star-like structure represents a characteristic

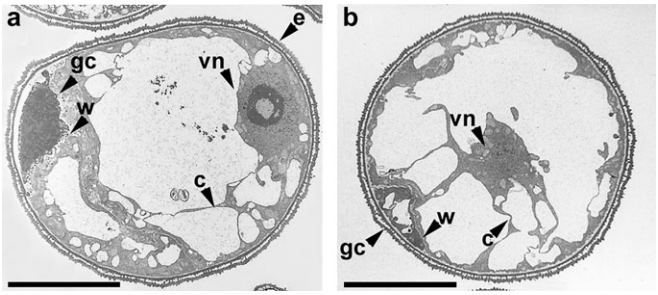

Fig. 3. Formation of star-like structure during initiation of barley androgenesis. (a) Asymmetric division of an enlarged microspore showing a small, condensed generative cell embedded in the cytoplasm of the large vegetative cell. The cell wall that separates the generative cell from the vegetative cytoplasm is attached to the intine, while the large vacuole in the vegetative cell is interspersed by cytoplasmic strands. (b) Evolution of star-like structure showing central positioning of the vegetative nucleus, while the generative cell remains attached to the intine. The vegetative cytoplasm shows numerous cytoplasmic strands radially oriented. c, Cytoplasmic strands; e, exine wall; gc, generative cell; vn, vegetative nucleus; w, cell wall. Scale bars = 15 μm.

morphological stage, following hormone or heat treatment to induce somatic embryogenesis in *Cichorium* (Dubois *et al*., 1991; Blervacq *et al*., 1995), and have been reported in isolated egg cells in culture (Kranz *et al*., 1995). Nevertheless, star-like morphology *per se* does not ensure that a cell will ultimately commit to the embryogenic pathway. According to Indrianto *et al*. (2001), the occurrence of star-like morphology is part of a dynamic process, where the time of occurrence will depend on the type of stress applied and the stage of microspore development. In barley androgenesis, enlarged microspores acquire embryo-like morphology within the first days after the onset of culture. Successful embryo formation, however, is restricted to a group of enlarged microspores that has the tendency to display star-like structures relatively later than the majority (Maraschin *et al*., 2005*a*). These results suggest that the period of star-like occurrence after the onset of culture is related to the embryogenic pathway of microspores.

Gene expression programmes during acquisition of microspore embryogenic potential

The analysis of biochemical and molecular changes during stress treatment to induce androgenesis has been a central point of research towards understanding the mechanisms involved in the reprogramming of microspores into embryos (reviewed by Mordhorst *et al*., 1997; Touraev *et al*., 1997). Most of the genes identified to be differentially expressed during stress treatment to induce androgenesis are involved with stress hormones, cellular protection from stress, sucrose–starch metabolism, and proteolysis. These results indicate that acquisition of androgenic potential largely relies on dedifferentiation, a process whereby existing transcriptional and translational profiles are probably

erased or altered in order to block pollen development and trigger the embryogenic route. The gene expression programmes that are associated with acquisition of embryogenic competence during androgenesis are highlighted in Fig. 2b.

Hormone-modulated gene expression

It is known that plant cells produce ABA in response to certain stresses such as osmotic shock, salinity, cold, and hypoxia (Zeevaart and Creelman, 1988). During androgenesis induction in barley by a mannitol stress treatment, higher regeneration efficiencies have been correlated to increasing levels of osmotic stress and ABA (Hoekstra *et al*., 1997). Upon initiation of wheat androgenesis, Reynolds and Crawford (1996) isolated a gene encoding an early cysteine-labelled class II metallothionein protein (*EcMt*). The expression of the *EcMt* gene is detected as early as 6 h after the onset of induction in auxin-containing media. The promoter region of the *EcMt* gene from wheat contains an ABA-responsive element, and its up-regulation during androgenesis is closely related to the peak of endogenous ABA production (Reynolds and Crawford, 1996). Further evidence has indicated that Ca^{2+} takes part in the ABA signalling transduction leading to *EcMt* gene expression, a process that might involve calmodulin (Reynolds, 2000). Members of the *ALCOHOL DEHYDROGENASE* (*ADH*) family are among the genes whose expression is modulated by ABA (Macnicol and Jacobsen, 2001). Interestingly, the induction of *ADH3* during stress treatment to induce barley androgenesis is correlated with high regeneration efficiencies, which in turn are associated with increased ABA levels (van Bergen *et al*., 1999; Maraschin *et al*., 2005*a*). Though it is not yet known whether *EcMt* and *ADH3* play regulatory roles during the acquisition of embryogenic potential, their relation to ABA suggests that an ABA signalling cascade may play an important role in the activation of specific gene expression programmes during initiation of androgenesis by stress. Kyo *et al*. (2000) isolated an embryogenic pollen-abundant phosphoprotein (NtEPc) from nitrogen-starved tobacco microspores. *NtEPc* encodes a protein that shows moderate homology to several type-1 copper-binding glycoproteins and to an early nodulin. *NtEPc* expression is restricted to the period of microspore stress treatment, and is induced by low pH and inhibited by cytokinin. These results indicate that, besides ABA signalling, other hormonal signalling cascades are likely to take part in the reprogramming of gene expression during androgenesis induction.

Genes involved in cytoprotection

Members of the heat shock protein (HSP) family have been reported to be highly expressed during initiation of androgenesis by heat and starvation (Pechan *et al*., 1991; Cordewener *et al*., 1994, 1997; Zarsky *et al*., 1995; Smykal and Pechan, 2000; Bárány *et al*., 2001), as well as during the initiation of somatic embryogenesis by auxin (Kitamiya *et al*., 2000). These results have led to the hypothesis that increased levels of HSPs may be associated with the acquisition of embryogenic potential. However, androgenesis in rapeseed can be induced by colchicine without altering the levels of HSPs (Zhao *et al*., 2003), suggesting that alterations in HSP subcellular localization may be associated with their regulatory roles. In agreement with this hypothesis, the phase of the cell cycle (Milarsky and Morimoto, 1986; Suzuki and Watanabe, 1992), and a heat shock treatment to induce rapeseed androgenesis (Binarova *et al*., 1997; Cordewener *et al*., 1997) have been reported to control HSP nuclear shuttling. Due to their chaperone activity, it is possible that HSPs play indirect roles in triggering androgenesis via controlling the subcellular localization of other key regulatory proteins, and/or via providing a higher level of thermotolerance (Schöffl *et al*., 1998). Another major component of stress-induced androgenesis appears to be related to the induction of *GLUTATHIONE S-TRANSFERASE* (*GST*) genes. *GST* genes encode proteins that are involved in several processes, including the detoxification of xenobiotics and protection from oxidative stress (Marrs, 1996). Members of the *GST* gene family are up-regulated during the initial stages of androgenic development in barley (Vrienten *et al*., 1999), as well as during auxin-induced somatic embryogenesis (Nagata *et al*., 1994; Thibaud-Nissen *et al*., 2003). Nagata *et al*. (1994) found that the induction of *GST* genes during somatic embryogenesis is auxin-regulated, indicating that ROS act as signalling molecules involved in inducing defence-related genes and hormone responses (Desikan *et al*., 1998; Pasternak *et al*., 2002). In agreement with this hypothesis, increased levels of ROS have been reported to enhance somatic embryogenesis in many plant species (Luo *et al*., 2001; Pasternak *et al*., 2002; Caliskan *et al*., 2004; Ganesan and Jayabalan, 2004). In barley, optimal androgenesis induction is obtained by a mannitol treatment of anthers. When mannitol is omitted during stress treatment, suboptimal regeneration efficiencies are achieved (Hoekstra *et al*., 1992; van Bergen *et al*., 1999). The levels of *GST* expression in barley microspores subjected to optimal and suboptimal stress treatments to induce androgenesis were found to be independent of the embryogenic potential associated with each treatment (Maraschin *et al*., 2005*b*). These results suggest that the roles of *GST* genes during acquisition of embryogenic potential are likely to be associated with protecting the cell against the harmful effects of ROS. However, one cannot exclude the possibility that the redox status of cells and the glutathione content may have important roles in developmental processes, especially in triggering cell division.

Genes involved in sucrose–starch metabolism

Gene expression during pollen development is separated into two phases: transcripts of the 'early' phase are detected

from meiosis until the first pollen mitosis, whereas transcripts from the 'late' phase accumulate from the first pollen mitosis onwards (Mascarenhas, 1990). Genes involved in starch biosynthesis belong to the class of 'late' genes, as starch accumulation takes place after the first pollen mitosis. *In vivo*, the repression of genes involved in starch biosynthesis has been reported to block pollen development (Datta *et al*., 2001, 2002). A similar mechanism may contribute to blocking gametophytic development during androgenesis induction *in vitro*. An array approach has shown that key genes involved in starch biosynthesis, such as sucrose synthase 1, phosphoglucomutase, UDP-glucose 4-epimerase, glucose-1-phosphate adenylystransferase, UTP-glucose-1-phosphate uridylyltransferase, and granule-bound starch synthase are down-regulated in microspores following mannitol treatment to induce barley androgenesis. The down-regulation of starch biosynthetic genes was shown to be parallel to the induction of a maltase gene and an invertase gene, which are involved in starch and sucrose breakdown, respectively (Maraschin *et al*., 2005*b*). These findings provide molecular evidence to support the hypothesis that the repression of starch biosynthesis may play an important role in blocking gametophytic development during androgenesis induction.

Proteolytic genes

Proteomics approaches have demonstrated that microspores show altered synthesis, phosphorylation, and glycosylation of proteins upon stress treatment to induce androgenesis (Kyo and Harada, 1990; Pechan *et al*., 1991; Garrido *et al*., 1993; Cordewener *et al*., 1994; Říhová *et al*., 1996). Many of these reports reveal that stressed microspores show an overall decrease in the protein levels, leading to the hypothesis that down-regulation of pollen-specific proteins or increased protein breakdown might play an important role in the dedifferentiation of microspores. This is in agreement with the fact that blocking pollen-specific gene transcription has a beneficial effect in initiating androgenesis (Harada *et al*., 1986).

In plant cells, starvation leads to transcription activation of the so-called 'famine genes', which encode proteins associated with the degradation of cellular components and with nutrient remobilization. During starvation, genes involved in carbohydrate remobilization are up-regulated in concert with enzymes involved in nitrogen recycling (Lee *et al*., 2004). Nitrogen recycling involves the degradation of proteins for nitrogen relocation, a process that comprises different classes of plant proteases and the ubiquitin-26S proteasome proteolytic pathway (Beers *et al*., 2004; Smalle and Vierstra, 2004). In somatic embryogenesis, cell dedifferentiation is accompanied by an increase in gene expression of proteases and proteins related to the ubiquitin-26S proteasome proteolytic pathway (Jamet *et al*., 1990; Thibaud-Nissen *et al*., 2003; Mitsushashi *et al*., 2004; Stasolla *et al*., 2004). Interestingly, the expression levels of genes encoding a ubiquitin-26S regulatory particle, cysteine protease 1 precursor, phytepsin precursor (aspartic protease), and the metalloprotease FtsH are correlated with the androgenic response of barley microspores (Maraschin *et al*., 2005*b*). These results indicate that proteases might be important for nitrogen relocation upon sugar depletion, a process that might result in the selective destruction of proteins associated with the previous differentiated state. This is in agreement with the role of the FtsH metalloprotease in protein turnover, as it is involved in degrading photosystem II reaction centre D1 protein upon its irreversible photooxidative damage (Lindahl *et al*., 2000). A mutational approach indicated that the *FtsH* metalloprotease gene is needed for the formation of normal, green choloroplasts (Yu *et al*., 2004). Chloroplast biogenesis is an important factor for the production of green plants from microspores, since in many species microspores often give rise to albino plants, reducing their use in plant breeding (Jähne and Lörz, 1995). Although it is not yet known whether the *FtsH* metalloprotease plays a role in chloroplast biogenesis during androgenesis initiation, these results indicate that protein turnover plays important regulatory roles during dedifferentiation processes. This is supported by increasing evidence that links proteolysis to several aspects of cellular regulation, including hormone signalling and cell cycle regulation (reviewed by Hellman and Estelle, 2004). The plant cell cycle is regulated by changes in the specificity and subcellular localization of cyclin-dependent kinases (CDKs), which in turn are modulated by cyclins, CDK-activating and -inhibiting kinases, and several CDK inhibitors (Criqui and Geschink, 2002). The half-life of many of these modulators is affected by the ubiquitin-26S proteasome proteolytic pathway (Geschink *et al*; 1998; Catellano *et al*., 2001; Capron *et al*., 2003; Lee *et al*., 2003; Ahn *et al*., 2004), linking protein degradation to cell cycle regulation in plants. Normal pollen development is characterized by tightly regulated events in the cell cycle. After the asymmetric division, the vegetative cell becomes arrested in the G_1 phase of the cell cycle, while the generative cell progresses into mitosis and divides again to produce two sperm cells. Induction of androgenesis by stress is able to overcome this developmentally regulated cell cycle arrest, as the vegetative cell re-enters S-phase during stress treatment, and microspores progress into G_2/M transition in culture (Touraev *et al*., 1996*a*). In this sense, the induction of components of the ubiquitin pathway and protease gene expression (Maraschin *et al*., 2005*b*) may be related to the regulation of mitotic progression during acquisition of microspore embryogenic potential. This hypothesis is further supported by the fact that proteolytic genes are activated prior to cell division-related genes during acquisition of embryogenic potential in somatic embryogenesis (Thibaud-Nissen *et al*., 2003; Stasolla *et al*., 2004).

Gene expression programmes during initiation of cell division

Master regulators of gene expression

As depicted above, differential screening approaches following stress treatment to induce androgenesis resulted in the identification of several genes and proteins associated with sucrose–starch metabolism, stress responses, proteolysis, and cytoprotection. Nevertheless, none of these approaches resulted in the identification of key regulatory genes clearly involved in the acquisition of microspore embryogenic potential, i.e. transcription factors and regulatory proteins. It is only when the stress-induced dedifferentiation phase is over that such genes are expressed, thus correlating with the period of MCS formation at the onset of culture (Fig. 2b). *BABY BOOM* (*BBM*), a member of the AP2/ERF family of transcription factors, has been isolated from androgenic rapeseed MCSs and is preferentially expressed during androgenesis and zygotic embryogenesis. Functional studies have shown that ectopic expression of *BBM* in rapeseed and *Arabidopsis* can lead to the spontaneous formation of somatic embryos on the leaves of young seedlings (Boutilier *et al*., 2002). *BBM* represents, therefore, the first androgenic-related gene identified so far to have a putative role in co-ordinating the phase of initiation of cell division during androgenesis. Interestingly, Boutilier *et al*. (2002) have shown that the ectopic expression of *BBM* is only capable of inducing embryogenesis on the leaves of young seedlings, while older plants do not show the same response. Taken together, these results suggest that a relatively undifferentiated cell state is important so that *BBM* can trigger embryogenic development, further supporting the idea that a period of dedifferentiation precedes cell division during induction of androgenesis and somatic embryogenesis. Another regulatory protein thought to play a role in cell division initiation during embryogenesis is AGAMOUS-like 15 (AGL15), a member of the MADS-domain family of transcription factors. Though the developmental role of AGL15 is still unclear, AGL15 has been shown to be translocated to the nucleus upon initiation of cell divisions during zygotic and somatic embryogenesis, apomixis, and androgenesis (Perry *et al*., 1999).

The *LEAFY COTYLEDON* genes, *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON2* (*LEC2*), and *FUSCA3* (*FUS3*), have been isolated from *Arabidopsis* mutant screen analysis and encode transcription factors involved in zygotic embryogenic development (Harada, 2001). Though mutant analysis indicates that *LEC1*, *LEC2*, and *FUS3* play a role in embryo maturation during later stages of embryogenesis, over-expression of *LEC1* and *LEC2* triggers somatic embryogenesis in vegetative tissues like *BBM* does (Bäumlein *et al*., 1994; Parcy *et al*., 1997; Lotan *et al*., 1998; Nambara *et al*., 2000; Stone *et al*., 2001). Therefore, it has been proposed that *LEC* transcription factors play key regulatory roles in co-ordinating the phase of embryogenic competence acquisition as well as the morphogenesis and maturation phases of embryogenesis (Harada, 2001). Similarly, WUSCHEL (WUS), a homeodomain protein that promotes a vegetative-to-embryonic transition (Zuo *et al*., 2002), is also involved in the specification of shoot and floral meristems during zygotic embryogenesis (Mayer *et al*., 1998). This indicates that the acquisition of embryogenic competence and embryo development are controlled by a spatial and temporal reprogramming of regulatory genes. The *PICKLE* (*PKL*) gene encodes a CHD3 protein, a chromosome remodelling factor which is ubiquitously expressed in *Arabidopsis*. During post-embryonic growth, *PKL* inhibits embryonic traits via transcriptional repression of seed storage proteins (Ogas *et al*., 1997) and *LEC* genes (Ogas *et al*., 1999; Rider *et al*., 2003), and therefore is a master regulator of embryogenesis. Though it is not yet known whether *PKL* plays a role in androgenesis, transcripts coding for seed storage proteins, such as members of the napin seed storage protein family, correlate with the initiation of androgenesis in rapeseed (Boutilier *et al*., 1994). This suggests a possible role for chromatin remodelling in the co-ordination of transcription during the context of a stress-induced developmental switch, especially in the de-repression of gene expression programmes associated with microspore embryogenic development.

The *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*) gene was first isolated from auxin-induced embryogenic carrot cell cultures and encodes a Leu-rich repeat transmembrane receptor-like kinase. In somatic and zygotic embryogenesis, *DcSERK* is transiently expressed during initiation of embryogenic development up to the globular stage (Schmidt *et al*., 1997). Ectopic expression of *AtSERK1*, the *Arabidopsis* homologue of *DcSERK*, has been reported to increase the efficiency of somatic embryogenesis initiation in *Arabidopsis* seedlings, indicating that higher levels of *AtSERK1* are sufficient to confer embryogenesis competence in culture (Hecht *et al*., 2001). Interestingly, high levels of *ZmSERK1* are detected in maize microspores at the competent stage for androgenesis induction and during initiation of MCS formation, indicating that a *SERK*-dependent signalling pathway might be involved in the acquisition of embryogenic competence and initiation of embryogenic development in microspores (Baudino *et al*., 2001). Similarly, initiation of somatic and zygotic embryogenesis takes place only from cell clusters expressing the *EP2* gene, which encodes a lipid transfer protein whose homologue *ECLTP* has also been demonstrated to accompany the initiation of barley androgenesis (Sterk *et al*., 1991; Toonen *et al*., 1997; Vrienten *et al*., 1999).

Cell–cell communication and secreted signal molecules

Differential screening approaches have resulted in the identification of two endosperm-specific genes, *ZmAE1*

and *ZmAE3*, in maize androgenic MCSs (Magnard *et al*., 2000). During *in vivo* zygotic embryo development, *ZmAE1* and *ZmAE3* are both transiently expressed during initiation of endosperm development in the embryo-surrounding region. During androgenesis, expression of *ZmAE1* and *ZmAE3* is detected only in 5–7-d-old MCSs, a period that coincides with the differentiation of a large cellular domain that shows coenocytic organization similar to that of the endosperm initials (Magnard *et al*., 2000). The identification of these genes is of particular interest since it suggests that androgenic MCS development requires endosperm-like functions which might be needed for the establishment of interactions that probably exist *in planta* between embryo and endosperm. In agreement with this hypothesis, the development of carrot somatic embryos relies on the presence of several secreted proteins (de Vries *et al*., 1988; van Engelen *et al*., 1991; van Hengel *et al*., 1998). EP3, an endochitinase protein secreted by non-embryogenic cells during carrot somatic embryogenesis, is also expressed in the endosperm during zygotic embryo development (van Hengel *et al*., 1998). In somatic embryogenesis, chitinase-modified arabinogalactan proteins present in the extracellular matrix have been demonstrated to control plant cell fate (van Hengel *et al*., 2001). Recently, it has been shown that androgenic MCSs progressively secrete proteins in culture which can sustain *in vitro* zygotic embryo development (Paire *et al*., 2003). Further characterization of the extracellular proteins secreted during maize androgenesis revealed that several proteins are glycosylated, including distinct arabinogalactan proteins. Interestingly, chitinases and other pathogen-related proteins are also transiently secreted into the media, and these conditioned media were able to rescue embryo development in tunicamycin-treated MCSs arrested at the multicellular stage (Borderies *et al*., 2004). This indicates that progression of embryogenesis relies on the perception of external signals which might be crucial for the activation of specific spatiotemporal developmental programmes during the making of an embryo.

Pattern formation

During zygotic embryo development, an initial asymmetric division establishes the apical–basal axis of the embryo via a reversal of auxin distribution during early embryogenesis (Jürgens, 2001; Friml *et al*., 2003). This opposes androgenic embryo development, where the establishment of an apical–basal axis takes place from the globular stage onwards (Hause *et al*., 1994; Maraschin *et al*., 2003*a*). During androgenesis, the first signs of pattern formation are visualized by periclinal divisions of the cells that surround the ELS, leading to epidermis differentiation (Telmer *et al*., 1995; Yeung *et al*., 1996). Following epidermis differentiation, rapeseed ELS proceed through heart- and torpedo-shape stages, in a similar way to zygotic embryos (Hause *et al*., 1994). An analogous situation is observed during somatic embryogenesis, where somatic embryo development parallels zygotic embryogenesis from the globular stage onwards (Zimmerman, 1993). The genetic analysis of zygotic embryonic pattern formation has recently been reviewed (Laux *et al*., 2004). The stereotyped sequence of embryonic developmental stages between different embryogenesis systems suggests that analogous molecular mechanisms of embryo patterning are shared between them (Dodeman *et al*., 1997). Further evidence to support this hypothesis is the similar spatial and temporal regulation of members of the 14-3-3 family of regulatory proteins prior to pattern formation in barley androgenic and zygotic embryos. In barley androgenesis, the expression of 14-3-3A in the outer layer of ELS precedes epidermis differentiation, while polarized 14-3-3C expression is correlated with the establishment of the scutellum during acquisition of bilateral symmetry. In the late embryogenesis stage, 14-3-3C expression is restricted to the scutellum and to a group of cells underneath the L_1 layer of the shoot apical meristem, prior to L_2 layer specification in both androgenic and zygotic embryos (Testerink *et al*., 1999; Maraschin *et al*., 2003*a*).

The gene expression programmes that are associated with each phase during androgenesis are highlighted in Fig. 2b, providing a comprehensive overview of the molecular mechanisms involved in microspore embryo formation.

Is there a role for programmed cell death (PCD) during androgenesis?

PCD is a genetically controlled mechanism that envisages the organized destruction of specific cell types and tissues (Lam, 2004). Zygotic and somatic plant embryogenesis are intimately associated with PCD, as this process is involved in the elimination of unneeded structures within the embryos (Mordhorst *et al*., 1997) and is essential for correct embryo patterning (Bohzkov *et al*., 2004; Suarez *et al*., 2004). Nevertheless, a role for PCD during androgenesis has not been explored until very recently. Studies on barley androgenesis indicate that PCD takes place on at least two levels: during induction of androgenesis by stress, and during the transition from MCSs into globular embryos.

PCD during androgenesis induction

One experimental approach to test the reversibility of initial stages of PCD has shown that agents which promote an oxidative burst can induce star-like morphology in tobacco protoplasts. After removal of the PCD-inducing agents, star-like structures were able to recover from the stress and start cell divisions (O'Brien *et al*., 1998). In animal systems, PCD signals are mediated by pleiotropic signal transductions, indicating that these pathways also have

roles in cell proliferation and differentiation (Green and Beere, 2001). The most common form of animal PCD, apoptosis, is regulated by a family of cysteine proteases called caspases. The caspase cascade is triggered by cytochrome *c* release from mitochondria, a process that involves several members of the Bcl-2 family of proteins (Bad, Bcl-xL, and Bax). Upon PCD stimuli, Bad is translocated from the cytoplasm to the mitochondria, where it associates with Bcl-xL and leads to cytochrome *c* release. Bax, in its turn, is a pro-apoptotic factor that is thought to accelerate this process (Gallaher *et al*., 2001). Members of the regulatory family of 14-3-3 proteins have been implicated in apoptosis signalling through their interaction with Bad, thereby preventing its translocation into the mitochondria and interaction with Bcl-xL. Recently, the proteolytic cleavage of the C-terminus of the human 14-3-3ε isoform has been shown to weaken its affinity to Bad, thereby leading to Bad translocation into the mitochondria and activation of the PCD pathway (Won *et al*., 2003). During androgenesis induction in barley, the proteolytic cleavage of the C-terminus of the 14-3-3A isoform is specifically associated with a population of non-enlarged microspores that dies during stress treatment (Maraschin *et al*., 2003*a*, *b*). The death of these cells displays characteristics of PCD, as visualized by the formation of DNA ladderings (Fig. 4). On the other hand, the population composed of enlarged microspores, which have acquired embryogenic potential, does not display DNA ladderings or 14-3-3A processing (Fig. 4; Maraschin *et al*., 2003*a*, *b*). These enlarged microspores are characterized by the expression of the *BAX INHIBITOR 1* (*BI-1*) gene (Maraschin *et al*., 2005*b*), the plant homologue of the human *BI-1* gene capable of suppressing Bax- and stress-induced PCD in plants (Kawai-Yamada *et al*., 2001; Chae *et al*., 2003). This indicates that a stress treatment to induce barley androgenesis activates PCD in non-enlarged microspores, while in enlarged ones it leads to the induction of cell divisions. Since most stress agents used to trigger androgenesis can induce PCD (Lam, 2004), it is likely that cell divisions may be induced by signalling pathways that cross-talk with those activated by PCD (Kuriyama and Fukuda, 2002). The final result might be related to the regulatory roles played by proteins like BI-1 and 14-3-3A. Interestingly, the processed form of 14-3-3A is also associated with PCD in barley tapetum upon normal pollen development (Wang *et al*., 1999; Maraschin *et al*., 2003*b*).

Since PCD plays important roles that are associated with the development and function of multicellular organisms (Lam, 2004), how can single cells such as microspores benefit from PCD? Answers for this question may arise from unicellular organisms, such as yeast (*Saccharomyces cerevisiae*). Ageing and stress can induce many yeast cells within a colony to die, a process that displays hallmarks of PCD and is controlled by molecular mechanisms that parallel animal and plant PCD (Madeo *et al*., 2002*b*).

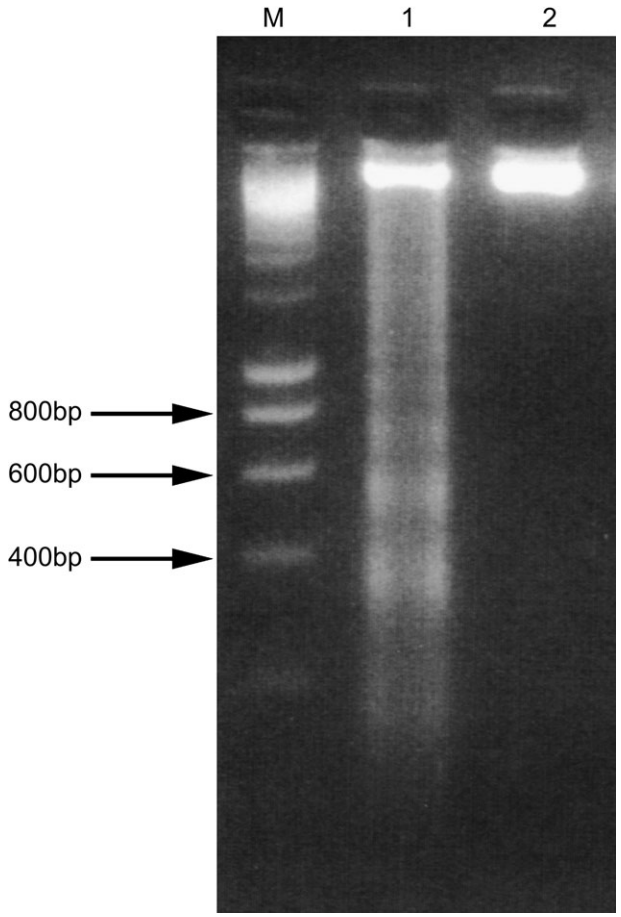

Fig. 4. Conventional DNA gel electrophoresis in enlarged and non-enlarged microspores after 4 d mannitol treatment to induce barley androgenesis. Lane 1, PCD in non-enlarged microspores as demonstrated by the formation of DNA laddering; lane 2, enlarged microspores with embryogenic competence; M, marker DNA.

A rapid, active suicide of these cells would spare metabolic energy for neighbouring cells, at the same time that it neatly destroys cells without any damage to the environment (Madeo *et al*., 2002*a*). As in yeast 'altruism', stress during barley androgenesis induction could possibly trigger the programmed removal of the 'weakest' cells, represented by the population of non-enlarged microspores, thereby contributing to the survival of the fittest, enlarged microspores. It will be a challenge to explore how the cell fate of enlarged microspores can be affected by PCD of the non-enlarged ones during barley androgenesis induction.

PCD during the transition from MCSs to globular embryos

The formation of MCSs from star-like microspores involves different developmental pathways that are defined by the symmetry of the first division and the fate of the daughter cells. The asymmetric division of the microspore nucleus resulting in a generative and a vegetative cell characterizes the A-pathway. In the A-pathway, MCSs are

formed from repeated divisions of the vegetative cell concomitantly to the death of the generative cell. In the B pathway, it is the symmetric division of the microspore nucleus that gives rise to MCSs (Sunderland, 1974). An alternative route to androgenesis is defined by the independent divisions of the generative and vegetative cells, giving rise to heterogeneous MCSs with two distinct cellular domains. Because heterogeneous MCSs originate from an initial asymmetric division, this pathway is regarded as a modification of the A-pathway (Sunderland *et al*., 1979). All the above-mentioned developmental pathways occur in most androgenic species, and the preponderance of one pathway over the other has been linked to the developmental stage of the cells and the type of stress applied (Sunderland *et al*., 1979; Zaki and Dickinson, 1991; Říhová and Tupý, 1999; Kasha *et al*., 2001; Kim *et al*., 2004). In rapeseed, MCSs are usually formed by the A- or B-pathway, and the early divisions of embryogenic microspores inside the exine wall appear to be random rather than regular (Hause *et al*., 1994; Telmer *et al*., 1995; Yeung *et al*., 1996). However, recent evidence shows that embryogenic microspores follow a very controlled pattern of cell divisions in wheat and maize, leading to the formation of specific cell domains within the exine: a cellularized domain composed of small cells, and a large domain composed of multinucleate cells. These domains have been compared with meristematic and endosperm initials during zygotic embryogenesis (Bonet and Olmedilla, 2000; Magnard *et al*., 2000). Though the vegetative and generative origins of these domains have not yet been established, small and large cell domains in barley MCSs developed via the modified A-pathway arise from divisions of the generative and vegetative cells, respectively (Maraschin *et al*., 2005*a*). The establishment of a cell tracking system has been crucial in determining that exine wall rupture in these embryos always takes place at the generative domain located at the opposite side of the pollen germ pore. During exine wall rupture, the generative cell domain is eliminated by PCD, and globular embryos are originated entirely from the vegetative domain (Maraschin *et al*., 2005*c*). In zygotic embryogenesis, the symmetry of the first division influences the differentiation and fate of the daughter cells, as the terminal cell gives rise to most structures of the embryo proper, while the suspensor is derived from the basal cell. In most species, the suspensor is eliminated by PCD in later stages of zygotic embryo development and it is not present in the mature seed (Jürgens, 2001). During carrot somatic embryogenesis, an initial asymmetric division also appears to seal the fate of the daughter cells, as the cytoplasm-rich cell differentiates into the embryo, and the vacuolated suspensor cell is eliminated by PCD (McCabe *et al*., 1997). These results highlight the importance of an asymmetric division during the initial steps of plant embryogenesis in defining different developmental fates, most probably by a mechanism that involves differential accumulation of mRNAs and morphogens, and distribution of organelles (Weterings *et al*., 2001; Bhalerao and Bennett, 2003; Friml *et al*., 2003).

During somatic embryogenesis in Norway spruce (*Picea abies* L. Karst), PCD is involved in the transition phase from pro-embryogenic masses to somatic embryo, and in the elimination of the embryo suspensor (Filonova *et al*., 2000). In this plant species, PCD is essential for correct embryo patterning and involves the activation of a caspase-6-like and a metacaspase protease (Bozhkov *et al*., 2004; Suarez *et al*., 2004). Despite the fact that canonical caspases have not yet been identified in plants, dying plant cells display caspase-like activity and a caspase-related family of proteins, called metacaspases, has been identified (Lam and del Pozo, 2000; Uren *et al*., 2000). During barley androgenesis, an increase in caspase-3-like activity has been correlated to PCD during the elimination of the generative cell domain in the transition from MCSs to globular embryos. PCD of the generative domain precedes exine wall rupture and is a condition for the release of globular embryos out of the exine wall (Maraschin *et al*., 2005*c*). It is conceivable that PCD might have a role in sculpting globular embryos by promoting exine wall removal and therefore allowing further embryonic development. Further molecular characterization of the events leading to the elimination of the generative cell domain in barley androgenic MCSs will help to elucidate the roles of PCD in exine wall rupture and in the transition from MCSs to globular embryos.

Concluding remarks

In recent years, there has been a considerable increase in the amount of information concerning the cellular and molecular aspects involved in androgenesis induction and embryo formation. The establishment of cell tracking systems has played a crucial role in pointing out the main morphological characteristics of embryogenic microspores, as well as in revealing the developmental pathways of induced microspores. The combination of cell tracking systems with biochemical and molecular markers has the potential to reveal more about the role of PCD, both during androgenesis induction and pattern formation, in microspore embryos. Due to the lack of genetic tools for the dissection of the signalling pathways leading to androgenesis induction, differential screening methods have often been used. These approaches resulted in the identification of several genes and proteins, which are markers of a developmental switch. Though the role of most of these markers during androgenesis remains to be determined, some marker genes, like *BBM*, are capable themselves of inducing embryogenic development. Such genes are often expressed after the activation of gene expression programmes associated with stress-response and cell

metabolism. This is not altogether surprising, as the activation of master regulators of embryogenesis, such as transcription and chromatin remodelling factors, is likely to involve several distinct signalling pathways which may be regulated by stress-induced proteolysis, oxidative burst, and changes in cell metabolism. Therefore, holistic approaches such as the integration of genomics, proteomics, and metabolomics, from the perspective of systems biology, have a great potential in revealing the interaction between different signalling cascades involved in triggering androgenesis. In terms of plant breeding, the key for increased regeneration efficiency during androgenesis will largely depend on the control of two main developmental switches, defined as the induction of microspore cell division and their ultimate commitment to the embryogenic pathway.

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