Summary
For breeding purposes, the evaluation of diversity in genetic pools and the establishment of homozygous lines are of critical importance. Homozygosity has been traditionally achieved by performing time-consuming and labor-intensive backcrosses. However, in the middle 1960’s, a new technique came into view: the production of double haploid plants from immature pollen grains (microspores). Prompted by certain stress signals, microspores can be switched from their normal pollen developmental pathway towards an embryogenic route of development, a process that is referred to as androgenesis. Due to the colchicine-induced or spontaneous chromosome doubling during the early stages of microspore embryo development, single haploid microspores give rise to fertile double haploid plants within a short period of time. The production of double haploids via androgenesis represents, nowadays, a powerful technique both for the production of hybrid seeds and the evaluation of genetic diversity.

Stress has been considered the main trigger for the androgenic switch in microspores. Depending on the plant species, and even the plant variety, different stress treatments are required to efficiently induce androgenesis. However, many agronomically important crops are still recalcitrant to androgenesis. Improvement in protocol development has been so far based on trial and error experiences, limiting further use of this biotechnology for breeding purposes. For both applied and fundamental research, it is of uttermost importance to understand how a highly specialized cell such as an immature pollen grain can be reprogrammed to become embryogenic. Barley (Hordeum vulgare L.) cv. Igri is considered a model plant for androgenesis studies due to the high regeneration efficiencies that are obtained. Efficient androgenesis in this cultivar is obtained by starvation and osmotic stress at the mid-late to late (ML-L) uninucleate stage of microspore development, which is achieved via a mannitol treatment of anthers.

In this thesis, barley androgenesis was used as a model system to gain insight into the molecular and cellular events that control the developmental switch of microspores into embryos. As shown by several experiments, microspore embryogenic development is divided into three main characteristic, overlapping phases: acquisition of embryogenic potential by stress, initiation of cell divisions and pattern formation. The main molecular and cellular events that characterize the different commitment phases of microspores into embryos are reviewed in chapter 1. This chapter presents an overview of the progress that the research on androgenesis induction has made in the recent years, emphasizing the phase of microspore embryogenic potential acquirement and initiation of cell divisions. In addition, this chapter draws a parallel between the molecular and cellular biology of androgenic development with that of the two most extensively studied model systems, somatic and zygotic embryogenesis. In chapter 2, time-lapse tracking of mannitol-stressed microspores
was used with the objective to identify the morphology of embryogenic cells, and to investigate the stress-induced microspore developmental pathways. Three developmental pathways were identified: developmental type I, or embryogenic pathway, was represented by the microspores that followed embryogenic cell divisions, formed multicellular structures and released globular embryos out of the exine wall; developmental type II was characterized by those microspores that followed embryogenic cell divisions, formed multicellular structures, but died during the transition to globular embryos; developmental type III comprised the microspores that were not triggered and died in the first days of tracking. The first morphological change associated with the embryogenic potential of microspores was represented by the migration of the nucleus towards the center of the cell (star-like structure), which represented a transitory stage between vacuolated enlarged microspores after stress and the initiation of cell divisions. The difference between type I and type II pathways was observed in the time they displayed star-like morphology. By combining viability studies with cell tracking, we showed that the release of globular embryos out of the exine wall in developmental type I was always preceded by the death of the cells positioned at exine wall rupture, which was at the opposite side of the pollen germ pore. This position-determined cell death process was found to be a marker for the transition from multicellular structures into globular embryos during barley androgenesis, as cell death was delayed or absent in developmental type II.

Since the position-determined cell death process during the transition from multicellular structures into globular embryos appeared to be an important step in the commitment of microspores to the embryogenic route, this process was characterized in detail in chapter 3. Morphological analysis of type I multicellular structures showed that these pro-embryos were composed of two different cellular domains: a large vegetative domain, and a small generative domain positioned at the opposite side of the pollen germ pore. During the transition between multicellular structures into globular embryos, the generative cell domain died and this process showed typical features of plant programmed cell death, a genetically controlled mechanism of cell suicide. Hallmarks of programmed cell death such as chromatin condensation, DNA degradation and an increase in the activity of caspase-3-like proteases preceded massive cell death of the generative cell domain. The orchestration of such a death program culminated with the elimination of the small generative domain, and further embryogenesis was carried exclusively by the large vegetative domain. These results show that programmed cell death is an important feature of the embryogenic pathway of barley microspores.

In chapter 4, the expression and tissue-specificity of three members of the 14-3-3 family of regulatory proteins were studied by using isoform-specific antibodies against 14-3-
3A, 14-3-3B and 14-3-3C. This chapter is composed of three parts, each concerning different aspects of 14-3-3 proteins in barley androgenesis. In part I, we demonstrate that androgenic globular embryos show a polarized expression of 14-3-3C, and a higher 14-3-3A expression in epidermis primordia. During later stages of androgenic embryo development, 14-3-3C was found to be specifically expressed in the scutellum and in a group of cells underneath the L1 layer of the shoot apical meristem, prior to L2 layer specification. These results suggest that differential expression of 14-3-3A and 14-3-3C precede pattern formation in androgenic embryos. In part II, the specific expression of the 14-3-3C isoform was demonstrated to be restricted to the L2 layer of the shoot apical meristem in both androgenic and zygotic embryos, as well as to L2 layer-derived cells of in vitro shoot meristematic cultures. In part III, the proteolytic cleavage of the C-terminus of the 14-3-3A isoform was demonstrated to be specific to anther walls and non-embryogenic microspores undergoing programmed cell death during androgenesis induction. Taken together, the results described in chapter 4 indicate that cell fate and pattern formation during barley androgenesis are associated with 14-3-3A post-translational modification and the spatial and temporal regulation of 14-3-3A and 14-3-3C expression.

In chapter 5, we have employed macroarray technology in combination with principle component analysis to identify gene expression profiles associated with the switch from pollen development towards the androgenic pathway. During the reprogramming of microspores, pollen-related genes were down-regulated, suggesting that stress acts in blocking pollen development. On the other hand, transcripts involved in sugar and starch hydrolysis, proteolysis, stress responses, inhibition of programmed cell death and signaling were induced. Further expression analysis of a subset of these genes revealed that the induction of genes encoding alcohol dehydrogenase 3 and proteolytic enzymes were associated with the androgenic potential of microspores, while the induction of genes involved in signaling and cytoprotection were probably part of stress responses.

The research described in this thesis has provided a substantial contribution towards understanding the mechanisms of androgenesis induction. The use of a cell tracking system in combination with biochemical markers has been crucial in pointing out the morphology of embryogenic microspores, and in identifying programmed cell death as an integral part of the developmental pathway of androgenic embryos. In addition, the markers identified in this thesis by cDNA array and 14-3-3 expression analyses represent useful tools for further analysis of stress-induced androgenesis and pattern formation in androgenic embryos. Understanding the role of these markers, as well as the role of programmed cell death during exine wall rupture and subsequent pattern formation represents a future challenge for the improvement of quality and yield of androgenic embryos.