Structural changes during the initiation of pollen embryogenesis in barley

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Dedication

This thesis is dedicated to my mother and the memory of my father.

This thesis would be incomplete without a mention of the support given me by my wife and to my kids how had suffered a lot because of my long absence during work.

Finally, this thesis is dedicated to all those who believe in the richness of science.

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DATA - DVD

A DVD of the movies of live-cell imaging

Abbreviations:

2,4-D	2,4-dichlorophenoxyacetic acid
APT24	Anther treatment in 0.4 M mannitol at 24°C in the dark for three days then starve isolated pollen in SMB1
APT4	Anther treatment in 0.4 M mannitol at 4°C in the dark for three days then starve isolated pollen in SMB1
BAP	6-benzylaminopurine
CCF	Conventional chemical fixation
DAPI	4′,6-Diamidin-2-phenylindol
EDTA	ethylenediaminetetraacetate
FS	Freeze substitution
GFP	Green fluoresence protein
HPF	High Pressure Freezing
HPF-CBY	High pressure freezing using cyanobacteria, nitrocelleose tubes and yeast
KBP	Kumlehn Barley Pollen medium
MES	2-morpholinoethanesulfonic acid
MWCF	Microwave assisted fixation
OsO_4	Osmiumtetroxid
PO	Propylene Oxide
SMBI	Starvation Medium Barley I

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1. Introduction

Plants display a remarkable potential for cellular totipotency. One important example is pollen embryogenesis which is a convert of a cell destined to produce a male gametophyte, i.e. a pollen grain, into an embryogenic cell which will develop into a sporophyte, i.e. an embryo (Touraev et al., 1997).

Although during micro-gametogenesis pollen grains are destined to undergo a process of differentiation to generate the male gametes (pollen gametogenesis pathway), cultured immature pollen of many plant species also can enter an alternate developmental pathway of deregulated cell proliferation. During this pathway, embryogenic development can occur, which entails the formation of mature plants with the haploid or doubled haploid set of chromosomes (Reynolds, 1997). Natural haploid embryos and plants, from female gamtophytic cells, have been described in about 100 species of angiosperms. However, there is no evidence that haploids can derive from the male gametophyte under natural conditions (McKone and Halpern, 2003). However, Pichot et al. (2008) mentioned that *Cupressus dupreziana* is the only plant in which progeny are produced by the apomictic development of pollen grains.

Haploids and doubled haploids can be obtained from male or female gametophytic cells. For some flowering plant species such as potato, both routes have been described. Doubled haploids can be obtained via three major methods namely pollen embryogenesis, gynogenesis and/or uniparental chromosome elimination.

Haploids are important to geneticists and plant breeders. The discovery of anther-derived haploid *Datura* plants in 1964 initiated great excitement in the plant breeding and genetics communities as it offered shortcuts in producing highly desirable homozygous plants (Riley, 1974; Maluszynski et al., 2003). Unfortunately, the expected progress extending the methods to other species was slow due to problems including genotype dependence, recalcitrance and the slow development of tissue culture technologies as well as a lack of knowledge of the underlying cellular processes (Riley, 1974). Recent years have witnessed great progress in the research and application of haploids in higher plants (Palmer et al., 2005; Maluszynski et al., 2003).

In most species, the available methods are not efficient enough or too much genotype-dependent for an economic use. The unique potential of pollen embryogenesis for basic

research and crop improvement is thus in strong contrast to the poor understanding of its underlying biological processes. The gain of comprehensive information on the initial mechanisms of pollen embryogenesis might stimulate a knowledge-based establishment of commercially useful doubled haploid technology in numerous plant species. The concept of this work encompasses contemporary structural analyses of pollen embryogenesis in comparison with pollen gametogenesis to identify the structural features underlying pollen embryogenesis.

1.1. Male gametogenesis

In order to understand pollen embryogenesis, it is useful to take a close look at normal pollen development. Microgametogenesis comprises events which lead to the conversion of unicellular pollen into mature microgametophytes containing two male gametes (called sperm or sperm cells). The first mitosis in pollen of angiosperms results in two structurally and functionally different nuclei, the generative and vegetative nucleus. This process is accompanied by an asymmetric cell division, which partitions the microspore into two daughter cells of unequal size (Tanaka, 1997; Twell et al., 1998; Borg et al., 2009). The smaller generative cell undergoes a second mitotic division resulting in the formation of two sperm cells, which during fertilization will fuse with the egg and central cell, respectively. The functional differentiation of pollen nuclei is accompanied by contrasting nuclear chromatin configurations. The generative nucleus is strongly heterochromatic, whereas the vegetative nucleus is typically strongly euchromatic reflecting its higher transcriptional activity (Honys and Twell, 2003). The nuclear differentiation occurs during or immediately after the first pollen mitosis (Terasaka and Tanaka, 1974). Soon after the asymmetric division of the pollen, the generative cell enters the S phase and then passes into G2, while the vegetative cell is arrested at G1 (Tanaka, 1997). Sangwan and Norrel (1987) performed an ultrastructural survey of the plastid types from the tetrad stage to early bi-cellular pollen grains in several angiosperms including grass species. They found that proplastids were specific to pollen grains of all species that undergo pollen embryogenesis, while amyloplasts were characteristic of those of recalcitrant species that failed to undergo pollen embryogenesis. In all species capable of pollen embryogenesis, starch accumulation started only at the

late bi-cellular stage, except for tomato and *Lilium* where it commenced prior to the first pollen mitosis. In recalcitrant species, starch accumulated throughout pollen development, i.e. starting in the tetrad stage and lasting until pollen maturation.

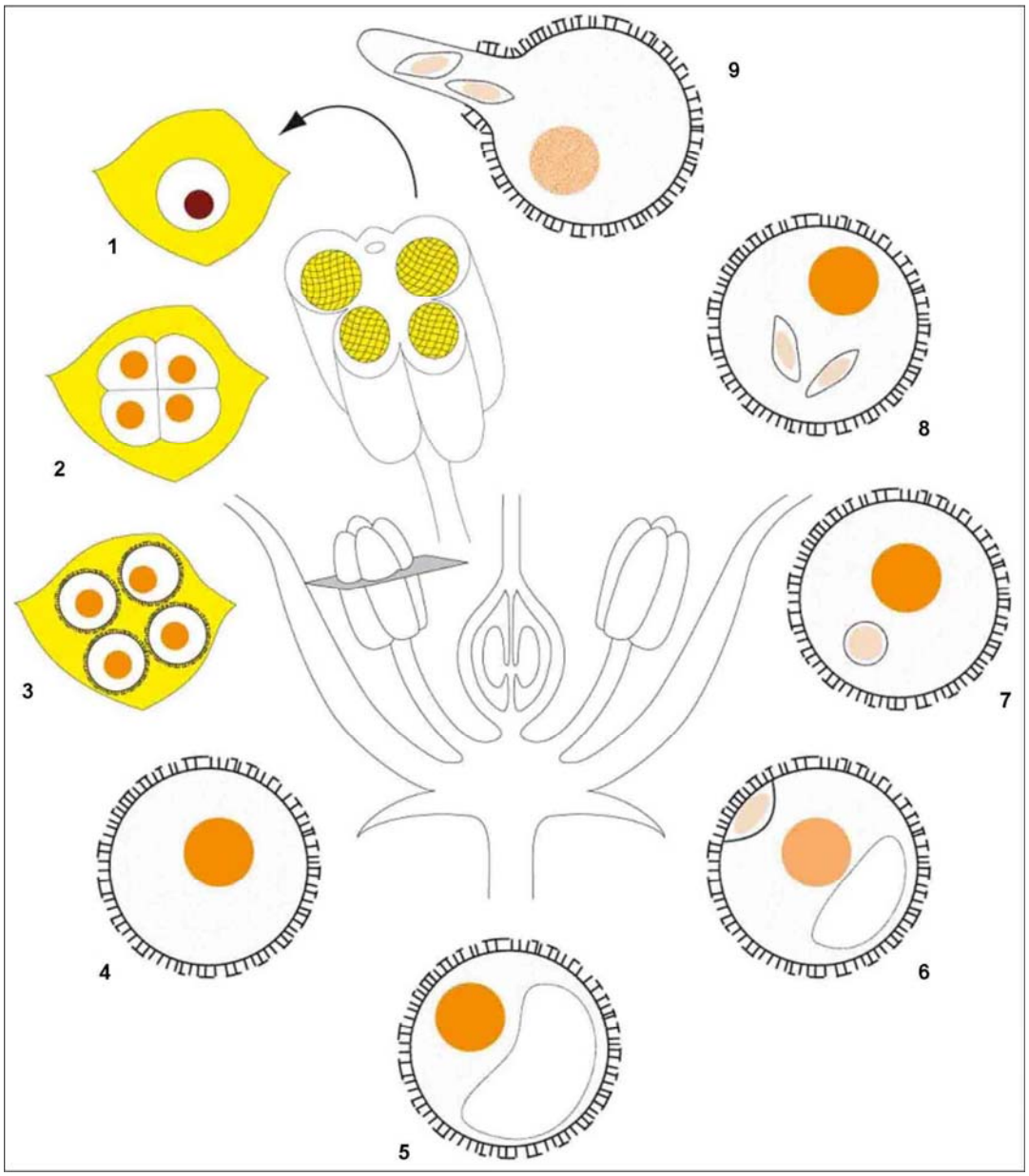

Fig. 1: Pollen gametogenesis in angiosperms. The formation and development of a pollen grain includes micro-sporogenesis (1-4) and micro-gametogenesis (5-9), respectively. (1) Pollen mother cells. (2) Tetrad of four haploid microspores enclosed by callose. (3) Microspores after exine wall formation are still arranged in tetrads. 4) deliberated microspore (5) Premitotic microspore with large central vacuole. (6) Bi-cellular pollen after first mitosis (asymmetric division). (7) Reduction of central vacuole. (8) Mature pollen after second mitosis (symmetric division of generative cell). (9) Germination of pollen tube (modified after Hess et al., 2009).

1.2. Pollen embryogenesis

It has been shown that several factors are able to change the normal development of **vacuolated immature pollen** from **pollen gametogenesis** into **pollen embryogenesis**. In vacuolated immature pollen, pollen embryogenesis has been induced by applying various treatments both *in vivo* and *in vitro*. These include nitrogen and carbon starvation (Touraev et al., 1996a; Hu and Kasha, 1999), cold (Hoekstra, 1996; Jahne et al., 1994; Hu and Kasha, 1999) or heat (Keller and Armstrong, 1979; Custers et at., 1994), as well as exposure to chemicals (Eady et al., 1995). These treatments have been applied to entire plants (Ouyang et al., 1989), spikes (Jahne et al., 1994; Hu and Kasha, 1999), anthers (Keller and Armstrong, 1979; Hu and Kasha, 1991; 1999) or to isolated vacuolated immature pollen (Custers et al., 1994; Indrianto et al., 1999). Upon these treatments, vacuolated immature pollen can undergo deregulated cell proliferation and change into what is called **embryogenic pollen** (Hoekstra el al., 1993). Stress seems to be an important stimulus for initiating this developmental program (Touraev et al., 1997). An understanding of its decisive role in this process was achieved by stress treatments applied to in vitro-cultured vacuolated immature pollen isolated directly from anthers of non-stressed plants. In *Brassica napus*, a high temperature treatment for 8 h at 32^{o}C was sufficient to induce up to 40% of isolated pollen to undergo successive cell divisions. Although cultured in a simple medium without growth regulators, thousands of plantlets can be obtained at a time. When retained at 18^{o}C, immature pollen developed into apparently normal mature pollen grains (Custers et al., 1994). Four stress pre-treatments have been shown to induce pollen embryogenesis at high frequencies: (i) cold temperature in barley (Roberts-Oehlschlager and Dunwell, 1990), rice (Cho and Zapata, 1988), maize (Gaillard et al., 1991), wheat (Gustafson et al., 1995) and many other species; (ii) high temperature in rapeseed (Custers et al., 1994), wheat (Touraev et al., 1996a) and tobacco (Touraev et al., 1996b); (ii) carbohydrate and nitrogen starvation in tobacco (Harada et al., 1986), barley (Hoekstra et al., 1992), rice (Ogawa et al., 1994) and wheat (Touraev et al., 1996a), and (iv) colchicine treatment in rapeseed (Zhao et al., 1996).

1.2.1. Brief history of doubled haploid production

In his 1974 review on 'The status of haploid research', Riley describes how the history of doubled haploids began with the observation of haploid production in the higher plant *Datura stramonium* L. (Jimson weed) reported by Bergner in 1921 (cited by Blakeslee et al., 1922). This was soon followed by similar discoveries in other species e.g. *Nicotiana tabacum* (Clausen and Mann, 1924) and *Triticum compactum* (Gains and Aase, 1926). The potential of haploids and doubled haploids in breeding and genetics was quickly realized and research was initiated to identify ways of improving the frequency of haploid production (Kimber and Riley, 1963). A wide range of methods was identified including parthenogenesis, pollen irradiation, selecting seed with twin embryos; sparse pollination, alien cytoplasm, wide hybridization and the use of certain genetic stocks (Kasha and Maluszynski, 2003). Chase (1952) is recognized as being among the first to incorporate haploids in a breeding program, in this case by inducing haploids in maize via parthenogenesis followed by doubling their chromosome complement to produce doubled haploids. A major breakthrough came with the generation of haploid plants from cultured anthers of *Datura* (Guha and Maheshwari, 1964; 1966). The method was tested and became established in many species, although less was known about the underlying mechanisms. Today isolated immature pollen culture is the method preferred most, though wide crossing and ovule culture are also deployed in producing haploids and doubled haploids. There has been resurgence in the applications of haploidy in higher plants (Forster et al., 2007) with published protocols for up to 200 species (Maluszynski et al., 2003b). In vitro culture of pollen or anthers has enabled the production of complete homozygous breeding lines within a much shorter time compared to traditional inbreeding (Morrison and Evans, 1988). Distant hybridization used for uniparental genome elimination as developed by Kasha and Kao (1970), is also used to produce haploids, but this method, as opposed to pollen embryogenesis, is not associated with spontaneous diploidization (Sadasivaiah et al., 1999).

1.2.2. Importance of haploid technology

In spite of its progress and benefits, conventional plant breeding based on classical genetics is still a developing technology (Cook, 1998: Schön et al., 2004). Yet, by itself it

may not continue to provide enough food to feed the world's population, which is expected to pass the 9 billion mark before the year 2050 (http://www.wpxi.com/news/27763073/detail.html). Genetic improvements in crop plants must be expanded, accelerated and carried out much more precisely and efficiently to meet the demands of the growing world population not only for more food, but also for greater diversity and higher quality of food, which need to be produced on less land, while protecting environmental and genetic resources. Meeting these multiple goals and expectations will be possible only through large widespread application of new tools of plant genetics, breeding and biotechnology.

Plant breeding depends on the commercial availability of homogenous material with guaranteed identical genotypes. Typically, time-consuming and costly procedures including conventional self and back crossing are used to generate these materials. A more efficient short cut for the production of homogenous plants is provided by the use of doubled haploid plants, which presents absolutely pure lines (Snape et al., 1988; Jähne and Lörz, 1995). Doubled haploids are useful for plant transformation, mapping genes and for the development of new markers in economically important crops (Choo, 1981; Gallais, 1990; Graner, 1996).

Research on doubled haploids accelerated in the last few decades with a growing number of successful applications (Forster et al., 2007). From the 1970s onwards there has been a steady rise in the number of new plant species in which doubled haploidy has been reported (Maluszynski et al., 2003b). Currently, there is much interest in applying doubled haploid technologies to neglected crops, particularly those of high value, e.g. species producing pharmaceutical and aromatic compounds, where large gains can be achieved within a short period of time (Ferrie, 2007). Doubled haploidy is the fastest route to homozygosity in plants. The core motivation for developing broadly applicable protocols to raise doubled haploids are at least three fold: (i) doubled haploids are useful in fixing traits rapidly in desirable combinations in one line or variety (Snape et al., 1988); (ii) doubled haploids facilitate hybrid breeding, and (iii) doubled haploids applied in marker studies speed up the development of mapping populations and marker/trait associations (Gallais, 1990; Graner, 1996; Forster and Thomas, 2005). As compared to inbreeds, doubled haploid lines can be evaluated earlier and with more accuracy and

confidence, especially in respect to quantitatively inherited traits such as yield and quality. Moreover, certain stages of doubled haploid cultures are useful as targets for inducing mutation (Devaux and Kasha, 2009). Doubled haploid also can be used to develop transgenic lines more rapidly from an original transformable line into commercial varieties. Gene transfer into embryogenic pollen allows the creation of homozygous transgenics in one step (Huang, et al., 1992; Harwood et al., 1996). Haploid structures induced from immature pollen in suspension constitute a perfect model to study plant cell cycle regulation, cell division and early embryogenesis. However, the advantages of doubled haploids for breeding purposes can be fully exploited only when efficient technologies are available in relevant species and genotypes (Devaux and Kasha, 2009).

Transformation in cultured barley pollen is progressing well using haploid systems (Jähne et al., 1994; Kumlehn et al., 2006). Jähne et al. (1994) were the first to report on the transformation of cultured barley pollen by particle bombardment. Of the five plants analyzed, all were reported to be homozygous for the transgene/trait of interest, yet experimental evidence was poor due to a too small number of T1 lines analyzed. The method of choice for transformation was initially particle bombardment of isolated immature pollen, since the co-cultivation of pollen with *Agrobacterium* required 2 days and the pollen viability was severely reduced during this period. Furthermore, the immature pollen wall was thought to prevent *Agrobacterium* from introducing its T-DNA into the target cells. However, Kumlehn et al. (2006) revised *Agrobacterium* procedures to transform cultures of barley pollen-derived embryos at the time when the multi-cellular structures just emerged from the pollen wall after 7-10 days of culture. Analysing transgene segregation in 20 transgenic lines, they found that 4 were homozygous for the transgene, while the remainder were hemizygous. They also obtained a number of transgenic haploid plants that could have doubled their chromosome number by colchicine treatment so as to make them instantly homozygous.

1.2.3. Technologies for the generation of doubled haploids

1.2.3.1. Uniparental genome elimination

In some genera, haploid plants can be obtained by uniparental genome elimination. This involves pollination with a genetically distant male partner (wide crossing) or a pollinator of the same genus having special genetic properties, e.g. haploid inducing genes. In both cases, fertilization takes place but male genetic material is eliminated from cells of the developing embryo at early stages of its growth. The resulting haploid embryos originate from the female partner, since the endosperm fails to develop properly. The cross needs to be rescued and cultured *in vitro* to produce haploid plants.

1.2.3.1.1. Wide Crossing

Wide crossing exploits haploidy from the female gametic line. It is a well known technique in the field of cereal and potato breeding (Laurie et al., 1990; Mujeeb-Kazi and Riera-Lizarazu, 1997). In some interspecific or intergeneric crosses of the *Poaceae* and *Pannicoidae,* fertilization is followed by paternal chromosome elimination from the hybrid embryo. In these crosses the endosperm is either not formed or poorly developed; therefore, such embryos do not mature in the caryopsis and embryo rescue and *in vitro* culture are necessary. (Laurie et al., 1990; Mujeeb-Kazi and Riera-Lizarazu, 1997). Intergeneric crosses are applied also in potato. However, the mechanism does not involve chromatin elimination but parthogenetic induction of the egg cell (Wędzony et al., 2009). Common examples of wide crossing are: i) bulbosum method in barley, ii) crosses with maize and its relatives, iii) maize doubled haploids by an inducer line and iv)potato doubled haploidy via crossing of *S. tuberosum* x *S. phureja*.

1.2.3.1.1.1. The Bulbosum Method

The '*bulbosum* method' was the first method in cereals based on wide crossing. The cross of barley (*Hordeum vulgare*) with a wild species, *Hordeum bulbosum*, resulted in the production of haploid *H. vulgare* plants (Kasha and Kao, 1970; Lange, 1971). Pollination with *H. bulbosum* also appeared to be effective for double haploided production in some wheat and triticale genotypes (Inagaki, 1985; Sitch and Snape, 1986). Crossing barriers between the majority of cultivated wheat and triticale genotypes and *H. bulbosum* limit

its wider application to other cereals (Wojciechowska and Pudelska, 1993; Inagaki and Tahir, 1995). Additional details are shown in subchapter (1.2.7).

1.2.3.1.1.2. Doubled haploidy of potato *via* crossing of *S. tuberosum* x *S. phureja*

Doubled haploids can be produced from tetraploid genotypes of *Solanum tuberosum* (cultivated potato) by pollination with the diploid potato species *Solanum* (Mendiburu et al., 1974; De Maine, 2003). In this method both male sperm cells of *S. phureja* take part in formation of functional endosperm, which triggers a parthenogenic development of the unfertilized egg cells. However, in *Solanum* species, pollen culture is more efficient than crossing with *S. phureja* (Jacobsen and Ramanna, 1994; Rokka, 2003).

1.2.3.1.1.3. Crosses of *Triticeae* with *Paniceae*

Crossing of wheat with maize (Zenkteler and Nitzsche, 1984) resulting in the formation of embryos raised the attention of plant breeders. Here the maize chromatin of the hybrid nuclei is eliminated during development of the hybrid embryo (Laurie and Bennett, 1989). In crosses between the members of *Pooideae* with maize the endosperm is formed only occasionally, while embryos abort as early as 5-6 days after fertilization (Laurie and Bennett, 1989; Wędzony and van Lammeren, 1996; Brazauskas et al., 2004). As a consequence embryos must be rescued and cultured *in vitro* within this time frame for haploid production.

Ushiyama et al. (1991) used pollen of wild Mexican corn-theosinte (*Zea mays* ssp. *mexicana*) in crosses with wheat. Sorghum (*Sorghum bicolor*) was utilized to pollinate hexaploid and tetraploid wheats (Riera-Lizarazu et al., 1992). Also, pearl-millet (*Pennisetum glaucums* synonym *Pennisetum americana*) was successfully applied in several experiments (Inagaki and Mujeeb-Kazi, 1995; Ohkawa et al., 1992; Mujeeb-Kazi and Riera-Lizarazu, 1997).

1.2.3.1.2. Inducer line

1.2.3.1.2.1. Maize doubled haploids by inducer line

Doubled haploids were also obtained in maize using inbred ‘inducer’ lines (Sarkar et al., 1994; Shatskaya et al., 1994; Chalyk 1999). Röber et al. (2005) developed and

characterized a new improved inducer line system. They found that 10% of progeny were haploids derived from the mother plant in addition it contains a color marker gene to easily select kernels containing haploid embryos among those containing diploid embryos.

1.2.3.1.2.1. Arabidopsis doubled haploids by inducer line

Ravi and Chan (2010) established a pioneer protocol for haploid production in *Arabidopsis thaliana* by manipulating, the centromere-specific histone CENH3. They showed that when a mutated genotype with *cenh3* null, expressing altered CENH3 proteins is crossed with wild type plants, chromosomes from the mutant are eliminated, thus producing haploid plants. In this case maternal or paternal haploids can be generated through reciprocal crosses. The theoretical explanation is that both centromeres of parent material interact unequally resulting in selective chromosome loss. Ravi and Chan (2010) assumed that since CENH3 is universal in eukaryotes, this method may be extended to produce haploids from other plant species.

1.2.3.2. Gynogenesis

Gynogenesis often was attempted as the alternative method in species where pollen embryogenesis fails. In gynogenesis, embryogenic development is induced in any cell of the female gamete. An advantage here is that re-programming is not required; the embryogenic pathway just needs to be switched on without proper fertilization (Keller and Korzun, 1996). It plays a limited role in cereals, although it was reported for certain genotypes of wheat (Zhu et al., 1981; Matzk, 1991; Comeau et al., 1992; Matzk et al., 1995), barley (Gaj and Gaj, 1996) and rice (Zhou and Yang, 1981). Grzebelus and Adamus (2004) screened a hundred onion genotypes to a standard procedure and revealed and they showed a high level of genetic variability in gynogenic response. Most of the regenerated plants appeared to be haploid and artificial chromosome doubling was required to produce the doubled haploids. Colchicine, oryzaline, trifluraline and aminoprophosmethyl were tested. Gynogenic origin of regenerated plants was confirmed by embryological studies of Musiał et al. (2001).

However, compelling evidence of the haploid or doubled haploid nature of the regenerants has not been provided and these methods did not enjoy any practical application.

1.2.3.3. Pollen embryogenesis

Pollen embryogenesis requires the complete re-programming of the developmental plan of the immature pollen to result in embryonic development rather than the formation of mature pollen grains. The vacuolated pollen is originally programmed to divide into two cells: a vegetative and a generative one. Subsequently, the generative cell gives rise to two male gametes (sperm or sperm cells), which can happen during pollen maturation itself or later during pollination in the pollen tube. The vegetative and generative cells that make up the mature pollen cell are highly specialized and have specific biological functions. The vacuolated immature pollen grains have a haploid chromosome complement, and under certain conditions and in many species, they are amenable to embryogenic induction. Vacuolated immature pollen is produced in large numbers inside plant anthers making them relatively easy to access and manipulate.

1.2.4. Stress and pollen embryogenesis

Abiotic stresses play a very important role in the induction of pollen embryogenesis as was first established for tobacco (Duckan and Heberle-Bors, 1976; Heberle-Bors and Reinert, 1981). Most protocols developed for both, mono- and dicotyledonous plants include low or high temperature either applied as a pre-treatment or employed at the beginning of culture in nutrient medium.

In the beginning of the 1990s the incubation of spikes at low temperatures of 4 to 7°C for a period of 3 to 4 weeks was regarded as a prerequisite for pollen embryogenesis induction in barley (Olsen, 1987; Ziauddin et al., 1990; Mordhorst and Lörz, 1993; Scott and Lyne, 1994; Evans and Batty, 1994; Salmenkallio-Marttila et al., 1995). Cold treatment of spikes for over 1 week was also applied to induce wheat (Gustafson et al., 1995; Ingram et al., 2000; Redha et al., 2000), triticale (Ślusarkiewicz-Jarzina and Ponitka, 1997; Marciniak et al., 1998; González and Jouve, 2000; Immonen and Robinson, 2000; Tuvesson et al., 2003; Wędzony et al., 2003) and rye (Immonen and

Anttila, 2000; Immonen and Tenhola-Roininen, 2003). Along with other treatments, the cold treatment favors the synchronization of the development of *in vitro* cultured pollen (Hu and Kasha, 1999).

Application of low temperature treatments was also proven to induce pollen embryogenesis in trees like oak (*Quercus sp*.) (Bueno et al., 2000; 2003; Raminez et al., 2004), apple (*Malus sp*.) (Höfer, 2003; 2004) and poplar (*Populus nigra*) (Andersen, 2003; Deutsch et al., 2004). It also favors pollen embryogenesis induction in some *Fabaceae* species (Zagorska and Dimitrov, 1995; Kaltchuk-Santos et al., 1997; Croser et al., 2004; De Moraes et al., 2004). Comparative studies by Bayliss et al. (2004) showed that low temperature stress gives better results in comparison to high temperature when applied to *Lupinus sp*.

The outstanding role of high temperature to induce pollen embryogenesis was also shown in the case of *Brassica napus* (Telmer et al., 1993; Custers et al., 1994; Binarova et al., 1997; Touraev et al., 1997; Indrianto et al., 1999; Smykal and Pechan, 2000). At a temperature of 25°C or lower, immature pollen in suspension continued their gametogenic development, while an 8 hour treatment at a higher temperature of 32°C was sufficient to embryogenesis. However, the duration of stress treatment has an impact on the frequency of the generation of albino plants appearing after regeneration (Ohnoutkovă et al., 2000; Zheng et al., 2001).

Indeed, temperature stress can be combined or replaced by other stress treatments, sometimes with better success than achieved with the protocols described above. For instance, the cold pre-treatment of spikes was replaced by heat, e.g. 33°C for 48-72 hours in wheat (Touraev et al., 1996a; b; Liu et al., 2002a; b). Elevated temperatures were routinely used for pollen embryogenesis induction in rapeseed (Pechan et al., 1991; Hause et al., 1993; Binarova et al., 1993; Boutilier et al., 1994; Telmer et al., 1995; Custers et al., 1999, 2001). Moreover, the induction of pollen embryogenesis was proven to be successful in a number of related *Brassica* species when high temperature protocols were applied (Duijs et al., 1992; Barro and Martin, 1999; Sato et al., 2002; Ferrie, 2003; Hansen, 2003).

Starvation stress is nowadays frequently applied to cereals in combination with a relatively short (3-5 days) incubation at low temperature (Touraev et al., 1996a; b;

Hoekstra et al., 1997; Caredda et al., 2000; Kasha et al., 2001; Li and Devaux, 2001; Wojnarowiez, et al., 2002; Jacquard et al., 2003; Cistué et al., 2003; Davies, 2003; Corronado et al., 2005; Kumlehn et al., 2006). An improvement in the pollen embryogenesis efficiency in maize was achieved by the application of a cold treatment (7°C) of tassels (Barnabás, 2003) or by pre-treatment at 14°C in a medium containing mannitol (Nageli et al., 1999; Obert et al., 2000; Zheng et al., 2003). Zheng et al. (2002, 2003) and Liu et al. (2002a; b) used the combination of growth regulators and a short cold treatment of spikes and/or starvation stress in mannitol with satisfactory results.

1.2.5. Genome doubling

The term spontaneous genome doubling is widely used to refer to doubling events under experimental conditions that have not been directedly designed to promote duplication. Segui-Simarro and Nuez (2008), Kasha (2005) and Kasha et al. (2006) have argued that such a process is far from spontaneous since many different *in vitro* or *ex vitro* factors may influence the duplication process. On the other hand, genome doubling is an event that also occurs spontaneously in nature and has been historically referred to as spontaneous or natural genome doubling (Jensen, 1974).

Seguí-Simarro and Nuez (2008) reviewed that across the literature four major mechanisms for plant genome doubling have been proposed (Jensen, 1974; d'Amato, 1984, 1989; Kasha, 2005) alternatively to the normal cell cycle: i) endoreduplication (DNA duplication without mitosis), ii) nuclear fusion (merging of coalescing nuclei into a larger nucleus, mixing both DNA contents), iii) endomitosis (mitosis in the absence of both mitotic spindle and nuclear envelope breakdown) and iv) c-mitosis (colchicine-induced collapse of the mitotic spindle and breakdown of the nuclear envelope). Endomitosis and nuclear fusions have been rarely documented in angiosperms (d'Amato, 1984) with the exception of the nuclear fusion events taking place during fertilization (West and Harada, 1993).

The spontaneous genome doubling that occurs during the *in vitro* culture of pollen (Jähne and Lörz, 1995), allows the use of pollen-derived plants directly for genetic improvement without colchicine treatment. In barley and wheat, spontaneous doubling frequencies between l8 and 85% of regenerants have been observed, resulting in completely fertile

doubled haploids (Hoekstra et al., 1993; Jähne and Lörz, 1995 Hu and Kasha, 1999; Indrianto et al., 1999). In *Datura*, it has been proposed that nuclear fusion was involved in genome doubling and that the combination of both mechanisms could explain a resulting ploidy level higher than diploid (Sunderland, 1974). Nuclear fusion of more than two nuclei has been used to explain the occurrence of triploids and higher ploidy levels in wheat (Hu and Kasha, l999). The production of completely fertile doubled haploid plants would indicate genome doubling to occur early in pollen culture (Kasha et al., 2001). Gonzalez-Melendi et al. (2005) presented a new method to prove that nuclear fusion is the major mechanism of spontaneous genome doubling during pollen-derived embryogenesis in barley. They have estimated the ploidy level of individual nuclei within young pro-embryos, as of the first embryogenic pollen division up to the formation of multinuclear structures, which are still surrounded by the exine. The method used was based on the measurement of the intensity of fluorescence after 4,6-diamidino-2-phenylindole dihydrochloride labeling (DAPI), nuclear size and the number of nucleoli per nucleus. This method using confocal laser scanning microscopy provided the opportunity to measure the ploidy of individual nuclei and avoids the overlapping of the fluorescence signal in multinuclear structures, which cannot be excluded using cytophotometer-based methods on other types of fluorescence microscopes. They showed that diploidisation is an ongoing process that can start after the first embryogenic division and continues in multicellular structures. 3D-reconstruction of image series showing entire pro-embryos and the observation of cross and longitudinal sections across stacks of optical sections together with correlative light and electron microscopy provided evidence for nuclear fusion as the main mechanism of diploidisation (Gonzalez-Melendi et al., 2005). Lee and Chen (1987) showed that fusion of the generative and vegetative nuclei in barley, which are initially physically separated by a wall, occurred in cultured pollen of barley. The generative cell appeared to play an active role in fusion as it elongated towards the vegetative nucleus, became detached from the pollen wall, and finally completely enclosed the vegetative nucleus. The generative cell wall disappeared before nuclear fusion took place. However, this case was not proven by any live-cell imaging or time lapse experiments.

Although genome doubling was presented to occur most likely through nuclear fusion, only live-cell imaging can provide unambiguous evidence for this process.

1.2.6. Morphological aspects and ultrastructural features

Based upon the comparison with pollen maturation, the identification of embryogenic pollen is required for a proper characterization and evaluation of pollen cultures, as well as for the selection of cells suitable for manipulation techniques such as microinjection and for an efficient isolation of certain cell types for detailed molecular analyses (Kumlehn and Lörz, l999). Sunderland and Wicks (197l) described the occurrence of pollen dimorphism in cultured tobacco anthers. This dimorphism was also found *in situ* in immature anthers of tobacco (Horner and Street, 1978). They suggested that embryos from anther culture were derived from one type of pollen grains called "S grains", which are smaller than the normal ones. They contain no starch or a cytoplasm which stains weakly with aceto-carmine. Olsen (199l) described the presence of a blue or red corona in cultured barley pollen and proposed that only the blue type produced immature-pollen-embryos. However, other studies showed no correlation between this sort of pollen grains and embryogenesis (Horner et at., 1978; Hoekstra, 1996). The identification of morphological indicators of pollen embryogenesis is still an important goal. The establishment of pollen tracking systems has been reported for barley (Bolik and Koop, 1991; Kumlehn and Lörz, 1999) and wheat (Indrianto et al., 200l). Various types of pollen have been reported to differ in their composition of the cytoplasm, the position of the nucleus, and the size and arrangement of the vacuoles. Bolik and Koop (1991) used a cultured pollen tracking system and described two basic kinds of barley pollen. Type A, with the nucleus being located at the cell periphery, and type B, with less cytoplasm and the nucleus located in the center of the immature pollen, thereby building so-called star-like structures. Similar structures have been reported in barley pollen cultures by Kumlehn and Lörz (1999) and in wheat by Indrianto et al. (2001).

In barley, only the type A was found to develop into embryos (Bolik and Koop, 1991; Kumlehn and Lörz, 1999). Indrianto et al. (2001) observed embryogenic development in type A pollen also in wheat, but the frequency was very low. Although some advances

have been made in recent years, it has as yet been not possible to establish cytological markers for the early identification of embryogenic pollen.

Defined changes in ultrastructure have been reported to accompany the reprogramming of the immature pollen to embryogenesis in dicot systems (Testillano et al., 2000), but little is known on the cellular characterization of this process in monocots (Ramírez et al., 2001). A few papers dealt with the structural alterations during embryogenesis and most of these done with *Nicotiana tabacum* and *Brassica napus* (Dunwell and Dunderland, 1974; Hause and Hahn, 1998; Rashid and Reinert, 1981; Rashid et al., 1982; Telmer et al., 1995; Zaki and Dickinson, 1990) and *Capsicum annum* L. (Barany et al., 2005). Attempts have recently been made to characterize specific genes expressed during pollen embryogenesis and to find cellular and molecular markers for this process (Cordewener et al., 1996; Sangwan and Sangwan-Norreel, 1996; Dunwell and Sunderland, 1974; Garrido et al., 1995; Reynolds, 1990; Sangwan-Norreel, 1978; Szakfcs and Barnabas, 1988; Zaki and Dickinson, 1990).

Ramírez et al. (2001) showed that most of the cells which responded to stress treatments were vacuolated immature pollen indicating that this developmental stage is responsive for embryogenesis induction in barley as well as in various dicot species (González-Melendi et al., 1996). At this stage, a large vacuole occupied a large proportion of the cell volume and a highly euchromatic nucleus was located at the cell periphery. The nucleus displayed one or two small nucleoli and the cytoplasm appeared comparatively clear. As a product of pollen mitosis I, vegetative cells contained large nuclei and a thin layer of peripheral cytoplasm; large vacuoles were still present at very early stages. The cytoplasm contained numerous ribosomes. The organelles and the nuclei exhibited a similar organization as in the vacuolated pollen. At later stages, subsequent divisions occurred and multicellular structures, still surrounded by the exine, were observed. Different cellular organizations appeared in these pro-embryos: some cells displayed a cytoplasm with a very large central vacuole, numerous small vesicles and small vacuoles. Other cells showed a dense cytoplasm containing abundant ribosomes and organelles and had a large nucleus located in the cell centre. The nuclei with one or two nucleoli contained small patches of heterochromatin. Caredda et al. (1999) showed that in vacuolated immature barley pollen, plastids were undifferentiated, occasionally

containing a single starch grain and a few thylakoids but no plastoglobules or prolamellar bodies. Plastid division was not detected at this stage, while after embryogenesis induced via cold pretreatment, the density of the spherical plastids decreased dramatically. The size of starch grains was reduced, whereas up to three thylakoids were detected. After 12 days of culture, two types of plastids were found: i) elongated plastids with starch and juvenile thylakoids, and ii) abnormal plastids, in which the envelope as well as the thylakoid organization were strongly modified. After 21 days, amyloplasts or plastids with diffusive starch content were observed.

According to the observations of Bone and Olmedilla (2000) the first embryogenic division in wheat pollen culture was symmetrical. After the induction of embryogenesis, there was a decrease in ribosome density within cells that had inactive nucleoli. This population was restored after initial embryogenic divisions. During the initial divisions, the embryogenic pollen grains did not appear to change in size compared to the non-induced immature pollen and the pollen wall remained intact. The exine did not undergo modification but the intine thickened, a feature that was proposed to be used as a structural marker of pollen embryogenesis. Upon embryogenic division, the cell walls contained numerous plasmodesmata. Another important feature of the embryogenic pollen with four or more cells was the presence of large vacuoles that displaced the nucleus and the cytoplasm toward the borders of the cell.

1.2.7. Barley as a model species for pollen embryogenesis in cereal crops

The production of haploids in barley has been widely used in breeding and the development of breeding methods. Initially, the Bulbosum method (Kasha and Kao, 1970; Lange, 1971) was employed to produce plants from female gametes only. On the other hand, anther culture enjoyed some improvement and has thus been widely used, but more recently, isolated immature pollen culture has been increasingly employed due to the higher regeneration efficiency that can be achieved (Devaux and Kasha, 2009). Thus, barley is now considered the cereal model crop species for haploid production and research (Sunderland, 1974; Kasha, 1974b; Pickering and Devaux, 1992; Jähne and Lörz, 1995; Thomas et al., 2003; Maluszynski et al., 2003; Devaux and Pickering, 2005; Kumlehn et al., 2006; Kasha, 2007).

Barley (*Hordeum vulgare* L.) is a diploid species ($2n = 2x = 14$) with a genomic designation of I while the chromosomes are described with the symbols 1H to 7H. This species is self-pollinating under most climatic conditions and therefore the cultivars are usually phenotypically and genetically uniform. In traditional breeding, these cultivars of barley were often achieved by inbreeding and subsequent selection lasting for several generations. In contrast to these approaches, haploid systems can achieve homozygosity in a single generation and therefore save much time in the breeding of new cultivars. Genetic uniformity is a requirement for the registration of new cultivars in many countries. Also here haploidy may be used in the final stages of breeding progress to achieve uniformity. Because of this, populations for molecular mapping studies are often derived as doubled haploids, particularly in barley. Haploids or doubled haploid lines are also valuable for genetic studies, in mutation and selection at the single cell level in cultures or in studying embryo development. In barley, the first haploids were produced by Clapham (1973) while Sunderland (1974) was a pioneer author in investigating the mechanism of haploid production. Barley was the first crop, in which large numbers of haploids could be produced from most genotypes when Kasha and Kao (1970) produced haploids from wide hybridization of *H. vulgare* with *H. bulbosum*. It was the 'Bulbosum method' that enabled researchers to produce doubled haploids and to evaluate the potential of haploidy in breeding programs (Choo et al., 1985). Both anther culture and immature pollen culture are heavily used in breeding programs.

It has been speculated that half of the barley cultivars now grown in central Europe were produced as doubled haploids (Devaux and Kasha, 2009; Wędzony et al., 2009). Breeders still use the Bulbosum method, particularly when genotypes were not amenable to anther or immature pollen culture.

Doubled haploid populations have been extensively utilized in molecular marker work in barley providing detailed chromosome maps. Barley pollen is often selected for biochemical and cytological investigations of embryogenesis (Devaux and Kasha, 2009). With the advance in molecular, biochemical and cytological tools, haploid research has shifted to the sequencing of DNA and transcriptome analyses that reveals hundreds of genes whose expression is associated with embryogenesis from immature pollen, and which can be assigned to various biochemical pathways (Devaux and Kasha, 2009).

1.2.8. Live-cell imaging

Imaging of live cells is a complex task even for experienced microscopists. However, many research questions can only be addressed using living cells. For example, studies of cellular dynamics of processes and structural components require live observation over time. Additionally, microscopy of GFP-labeled proteins in living cells is both more simple and less prone to artifacts than microscopy of fixed cells. A major goal during the performance of live-cell imaging experiments is to maintain the cells in a healthy state with normal function while they are on the microscope stage.

The first live-cell imaging chambers were designed and built shortly after mammalian cell culture techniques were developed in the early twentieth century. By the 1950s and 1960s, more sophisticated live-cell imaging chambers were designed for high-resolution investigations that incorporated the emerging techniques of phase and differential interference contrast (DIC) microscopy. Of major importance in terms of culture chamber design is to guarantee that the cultured cells are healthy and undergoing growth on a normal timescale, as well as providing an adequate optical window for the microscope to ensure that imaging of the culture can be conducted at high enough numerical aperture to meet the resolution demands of the experiment.

Specimen chambers are an integral and critical part of live-cell imaging. They should permit surface-sterilization and isolation from the laboratory environment. On the other hand, the culture chamber should also offer uncomplicated access to the cells if the investigation involves e.g. microinjection, addition of reagents (such as drugs or metabolites), physical manipulation of the cells, or alterations as to the culture medium. Occasionally, it will be necessary to adapt existing chambers or design them to accommodate the specific demands of a particular experiment.

The use of fluorescence, either in the form of autofluorescence, fluorescent dyes, or as fluorescent proteins produced in transgenic organisms greatly facilitates the visualization of the dynamics and interactions of intracellular molecules. However, fluorescence live-cell imaging is limited by photobleaching and phototoxicity induced by the excitation light (Hoebe et al., 2007). Minimization of the excitation-light dose directly impairs image quality because of reduced fluorescence (Sheppard et al., 1995). Finding the proper balance between image quality (high light dose) and cell viability (low light dose) is a

major challenge in live-cell imaging. In the end however, observing a biological event as it unfolds in the living cell provides unique insight into the nature of the phenomenon under study (Shaw, 2006). Among the most important routine considerations for live-cell imaging that must be addressed are temperature, oxygenation, humidity, osmolarity, pH (medium buffering), phototoxicity, the laboratory environment, microscope focus drift, fluorescence signal strength and resolution.

Due to the lack of sophisticated time-lapse experiments, some important information on pollen embryogenesis is yet to be uncovered. Multidimensional live-cell imaging of pollen embryogenesis using short time intervals of wild type and transgenic pollen with GFP-labeled nucleus is required. This allows the monitoring of dynamic processes of a sample under physiological conditions over a prolonged period of time. The combination between DIC bright field and laser scanning confocal microscopy provides the advantage to study pollen embryogenesis in a much more straightforward way and hence facilitates the understanding of the dynamic cellular behavior and organization of the organelles. By tracking back the time-lapse data, it will be possible to identify the cell types competent to pollen embryogenesis and answer questions like how spontaneous genome doubling occurs.

1.2.9. Tracking of pollen types during culture

Several pathways of pollen embryogenesis have been proposed, which can lead to the formation of multicellular structures from vacuolated immature pollen (Sunderland and Evans, 1980; Hu and Kasha, 1999): i) The A-pathway: an asymmetric pollen mitosis I with successive mitotic divisions of the vegetative or generative nucleus, or of both vegetative and generative nuclei, can result in embryogenesis. ii) The B-pathway: a symmetric pollen mitosis I leading to two similar vegetative nuclei. Their subsequent fusion and mitotic divisions can lead to a spontaneously doubled haploid multicellular structure. iii) The C-pathway: fusion of the vegetative and generative nuclei which then continue mitotic divisions resulting in the formation of a callus or embryoids. However, Maraschin et al. (2005) showed by time-lapse studies of cultured barley immature pollen the existence of three developmental types. One part of the immature pollen developed into embryo-like structures, a second part formed multicellular structures but failed to

pursue embryo-like development and a third part of the immature pollen finally followed gametophytic divisions, accumulated starch and died in the first days of tracking. These pathways often vary among the species were studied but the presence of more than one pathway has regularly been observed within a culture (Hu and Kasha, 1999; Kasha et al., 2001). Zaki and Dickinson (1991) reported that only the B-pathway was observed in canola and they proposed that a symmetric mitotic division of pollen was a key factor in embryogenesis. In many plant species, a deviation from the normal asymmetric divisions, typically of the first gametophytic pollen mitosis, accompanied the induction of embryogenesis (Sangwan and Sangwan-Nonel, 1987; Sunderland and Huang, 1987; Zaki and Dickinson, 1990; 1991; Simmonds and Keller, 1999; Bonet and Olmedilla, 2000). It has been suggested that the change in division symmetry might be sufficient to induce pollen embryogenesis (Zaki and Dickinson, 1990; Telmer et al., 1993, 1995). Induction of symmetrical divisions at pollen mitosis I using microtubule inhibitors or centrifugation (Tanaka et al., 1981) results in the formation of two equally sized cells, neither of which possesses the condensed nuclear chromatin characteristic of the generative cell (Twell et al., 1998). Cell fate monitoring in tobacco pollen matured in vitro after colchicine treatment showed that the pollen-specific lat52 promoter was activated in both daughter cells following symmetric division (Eady et al., 1995). Despite the arguing of some authors, division symmetry does not appear to be causally related to the initiation of pollen embryogenesis. For example, in the absence of a stress treatment, markers of gametophytic development (i.e. *lat52-gus* expression and pollen germination) are expressed in cultured tobacco pollen even after induction of symmetric divisions (Eady et al., 1995; Touraev et al., l995). Both symmetrical (Ouyang et al., 1973; Zheng and Ouyang, 1980; Hu and Kasha, 1999; Bonet and Olmedilla, 2000) and asymmetrical (Hassawi et al., 1990; Hu and Kasha, 1999) pollen mitosis I have been reported in wheat embryogenic pollen. Different culture conditions and treatments used seem to be more important in the definition of pollen mitosis I (Hu and Kasha, 1999; Kasha el al., 2001). Hu and Kasha (1999) examined the influence of several anther and spike treatments on pollen embryogenesis and found a clear effect of treatment on pollen mitosis I whether to be symmetric or asymmetric. Using mannitol treatment of wheat anthers for 7 days at 28^{o}C, mainly symmetrical pollen divisions were found (Hu and Kasha, 1999).

Asymmetrical pollen mitosis I has frequently occurred after cold pretreatment (Hu and Kasha, 1999; Kasha et al., 2001).

1.3. Samples preparation for microscopic examination

Excellent sample preparation and fixation is a prerequisite for all types of microscopy. The goal is to preserve tissue and cellular structures as faithfully as possible under physiologically active conditions at the best possible resolution. In the post-genomic era, the cutting edge research is moving towards the mapping and visualization of dynamic cellular processes or structural changes *in vivo*. The ultrastructural preservation of different stages of pollen gametogenesis or embryogenesis with highly vacuolated cells or multicellular structures surrounded by a poorly penetrable cell wall presents a major challenge.

Chemical fixation is based on selective cross-linking of molecules. Due to the relatively slow penetration of the fixatives, a possible dislocation of macromolecules and thus a possible change of ultrastructure can not be excluded (Mersey and McCulley, 1979). Kääb et al. (1999) and Heumann (1992) showed that microwave-assisted chemical fixation (MWCF) provided preservation of morphological structures within seconds. The energy band of the electromagnetic spectrum of the microwave causes rotation of dipolar molecules (i.e. water, glutaraldehyde, proteins), which leads to a quick and homogeneous temperature increase on non-conductive materials within the radiated sample. As a result chemical reactions, including fixations, are speeded up considerably (Heumann, 1992; Giberson et al., 1995). Using the microwave radiation, fixation times of chemical fixatives such as formaldehyde and glutaraldehyde (Leong and Leong, 1997), can be reduced to seconds, which significantly improves the resolution of fine structures (Heumann, 1992). This form of non-ionizing radiation is employed to accelerate numerous procedures embracing all aspects of the preparation of tissues for optical microscopy and ultrastructural examination and does not have any deleterious effects on histochemical staining and immunological labeling efficiency (Leong et al., 1985).

So far there is no conclusive explanation available for the precise physicochemical events that influence the rapid fixation of specimens exposed to microwave radiation (Login et al., 1987). Benhamou et al. (1991) showed that the diffusion of the fixative into tissues

and cells is greatly enhanced by the instantaneous thermal effect of microwave radiation. Leong and Sormunen (1998) showed that heating of a 4% formaldehyde solution to 60-70°C hastens the fixation process. However, it is also speculated that, in addition to the thermal component, electric fields may influence the penetration rate of fixatives. Support for this assumption comes from observations, that samples fixed without microwave irradiation at a temperature similar to that reached during microwave exposure, did not yield a similar ultrastructural preservation (Hopwood et al., 1984; Leong et al., 1985).

Although chemical fixation and in particular MWCF is very useful for morphological and histological aspects, these advances have pushed the limits of chemical fixation and allowed generating reliable data for high resolution microscopy, resulting in an increased interest in cryofixation for the sake of ultra-rapid immobilization of biological structures.

One of the most challenging techniques in sample preparation for the preservation of ultrastructure and macromolecular assemblies *in vivo* is high pressure freezing (HPF) followed by freeze substitution (FS). This process can overcome the limitations of chemical fixation by immobilizing all molecules within milliseconds (Gilkey and Staehelin, 1986; Moor, 1987; McDonald, 1999; Vanhecke and Studer, 2009). The ultra-rapid freezing process vitrifies cellular water without the formation of ice crystals, or forms a large number of very small ice crystals (1 nm or less), so that they do not create significant distortions for many electron microscopy studies. During freeze substitution this vitrified water is replaced by an organic solvent. Simultaneously, by adding fixatives such as osmium or glutaraldehyde to the freeze substitution medium, the ultrastructural preservation and contrast can be enhanced. The low temperature allows fixatives to infiltrate the specimen while suppressing cross-linking reactions. The warming up of well infiltrated specimens results in an homogenous fixation avoiding diffusion gradient-related artifacts (Giddings, 2003; Buser and Walter, 2007). HPF followed by FS has been suggested as an improved sample preparation method where the aim is to characterize the molecular content and structure of macromolecular assemblies in cells that are complicated to be fixed (McDonald and Morphew, 1993, McDonald, 1995), such as immature pollen with exine and intine. Taken together, the employment of HPF-FS

preserves **ultrastructural** changes of pollen embryogenesis in their native state and with **high** spatial and **temporal** resolution.

1.4. Aims and scope of the thesis

The importance of pollen embryogenesis and doubled haploid technology with its unique potential for basic research and crop improvement are in strong contrast to the poor understanding of their underlying biological processes. Little is known about the cellular particularities of this process in general and more specifically in the economically important monocots. For barley, which is considered a model of cereal crops for pollen embryogenesis research, detailed structural investigations are lacking.

Therefore, the scope of this research was to conduct **a comparative structural analysis of barley pollen embryogenesis and gametogenesis** in order to **identify structural markers and features of the embryogenic pathway** i.e. plastid differentiation, first pollen mitosis and the mechanism of spontaneous genome doubling. To understand the initial mechanisms of the process, it was essential **to first establish appropriate sample preparation protocols for basic histological and high-resolution ultrastructural analysis of barley pollen.** Simultaneously, a **protocol for live-cell imaging had to be established to visualize cell dynamic processes of pollen embryogenesis** and to characterize the initial mechanisms of this developmental process.

To meet the individual aims, the following approaches were conducted:

- In order to identify structural and ultrastructural features of pollen embryogenesis with high spatial and temporal resolution at a close-to-native state, a protocol of high pressure freezing was established.

- To describe the different pollen types and follow the development of an individual pollen during the process of embryogenesis, live-cell imaging in a custom-made chamber was performed to host cultured pollen over a long period of time (between 2 weeks to a month).

- To monitor the behavior of nuclei and study the contribution of generative and/or vegetative nuclei to pollen embryogenesis, a transgenic line was generated that accumulates GFP in the pollen nucleus.

- To achieve high induction of pollen embryogenesis for successful live-cell imaging experiments, a new protocol was set up for a sequential treatment of anthers and isolated immature pollen.

The thesis is composed of four main subchapters. The first subchapter (3.1) deals with the comparison of pollen structure preservation by conventional chemical fixation, microwave-assisted fixation and high pressure freezing fixation methods. In addition, it shows how cyanobacteria were introduced to improve the protocol of HPF to gain the best possible preservation of ultrastructure of embryogenic and gametophytic pollen. The second subchapter (3.2) deals with the comparison of structural and ultrastructural features of pollen embryogenesis vs. gametogenesis in order to identify the early structural markers of induced pollen. The third subchapter (3.3) covers the generation of transgenic barley with GFP-labeled nuclei. Eventually, the fourth subchapter (3.4) presents a protocol that results in pollen embryogenesis with improved efficiency, different developmental types of pollen during live-cell experiments and the mechanism of spontaneous genome doubling.

2. Materials and methods

2.1. Immature pollen isolation and culture

2.1.1. Donor plants and growth condition

Barley (*Hordeum vulgare* L.) plants cv. 'Igri' (Saatzucht Ackermann, Irlbach, Germany) were germinated in a growth chamber (14/12°C day/night, 16 h light cycle), followed by 8 weeks vernalization treatment (2°C, 9 h light cycle) and cultivation in a climate controlled glasshouse (18/14°C day/night, 16 h light cycle). Artificial illumination was provided by SON-TAgro lamps (Philips, Hamburg, Germany) at about 200 W/m^2.

2.1.2. Nutrient media for embryogenic pollen culture

The culture media used were prepared with autoclaved macronutrients and sterile-filtered stock solutions (micronutrients, vitamins, NaFeEDTA). The pH of the media was adjusted with NaOH and HCl solutions. Media were sterilized by filtration through a Nalgene 0.2µm filter (Rochester, USA). All chemical solutions in this study were prepared using purified Milli-Q water (Millipore) with a resistance of 18 MΩ.cm.

2.1.2.1. Starvation medium

The Starvation Medium Barley 1 (SMB 1) described by Corronado et al. (2005) to stress immature pollen is made up of 0.4 M maltose, 1 mM $CaCl_2$, 1 mM NH_4Cl, 1x KBP-Micro minerals (50 mM $MnSO_4$, 50 mM H_3BO_3, 25 mM $ZnSO_4.7H_2O$, 0.5mM $Na_2MoO_4.2H_2O$, 0.1 mM $CuSO_4.5H_2O$, 0.1$CoCl_2.6H_2O$, 1mM KI), 4 µM benzyladenine (BA) and 2 mM morpholinoethanesulfonic acid, pH adjusted to 5.5.

2.1.2.2. Induction medium

The Kumlehn Barley Pollen medium (KBP, Kumlehn et al., 2006) in which immature pollen were incubated after their stress treatment was composed of KBP-Macro minerals (20 mM NH_4NO_3, 400 Mm KNO_3, 50 mM KH_2PO_4, 60 mM $CaCl_2.2H_2O$, 20 mM $MgSO_4.7H_2O$), 1x KBP-Micro minerals, 75 µM NaFeEDTA, 4 µM BAP, 0.25 M Maltose, 3 mM glutamine and 1x KAO and MICHAYLUK VITAMIN SOLUTION-100x (Sigma) with pH adjusted to 5.9.

2.1.2.3. Immobilization medium

Immobilization medium used during live-cell imaging was composed of double-concentrated filter-sterilized SMB1 medium which was mixed and homogenized at room temperature with double concentrated autoclaved agarose gel (12g/l). Immobilization media were always prepared immediately before usage.

2.1.3. Spikes collection and immature pollen isolation

After a cytological determination of developmental stages of the spike according to the method of He and Ouyang (1984), a correlation was established between the spike phenotype and the developmental stages of the pollen. For this purpose, spikes of a wide developmental range were used. Samples were collected from the middle third of a spike. Only spikes that contained highly vacuolated immature pollen, a stage only found immediately before and after the first pollen mitosis, were used for further experiments. The corresponding spike phenotype was when the tips of the awns had just emerged from the sheath of the flag leaf (Fig. 1a). The central two-thirds of such spikes florets were used for immature pollen isolation and culture.

Immature pollen was isolated and cultured according to Kumlehn et al. (2006) with minor modifications. Harvested tillers were transported to a horizontal laminar flow bench and sprayed with 70% ethanol for 30 seconds to sterilize their surface. They were then dried by sterilized paper tissue. All subsequent steps were carried out under aseptic conditions. Spikes were removed from the flag leaf sheath and awns were cut off. A total of 8 to 10 spikes were cut into segments of about 1 cm and transferred into a Waring blender (Eberbach, Ann Arbor, MI, USA) pre-cooled to 4°C in a refrigerator (Fig. 2b). Tissues were homogenized in 18 ml of ice-cold 0.4 M mannitol by blending twice for 5 s at 'low' speed. The homogenate was poured through a 100-µm nylon mesh (Wilson, Nottingham, UK) into a plastic container on ice (Fig. 2c). The Waring blender was washed with 10 ml 0.4 M mannitol which was collected and poured through 100-µm nylon mesh. The debris retained on the mesh was gently squashed by forceps (Fig. 2d) and transferred back into the blender. The homogenization process was repeated a second time now using 12 ml of ice-cold 0.4 M mannitol solution. Also now the Waring blender was rinsed afterwards with 10 ml of 0.4 M mannitol solution which was collected and filtered through 100-µm

nylon mesh. The pooled immature pollen suspensions were transferred into a 50 ml tube. To reduce the number of potentially damaging centrifugation steps (see Kumlehn et al., 2006) excess liquid of the suspension was carefully pipetted off through a 30 µm nylon filter fixed on top of a cut off end of a 1 ml pipette tip (Fig. 2e). The pellet thus obtained was re-suspended in 5 ml ice-cold 0.55 M maltose in a 15 ml tube. The suspension was carefully overlaid with 1.5 ml 0.4 M ice-cold mannitol (Fig. 2f, g). After density gradient centrifugation at 4°C at 100 ***g*** in a swing-out rotor for 10 min, a nearly pure population of

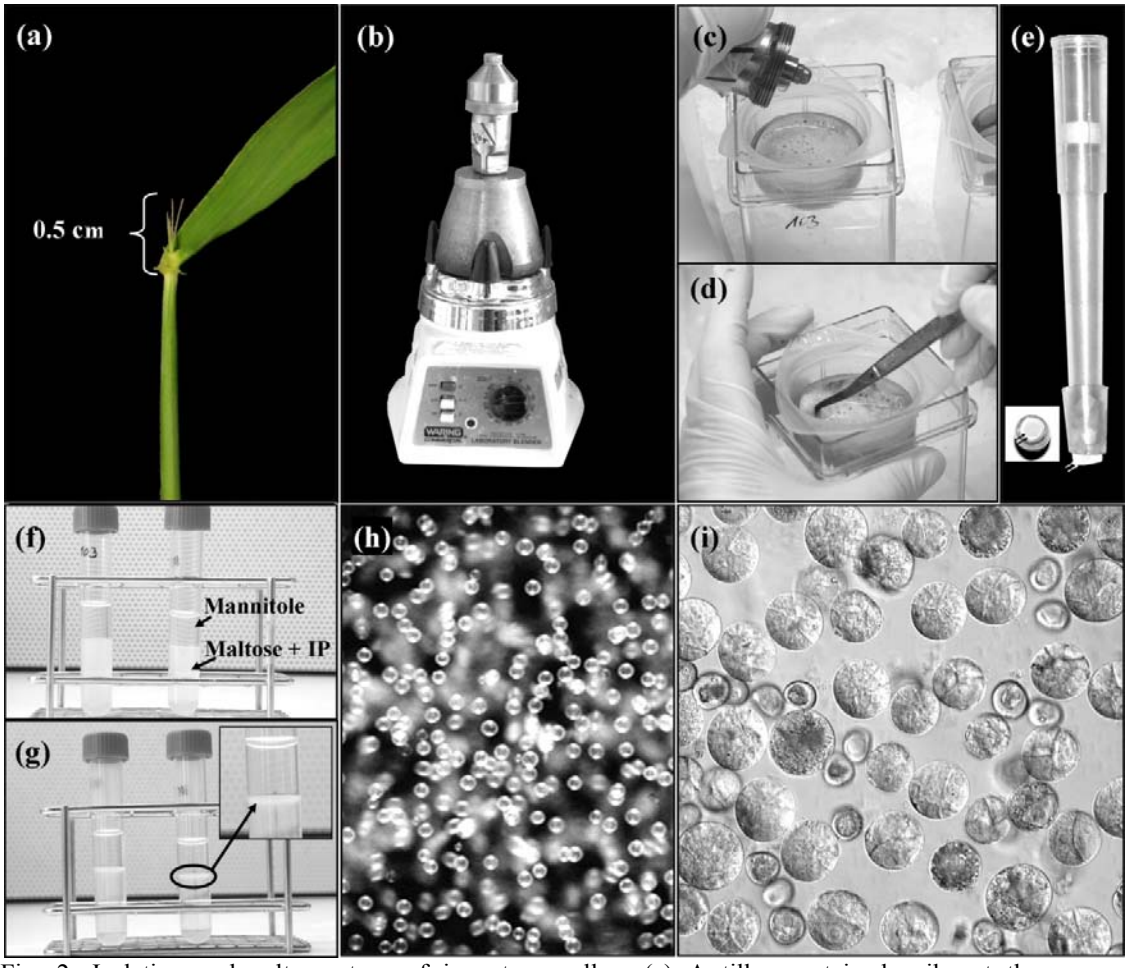

Fig. 2: Isolation and culture steps of immature pollen. (a) A tiller contained spike at the proper developmental stage (bracket indicates the length of awns outside the flag leaf sheath). (b) Waring blender (Eberbach, Ann Arbor, MI, USA) used to homogenize spike tissue. (c) Filtering of the homogenate over a 100 µm mesh nylon filter, collecting the filtrate in a plastic container on ice. (d) Gently squashing of the debris retained on the filter by forceps. (e) 30 µm nylon filter fixed on top of a cut off end 1 ml pipette tip (f) 15 ml tube with the 0.55 M maltose solution containing the immature pollen overlaid with 1.5 ml 0.4 M mannitol. (g) After centrifugation, viable vacuolated immature pollen has accumulated at the interface. (h) Suspension of potentially embryogenic immature pollen in starvation medium immediately after isolation. (i) Multicellular structure, 7 days in KBP culture. IP, immature pollen.

highly vacuolated immature pollen was collected from the interphase (Fig. 2g) and transferred into a 50-ml tube. The final volume was increased to 20 ml with 0.4 M mannitol. Prior to the final pelleting two 20-µl samples were taken to assess the total number of pollen using a haemocytometer (Paul Marienfeld, Lauda-Königshofen, Germany). After centrifugation, excess medium was discarded as described previously. Starvation medium was added to obtain a population density of $2x10^5$ pollen per ml (Fig. 2h).

2.1.4. Embryogenic pollen culture

Viable immature pollen was cultured mainly at 200000 cells/ml in SMB1 medium in 35-mm petri dishes. After stressing immature pollen in SMB1 medium for 2 days at 14°C in the dark, SMB1 was pipetted off through a 30 µm nylon filter fixed at the cut-off end of a 1 ml pipette tip and replaced by KBP medium. Care was taken to let the cells not dry out. Cultures were incubated at 25°C in the dark.

For the correct developmental stage wheat plants were used in which the spikes were out of the flag leaf sheath by 3 to 4 cm. Spikes were surface sterilized by spraying with 70% ethanol for 30 seconds and dried on sterile paper tissue in a Horizontal Laminar Flow Bench. After removing the glumes of the florets, ovaries were isolated, cut longitudinally followed by incubation in KBP medium for 2 to 3 days at 10°C in dark before co-culturing with immature pollen.

2.2. Fixation of immature pollen for microscopic analysis

To study pollen embryogenesis, cultured immature pollen at various developmental stages ranging from 0 to 12 days of culture was prepared for fixation. Prior to either CCF, WMCF or HPF, the immature pollen was concentrated into a pellet in 2 ml Eppendorf tubes by careful pipetting off excess of culture medium through a 30 µm nylon filter (see above) while at the same time taking care not to let the pellet dry out. This step proved superior to centrifugation which could result in a significant portion of damaged cells. In the subsequent steps, however, after the immature pollen had been fixed, the cells were pelleted by centrifugation at 1000 rpm for 3 min. at room temperature. The pellets were loosened up by gently tapping the Eppendorffs. Under these conditions and despite their

high concentration, immature pollen remained in good physiological conditions until fixation.

Also in the study of pollen gametogenesis, fresh pollen grains were isolated at different developmental stages of pollen maturation according to spikes morphology. Spikes were collected at booting, heading and anthesis stages (Zadoks et. al., 1974). Pollen grains were isolated from the middle third of the spikes. For basic histological studies chemically fixed pollen was used. This chemical fixation was carried out either at room temperature or in a laboratory microwave oven. For ultrastructural studies, the fixation procedure of choice was HPF.

2.2.1. Conventional chemical fixation, dehydration and resin embedding

Isolated immature pollen was fixed in 50 mM cacodylate buffer (pH 7.3) containing 2% (v/v) glutaraldehyde and 2% (w/v) formaldehyde at RT for 2h. After three time washes with cacodylate buffer and aqua dest., the samples were post-fixed with 1% (w/v) OsO_4 in H_2O for 30 min. in dark (White et al., 1979; Langford and Coggeshall, 1980 and 1981). After three washes with aqua dest., samples were dehydrated in an ethanol series of ascending concentration (Table 1) followed by two additional incubations in 100% propylene oxide (PO) for 1 h to facilitate the subsequent infiltration with Spurr resin (Plano GmbH, Marburg, Germany). Spurr resin infiltration (Spurr, 1969) was performed as shown in Table 1. After the infiltration procedure was completed samples were transferred into BEEM capsules and kept for 6 h in fresh resin followed by polymerization over night in an oven at 70°C (Table 1).

Table 1: Protocol for conventional chemical fixation, dehydration and resin embedding of barley pollen

Process	Chemical	Temperature	Duration
Primary fixation	2% (v/v) glutaraldehyde and 2% (w/v) formaldehyde in 50 mM cacodylate buffer (pH 7.3)		2 h
Washing step	Three times with 50 mM cacodylate buffer (pH 7.3)		5 min
			5 min
			5 min
Washing step	Three times with aqua dest.		5 min
			5 min.
			5 min
Post-fixation	1% (v/v) osmium tetroxide in aqua dest.		30 min
Washing step	Three times with aqua dest.		5 min
			5 min
			5 min
Dehydration	30% (v/v) Ethanol	21°C	1 h
	40% (v/v) Ethanol		1 h
	50% (v/v) Ethanol		1 h
	60% (v/v) Ethanol		1 h
	75% (v/v) Ethanol		1 h
	90% (v/v) Ethanol		1 h
	100% Ethanol		1 h
	100% Propylene oxide		1 h
Infiltration	25% (v/v) Spurr/ Propylene oxide		2 h
	50% (v/v) Spurr/ Propylene oxide		2 h
	75% (v/v) Spurr/ Propylene oxide		2 h
	100% Spurr		6 h
Polymerization	100% Spurr	70°C	24 h

2.2.2. Microwave assisted chemical fixation, dehydration and resin embedding

Login et al. (1987) demonstrated that microwave energy can be used in conjunction with chemical cross-linking agents like aldehydes to rapidly fix biological tissue and cells for microscopy. To take advantage of this time-saving procedure, isolated immature pollen was chemically fixated in a microwave as described in Table 2. Microwave irradiation was delivered through a dedicated laboratory microwave oven (Model PELCO BioWave® 34700-230, Ted Pella, Inc, CA, U.S.A.).

Table 2: Protocol for microwave-assisted chemical fixation, dehydration and resin embedding of immature pollen

Process	Chemical	Irradiation	Time	Vacuum
Primary fixation	2.0% (v/v) glutaraldehyde and 2.0% (w/v) formaldehyde in 50 mM cacodylate buffer (pH 7.3)	150 W	1 min.	--
		0 W	1 min.	--
		150 W	1 min	--
		0 W	1 min	--
		150 W	1 min.	15 mm Hg
		0 W	1 min.	15 mm Hg
		150 W	1 min.	15 mm Hg
		0 W	1 min.	15 mm Hg
Washing step	Three times: 1x with 50 mM cacodylate buffer (pH 7.3) and 2x water	150 W	45 Sec.	--
		150 W	45 Sec.	
		150 W	45 Sec.	
Post-fixation	1% (v/v) osmium tetroxide in aqua dest.	0 W	1 min.	15 mm Hg
		80 W	2 min.	
		0 W	1 min	
		80 W	2 min	
Washing step	Three times with aqua dest.	150 W	45 Sec.	--
		150 W	45 Sec.	
		150 W	45 Sec.	
Dehydration	30% (v/v) Ethanol	150 W	45 sec.	--
	40% (v/v) Ethanol	150 W	45 sec.	--
	50% (v/v) Ethanol	150 W	45 sec.	--
	60% (v/v) Ethanol	150 W	45 sec.	--
	75% (v/v) Ethanol	150 W	45 sec.	--
	90% (v/v) Ethanol	150 W	45 sec.	--
	100% Ethanol	150 W	45 sec.	--
	100% Propylene oxide	150 W	45 sec.	--
Infiltration	30% (v/v) Spurr/ Propylene oxide	350 W	3 min.	5 mm Hg
	40% (v/v) Spurr/ Propylene oxide	350 W	3 min.	5 mm Hg
	50% (v/v) Spurr/ Propylene oxide	350 W	3 min.	5 mm Hg
	60% (v/v) Spurr/ Propylene oxide	250 W	3 min.	5 mm Hg
	60% (v/v) Spurr/ Propylene oxide	250 W	3 min.	5 mm Hg
	60% (v/v) Spurr/ Propylene oxide	250 W	3 min.	5 mm Hg
	60% (v/v) Spurr/ Propylene oxide	250 W	3 min.	5 mm Hg
	100% Spurr	250 W	3 min.	5 mm Hg
	100% Spurr	250 W	3 min.	5 mm Hg
Polymerization: 100% Spurr at 70°C for 24 h				

2.2.3. Cryo-fixation using high pressure freezing

To obtain preservation of the ultrastructure at highest resolution, immature pollen was high pressure frozen followed by freeze substitution (McDonald and Morphew, 1993). The establishment of a successful HPF protocol for pollen was essential to obtain high structural resolution. A number of specimen fillers were tested for their ability to avoid

freezing artifacts during HPF. Eventually a method was established using cyanobacteria as filler in nitrocellulose capillaries to improve ultrastructural preservation of immature barley pollen upon high pressure freezing (Daghma et al., 2011).

2.2.3.1. Cultivation of Cyanobacteria

Cyanobacteria (*Synechocystis 6803* or *Synechcoccus 7942*) were used as space filler in between the pollen grains during HPF. As described by Ferris and Hirsch (1991) both cyanobacterial strains were grown in BG-13 medium consisting of (per liter) :$NaNO_3$ (1.5 g), $NaHCO_3$ (1.7 g), K_2HPO_4 (31 mg), $MgSO_4$ $7H_2O$ (75 mg), $CaCl_2$ H_2O (36 mg), Na_2CO_3 (20 mg), citric acid (6 mg), ferric ammonium citrate (6 mg), disodium magnesium EDTA (1 mg), H_3BO_3 (2.86 mg), $Mn_2Cl_2.4H_20$ (1.81 mg), $ZnSO_4.7H_2O$ (220 µg), $Na_2MoO_4.2H_2O$ (390 g), $CuSO_4.5H_2O$ (80 µg), and $CoCl_2.6H_20$ (40 g). Under an atmosphere of 5% (v/v) CO_2 the pH of the BG-13 medium typically was 7.5 to 7.6. Media were sterilized by autoclaving for 20 min at 15 lb/in^2. Cultures were incubated on a shaker at 180 to 200 rpm and kept at 30°C under continuous illumination with 40-W cool-white fluorescent lamps at an irradiance of 3 to 5 klux.

2.2.3.2. Cultivation of yeast

The wild-type *Arxula adeninivorans* strain LS3 (originally isolated from wood hydrolysates in Siberia) was an additional essential element in the high pressure freezing protocol established. *Arxula* was grown under non-selective conditions on a yeast solid rich medium for 3 to 4 days, before inoculation into liquid minimal medium according to Tanaka et al. (1967). The liquid culture was incubated for 2 days under orbital shaking at 30°C and 180 rpm until an optical density (OD_{600nm}) of 20 was reached. The solid rich media contained 0.5% Pepton, 0.5% yeast extract, 1% glucose and 2% Bacto-Agar in aqua dest.. The pH was adjusted to 6.5 and the medium was autoclaved for 20 min. at 121^{o}C.

Yeast minimal medium contained (per liter) 3.7 g $NaNO_3$, 6.75 g KH_2PO_4, 1.75 g K_2HPO_4, 2 mg Ca $(NO_3)_2.4H_2O$, 0.2 mg $FeCl_3.6H_2O$ and 1 ml component II (100 ml component II contains 50 mg H_3BO_4, 10 mg $CuSO_4.4H_2O$, 10 mg KI, 40 mg

$MnSO_4.4H_2O$, 40 mg $ZnSO_4.7H_2O$, 20 mg Na_2MoO_4 and 10 mg $CoCl_2$). After adjusting the pH to 7 the medium was autoclaved for 20 min. at 121°C.

After autoclaving, 0.5% vitamin mix stock solution and 2% carbon source (glucose or sorbitol) were added to the minimal liquid medium. Vitamin mix stock solution contained 40 mg C-D-pantothenat, 40 mg thiamindichlorid, 10 mg nicotinic cid, 40 mg pyridoxin, 0.4 mg biotin and 400 mg inosit in 100 ml aqua dest.. All chemicals used for preparing the yeast medium were purchased from SIGMA.

Before HPF, in Falcon tubes approximately 15 ml of cyanobacteria culture and yeast culture were separately centrifuged for 3 min. at 2000 rpm (Eppendorf Centrifuge-5403, Universal Labortechnic, Leipzig, Germany) at room temperature. Excess medium was pipetted off and discarded. Pellets were taken up in 2 ml KBP medium and transferred into 2 ml Eppendorf tubes. After three more washes for 5 min. the pellets were resuspended once more in 2 ml KBP medium. Then 200 µl suspension of cyanobacteria and yeast respectively were transferred into 200 µl yellow pipette tips with sealed ends, and centrifuged for 3 min. at 2000 rpm to generate very dense pellets of cyanobacteria and yeast (Fig. 3a).

2.2.3.3. High pressure freezing and freeze substitution

HPF was performed in a Leica EM HPF (Leica Microsystems, Bensheim, Germany). Crucial for the successful HPF of a cell suspension is the reduction of the aqueous phase of the culture medium, here called void volume, to a minimum. Several methods were tested to achieve the most optimal conditions. Initially the 0.15 standard aluminum platelets (Engineering Office M. Wohlwend GmbH, Switzerland) were filled with a pellet of concentrated immature pollen. In an adaptation of this method the pellet of immature pollen was first mixed 1:1 with a pellet of fresh cyanobacteria before being applied into the aluminum platelets. An alternative approach was the use of nitrocellulose capillaries (Cat. No. LH 01843 VN, BAL-TEC, Balzer, Switzerland). Immature pollen was loaded into capillaries through capillary force. Capillaries were loaded with pure immature pollen pellet or with a 1:1 mixture of immature pollen and cyanobacteria pellets. Once loaded, the nitrocellulose capillaries were cut into pieces of approx. 2.0 mm in length and transferred into the aluminum platelets.

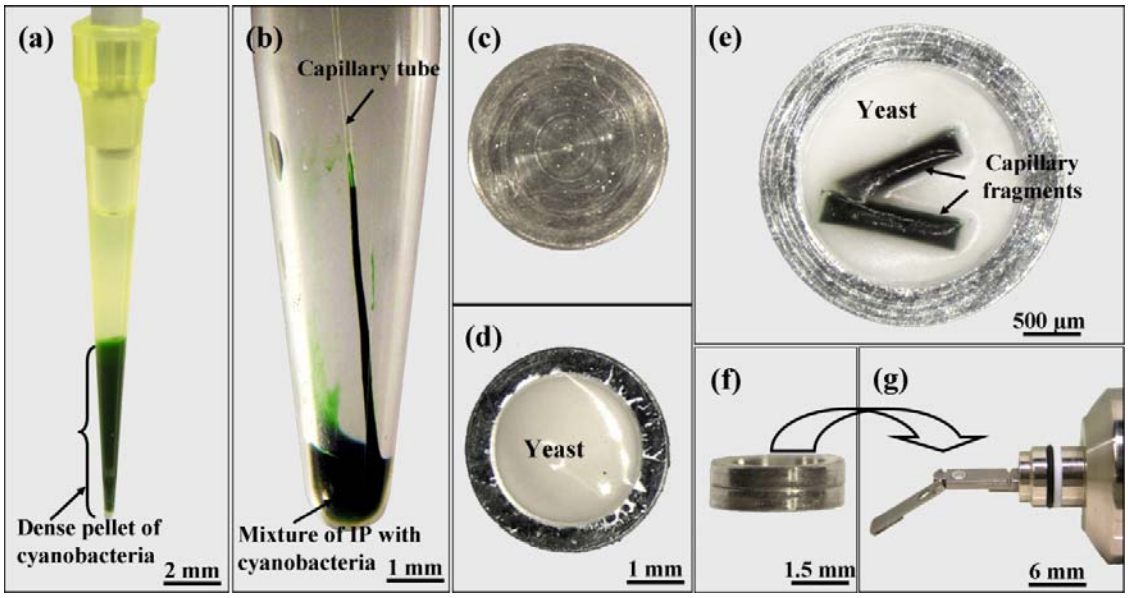

Fig. 3: Loading a mixture of immature pollen and cyanobacteria into nitrocellulose tube before high pressure freezing. (a) Dense pellet of cyanobacteria. (b) Mixture of a dense pellet of cyanobacteria and concentrated immature pollen being loaded into capillary tube. (c) A 0.3 mm aluminum cover. (d) A 0.15 mm aluminum platelet filled with fresh yeast paste. Note the shiny surface of the paste, indicating correct moisture conditions. (e) A 0.15 mm aluminum platelet loaded with filled capillary fragments embedded in yeast. (f) Covered sample. (g) Sample loaded into the HPF specimen holder prior to HPF. IP, immature pollen.

Table 3: Freeze substitution and resin infiltration of immature barley pollen after high pressure freezing

Freeze substitution			
98%	Acetone + 1% GA	-90°C	46 h
98%	Acetone + 1% GA	-90°C to -70°C	5 h
98%	Acetone + 1% GA	-70°C	24 h
98%	Acetone + 1% GA + 1% Os	-70°C to -50°C	5 h
98%	Acetone + 1% GA + 1% Os	-50°C	24 h
98%	Acetone + 1% GA + 1% Os	-50°C to -35°C	3 h
98%	Acetone + 1% GA + 1% Os	-35°C	24 h
98%	Acetone + 1% GA + 1% Os	-35°C to 20°C	6 h
100%	Acetone	RT	1 h
100%	Acetone	RT	1 h
Propylene oxide		RT	1 h
Infiltration with Spurr's resin and polymerization			
25%	Spurr / Acetone	RT	6h
50%	Spurr / Acetone	RT	6h
75%	Spurr / Acetone	RT	6h
100%	Spurr	RT	Over night
Polymerization		70°C	24 h

The empty space around these capillary pieces was completely filled up either with hexadecene or with a fresh yeast pellet paste (Fig. 3e). In case of using the latter method one has to make sure that the yeast does not dry out. Note the shiny surface of the paste (Fig. 3d). After covering the samples with the flat side of a 0.30 mm platelet, they were placed in the Leica EM HPF and high pressure frozen under ca. 2000 bar. Samples were transferred to an automated FS unit (Leica Microsystems, Bensheim, Germany) and incubated with 1% (v/v) glutaraldehyde in acetone at -90^{o}C (Table 1). The small capillary pieces were removed from the platelets and cleared of remaining yeast or hexadecene to facilitate the FS process. Freeze substitution, Spurr's resin infiltration and polymerization was performed as shown in Table 3.

2.2.4. Trimming of resin blocks

Blocks with the embedded immature pollen were trimmed with a Leica EM TRIM (Leica Microsystems, Bensheim, Germany) device. Since the shape of the trimmed sample has a profound effect on the sectioning characteristics, trapeze-like structures with parallel edges at the top and bottom are required.

2.2.5. Sectioning of resin blocks

Trimmed blocks were cut with a Leica Ultracut ultramicrotome (Leica Microsystems, Bensheim, Germany). The rough surface after the trimming was precut with glass knife. Semi-thin (2 µm) and ultra-thin (70 nm) sections were cut with diamond knifes ultra 45° and ultra 35° respectively (Diatome, Weinheim, Switzerland). Single semithin or serial sections were collected on poly-L-lysine coated slides (SIGMA) and incubated for 2-5 min on a heating plate set at 80°C. Ultratin sections were collected on 75 mesh hexagonal copper grids (Agar Scientific Ltd., Stansted, United Kingdom) and stored in grid boxes.

2.2.6. Staining and contrasting

2.2.6.1. Histological staining

For histological observations semi-thin sections were post-stained with 1% (v/v) methylene blue / 1% (v/v) Azur II in 1% (v/v) aqueous borax for 2 minutes on a heating

plate set at 60°C. After washing, the slides were dried and cover slipped with Entellan embedding medium (Merck, Darmstadt, Germany). Sections were examined in a Zeiss Axiovert 135 microscope. Digital images were made with a Zeiss Axiocam (type HRc) camera.

2.2.6.2. Contrasting of ultra thin sections

For electron microscopy ultra-thin sections were contrasted in LEICA EM STAIN (Leica Microsystems, Vienna, Austria) with 0.5% uranyl acetate (Ultrostain 1, Laurylab, Sain-Fons, France) for 30 min followed by an incubation in 3% lead citrate (Ultrostain 2, Laurylab, Sain-Fons, France) for 90 sec. Ultrastructural observations were made in a Tecnai 20 Sphera G2 transmission electron microscope (FEI, Eindhoven, Netherlands) at 120kV.

2.2.6.3. Fluorescence labeling

To visualize nuclei during the development of immature pollen into multicellular structures, either chemically fixed or unfixed immature pollen were stained with a solution of 1 µg/ml DAPI in aqua dest. supplemented with 1% (v/v) triton X100 for 10 min. In an alternative approach the staining was performed in a laboratory microwave oven (PELCO BioWave® 34700-230, Ted Pella, Inc, CA, U.S.A.) using 150 W irradiation and 15 mm Hg vacuum for 1 min. After washing in aqua dest., immature pollen samples were mixed on a slide with a 1:1 mixture of glycerol/PBS, covered with a coverslip and sealed with nail varnish without squashing. Samples were observed with an Enterprise II UV laser using a 354 nm excitation line in combination with a 375-475 nm band-pass filter using a LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany).

2.2.7. 3D reconstruction of embryogenic pollen

For 3D reconstruction of entire pollen series of semi-thin sectioning (2 µm) was performed. The collected sections were histologically stained as previously described. After selecting cells of interest, serial sections of the region of interest were imaged using a Zeiss Axiovert 135 (Fig. 4) and used for 3D reconstruction. The contrast of individual

recordings was optimized with Photoshop version CS3, reconstructions were performed with ImageJ software from NIH-Image (http://rsbweb.nih.gov/ij/).

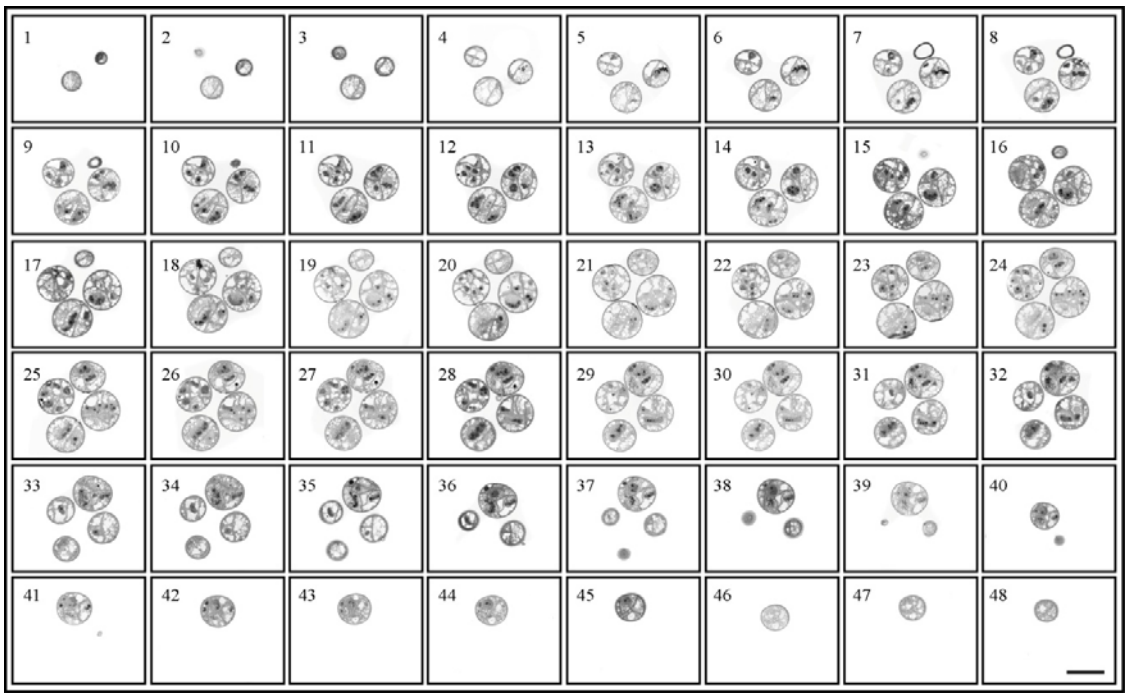

Fig. 4: Serial sectioning of immature pollen. Histological stained sections from 6 days old immature pollen were used for 3D reconstruction. Numbers on figure refer to the section number.

2.3. *Agrobacterium*-mediated gene transfer into embryogenic pollen

2.3.1. Cultivation and manipulation of *Agrobacterium*

Agrobacterium tumefaciens strain LBA4404 (Komari et al., 1996) was grown on CPY medium (Komari et al., 1996) which contains 5 g/l sucrose, 500 mg/l $MgSO_4$, 5 g/l peptone and 1 g/l yeast extract, pH 7.2. Medium was solidified with 0.8% agar and autoclaved at 120°C for 20 min. After autoclaving, the antibiotics spectinomycin (100 µg/ml) and tetracycline (10 µg/ml) were added. Liquid culture of A. *tumefaciens* was grown on 28°C in Erlenmeyer flasks on a shaker at 180 rpm. Before co-culture with embryogenic pollen, standard glycerol stocks were prepared by mixing 200 µL from a single *Agrobacterium* colony-derived culture at OD_{600} 2.0 to 2.5 with 200 µL of 15% glycerol. The stock was stored at -80°C until use. A single tube of cryo-stock was thawed

and added to 10 ml of CPY medium in a 100-ml Erlenmeyer flask and grown over-night at 28°C on a shaker at 180 rpm.

The E. coli strain DH10B was grown at 37°C on LB-medium as described by Silhavy et al. (1984).

2.3.2. Transformation vector

For the generation of transgenic barley plants, the hypervirulent *A. tumefaciens* strain LBA4404 was used carrying the binary vector pGH252n (Hensel et al., unpublished). The plasmid pGH252n includes an *HPT* selectable marker gene driven by the maize *UBIQUITIN 1* promoter with the first intron (Christensen and Quail, 1996), an *NLS-sgfp* (S65T) reporter gene (Chiu et al., 1996) driven by the same promoter as the *HPT* gene and the vector backbone from p6U (DNA-Cloning-Service, Hamburg, Germany) with its borders derived from a nopaline Ti plasmid. The binary vector was introduced into *A. tumefaciens* by electroporation.

2.3.3. Generation of transgenic plants

According to Kumlehn et al. (2006), six to eleven days after immature pollen isolation, the KBP medium was removed from the embryogenic pollen cultures using a disposable pipette and replaced by 1 ml of CK medium (Table 4). The population density of the *Agrobacterium* pre-culture was determined using a spectrophotometer and an appropriate volume was added to a 1 ml culture of embryogenic immature pollen to obtain a bacterial density of 10^8 cfu/ml, if not stated otherwise. Dishes were incubated at 21°C under shaking at 65 rpm. After 48 h of co-culture, the medium was removed by a disposable pipette; the pollen was washed in 0.4 ml of ASt medium and subsequently cultured in 1.1 ml of ASt medium supplemented with selective agents (Table 4). After 7 days of incubation at 26°C with shaking at 65 rpm, the ASt medium was replaced by 1.5 ml of S medium (Table 4) for further development. Sealed culture dishes were kept at 26°C under shaking at 65 rpm. S medium was renewed every 7 days. Four weeks after co-culture, grown calli were plated onto K4NBCAx medium (Table 4) for regeneration. After 1 week of incubation at 26°C in the dark, the plates were transferred into the light. After 4 weeks on K4NBCAx, the developing structures were transferred into plastic containers

Table 4: Media used for *Agrobacterium*-mediated transformation of barley pollen cultures and subsequent regeneration of transgenic plants

Media / Component (mg/L)	**KBP**	**CK**	**ASt**	**S**	**K4NBCAx**
NH_4NO_3	80	80	80	80	80
KNO3	2022	2022	2022	2022	3640
$KH2PO_4$	340.2	6465	3658	340	340
K_2HPO_4	-	871	108.9	-	-
$CaCl_2 \cdot 2H_2O$	441	441	1470	441	441
$MgSO_4 \cdot 7H_2O$	246	246	246	246	246
NaFeEDTA	27.5	27.5	27.5	27.5	27.5
$MnSO_4 \cdot 4H_2O$	11.2	11.2	11.2	11.2	11.2
H_3BO_3	3.1	3.1	3.1	3.1	3.1
$ZnSO_4 \cdot 7H_2O$	7.2	7.2	7.2	7.2	7.2
$Na_2MoO_4 \cdot 2H_2O$	0.12	0.12	0.12	0.12	0.12
$CuSO_4 \cdot 5H2O$	0.025	0.025	0.025	0.025	1.25
$CoCl_2 \cdot 6H_2O$	0.024	0.024	0.024	0.024	0.024
KI	0.17	0.17	0.17	0.17	0.17
Retinol	0.01	0.01	0.01	0.01	-
Thiamine-HCl	1.0	1.0	1.0	1.0	10.0
Nicotinic acid	1.0	1.0	1.0	1.0	1.0
Riboflavin	0.2	0.2	0.2	0.2	-
Ca-pantothenate	1.0	1.0	1.0	1.0	-
Folic acid	0.4	0.4	0.4	0.4	-
Pyridoxine-HCl	1.0	1.0	1.0	1.0	1.0
Cobalamine	0.02	0.02	0.02	0.02	-
Ascorbic acid	2.0	2.0	2.0	2.0	-
Calciferol	0.01	0.01	0.01	0.01	-
Biotin	0.01	0.01	0.01	0.01	-
Cholin chloride	1.0	1.0	1.0	1.0	-
p-Aminobenzoic acid	0.02	0.02	0.02	0.02	-
Myo-inositol	100	100	100	100	-
Glutamine	438	-	146	-	-
Maltose-H_2O	90000	90000	90000	90000	36000
BAP	0.9	0.45	0.45	0.225	0.225
2,4-D	-	-	2.21	-	-
Acetosyringone	-	98.1	98.1	-	-
Cefotaxime	-	-	125	125	125
Amoxicillin	-	-	100	100	100
Bialaphos	-	-	24.8	33	-
Hygromycin	-	-	26.4	52.8	-
MES	-	1952	1952	1952	-
Phytagel	-	-	-	-	4.000
pH	5.8	5.9	5.0	5.5	5.8

BAP, 6-benzylaminnopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; EDTA. ethylenediaminetetraacetate; MES, 2-morpholinoethanesulphonic acid.

with the same medium and cultured for another 4 weeks to generate plantlets which were then potted in soil. GFP expression in callus, root tips, leaves and pollen was visualized by CLSM using a 488 nm laser line for excitation. GFP signals were detected with a 505-530 nm bandpass filter.

2.3.4. Ploidy level analysis and colchicine treatment

Ploidy level of regenerants was assessed using a flow cytometer (Partec GmbH, Münster, Germany) which measures total DNA content of individual nuclei. Nuclei were stained with CyStain UV (Partec GmbH, Münster, Germany) according to the manufacturers instructions.

To induce the formation of double haploids the single haploid plants were treated with the mitotic inhibitor colchicine (Luckett, 1989; Takamura and Miyajima, 1996). For this purpose, haploid plants which had developed at least 2 tillers were removed from the soil and the roots were carefully washed with tap water, cut back to 1 cm and immersed in aqueous colchicine solution 0.1% (v/v), 0.8% (v/v) dimethyl sulfoxide (DMSO), and 0.05% (v/v) Tween-20) at room temperature in dark for 5 h. After removal from the colchicine solution the roots were carefully rinsed with running tap water for a few minutes. Treated plants were transplanted in soil. After re-establishment, plants were vernalized in a cold room at 2^{o}C and 8 h day length for 6 weeks. Grains of doubled haploids were harvested at full maturity.

2.3.5. Molecular analysis of transgenic plants

Genomic DNA prepared from leaf material (Palotta et al., 2000) was analysed by standard PCR using the primers for the coding sequence of *HYGROMYCIN* GH-HYG-F1 (5'-GATCGGACGATTGCGTCGCA-3') and GH-HYG-R2 (5'-TATCGGCACTTTGCATCGGC-3'), or *gfp* GH-GFP-F1 (5'-GGTCACGAACTCCAGCAGGA-3') and GH-GFP-R2 (5'-TACGGCAAGCTGACCCTGAA-3'). For gel blot analysis genomic DNA was digested with *Hind*III, separated in 0.8 per cent (w/v) agarose gel (30 µg per lane), and blotted onto Hybond N+ membrane (Amersham, Braunschweig, Germany) by capillary transfer under alkaline conditions according to the manufacturer's instructions. Membranes were hybridized with the DIG-labelled *HYG* probe according to the manufacturer's instructions (Roche, Mannheim, Germany). Hybridisation and detection was performed according to the protocol for non-radioactive southern blot experiments (Roche, Mannheim, Germany).

2.3.6. Analysis of reporter gene expression using confocal laser scanning microscopy

Samples from T0 transgenic plants leaf, root and pollen were analysed for the presence of GFP with a LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany) as described under 2.3.3. Auto-fluorescence of chlorophyll was detected with a 488 nm laser line in combination with a 650 nm long-pass filter.

2.4. Live-cell imaging

2.4.1. Induction of pollen embryogenesis in a GFP reporter line

Spikes of transgenic plants were collected at the appropriate developmental stage as described previously (see 2.1.3.). Pollen embryogenesis was induced by stress treatment, i.e. immature pollen was isolated from the anthers immediately after the collection of spikes and cultured in SMB1 medium for 2 days at 24^{o}C in dark after which SMB was replaced by KBP medium (see 2.1.5.). Alternatively, whole anthers were isolated by a forceps under aseptic conditions and incubated in 0.4 M mannitol for 3 days in the dark either at 4°C (APT4) or at 24^{o}C (APT24). Following isolation, the anther-derived immature pollen was cultured in SMB1 for 1 day at 24°C in the dark before transferring to KBP medium as formerly described (see 2.1.5.). For each of the three treatments, two biological replicates, each containing three technical replicates, were used to assess the total number of multicellular structures (MCSs), gametophytic pollen (GP) and pollen survival after 2 weeks of culture in KBP medium.

SigmaStat software was used for statistical analysis. A pairwise comparison of the treatments with regard to the proportion of all considered characters was conducted. One-Way Analysis of Variance for each character was performed. Normality and equal variance tests were passed for all measured characters. Analysis was done at an overall significance level 0.05.

2.4.2. Isolation of transgenic immature pollen after anthers treatment

After three days of treatment in 0.4 M mannitol either at 4°C or at 24°C, the anthers were transferred into a 50 ml falcon tube filled with 20 ml 0.4 M mannitol. Two 20x8 mm magnetic stirrer bars were placed into the tube and the suspension stirred 3 times for 2-3 min at maximum speed until the mannitol solution became turbid and greenish. As the magnetic bars moved freely in the tube they, caused irregular stirring which effectively threshed the immature pollen out of the anthers. Afterwards the suspension was filtered through a 100 µm mesh to withhold any anthers debris. Further isolation steps were done as described previously (see 2.1.3)

2.4.3. Construction of a live-cell imaging chamber

The experimental set-up for a live-cell imaging chamber with excellent optical properties should allow the specimens to be maintained for several weeks under optimized culture conditions. Since the work had to be performed within the facilities of the IPK microscopy core facility service unit, the imaging chamber also had to be small and easily exchangeable. These demands called for a custom-made construction. The final design was based on a 2-wells Chambered Coverglass (Thermo Scientific-Cat. No. 155380) in combination with a 12 mm Millicell Cell Culture Insert with 0.4 µm hydrophilic PTFE membrane (Millipore-Cat. No. PICM01250) and a Countess® Cell Counting Chamber Slide (Invitrogen, Cat. No. C10315) (Fig. 5). All steps were performed under aseptic conditions. The manufacturing of a single live cell imaging unit started with two Millicell Cell Culture Inserts from which the 0.4 µm hydrophilic PTFE membranes were carefully removed (Fig. 5b, c) and stored under sterile conditions. From a Countess® Cell Counting Chamber Slide the plastic mask was removed (Fig. 5e) and its size adjusted in order to have it fit precisely inside the well of a Chambered Coverglass (Fig. 5g). Using a heated scalpel blade a hole of approximately 7x7 mm was made in the middle of the plastic mask. Having this done, one of the previously isolated 0.4 µm hydrophilic PTFE membrane was placed over the hole in the plastic chamber. Using a heated forceps the rim of the PTFE membrane was melted fixed to the plastic chamber (Fig. 5f).

In the next step, 1 ml of immature pollen culture was pipetted in a 6 cm diameter plastic petri dish. With a 30 µm mesh-coated pipette tip 900 µl medium was removed leaving 0.1 ml of highly concentrated immature pollen (Fig. 6c). Due to the hydrophobic nature of the plastic, this droplet retained a convex surface shape which is crucial for the successful isolation of healthy immature pollen. By leaving the droplet undisturbed for 3

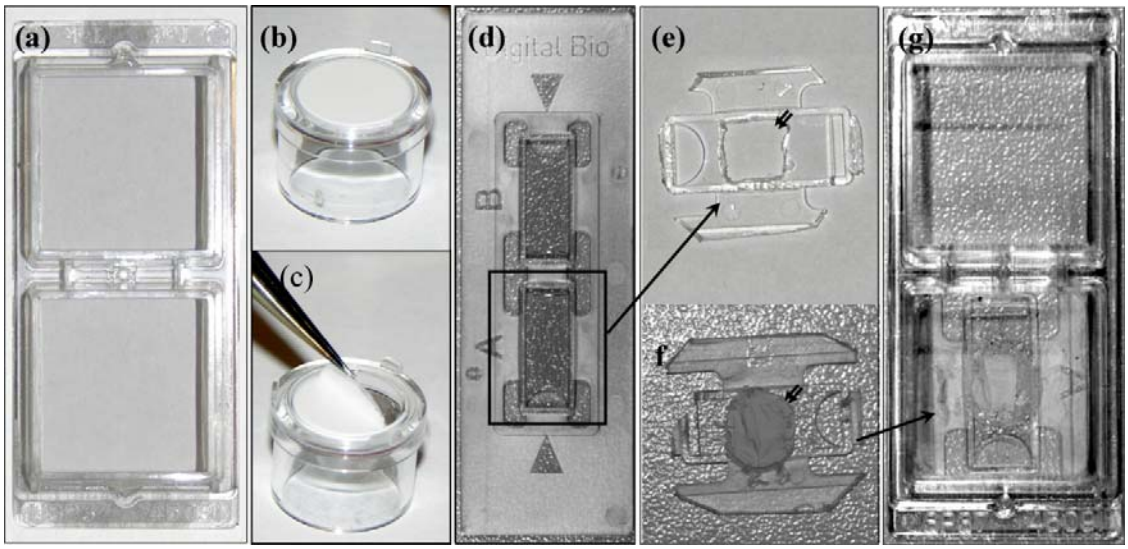

Fig. 5: Parts used to construct the custom made live cell imaging chamber. (a) Lab-Tek™ 2 wells chambered coverslip. (b) 12 mm Millicell Cell Culture Insert with 0.4 µm hydrophilic PTFE membrane. (c) Membrane being detached by a forceps. (d) Countess® Cell Counting Chamber Slides with plastic masks. (e) Plastic mask with fabricated hole. (f) Plastic mask with hole covered by 0.4 µm hydrophilic PTFE membrane. (g) Plastic mask placed inside Chambered Coverglass.

to 5 min., dead cells sunk to the bottom while viable vacuolated immature pollen remained in suspension or, depending on the extent of vacuolization, accumulated near the surface of the droplet.

With the help of a forceps the remainder of the two detached 0.4 µm hydrophilic PTFE membranes was carefully placed over the droplet of concentrated immature pollen suspension for 2 to 3 seconds. When the membrane was lifted, a small aliquot of medium was retained containing a substantial amount of healthy immature pollen. With a piece of filtering paper the amount of culture medium was reduced as much as possible while taking care not to loose the cells or let them dry out on the membrane. The membrane with the concentrated immature pollen was quickly transferred into a well of a Chambered Coverglass (Fig. 5c). Again, care was taken to avoid the inclusion of small

air bubbles while the membrane was placed carefully face-down. At this stage a thin layer of highly concentrated immature pollen had been created between the PTFE membrane and the glass coverslip of the 2 wells Chambered Coverglass (Fig. 5c). Subsequently the PTFE membrane was covered with a droplet of SMB1 semi-solidified with agarose gel (Fig. 6d). Until now all working steps had to be done very fast to avoid drying out the cells. The covering of the membrane by semi-solidified SMB1 medium effectively protected the immature pollen against this risk.

At this stage the pre-prepared plastic mask with PTFE membrane was placed on top of the agarose gel droplet in the Chambered Coverglass (Fig. 6e, f). Finally 1 ml of immature pollen culture was added to maintain the recommended population density for immature pollen culture (Coronado et al., 2005). Finally the chamber was covered with a plastic lid (Fig. 6h). After it was discovered that even with this lid, evaporation was substantial during observation, it was decided to cut off a small square of about 1.5 x 1.5 cm in the plastic lid. The hole allowed the refilling with distilled sterilized water to compensate for evaporation losses. This was done on a daily basis to maintain a correct culture volume and pollen concentration. To minimize the risk of culture contamination, the hole was sealed with a sterile coverslip, which was exchanged on a daily basis (Fig. 6h). Great care was taken to smoothen the edges of the hole in the lid. If this was omitted, coverslips would not seal well and evaporation could be even worse than before.

The live cell imaging chamber with immature pollen culture could now be transferred to the microscope to start the time laps imaging. The schematic design of the live-cell imaging chamber is illustrated in Fig. 6. All time lapse observations were performed in a darkened room. Although officially not temperature controlled, by the appropriate settings of heating and air conditioning, it was possible to maintain a near to constant temperature of 25°C in the microscope room. Microscopical observations were performed on a Zeiss LSM 510 META microscope in combination with LSM 3.2 or LSM 4.2 software.

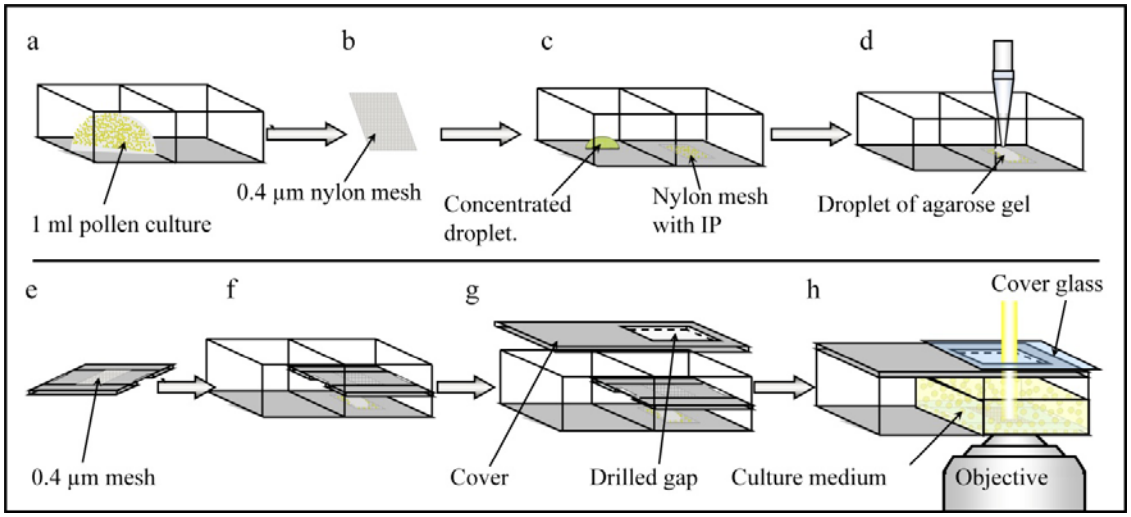

Fig. 6: Schematic drawings of a custom-made live-cell imaging chamber used for time laps recording of immature pollen culture. (a) Two wells Lab-Tek™ Chambered Coverslip with 1 ml immature pollen culture suspension. (b) Detached 0.4 µm hydrophilic PTFE membrane. (c) Chambered Coverslip with droplet of concentrated immature pollen culture on the left side and 0.4 µm hydrophilic PTFE membrane with immature pollen underneath on the right. (d) Covering of the 0.4 µm membrane with SMB1 semi-solidified in agarose gel. (e) Plastic mask with 0.4 µm membrane. (f) Plastic mask with 0.4 µm membrane placed on top of the agarose covered by a primary membrane holding the immature pollen. (g) Covering the Chambered coverslip. h) After adding 1 ml culture pollen and covering the hole with cover glass the live-cell imaging chamber is ready for microscopic observation. IP; immature pollen.

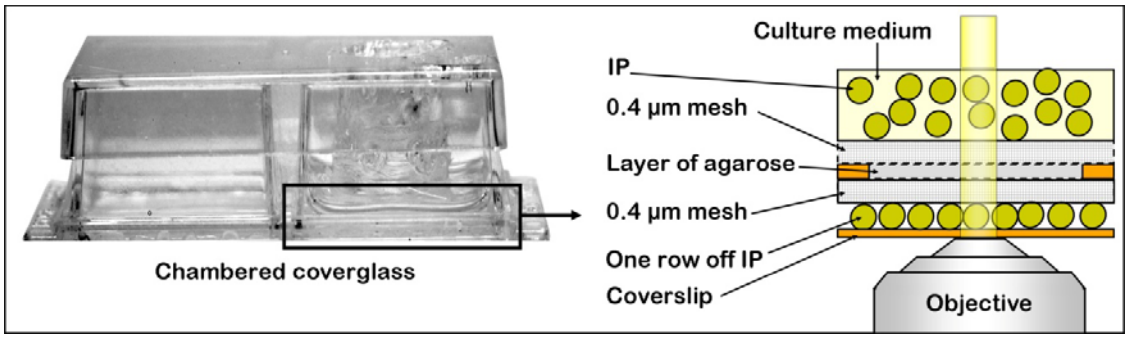

Fig. 7: Schematic drawing of the experimental set-up to monitor the development of isolated immature pollen.

2.4.4. Trapping of immature pollen for live-cell imaging

Since vacuolated immature pollen does not sink in culture medium, immobilization is a prerequisite for time-lapse recording. When mixed and immobilized in low-melting agarose, the immature pollen became distributed throughout the medium, resulting in only a few cells to be in the same optical plane. After a number of trial and error experiments, the best way of immobilizing cells within a narrow optical range was found

to be adhering and thus trapping the immature pollen under a 0.4 µm membrane. This membrane was covered by a small droplet of agarose gel and a secondary 0.4 µm membrane to ensure permanent immobilization of the cells under observation which are now still in liquid medium under the primary 0.4 µm membrane (Fig. 7). This set-up also facilitates the nutrient exchange through the liquid medium in a convenient way for the cells.

2.4.5. Monitoring of pollen development

Wild type immature pollen was isolated and cultured in starvation medium for 24 hours before being transferred to the microscope for live cell imaging. One day after the start of the microscopic observation, starvation medium was pipetted off using a 1 ml pipette tip with a 30 µm nylon mesh cover, and replaced by 1 ml KPB medium. Developmental progress of the immature pollen in culture was observed for up to 28 days on end using a HeNe 633 laser line. Z-stacks of 9 images with a spacing of 4 µm were acquired every 3 min.

Cultures of transgenic immature pollen expressing GFP with a nuclear targeting signal were observed over a time period for up to 2 weeks. Time lapse observation started 1 day after isolation. Also here the starvation medium was replaced by KBP medium. GFP was excited with the 488 nm laser line from an argon-krypton laser. Parallel to the fluorescence recording, DIC images were acquired. Z-stacks of 9 to 11 images with a spacing of 4 to 5 µm were taken every 3 min. To reduce the risk of bleaching, laser intensity was always kept below 4% emission. The GFP has a major excitation peak at a wavelength of 488 nm and its emission peak is at 510 nm.

3. Results

3.1. Comparative analysis of pollen fixation protocols

Kumlehn and Lörz (1999) showed that, when isolated immature barley pollen is cultured in SMB1, the preprogrammed gametophytic development can be switched to embryogenic competence. The efficiency of pollen embryogenesis can be substantially enhanced, when after 2 days in SMB1 immature pollen is transferred into KBP medium containing longitudinally cut wheat ovaries, which act as feeders (Corronado et al., 2005). The liquid culture conditions used here allowed a continuous optical recording by live-cell imaging of the structural changes taking place during this process and were thus selected to optimize the fixation protocols.

A main issue in this study on pollen embryogenesis was to identify earliest structural and ultrastructural differences between pollen embryogenesis and pollen gametogenesis. Since all initial changes are subtle, great care was taken to adapt the fixation and embedding protocols in order to produce minimal ultrastructural artefacts. It must be kept in mind that the sample material was extremely heterogenic. The starting material typically consisted of the highly vacuolated uni-nuclear or early bi-cellular pollen. In case of the gametophytic pathway, this immature pollen could develop into mature pollen with dense cytoplasm. If the embryogenic pathway was followed, a multicellular organization arose containing cells with different levels of vacuolization. As a consequence, each culture eventually contained a mixture of highly and poorly vacuolated, uni-cellular and multicellular structures. The challenge was to develop a fixation protocol that would meet the varied demands for the optimal fixation of these different developmental stages. Therefore, three different fixation methods were applied: CCF, MCF and finally, high HPF-FS.

The most critical factor in all fixation protocols is the time period required for sample fixation; the faster the fixation the more reliable is the preservation of cellular structure. In CCF, fixation depends on the diffusion rate of the fixatives and is therefore relatively slow. With the introduction of the microwave, the CCF could be vastly accelerated thus limiting the extent of artifacts and improving the ultrastructural preservation. In the end, however, the MWCF remains a chemical fixation with the limitations associated to it.

Fastest fixation can only be achieved by cryo-fixation. Since the Melzer lab possessed a fair amount of experience in the HPF of yeasts and bacteria (Agarwal et al., 2009), it was decided to spend most of the efforts on developing a HPF protocol specifically adapted to pollen suspensions and compare it with CCF and MWCF since their was no known protocols of pollen cryofixation.

3.1.1. Optimization of a protocol of high pressure freezing for pollen

In the absence of cyanobacteria as a biological filler, the HPF procedure of pelleted immature pollen resulted in a very poor preservation of the ultrastructure (Fig. 8a, e). This was independent on whether the pollen was loaded directly into the aluminium platelets or into capillary tubes. The histological analysis of the embedded and sectioned material showed that the apparently compact pellets of immature pollen inside the nitrocellulose tube contained in fact large intercellular spaces (Fig. 8a). Evidently a substantial part of the volume of the immature pollen pellet consisted of a liquid phase which is a source of ice crystal formation and thus explains the distorted structures observed. To reduce the amount of free liquid, the immature pollen suspension was mixed with cyanobacteria to occupy the intercellular volume. As a consequence, ultrastructural preservation after HPF was substantially improved and the histological analysis confirmed that the cyanobacteria effectively occupied most of the intercellular spaces. Despite these positive results, the method had to be improved because during the subsequent FS treatment, the immature pollen-cyanobacteria pellets frequently disintegrated into small fragments which were difficult to retrieve. To keep the pellet of pollen and cyanobacteria in a well-defined volume, the samples were loaded into capillaries. The loading of capillaries depends on capillary forces and thus on the composition of the suspension. Best results were obtained when pellets of immature pollen and cyanobacteria were mixed at a ratio of 1:1. The loading process of a capillary was found better not to exceed 3 min to avoid detrimental effects on the viability of the immature pollen. The capillaries not only served to contain the sample in space, a favourable side-effect of the capillary was a more dense packing of the cellular components as compared to when mixtures where loaded into aluminium platelets. In this respect, the cyanobacteria were also beneficial as packing of pure immature pollen was

not nearly as dense as when cyanobacteria were included. All HPF samples without cyanobacteria typically showed clear evidence of damage due to ice crystal formation during the freezing process. This became evident already at low magnification in the light microscope in the form of an intensely methylene-blue staining of the immature pollen (Fig. 8b). When immature pollen was mixed with cyanobacteria, it remained largely spherical after HPF and their cytoplasm stained less intense with methylene blue (Fig. 8d). These differences in preservation became more pronounced at the ultrastructural level. In the absence of cyanobacteria, the cytoplasm of high pressure frozen pollen was

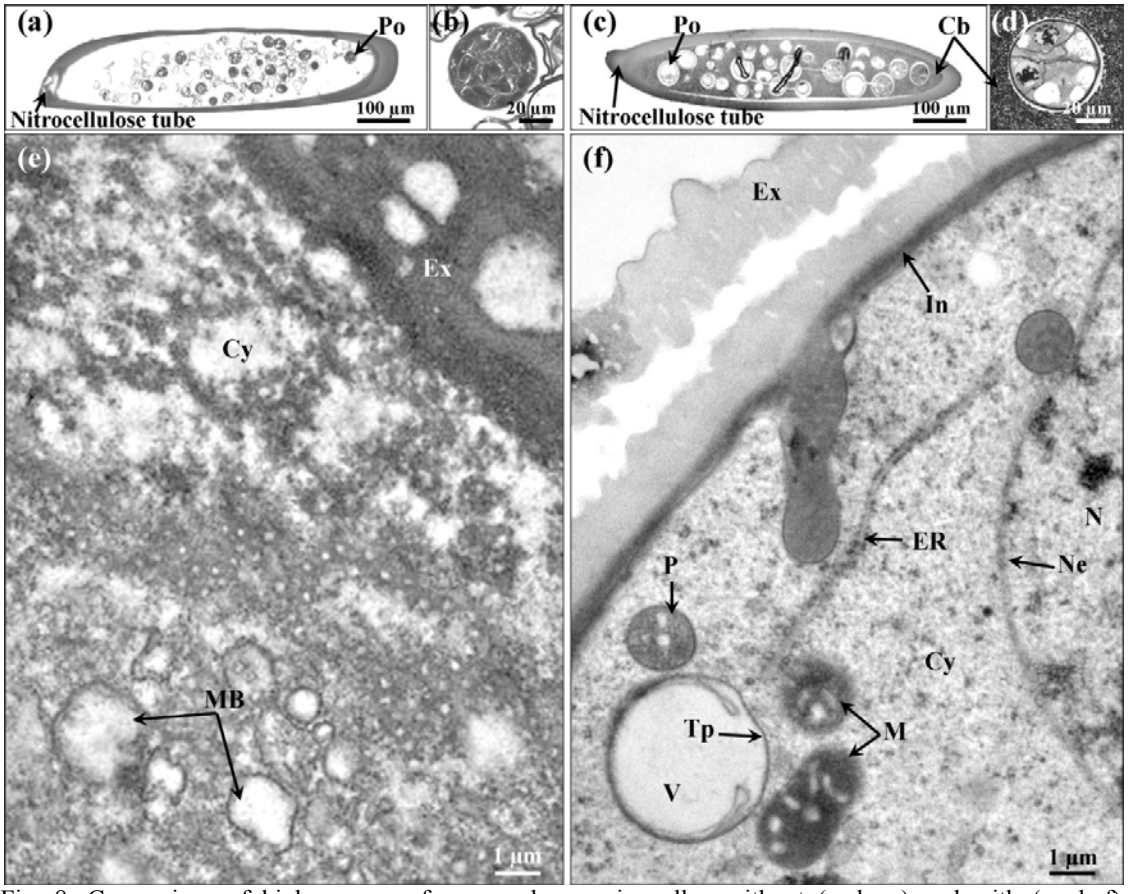

Fig. 8: Comparison of high pressure frozen embryogenic pollen without (a, b, e) and with (c, d, f) cyanobacteria as filler into nitrocellulose tubes. (a) Light microscope image of section of a capillary tube filled by pollen in the absence of cyanobacteria. (b) Immature pollen after HPF in absence of cyanobacteria. (c) Light microscope image of a section of a capillary tube filled by a mixture of pollen and cyanobacteria. (d) Immature pollen surrounded by cyanobacteria after high pressure freezing. (e) High magnification of the cytoplasm of embryogenic pollen after HPF without cyanobacteria. (f) High magnification of the cytoplasm of embryogenic pollen after HPF with cyanobacteria. Cy, cytoplasm; Cb, cyanobacteria; ER, endoplasmic reticulum; Ex, exine; In, intine; M, mitochondria; MB, microbody; N, nucleus; Ne, nuclear envelope, P, plastid; Po, pollen; Tp, tonoplast; V, vacuole.

interspersed with electron transparent spots and areas of a coarse and granular appearance. Vesicular structures were often damaged and membranes were irregular and distorted (Fig. 8e). Interestingly, already the application of a yeast paste to fill the aluminium platelet cavity improved ultrastructural preservation. These observations indicated that the combined application of capillaries, cyanobacteria and yeast (HPF-CBY) resulted in a significantly improved ultrastructural preservation.

Using whole barley anthers (Fig. 9) demonstrates that this protocol of sample preparation presented here may be useful not only in the fixation of cell suspensions but also of complex tissues.

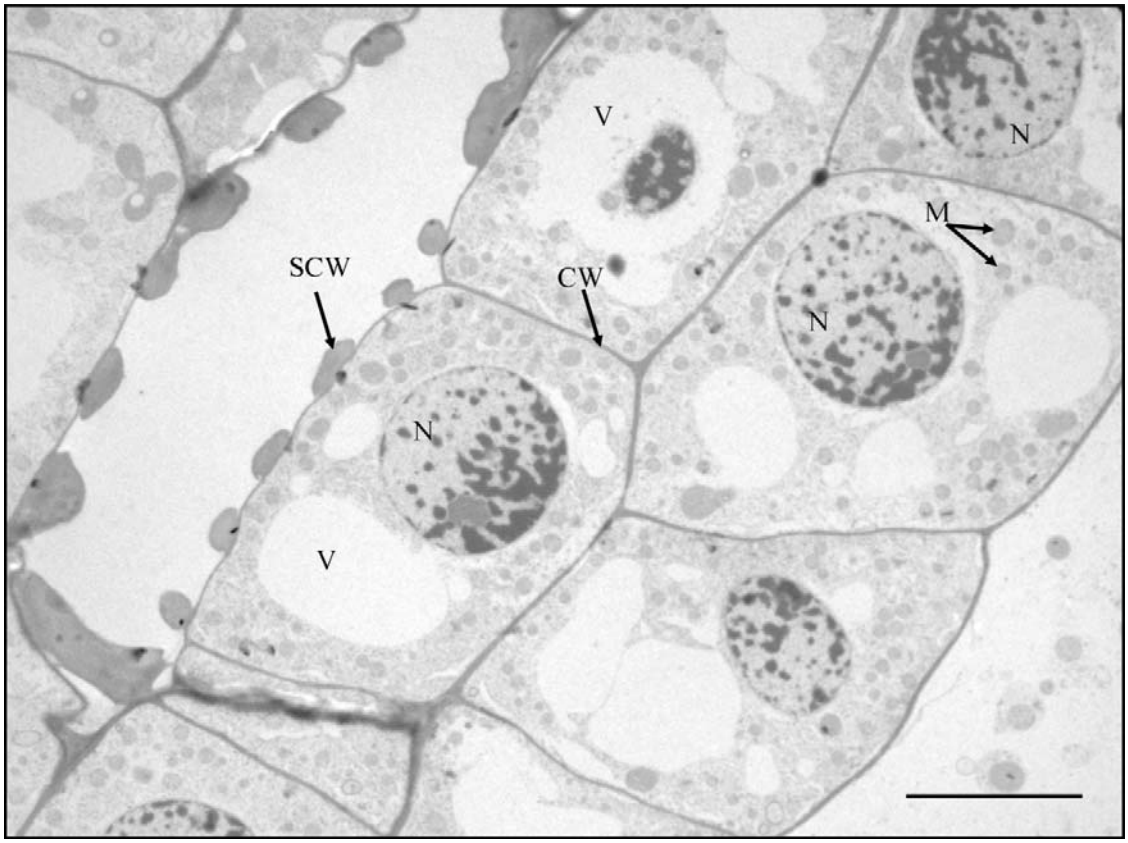

Fig. 9: TEM micrograph of barley anther after high pressure freezing using cyanobacteria as biological filler. CW, cell wall; M, mitochondria; N, nucleus; SCW, secondary cell wall; V, vacuole. Bar = 20 µm.

3.1.2. Comparison of pollen fixation protocols by light and transmission electron microscopy

The structure and ultrastructure of CCF or MWCF treated samples differed from that of HFP-CBY samples in several important aspects. The light microscopy images of

vacuolated pollen specially were inadequate to show significant qualitative differences between the fixation protocols applied (Fig. 10a, c, e). In case of multicellular structures, CCF and to a slightly lesser degree MWCF treated samples showed clear distortions in the cytoplasm when compared to the preservation seen after HPF-CBY (Fig. 10b, d, f). The transmission electron microscopy images of pollen cytoplasm showed high distortion of the organelles fixed by CCF compared to the other protocols (Fig. 10g, h, i). Therefore, a more detailed comparison of pollen ultrastructure fixed by MWCF and HPF-CBY was performed.

Qualitative differences among the different fixation protocols were best seen at ultrastructural level. The least satisfying were the results after CCF (Fig. 10g). Mitochondria were among the few organelles that could be identified, Golgi bodies and endomembranes were highly distorted. Furthermore, the strong contrasts between organelles and cytoplasmic matrix were a clear sign of a significant extraction of some cytoplasmic content (e.g. lipids) having taken place during and after the fixation

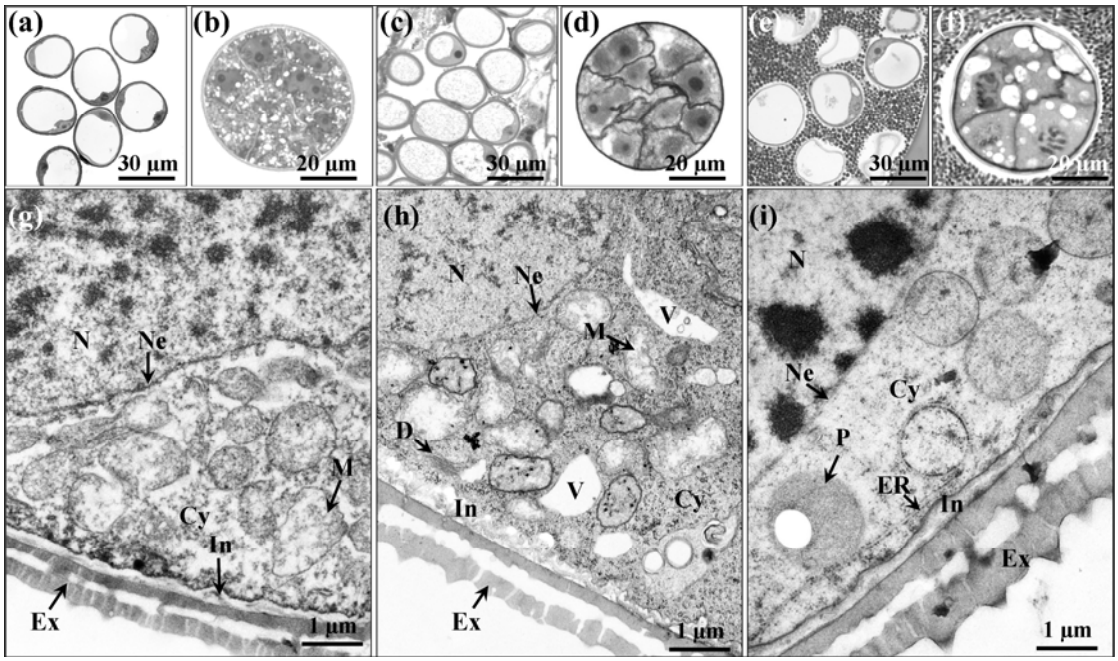

Fig. 10: Comparison of structural preservation of cultured pollen after conventional chemical fixation (a, b, g), microwave-assisted fixation (c, d, h) and high pressure freezing (e, f, i). (a, c, e) Light microscopy images of vacuolated immature pollen. (b, d, f) Light microscope images of multicellular structures after 7 days of induction. (b, e, h) Transmission electron microscopy images of cytoplasm from multicellular structure after 7 days of induction. Cy, cytoplasm; ER, endoplasmic reticulum; M, mitochondria; N, nucleus; Ne, nuclear envelop; V, vacuole.

procedure (Fig. 10g). The same fixation medium gave much better results when used in the MWCF procedure. Here, the cytoplasm was more homogenous with organelles standing out less clear from the cytoplasm which is sign of significantly less extraction of the cytoplasmic material (Fig. 10h, 11c, d, g, h). Though organelles were better preserved, endiplasmic reticulum and Golgi were bloated and the mitochondrial cristae were often damaged (Fig. 11c, d).

HPF-CBY showed superior preservation of the cellular ultrastructure. At all developmental stages, there was no obvious compression or deformation of delicate structures like endomembranes, nuclear envelopes or the tonoplast (Fig. 10i, 11e, f, i, j). Organelles were evenly distributed and the cytoplasmic matrix appeared less granular than in either CCF or MWCF, implying a further reduction in the extraction of cytoplasmic compounds e.g. lipids and free ions (compare Fig. 10g, h, i).

Exemplary of the improved ultrastructural preservation after HPF-CBY was the Golgi apparatus. MWCF procedures almost invariably induce a swelling of the cisternae (Fig. 11c, see also Mollenhauer et al., 1988). As such, chemical fixation can be considered unsuitable when dynamic structures like Golgi apparatus are investigated. After HPF-CBY the Golgi cisternae were narrow and of constant width (Fig. 11e).

Rough endoplasmic reticulum (RER) and nuclear membranes were also much better preserved by HPF-CBY. After CCF the RER was often distorted beyond recognition while after MWCF, despite an improved preservation, the RER usually showed undulating membranes and a bloated lumen (Fig. 11d). In contrast, in HPF-CBY-treated samples the membranes of the RER appeared as parallel straight lines with an internal lumen of approximately 30 nm in width (Fig. 11f). The double layered nuclear membranes were undulated and difficult to be distinguished as a double membrane in MWCF samples (Fig. 11h), while the double layers were identified after HPF-CBY as well as the presence of nuclear pore complexes (Fig. 11j). Though mitochondria were also relatively well preserved by MWCF (Fig. 11g), their double layered membrane and their internal cristae were much clearer after HPF-CBY (Fig. 11i).

The superior performance of HPF-CBY over chemical fixation protocols was further demonstrated by the preservation of microtubules. Microtubules are dynamic but instable structures that especially in plant cells can quickly disintegrate (Lloyd and Chan, 2002;

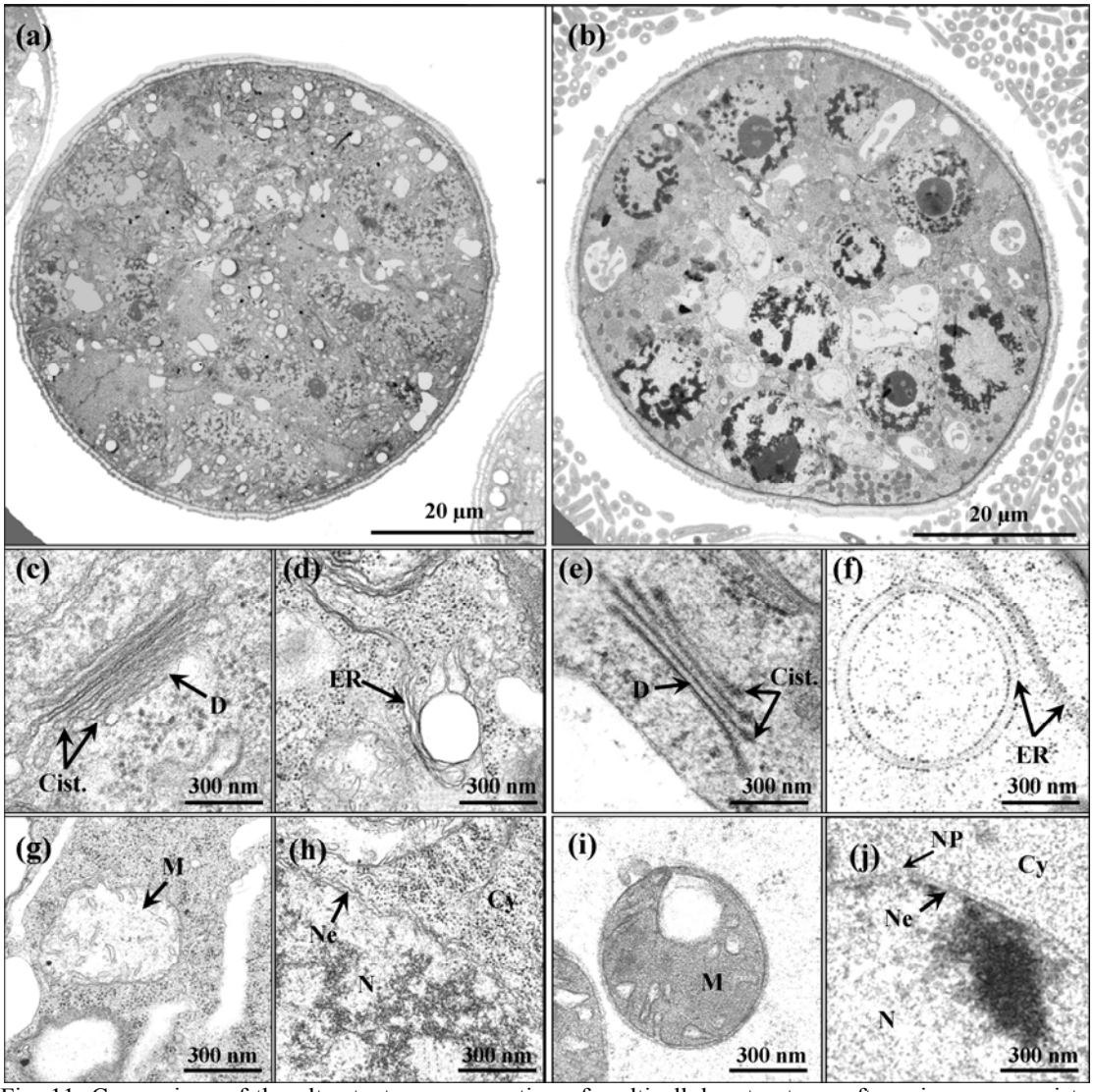

Fig. 11: Comparison of the ultrastucture preservation of multicellular structures after microwave-assisted chemical fixation and high pressure freezing. (a, c, d, g, h) Microwave-assisted chemical fixation. (b, e, f, i, j) High pressure freezing. Note the overall granular appearance of the cytoplasmic matrix after microwave-assisted fixation compared to the rather homogenous impression after high pressure freezing. (c, e) Golgi apparatus. (d, f) Endoplasmic reticulum. (g, i) Mitochondria. (h, j) The nuclear envelope. Cy, cytoplasm; D, dictyosomes; ER, endoplasmic reticulum; Ex, Exine; In, Intine; M, Mitochondria; N, nucleus; Ne, Nuclear envelop; NP, nuclear pore; Tp, tonoplast; V, Vacuole.

Moores, 2008). Actually, the chemical fixation protocols applied here were not suitable to specifically preserve microtubules (He and Wetzstein, 1997) and as such it was no surprise that in samples of both, CCF and MWCF, microtubules were poorly preserved or even not visible. By the shear swiftness of fixation HPF successfully preserved microtubules (Fig. 12). Nevertheless, few of the cells within a capillary showed partial

freezing artifacts due to ice crystal formation (Fig. 13), or some cells appeared to be poorly frozen or damaged after HPF-CBY.

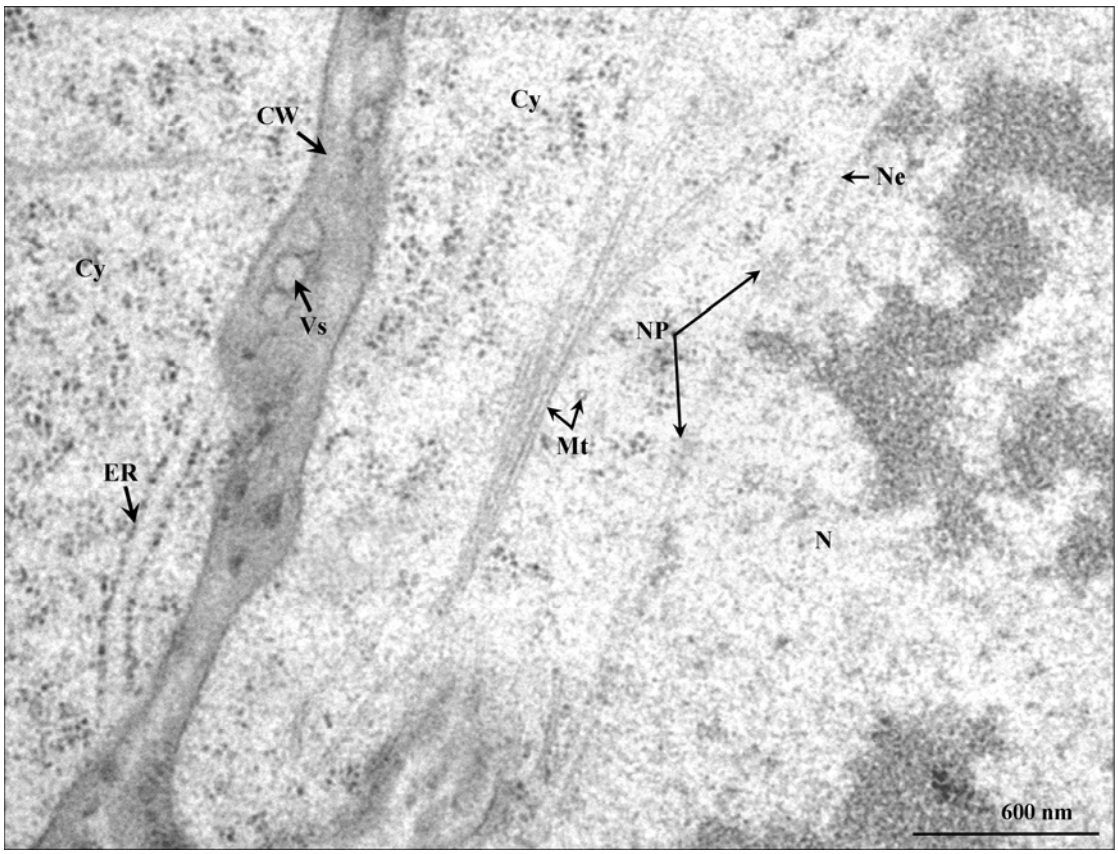

Fig. 12: TEM micrograph of cytoplasm of a multicellular structure (8 days) after high pressure freezing. Cell walls are of varying thickness with vesicular inclusions. Microtubules are clearly recognizable as are the multiple nuclear pore complexes in the double layered nuclear envelope. Cy, cytoplasm; ER, endoplasmic reticulum; Mt, microtubule; N, nucleus; Ne, nuclear envelop; NP, nuclear pore.

3.2. Structural and ultrastructural comparison of pollen embryogenesis vs. gametogenesis

3.2.1. Structural changes during gametophytic and embryogenic development of pollen

To induce embryogenesis, immature pollen was cultured in SMB1 starvation medium followed by incubation in KBP nutrient medium. Regular analysis of the culture by light microscopy revealed that the highest response was found in populations which predominantly consist of highly vacuolated immature pollen (Fig. 14b, c, s). This

observation is in agreement with an earlier study by Ramirez et al. (2001) in which this developmental stage was found to be most sensitive to the induction of embryogenesis in barley. In various other dicot species highly vacuolated immature pollen were also found to be the most efficient developmental stage for the induction of embryogenesis (González-Melendi et al., 1996).

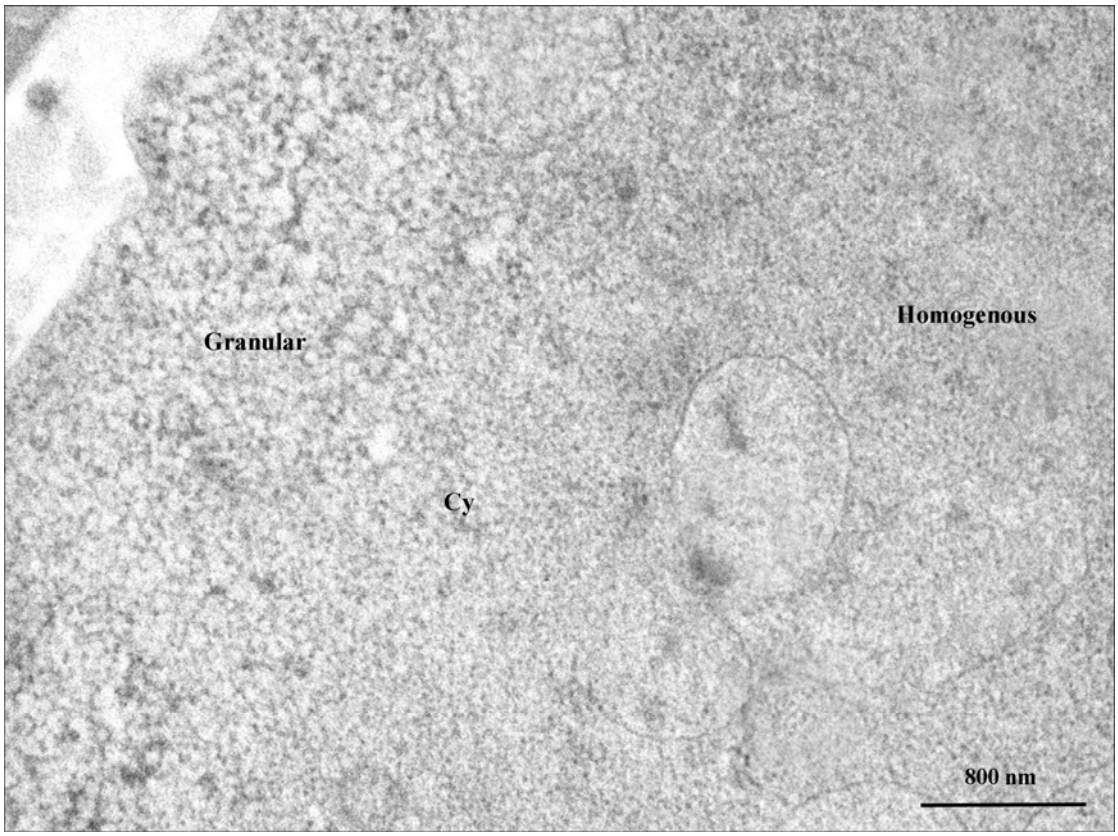

Fig. 13: TEM micrograph of freezing artifacts due to ice crystal formation during high pressure freezing. Partial distortion of the cytoplasm of an immature pollen. Due to the artefacts appears the cytoplasm left appears more granular in contrast to the homogenous preserved cytoplasm to the right. Cy, cytoplasm.

The current study showed that due to the large central vacuole, the cytoplasm containing organelles and nucleus is constricted to a thin layer at the cell periphery (Fig. 14b, c, s). Under conditions that trigger pollen embryogenesis, the first mitotic processes took place 1 to 2 days after the transfer of the immature pollen into the KBP medium. At this early stage of embryogenesis the binuclear embryogenic pollen are still characterized by large central vacuole and a peripheral cytoplasm (Fig. 14e, f, t). Under pollen embryogenesis-triggering conditions, the first pollen mitosis often gives rise to two identical nuclei (Fig.

14e, f, t). Ultrastructural analysis confirmed that a symmetric cell division had occurred (Fig. 19c, d). In size and chromatin distribution pattern, as analyzed by DAPI staining, these nuclei are similar to the nuclei of uni-nucleate pollen (Fig. 14b, c, s) or the vegetative nucleus of a bi-nuclear gametophytic pollen (Fig. 14d). In the case of gametophytic pollen, however, the outcome of the first mitosis is a large vegetative nucleus and a small generative nucleus (Fig. 14d) followed by an asymmetric cell division creating a larger vegetative cell and a smaller generative cell (Fig. 14d). Initially this generative cell locates to the pollen periphery, it later detaches and is then found in the cytoplasm of the vegetative cell (Fig. 14j). During the second pollen mitosis, which is typically followed by a symmetric cell division, the generative cell formed two sperm cells, marking the final stage of gametophytic development (Fig. 14m, p).

After the first division, the next developmental stage of the embryogenic pollen is the four-nuclei stage (Fig. 14i) indicating that for some time divisions occur synchronously. This ongoing mitotic activity results in multicellular structures. Still, the individual embryogenic structures differ greatly in their developmental progress. After two weeks in KBP medium, the first embryogenic pollen breaks out of the original exine of the pollen marking the end of the embryogenesis and the start of the development of pro-embryos (Fig. 14n, o, q, r). By the end of embryogenesis the structural and ultrastructural differences with the process of gametogenesis had become very pronounced. While gametophytic pollen accumulated starch before and after the first mitosis and during pollen maturation (Fig. 14g, j, m, p), starch was not found during pollen embryogenesis at least in light microscopy (Fig. 14h, i, k, l, u, v). Moreover, the intine remained thin during embryogenesis but significantly increased in thickness during gametogenesis (Fig. 14 p).

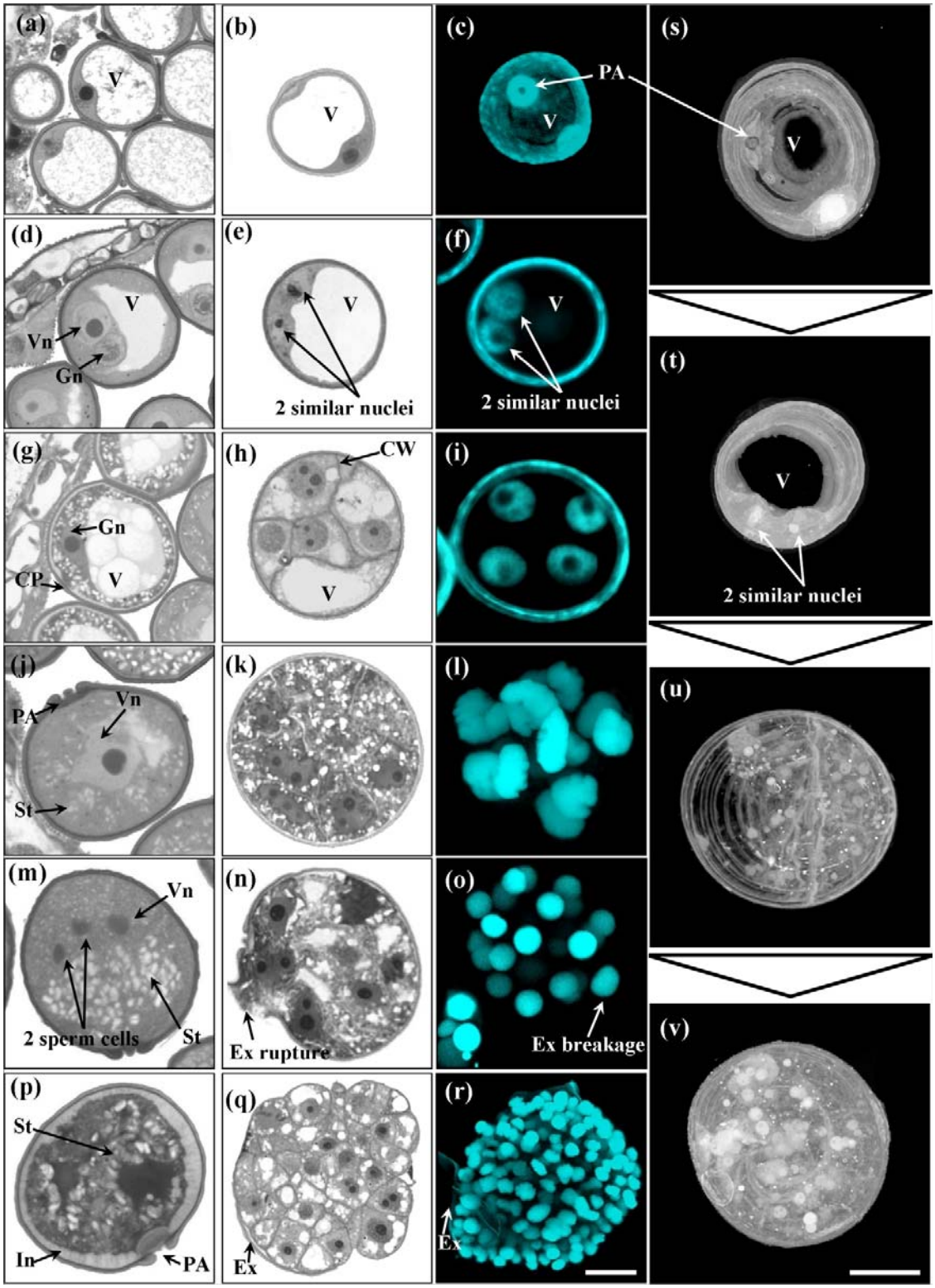

Fig. 14: Comparison of gametophytic and embryogenic developmental pathways. (a, d, g, j, m, p) Light microscopy images of different stages of gametophytic development. (b, e, h, k, n and q) Light microscopy images of different stages of embryogenic development. (c, f, i, l, o, r) CLSM images of DAPI-stained nuclei in embryogenic pollen at different stages.

Taken together, early stages of embryogenesis differ from gametogenesis in several aspects: : i) first gamtophytic mitosis is asymmetric, whereas first embryogenic mitosis was predominantly symmetric, ii) gametophytic pollen accumulates starch gradually between end of uni-nucleate stage till pollen maturation, while there is no visible starch during embryogenic pollen development. iii) gamtophytic pollen run always two cycles of mitosis, while embryogenic pollen perform numerous cycles of mitosis. iv) matured pollen contained a thick intine, whereas embryogenic pollen retained a thin intine. v) gametophytic pollen showed a continuous decrease of the central vacuole after first mitosis till disappeared at maturation, conversely embryogenic pollen kept a large vacuole even after several cycles of mitosis, albeit associated with a reduction of its size, and daughter cells of multicellular structures contained small vacuoles.

3.2.2. Structural changes inductive for spontaneous genome doubling

Genome doubling and the resulting formation of diploid plants is an important aspect in pollen embryogenesis (Kasha et al., 2001). So far, most of the available information about diploidization during pollen-derived embryogenesis has been gained from flow-cytometric analyses of somatic tissues from regenerated plantlets (Li and Devaux 2003). More recently, using DAPI stained nuclei and CLSM, Gonzalez-Melendi et al. (2005) showed that spontaneous genome doubling during barley pollen embryogenesis predominantly derives from nuclear fusion. Assuming that this process may involve the fusion of haploid nuclei, immature pollen at different developmental stages were microwave-fixed and DAPI-stained to visualize the nuclear morphology during pollen embryogenesis. Fig. 15 shows embryogenic pollen with various nuclear organizations. The first mitosis resulted in two similar nuclei (Fig. 15a-d). Cell wall separating the two nuclei was not observed under DIC (Fig. 15a-d). This suggested that cytokinesis was disturbed or not taking place. Either situation would allow a close association of the two nuclei as observed in Fig. 15b to d. If a cell wall is absent or incomplete these nuclei may adhere to each other. In Fig. 15a, the two nuclei were in close proximity to each other and in Fig. 15b a long flat contact zone between the nuclei. Fusion of the nuclear envelope

Continue Fig. 14: (s, t, u, v) Reconstructed 3D images derived from serial sectioned embryogenic pollen. CW, cell wall; Ex, exine; Gn, generative nucleus; In, intine; N, nucleus; Ne, nuclear envelope; n, nucleoli; PA, pollen aparature, St, starch; Vn, vegetative nucleus. Bar = 10 µm.

(Fig. 15c) at this stage could then mark the start of DNA fusion. Indirect support for this hypothesis came from DAPI-stained nuclei at later developmental stages.

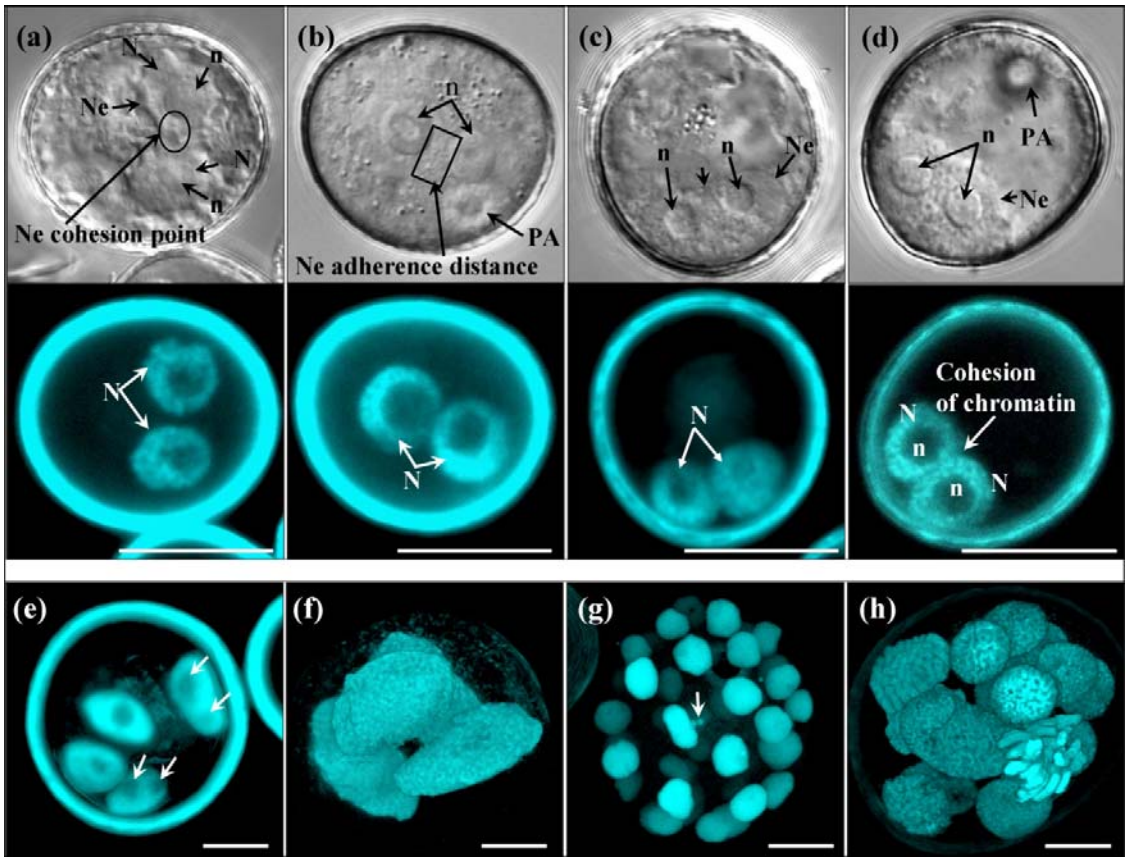

Fig. 15: Possible mechanism of spontaneous genome doubling during pollen embryogenesis. DIC in gray scale, DAPI staining in blue. (a-d) Embryogenic pollen after first mitosis contained two nuclei similar in shape and size. Cell wall was not detected in DIC. (a) Daughter nuclei are still clearly separated. (b) and (c) however, they are lying in close vicinity. (d) There seems to be direct contact, DIC image showed both nuclei enclosed by one nuclear envelop. (e-h) Three dimensional confocal images of early (e-f) and late multicellular structure (g-h). (e) Two of the nuclei contain 2 nucleoli (arrows). (g) Presence of micronuclei (arrows) indicating nuclear instability. (f, h) Multicellular structure with large sized nuclei. N, nucleus; Ne, nuclear envelope; n, nucleoli; PA, pollen aparature, Bars = 20 µm

In the early developmental stages, all nuclei were characterized by the presence of a single nucleolus (Fig. 14a, b, c, s; Fig. 15a, b), while nuclei after fusion contained two nucleoli (Fig. 15d, e). As it was shown that nucleus size was doubled after fusion (Fig. 15a-d), the investigation of later stages showed that nuclear size varied among the investigated embryogenic pollen, even though they were taken at similar stages (Fig. 15e-h). These differences in size might have been caused by nuclear fusion.

It was also observed that generative cell occasionally remained viable for some time and underwent a mitosis (Fig. 16). This raised the question whether the generative cell contributes to pollen embryogenesis.

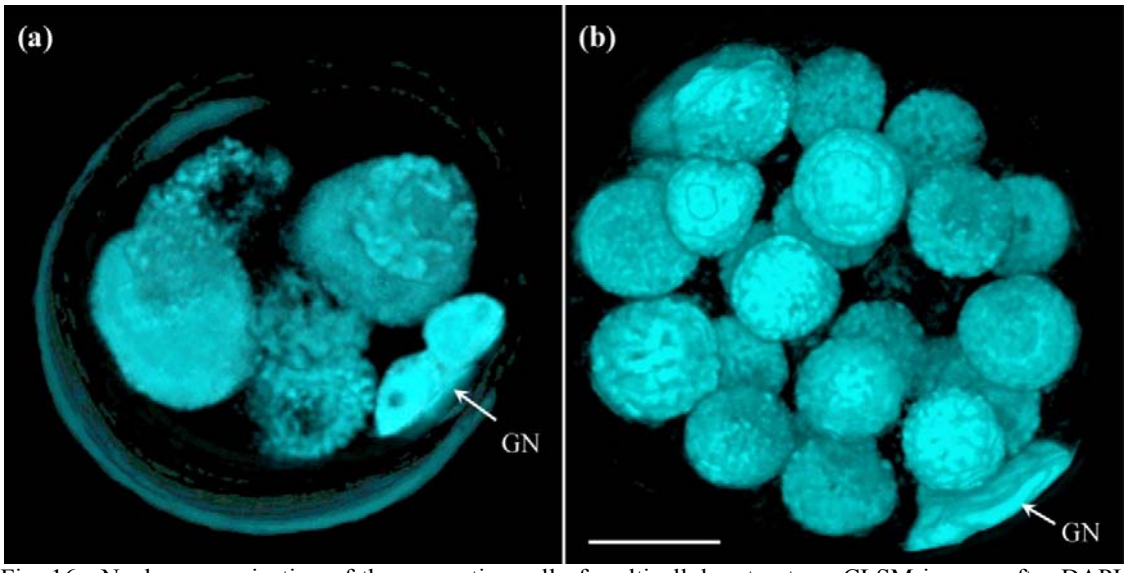

Fig. 16: Nuclear organization of the generative cell of multicellular structure. CLSM images after DAPI staining. Generative nuclei are recognizable by their ellipsoid shape, heavier staining and their peripheral localization. The generative cell in (a) has undergone mitosis and produced two nuclei. (a) 5 days multicellular structure. (b) 8 days multicellular structure. Bar = 25 µm.

3.2.3. Ultrastructure changes during pollen development

The cellular organization during the pollen gametogenesis and embryogenesis was analyzed in order to compare both pathways in respect to the ultrastructural changes. Starting point of the study was the highly vacuolated uni-nucleate pollen which, depending on growth conditions, is capable of either entering the embryogenic or preceding the gametophytic pathway. This uni-nucleate pollen is characterized by a large central vacuole forcing the cytoplasm to occupy a thin layer at the cell periphery. In this particular type of immature pollen, the cytoplasm appeared very smooth and contained only a small amount of endoplasmic reticulum as well as a few organelles like mitochondria and the Golgi apparatus (Fig. 17). Plastids were not identified and starch accumulation was absent at this stage (Fig. 17). The nucleus was strongly euchromatic with only minor patches of heterochromatin. Its oval shape was probably due to elevated

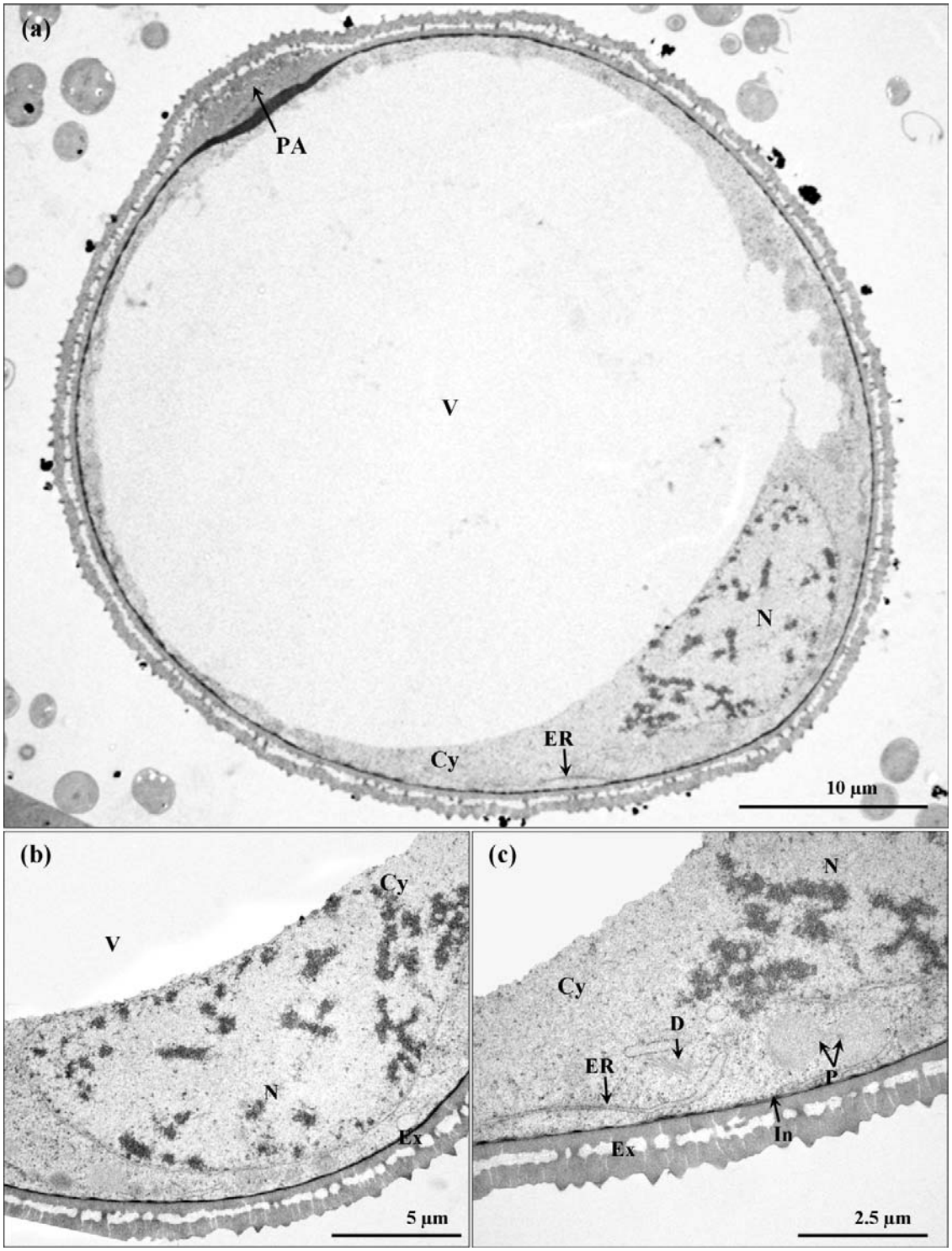

Fig. 17: Ultrastructure of highly vacuolated uni-nucleate immature pollen immediately after isolation. (a) Uni-nucleate immature pollen. (b) Oval nucleus. (c) Cytoplasm containing a few organelles and a thin intine. Cy, cytoplasm; D, dictyosomes; ER, endoplasmic reticulum; Ex, exine; In, intine; N, nucleus; P, plastid; V, vacuole; PA, pollen aparature.

turgor pressure of the large central vacuole (Fig. 17b). The intine appeared as a very thin layer underneath the exine (Fig. 17c).

3.2.3.1. Pollen gametogenesis

Structural changes at the commencement of the gametophytic pathway were difficult to identify. The first visible change was the gradual increase of the volume of cytoplasm and the amount of organelles (Fig. 18a, b). In the further course of gametophytic development, the cytoplasm became highly enriched in endoplasmic reticulum, mitochondria and densely packed ribosomes, giving the cytoplasm a coarse look (Figs. 18a-d; 20c, d). Plastids appeared early on, and almost from the start they were containing starch granules (Figs. 18b; 23c, d). Initially, this developing pollen still possessed a large central vacuole and the cytoplasm was devoid of small vacuoles which were a typical feature of embryogenic pollen before first mitosis (Fig. 19a, b). The first mitosis within the gametophytic pollen produced two dissimilar nuclei along with an asymmetrical cell division resulting in a generative and vegetative cell (Fig. 18c, d).

Whereas the generative nucleus contained extensive amounts of heterochromatin, the vegetative nucleus was strongly euchromatic with only minor patches of heterochromatin (Fig. 18d). Though the generative cell was embedded in the cytoplasm of the vegetative cell, plasmodesmata appeared to be absent from the wall of the generative cell. The most prominent effect of the pollen maturation process was the massive accumulation of starch granules in the plastids that later transformed into amyloplasts (Fig. 18e, f). During this time the intine became markedly thicker with many vesicle-like inclusions (Fig. 18e; 22d), while the thickness of the exine remained virtually unaltered (Figs. 19e, f; 22b).

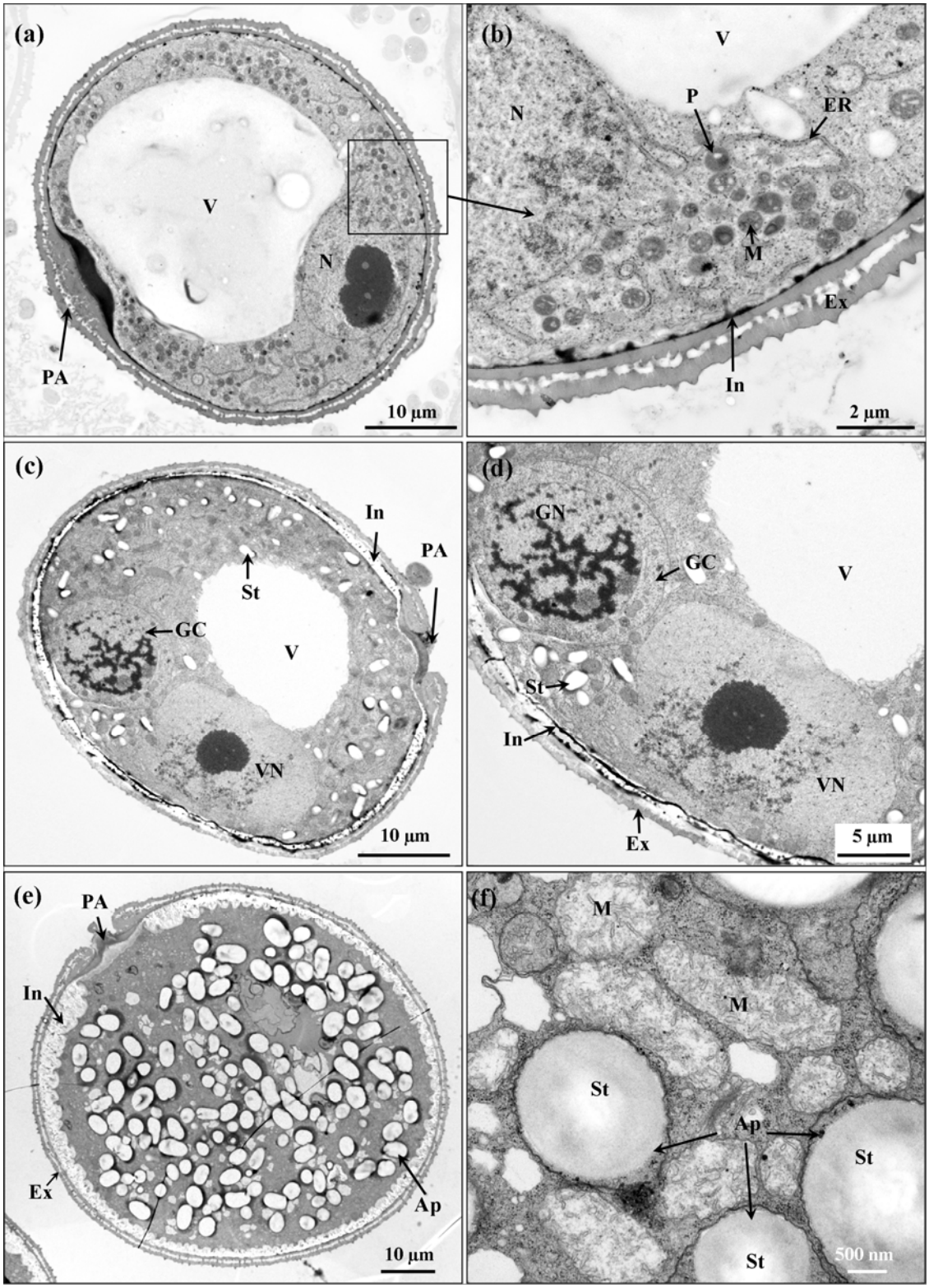

Fig. 18: TEM micrographs of gametophytic pollen development. (a) Uni-nucleate pollen with cytoplasm densely packed with organelles and large central vacuole. (b) Cytoplasm with abundant mitochondria, endoplasmic reticulum and occasional plastids. (c) Bi-cellular pollen after first mitosis with a small central vacuole and a thick intine. (d) Detail of a generative cell and a vegetative nucleus.

3.2.3.2. Pollen embryogenesis

After induction of embryogenesis, the first ultrastructural change observed was the appearance of numerous small vacuoles in the otherwise still thin layer of peripheral cytoplasm (Fig. 19a, b). This cytoplasm was surrounded by a thin intine and relatively poor in organelles (Fig. 19a, b), especially when compared to the gametophytic pollen before the first mitosis (Fig. 18a, b). The first mitosis in the embryogenic pollen resulted in two nuclei which, based on their size and chromatin patterning, were identical. In many cases the two nuclei were not separated by a visible cell wall indicating that karyokinesis was then not followed by cytokinesis (Fig. 19c, d). At this stage, the cytoplasm started to gain in volume and contained more endoplasmic reticulum and mitochondria than before. A consequence of this process was the gradual reduction in size of the central vacuole (Fig. 19c). The intine wall appeared unaltered or only slightly thickened (Fig. 19d) and plastids with starch were not identified (Fig. 23a, b) unless at very late stages of development (Fig. 19e, f).

In subsequent stages the two nuclei of the embryogenic pollen proliferated and gave rise to multicellular structures with small cells and a dense cytoplasm, still confined within the original pollen wall that showed no significant thickening when compared to early stages of embryogenesis (compare Figs. 17a, c; 19e, f). All multicellular structures showed similar ultrastructural organization with euchromatin-rich nuclei and a dense cytoplasm containing various vacuoles of different sizes. Due to the comparatively lower density of mitochondria and endoplasmic reticulum, the cytoplasm as a whole always appeared less dense than that of mature pollen (Fig. 20a, b). An interesting observation was that the Golgi apparatus in the embryogenic pollen on average contained fewer cisternae than those from gametophytic pollen (Fig. 20e, f). The individual cells within the multicellular structures were connected by primary plasmodesmata which were clearly visible in the newly formed cell walls (Fig. 21). In the late stages of multicellular structure, just before the breakage of the exine, individual plastids contained large starch granules (Fig. 19e, f).

Continue Fig. 18: (e) Mature pollen showing large amounts of starch grains and a thick intine. (f) Cytoplasm of mature pollen in detail revealing dense packing of amyloplasts and mitochondria. Ap, amyloplast; ER, endoplasmic reticulum; Ex, exine; GC, generative cell, Gn, generative nucleus; In, intine; M, mitochondria; N, nucleus; P, plastid; PA, pollen aparature, St, starch; V, vacuole; Vn, vegetative nucleus.

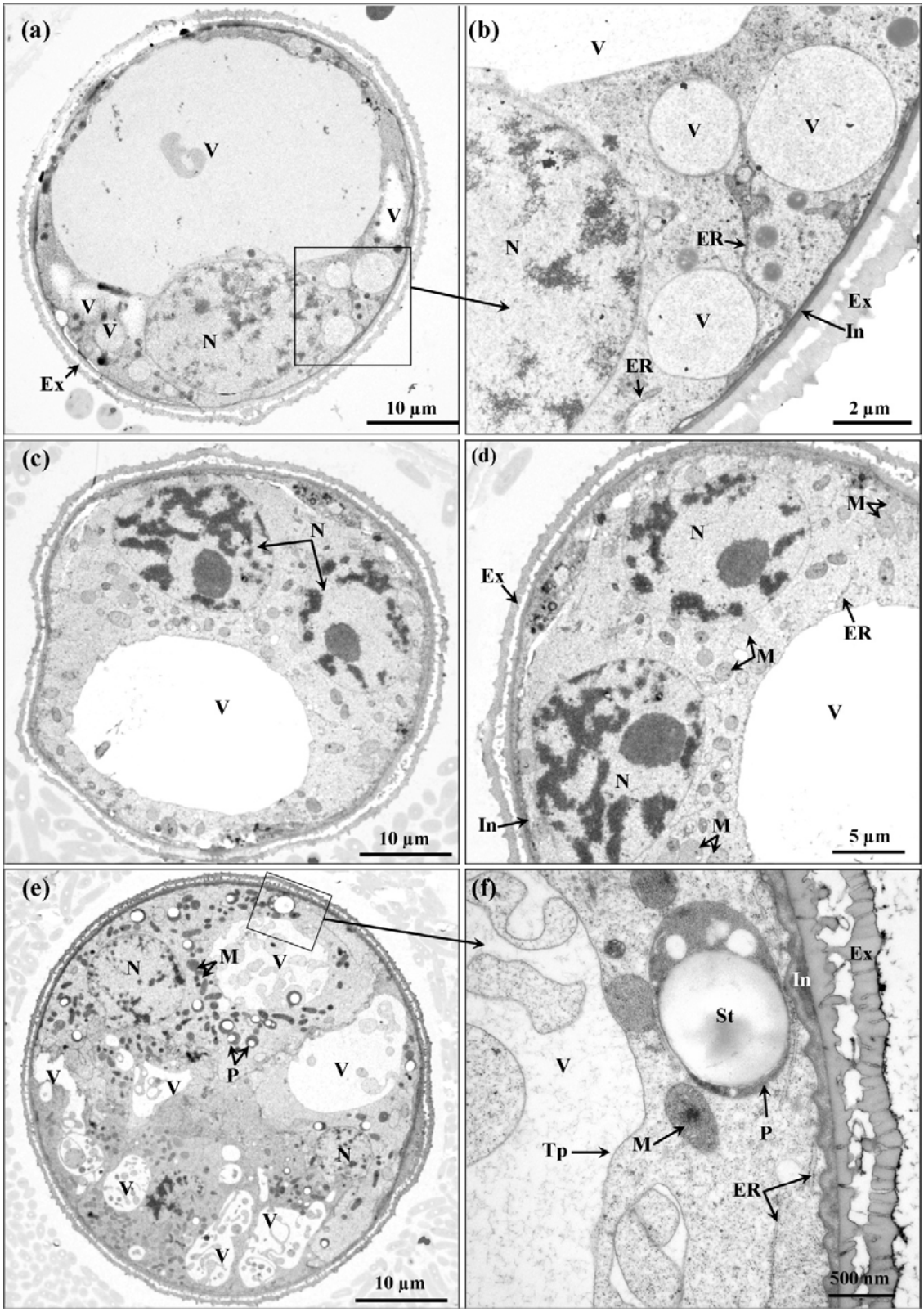

Fig. 19: TEM micrographs of embryogenic pollen development. (a) Uni-nucleate pollen 1 day after induction with increased volume of cytoplasm and a spherically shaped nucleus.

More details about plastid differentiation are shown in Fig. 24. Embryogenic pollen always contained proplastids (Fig. 24c, e), gametophytic pollen were characterized by more differentiated starch grain containing plastids (Fig. 24b, d). Ultrastructural observations showed that starch accumulation and amyloplast formation already take place before the first gametophytic mitosis and continued till pollen maturation (Fig. 18a, c; Fig. 23c, d). In contrast to this, pollen that formed multicellular structure never accumulated starch until the late stages of multicellular structures (Figs. 19; 23a, b).

It could not be identified precisely at what stage the embryogenic pollen started to accumulate starch grains, but whenever they were observed, it was generally after the second mitosis and at least prior to the breakage of the exine restricted to a small sub-population of the investigated cells. Common to both the gametophytic and embryogenic pathways is a large central vacuole of the premitotic stage of uni-nucleate immature pollen. Gametophytic pollen decreased the size of the central vacuole after first mitosis (Fig. 18c) while vacuoles disappeared at later stages of maturation (Fig. 18e; 22c; 23c). Details of vacuole behavior during pollen embryogenesis are shown in Fig. 25. Young microspores isolated at the booting swollen stage of spike (Zadoks et al., 1974), contained a lot of small vacuoles that differed in size and a localized nucleus at the center (Fig. 25a). The premitotic uni-nucleate immature pollen, which is the proper stage of embryogenic induction, displayed a large central vacuole while small vacuoles disappeared from the thin cytoplasm (Fig. 25b). Before and after the first embryogenic mitosis, in addition to the large central vacuole, several small vacuoles were distributed throughout the cytoplasm and some of them were dumbbell-shaped (Fig. 25c). Daughter cells of the multicellular structures at 4 to 5 cells stage contained a large central vacuole with only a few small vacuoles in the cytoplasm (Fig. 25d). By contrast, late multicellular structures showed a large number of small vacuoles in all daughter cells. Some of them contained cytoplasmic inclusions and some other were dumbbell-shaped (Fig. 25e).

Continue Fig. 19: (b) Cytoplasm containing multiple small vacuoles. (c) Bi-nuclear embryonic pollen 1 day after induction with two identical sister nuclei. (d) Detail of sister nuclei were not separated by a cell wall. (e) Multicellular structure (12 days after induction) at late stage before exine rupture contained plastids with starch granules and small vacuoles. (f) High magnification of the cytoplasm showing mitochondria and plastids. A large plastid contained a starch granule. ER, endoplasmic reticulum; Ex, exine; In, intine; M, mitochondria; N, nucleus; P, plastid, St, Tp; tonoplast; starch; V, vacuole.

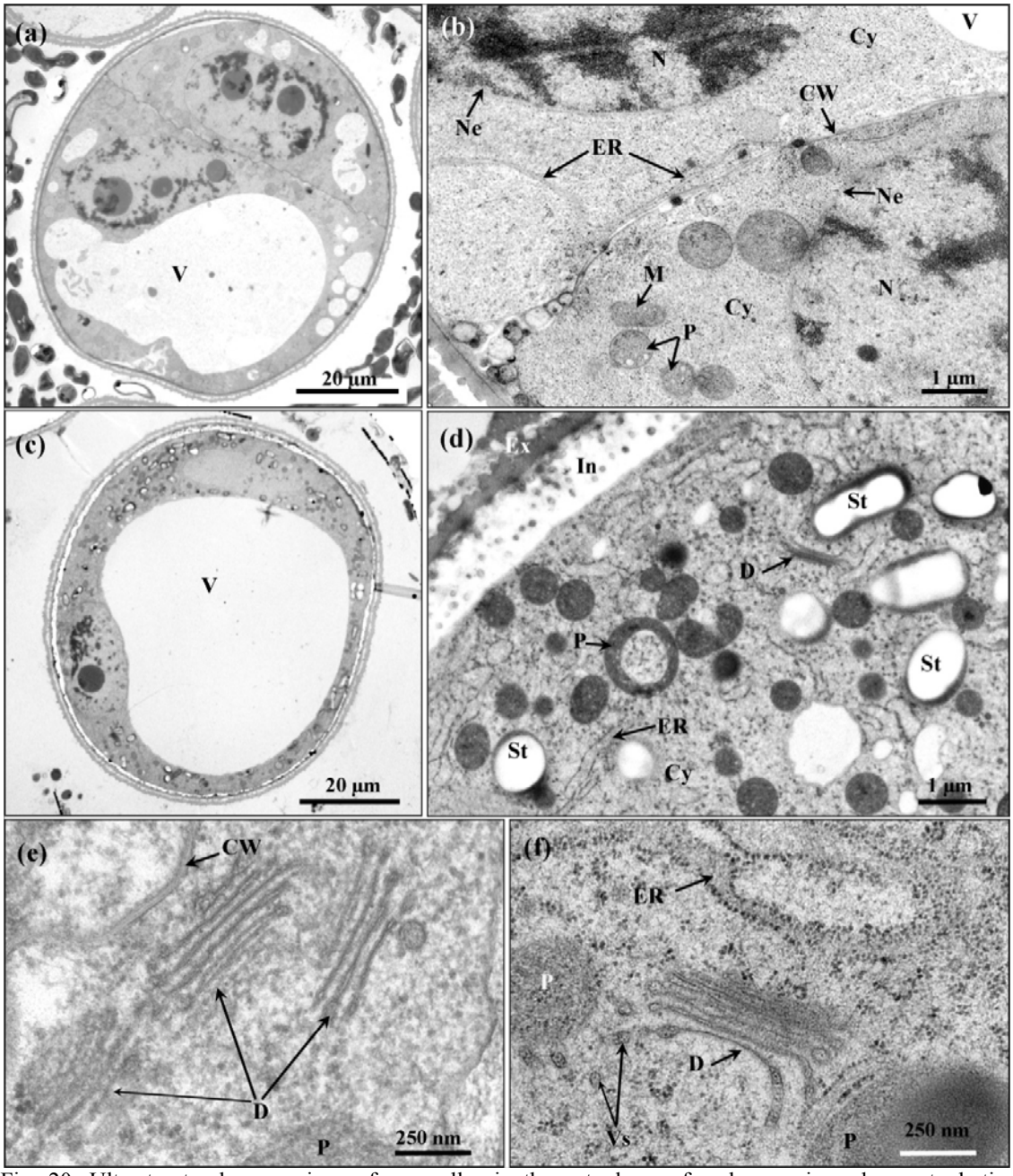

Fig. 20: Ultrastructural comparison of organelles in the cytoplasm of embryogenic and gametophytic pollen. (a, b, e) Embryogenic pollen. (c, d, f) Gamtophytic pollen. (a) Bi-nuclear pollen (3 days). (b, e) Cytoplasm in detail with a few mitochondria, endoplasmic reticulum, Golgi apparatus (e) amid ribosomes (black dots). (c) Gametophytic pollen before first mitosis with dense cytoplasm. (d, f) Cytoplasm in detail with dense network of rough endoplasmic reticulum, plastids with starch granules, mitochondria and dictyosomes. Cy, cytoplasm; D, dictyosome; ER, endoplasmic reticulum; Ex, exine; In, intine; M, mitochondria; N, nucleus; Ne, nuclear envelope; P, plastid; St, starch; V, vacuole.

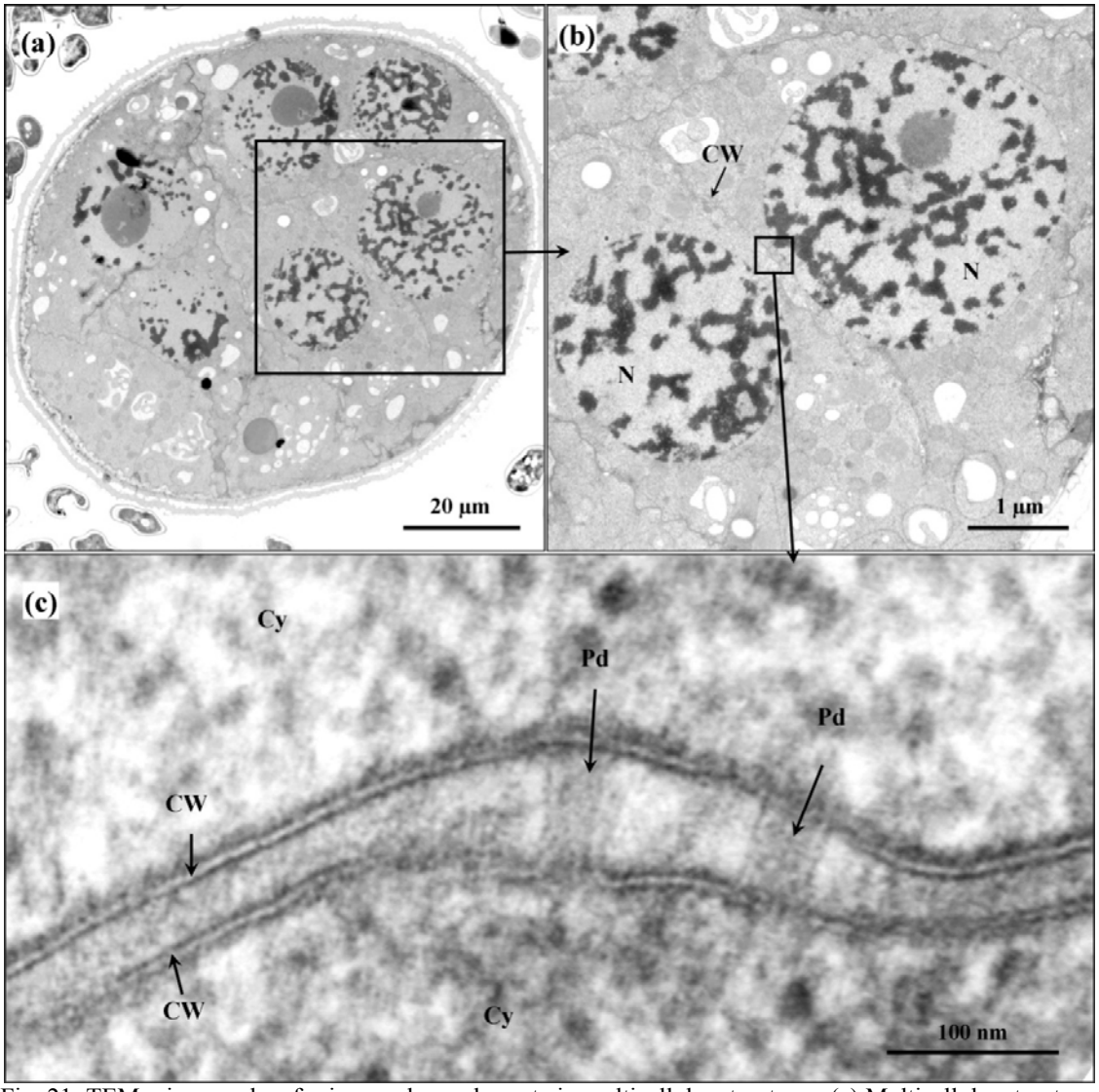

Fig. 21: TEM micrographs of primary plasmodesmata in multicellular structures. (a) Multicellular structure (13 days). (c) Detailed view of primary plasmodesmata between the new developed cell wall of two cells shown in (b). CW, cell wall; N, nucleus; Pd, Plasmodesmata.

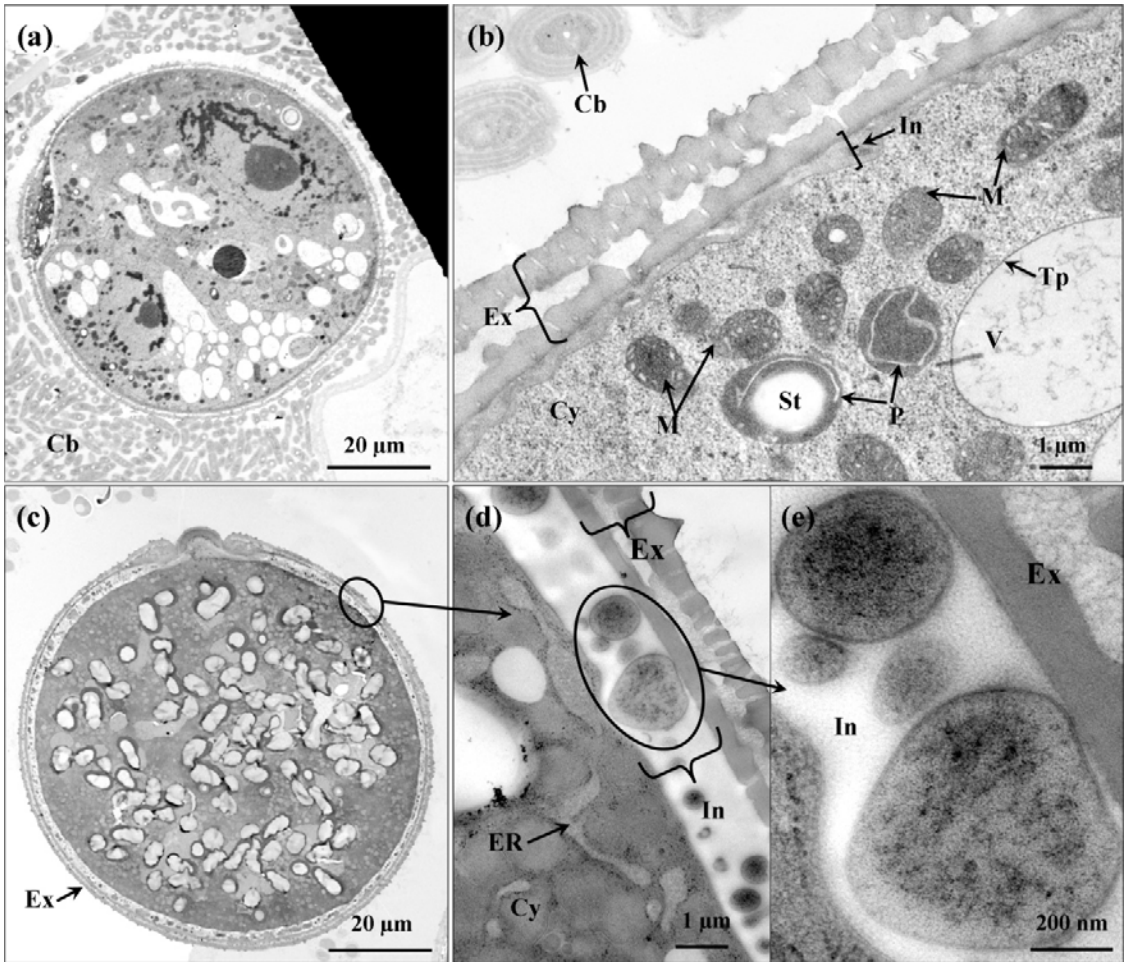

Fig. 22: Ultrastructural comparison of the intine of embryogenic and gametophytic pollen. (a, b) Multicellular structure (10 days) with a thin intine (detail in b). (c-e) Mature pollen after anthesis containing a clearly thickened intine with vesicle inclusions (detail in e). ER, endoplasmic reticulum; Ex, exine; In, intine; M, mitochondria; P, plastid; St, starch; Tp, tonoplast; V, vacuole.

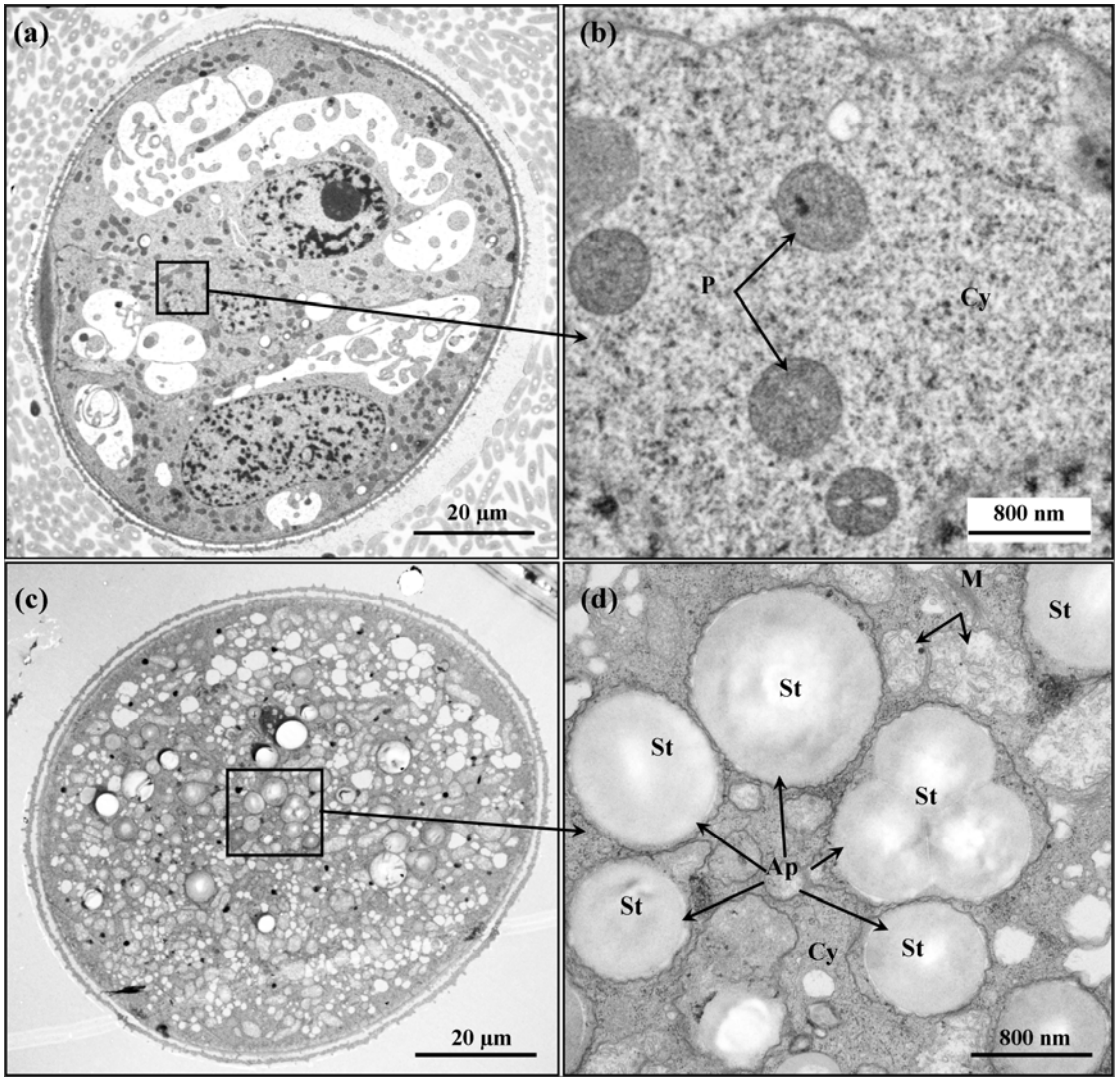

Fig. 23: Ultrastructural comparison of starch content and plastid type of late developmental stages of embryogenic and gametophytic pollen. (a, b) Multicellular structure (10 days) with rich cytoplasm and plastids not showing any starch accumulation (detail in b). (c, d) Mature pollen after anthesis with a massive accumulation of starch granules in amyloplasts (detail in d). Ap, amyloplast; P, plastid; St, starch.

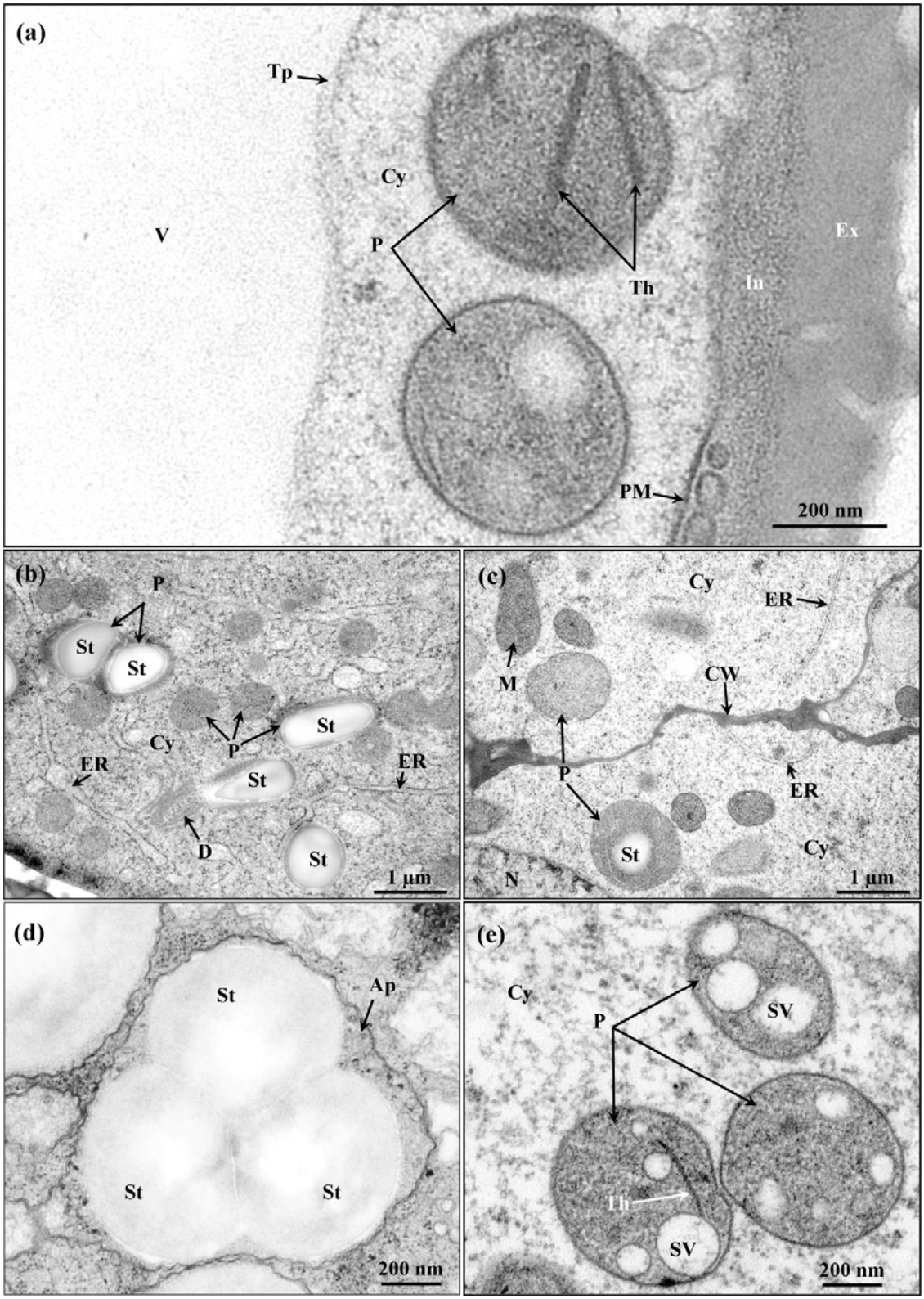

Fig. 24: TEM micrographs of plastid types in pollen embryogenesis and gametogenesis. (a) Proplastids in fresh isolated immature pollen. (b) Plastids with large starch grains in gametophytic pollen before first mitosis. (c) Plastids in early multicellular structure (5 days) without or with a single small starch grain.

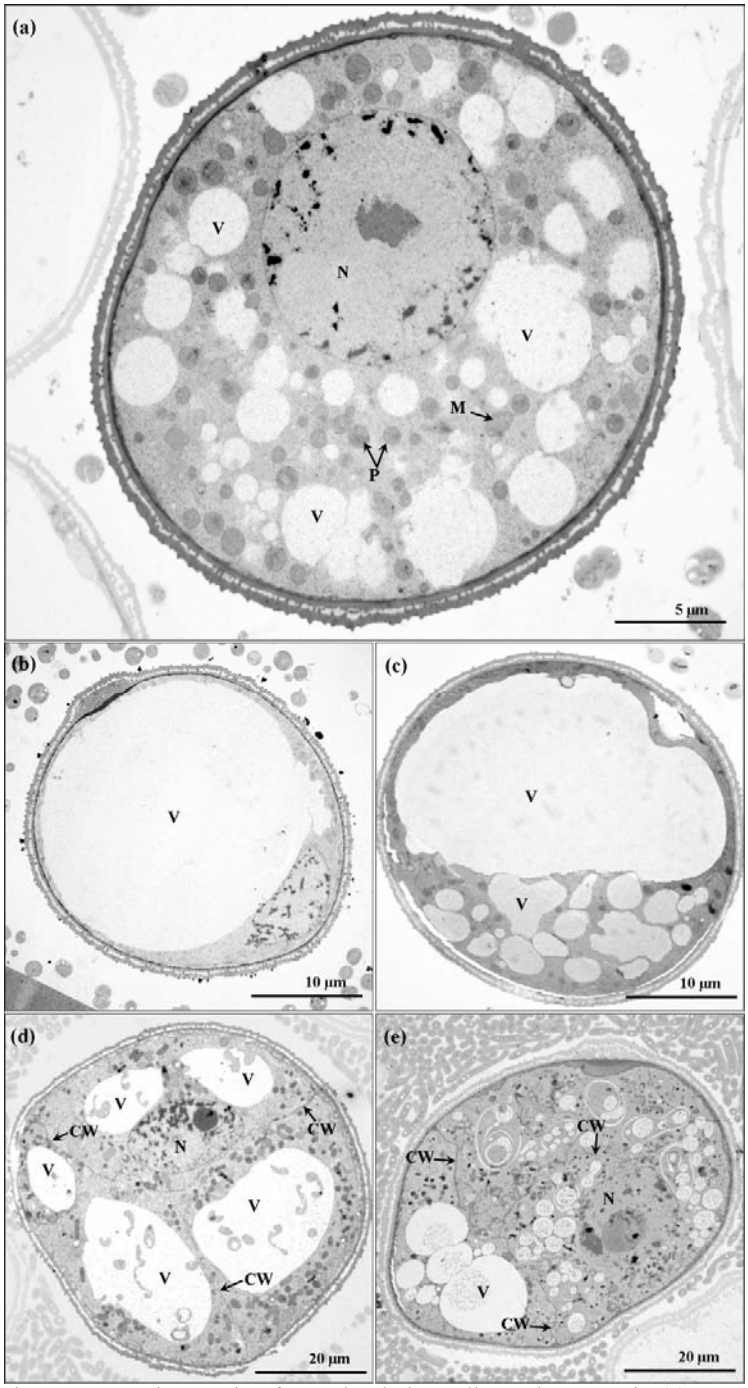

Fig. 25: TEM micrographs of vacuoles during pollen embryogenesis. (a) Young microspore isolated from young spike at booting swollen stage containing multiple small vacuoles. (b) Uni-nucleate immature pollen with large central vacuole. (c) Embryogenic pollen with a large central and multiple small vacuoles (1 day after induction). (d) A multicellular structure containing few relatively large vacuoles in daughter cells (4 days) (e) A multicellular structure containing numerous small vacuoles (8 days). CW, cell wall; M, mitochondria; P, plastids; V, vacuoles.

Continue Fig. 24: (d) Amyloplast in mature pollen after anthesis. (e) Plastids with storage vacuoles in late multicellular structure (10 days). Am, amyloplast; Cy, cytoplasm; CW, cell wall; D, dictyosomes; ER, endoplasmic reticulum; Ex, exine, In, intine; M, mitochondria; N, nucleus; P, plastids; PM, plasma membrane; St, starch; SV, storage vacuole; Th, thylakoids; Tp, tonoplast; V, vacuole.

3.3. Generation of transgenic plants with GFP-labeled nuclei

3.3.1. Gene transfer into embryogenic pollen

An important aspect of the present study was to follow the fate of the nuclei during embryogenesis. After preliminary process-oriented investigations with wild type material it turned out that plain differential interference contrast was inadequate without the help of specific markers. Therefore, the transgenic plants constitutively expressing a *SV40 NLS:GFP* construct were generated. To this end, embryogenic pollen were used as gene transfer recipient tissue, and 71 T_0 regenerants were obtained. These plants were analysed with respect to their ploidy level, the presence of the transgene, and the accumulation of the transgene product.

3.3.2. Ploidy level and molecular analysis of transgenic plants

The ploidy level of the regenerants showed that the vast majority were either haploid (42.2%) or diploid (47.9%). Because of space limitations in the greenhouse, five spontaneously doubled and 20 haploid lines (the latter after colchicine treatment) were kept and grown to maturity. Eighty-five percent of the colchicine-treated haploid plants set grains (17 plants). PCR analysis with primers specific for the selectable marker gene *HPT* showed that all obtained 22 doubled haploid plants contained the corresponding fragment. Further CLSM examination of different tissues (i.e. root tips, leaves, pollen) showed that only one single line out of these 22 did accumulate detectable amounts of GFP in its nuclei (Fig. 26).

Immature pollen of the selected plant was isolated and cultured to induce pollen embryogenesis and the eventual production of doubled haploid plants. The 27 T_1 regenerants that were produced varied in their ploidy level and comprised of 7 tetraploid, 5 triploid, and 15 diploid plants. Progenies of 7 randomly selected diploid plants were grown for live cell imaging studies. To confirm the integration of the *HPT* and *NLS:gfp* genes in the plant genomic DNA, four T_2-families were selected at random for PCR and southern blot analysis (Fig 27). As expected, all plants proved PCR-positive for both *HPT* as well as *GFP* and were therefore considered to be homozygous as to the T-DNA (Fig. 27b).

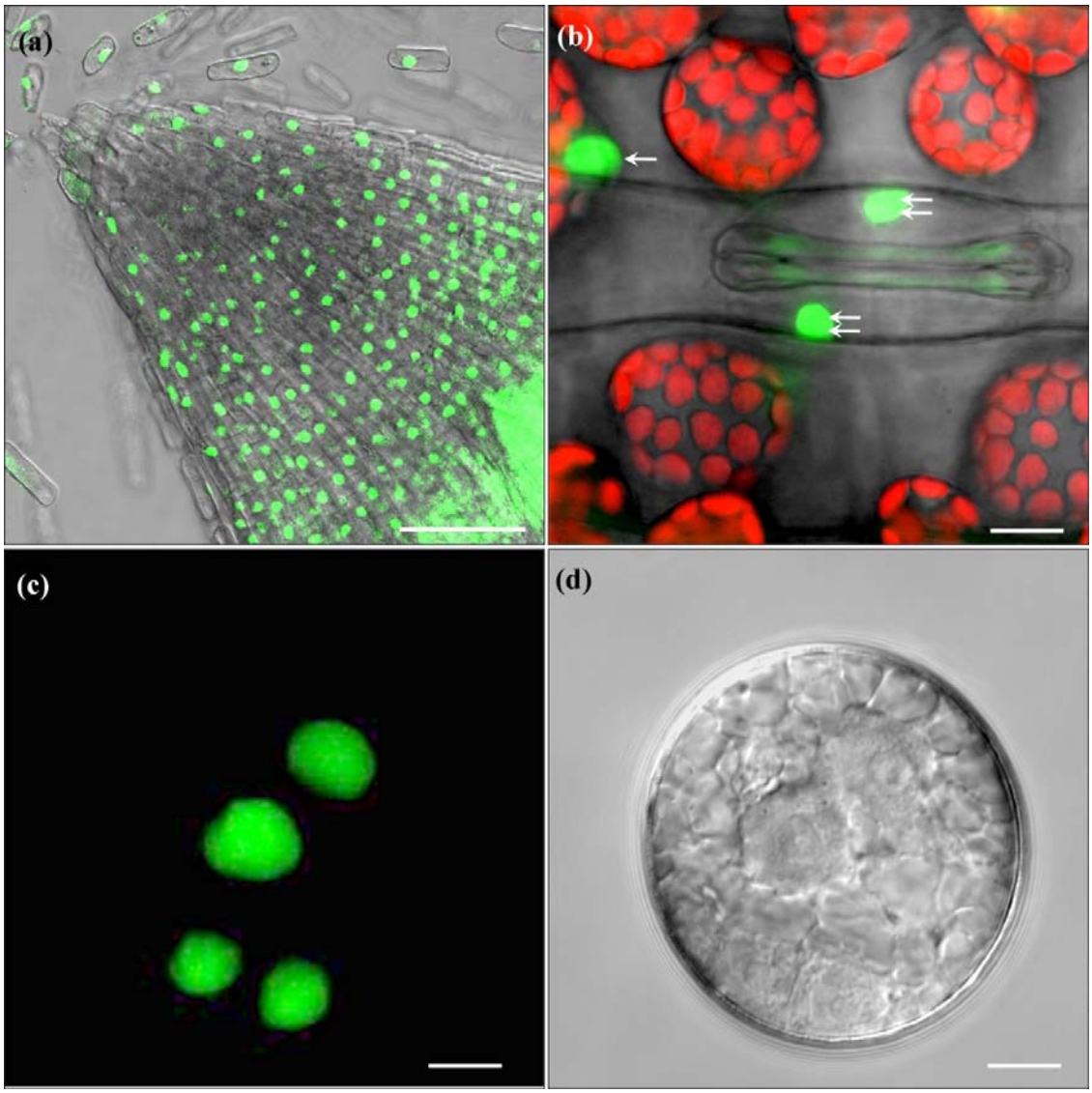

Fig. 26: Fluorescence microscopic analysis of T_1 regenerants of barley expressing the *SV40-NLS:GFP* gene. (a) GFP accumulation in the nuclei of a root tip. (b) Leaf tissue with GFP in the nuclei stomata (arrows) and epidermis cells (arrow). Chlorophyll autofluorescence is shown in red. (c, d) Transgenic immature pollen after the second pollen mitosis. (c) GFP in the nuclei. (d) corresponding DIC image.

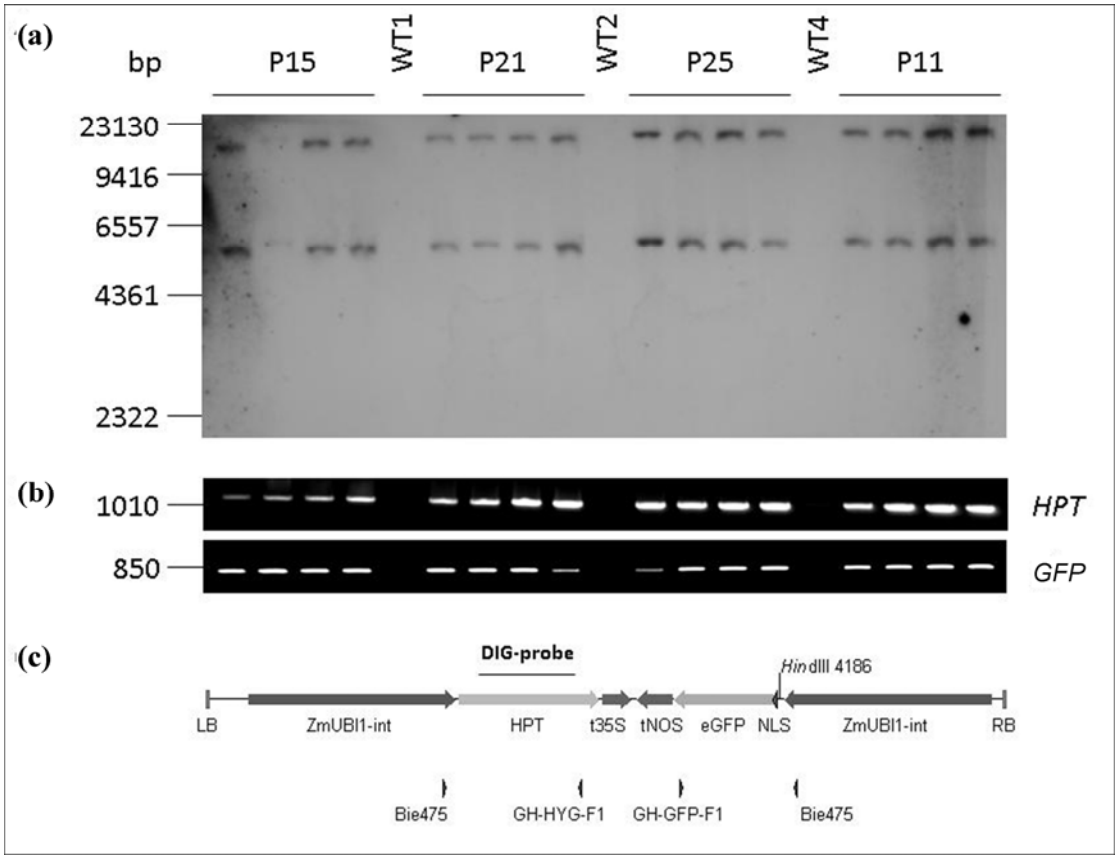

Fig. 27: Southern blot and PCR analyses of plants of four randomly selected T_2-families harboring the *NLS:gfp* construct. (a) Southern blot analysis of *Hind*III-digested genomic DNA hybridized with an *HPT*-specific probe. (b) PCR analysis with primer pairs specific for the *HPT* (upper) and the *GFP* (lower) genes. (c) Map of the T-DNA with primer pair and hybridisation probe positions indicated. WT, wild type cv. 'Igri'.

3.4. Live-cell imaging of cultured pollen

Pollen embryogenesis, as shown above, is a process, starting with an inductive treatment of immature pollen and ending with the formation of multicellular structures that more or less resemble zygotic embryos. Under appropriate conditions, this process takes about two weeks. Both starting material and cultures were far from being homogenous. The former often contained a mixture of uni- and early bi-nuclear immature pollen, while the latter was a complex mixture of multicellular structures, gametophytic pollen (GP), viable but undeveloped pollen and dead cells. Conventional light microscopy proved to be inadequate to establish which cells eventually produce what kind of structures and when the major cellular changes appear to happen. The employment of live-cell imaging, however, was accompanied by a number of technical obstacles that had to be solved first. Among the most significant technical challenges for performing successful live-cell

imaging experiments was the development of a protocol for the efficient induction of pollen embryogenesis and maintaining the cells in a healthy state on the microscope stage while being illuminated by white and UV-light.

3.4.1. Induction of embryogenesis in transgenic pollen

Different protocols for stress treatments were tested for their ability to induce pollen embryogenesis. In every treatment, immature pollen of five spikes was isolated and cultured. In the first experiments freshly isolated immature pollen were starved in SMB1 for 2 days at 24°C (SMB1 protocol). In an alternative protocol excised anthers were incubated in 0.4 M mannitol in the dark for three days either at 4°C (APT4) or at 24°C (APT24). After incubation of anthers, immature pollen was isolated and starved for one day in SMB1 medium. Immature pollen of the different treatments was transferred to KBP medium for further development.

Best results in terms of the yield of isolated immature pollen per 5 spikes were obtained through direct isolation (SMB1 protocol), while APT4 gave better results than the APT24 treatment. After immature pollen had been cultured for two weeks in KBP medium, the number of multicellular structures, gametophytic pollen and the total proportion of surviving pollen was determined (Fig. 28; 29). Although both APT4 and APT24, as compared with the SMB1 treatment, led to a higher percentage of pollen with starch accumulation that is indicative of the gametic developmental pathway, it was found that the APT4 treatment resulted in the highes pollen survival and efficiency of multicellular structure formation of all treatments tested. In turn, SMB1 resulted in the lowest percentage of survival and multicellular structures formed. As a consequence of its efficiency in pollen embryogenesis, the APT4 treatment became the method of choice for the live cell imaging experiments.

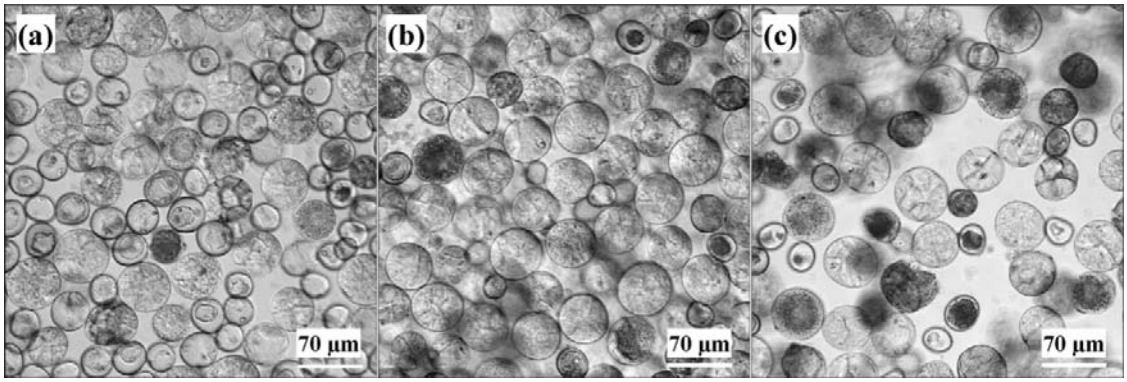

Fig. 28: Multicellular structure population after different stress protocols to induce pollen embryogenesis, two weeks in KBP medium. (a) SMB1. (b) APT4. (c) APT24.

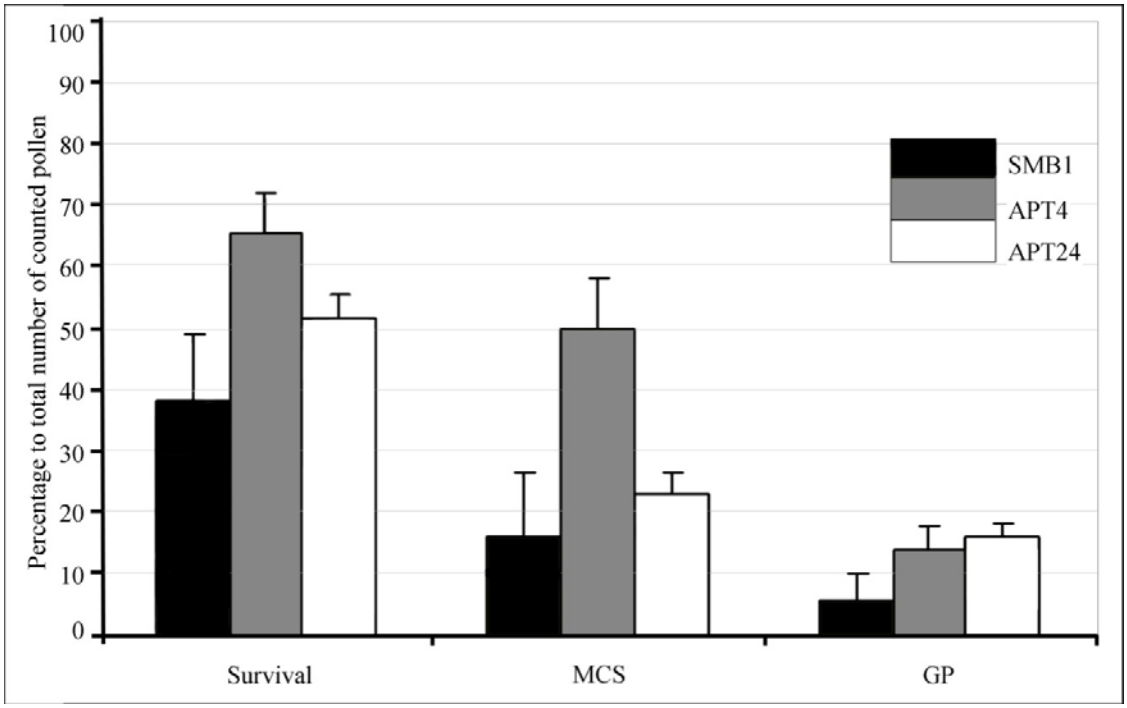

Fig. 29: Effect of the SMB1, APT4 and APT24 protocols to induce pollen embryogenesis in immature microspore cultures. Cells were analyzed after 2 weeks of culture in KBP medium for induction of pollen embryogenesis. Survival has been described by the total number of viable structures. MCS, multicellular structures; GP, gametohytic pollen.

3.4.2. Setup of live-cell imaging chamber

Live-cell imaging was performed on a CLSM Zeiss LSM 510 META equipped with an inverted microscope. The latter feature is of great advantage because the object of study was as close to the objective as possible. The combination of an inverted microscope with chambered coverslides appealed to this paradigm.

Kumlehn and Loerz (1999) and Indrianto et al. (2001) showed that live-cell imaging of

pollen embryogenesis is possible when cells are spaced at distances that avoid overgrowth by neighboring cells as well as the optical interference of other cells in the background or foreground. Nevertheless, a minimal cell density is required to condition the medium for successful embryogenesis. The culture medium presented a hitherto unforeseen problem. Due to the large vacuole, immature pollen tended to float and did not settle at the bottom of the coverslip. It thus became a demand to device a format that would restrain the cells at the surface of the coverslip.

After a period of trial and error, a protocol was established that started with the pipetting of 1 ml immature pollen suspension on a plastic surface followed by concentrating the pellet into around 100 µl. With time the highly vacuolated immature pollen accumulated at the liquid-air interface and could be collected by placing a small piece of hydrophilic PTFE membrane on top of the droplet to which the vacuolated cells would adhere. The thus concentrated cells of interest were placed with the PTFE membrane on top into a chambered cover slip.

By using the inverted microscope, detailed recordings were feasible, since the cells of interest had been trapped directly on the coverslip of the custom made chamber (Fig. 30). This setup allowed using the recommended cell density of pollen culture (i.e. 200,000 per ml) with high optical visualization and maintaining the cells under normal growth conditions for up to 30 days.

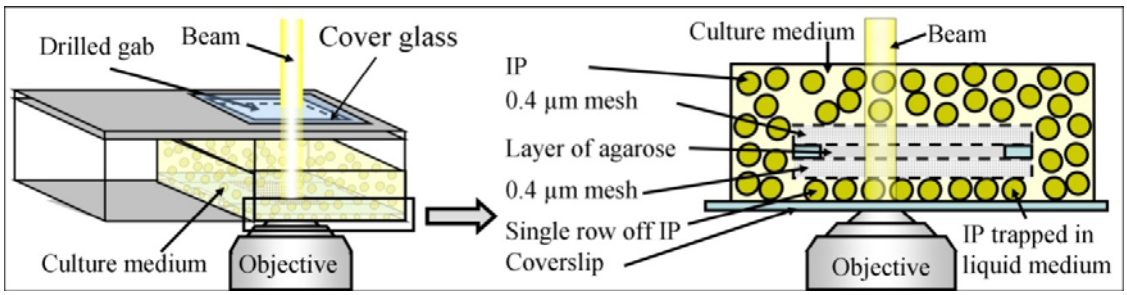

Fig. 30: Schematic drawings of the custom-made live-cell imaging chamber. IP, immature pollen.

3.4.3. Monitoring of cultured immature pollen

3.4.3.1. Examination of wild type pollen

Microscopic observations of immature pollen in culture were started at the end of the starvation phase. Observations were made with a 20x objective (NA 0.75) which was an optimal trade-off between resolution and monitoring a useful number of pollen grains. Nevertheless, the number of pollen that could be followed in a single experiment was rarely more than a handful (4-5 cells). Therefore, numerous live cell imaging trials had to be carried out to obtain conclusive data. In the confined space of the culture chamber and the absence of agitation, development of the cultured pollen was significantly delayed compared to standard culture conditions. Therefore, a single series monitoring the development of cultured immature pollen till the multicellular structure stage lasted up to 28 days. Despite this slow development that entailed a prolonged incubation time, immature pollen cultures often retained a high level of viability emphasizing the appropriation of the culture chamber set-up.

Initial experiments involved non-fluorescent, wild-type immature pollen, which was studied using DIC to improve the contrast of structural details. Fig. 31 shows a typical initial live-cell imaging experiment of cultured immature pollen. The fate of five vacuolated uni-nucleate pollen grains was studied for seven days. Z-stacks of seven optical sections with 4 µm spacing were recorded each 10 min resulting in a total number of 3570 recorded images. Two of the pollen, No. 4 and 5, died between days 3 and 5 of the experiment (Fig. 31f, g). The other three pollen survived for seven days before they also succumbed. Of these three pollen, only No. 2 underwent embryogenic development and completed several cycles of cell division, the first of which occurred on day 4 of the experiment (Fig. 31h). The pollen No. 1 and 3 remained stationary and did not show embryogenic development. These first recordings often had a low optical quality. This was due to the gradual condensation of water droplets in the live-cell imaging chamber which caused a diffraction of the light beam associated with poor DIC images. This problem was overcome by the introduction of a hole in the top lid of the live-cell imaging chamber covered by a coverslip which allowed sufficient evaporation and avoidance of water droplet formation.

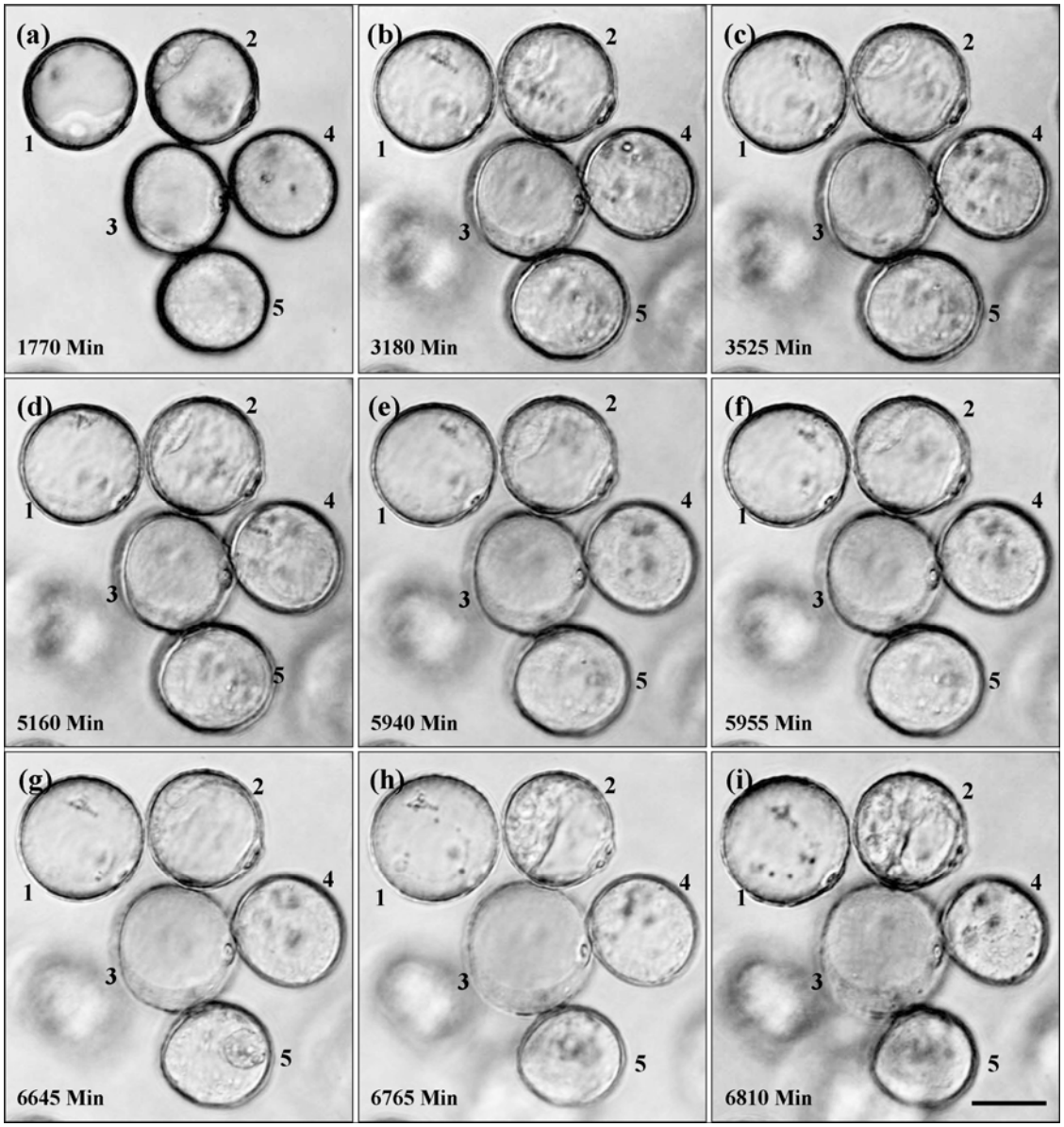

Fig. 31: Live-cell imaging of immature pollen wild-type over 7 days. Pollen No. 1 to 3 survived without development. Pollen No. 2 underwent embryogenic development with a first mitosis taking place on day 4 (e-i). Pollen No. 4 and 5 died and collapsed between days 3 to 5 (f-i).

Fig. 32 shows one of the advances live-cell imaging experiments with cultured immature wild-type pollen. Four vacuolated uni-nucleate pollen grains were observed over a time-span of 30 days. Z-stacks of nine optical sections with 5 µm spacing were recorded every 3 min giving a total number of 91242 recorded images. After two days the first mitotic event was recorded in pollen no. 1. Nuclear division occurred at the opposite side of pollen aperture and resulted in two nuclei of identical size (Fig. 32b).

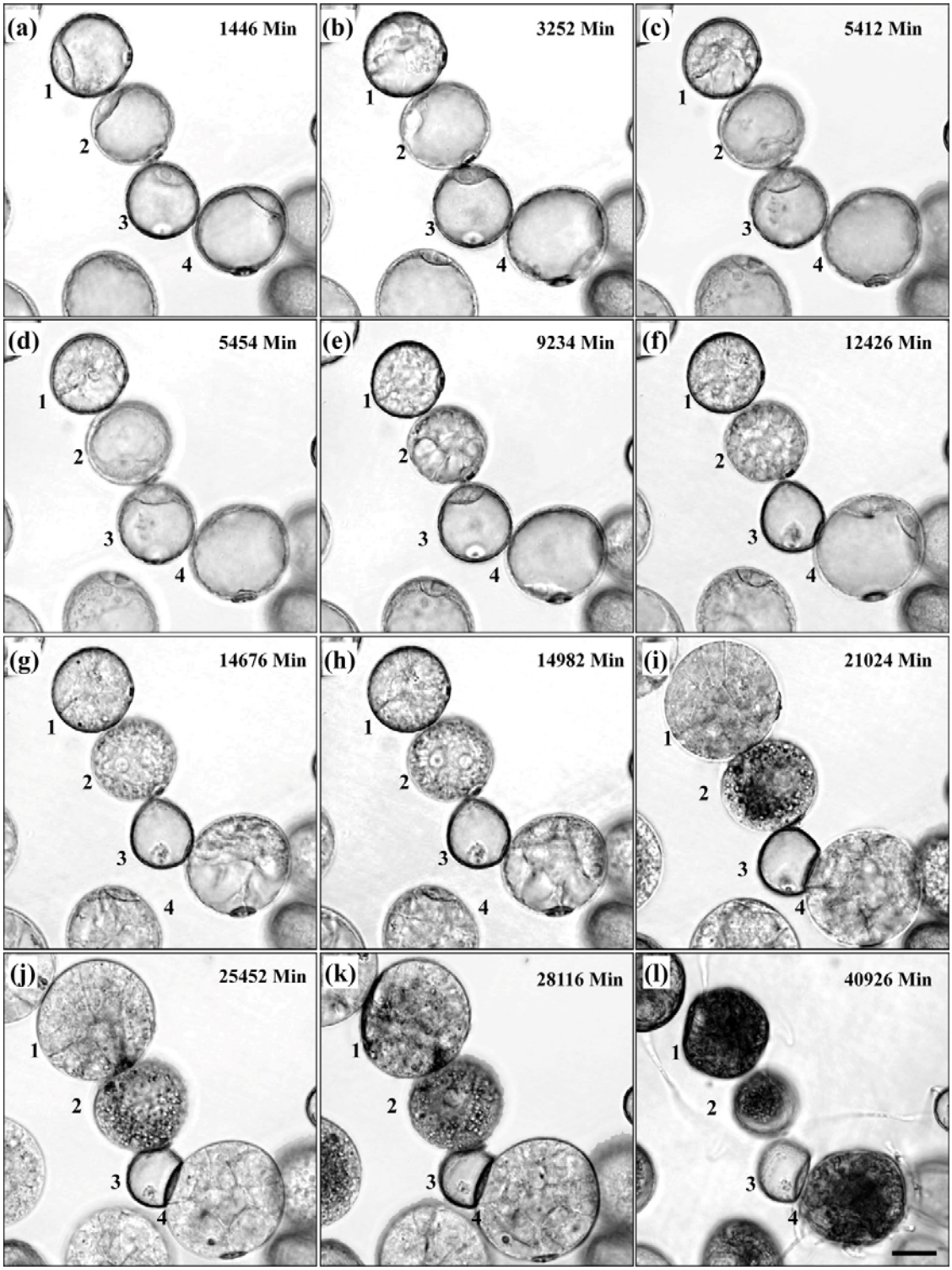

Fig. 32: Live-cell imaging of immature pollen over 30 days. Pollen No. 1 and 4 underwent embryogenic development and reached the multicellular structure stage (a-l). After dividing just once (e-f), pollen no. 2 remained stationary and started to accumulate starch granules (g-k). Pollen no. 3 showed no sign of development and died after 7 days (d-f). Fungal infection led to death of all cells by day 30 (l).

In the course of the following days a further string of synchronized cycles of mitosis was observed in pollen no. 1 which led to the generation of a multicellular structure with small vacuoles. Despite high mitotic activity, the diameter of pollen no. 1 remained constant over the first 14 days of the observation (Fig. 32c-h) before increasing significantly in the period between days 14 and 28, before the exine wall was broken. In pollen no. 2, the first mitotic event was observed after 3 days of culture (Fig. 32b). This pollen retained its large vacuolar space approx. 10 days of culture (Fig. 32c, d), then vacuolar compartmentalization started (Fig. 32e, f). No further mitosis was observed in pollen no. 2 which showed little increase in size over the entire time of observation. From day 15 onwards, this pollen started to accumulate starch granules (Fig. 32h). Pollen no. 3 did not show any developmental changes and collapsed on day 7 (Fig. 32f). The last in this series, pollen no. 4, first increased in size (Fig. 32a-e) before performing a first mitosis after 7 days in culture resulting in two similarly sized nuclei (Fig. 32f). Several additional synchronized cycles of mitosis were recorded which turned pollen no. 4 into a multicellular stucture of steadily increasing size (Fig. 32g, k). Also here no rupture of the exine wall was observed in the first 28 days of observation. This particular experiment had to be terminated on day 30 after a fungal infection occurred. Two days after the detection of growing hyphae the entire culture had died (Fig. 32l). From results like these it became evident that cultures in the live-cell imaging chamber were prone to a delay in development compared to culture under optimal conditions.

3.4.3.2. Examination of transgenic immature pollen

After optimizing the custom-made chamber for live cell imaging, it was used to visualize transgenic immature pollen during embryogenesis. In five experiments starting on day 0 after isolation, the developmental progress of a total of 71 individual immature pollen grains was analyzed. Pollen that died within the first hours after isolation were not further considered. At the beginning of the recording period, only one of these immature pollen grains was in the bi-cellular developmental stage and the other 70 were uni-nucleate. With a diameter of 40 µm the bi-cellular immature pollen was distinctly larger than the uni-nucleate pollen which were about 30 µm in diameter.

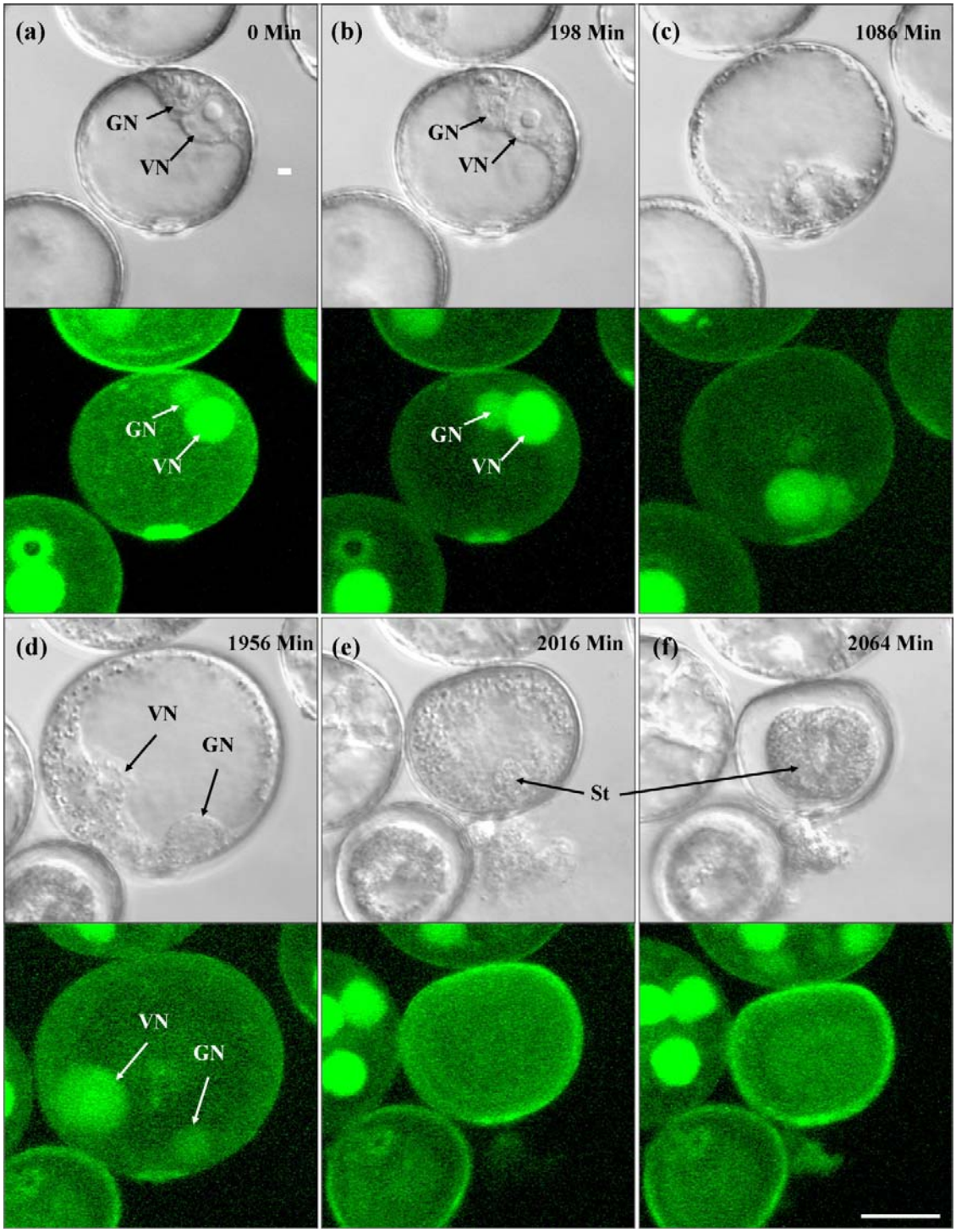

Fig. 33: Time-lapse of bi-cellular pollen fate in culture. CLSM showing DIC and nuclear GFP fluorescence in parallel. (a) Bi-cellular pollen with large vacuole, a large vegetative and a smaller round-shaped generative nucleus. (b-d) Distinct size increase and progressive accumulation of starch granules, translocation of the generative cell towards the site of the aperture. (e, f) Cell death indicated by the disappearance of the GFP and a general collapse of cell and cytoplasm. GN, generative nucleus; St, starch granules; VN, vegetative nucleus. Bar = 20 µm.

An interesting feature of this bi-cellular pollen was the size and shape of its nuclei, in particular that of the presumed generative nucleus which, though distinctly smaller, remained round (Fig. 33a-f). With time, the bi-nucleate pollen grain increased in size without showing any further mitosis divisions. This pollen grain started to accumulate increasing amounts of starch (Fig. 33c, d) until it died as indicated by a collapse of the cytoplasm on second day of culture (Fig. 33d-f). The remaining 70 uni-nucleate immature pollen grains showed various patterns of development. All of these individuals were initially characterized by the presence of a large central vacuole.

Based on the first mitosis (symmetric or asymmetric) and the final fate of the immature pollen, Table 5 shows that five different developmental pathways could be discerned over time. Based on the final fate, further differentiations within the symmetric and asymmetric pathway resulted in a total number of 9 different developmental types as shown in Table 5.

Table 5: Different types of pollen development as observed in live-cell imaging experiments during the initial 7 days of culture in KBP medium

Types	Symmetric			Asymmetric			Micronuclei	Large pollen	Undeveloped
	I	II	III	IV	V	VI	VII	VIII	IX
No. of pollen	38	2	3	3	2	5	1	2	14
Percentage	54.3	2.8	4.3	4.3	2.8	7.1	1.4	2.8	20

The majority of the uni-nucleate pollen started their development with a symmetrical division (61.1%). At this stage, three different types of development followed. 54.3% of the total uni-nucleate pollen followed the type I pathway, while 2.8% followed a type II pathway and 4.3% was identified as type III pathway (Table 5). In type I, immature pollen went through successive cell divisions and eventually formed multicellular structures (Fig. 34). Under the present culture conditions, exine rupture was not observed prior to day 14. The nucleus of the type I cells contained a single nucleolus (Fig 34a) and migrated to a more central position within the cell before division (Fig. 34a-c). The vigorously moving nucleus which increased in size and became spherical, remained in its central position by cytoplasmic strands that run between the cellular periphery and the nuclear periphery (Fig. 34b, c). These cytoplasmic strands were always the final structural markers before division of the nucleus. The first mitosis resulted in a symmetric division (Fig. 34a-e). Throughout this process, the cells themselves did not

increase in size, albeit a clear expansion of the cytoplasm was observed at the same time (Fig. 34a-f). This type of pollen did not show starch accumulation. A second symmetric and synchronized mitosis took place in both daughter cells still within the first day of culture in KBP medium (Fig. 34f, g) indicating successive cell proliferation towards the formation of a multicellular structure.

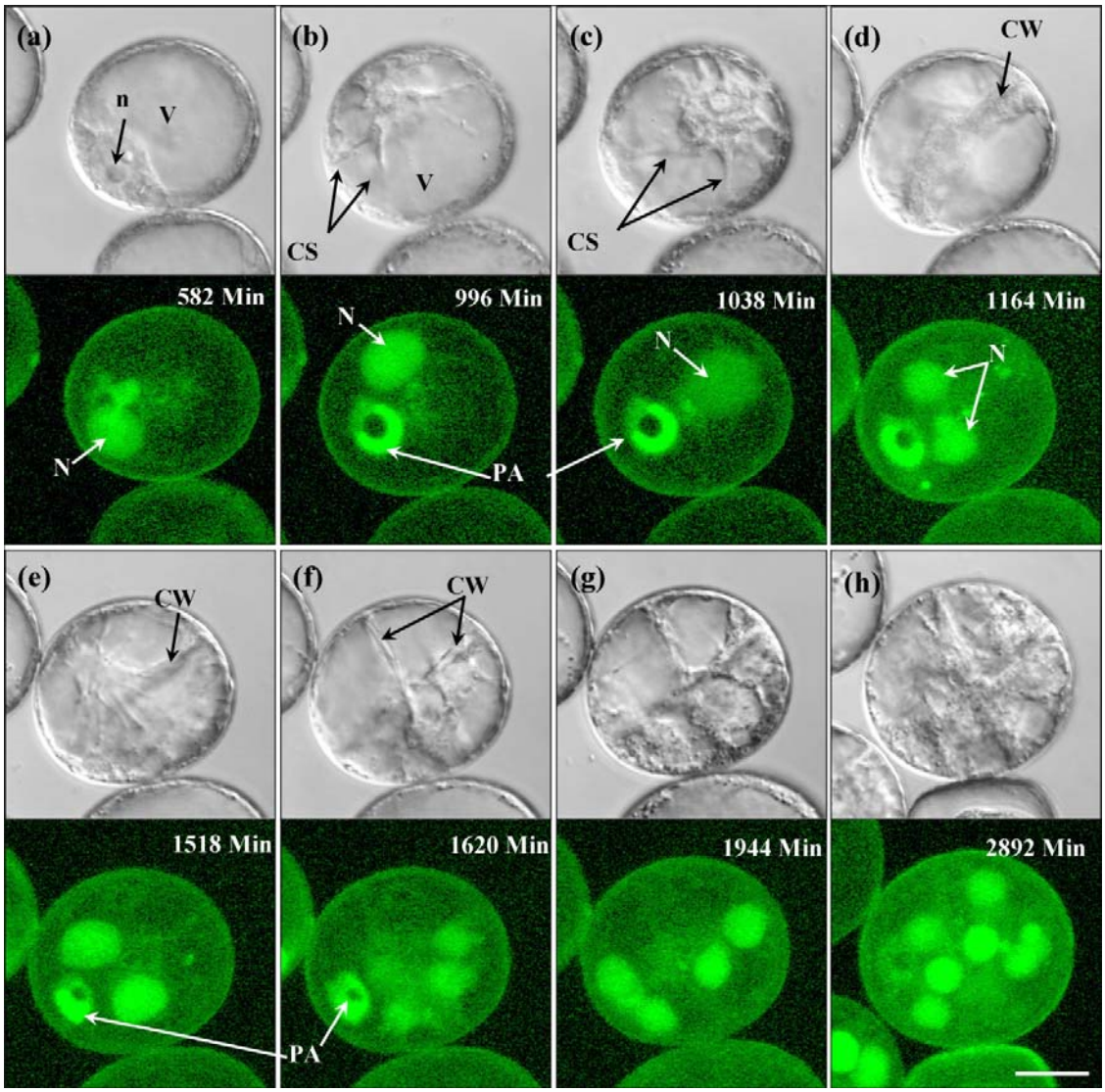

Fig. 34: Time lapse of type I development (embryogenic pollen). CLSM images shown in DIC and nuclear GFP fluorescence. (a) Uni-nucleate pollen grain with the nucleus close to the pollen aperture. (b, c) Migration of the nucleus to a semi-central position and formation of cytoplasmic strands. The blurred fluorescence signal seen in c indicates the disruption of the nuclear envelope before mitosis. (d) Newly formed cell wall (DIC) and two daughter nuclei (GFP) after mitosis. (e) Appearance of cytoplasmic strands indicating imminent second mitosis. (f, g) Newly formed cell walls (DIC) separate four cells (GFP) contained within the original exine. (h) Additional cycles of mitosis have created multicellular structure. CS, cytoplasmic strands; CW, cell wall; N, nucleus; n, nucleolus; PA, pollen aparature. Bar = 20 µm.

Type II was characterized by a first apparently symmetric division but did not show any further mitotic activity afterwards (Fig. 35).

Another early difference to the type I development was the behavior of the nucleus which prior to mitosis did not move to a central position but remained peripheral, opposite to the pollen aperture (Fig. 35a-e). Furthermore, also the cytoplasm remained largely peripheral and did not form cytoplasmic strands (Fig. 35a-e). Despite the formation of two nuclei of equal size and shape, the DIC recordings did not show any sign of cell wall formation (Fig. 35d-g). This absence of a median cell wall was further supported when the two daughter nuclei moved side by side away from their position opposite to the pollen aperture approximating the latter (Fig. 35f-j). The cell gradually increased in size (Fig. 35a-l) and after three days in KBP medium, starch granules started to accumulate (Fig. 35k). Shortly afterwards, the cells died as indicated by a sudden decrease of pollen size and disappearance of GFP from the nucleus, eventually resulting in a collapse of the cytoplasm (Fig. 35m-o).

Type III development also started with a nucleus located opposite to the pollen aperture at the time of cell division. Mitosis took place in the periphery of the cell without the formation of cytoplasmic strands (Fig. 36a-c). In contrast to type II development, however, the resulting cell division was clearly symmetrical with the two daughter nuclei moving into the center of the cell, separated by a median cell wall (Fig. 36d-o). Although the daughter nuclei left the cellular periphery and cytoplasmic strands were formed (Fig. 36h-j), no further mitotic divisions occurred as it was the case in type I development. Similar to type I, in type III cells did not increase in size over time, but similar to type II they gradually accumulated starch granules (Fig. 36g-o). Cells showing the type III development normally remained viable for more than two weeks, i.e. during the entire time of observation as indicated by the consistent presence of GFP in the nucleus (Fig. 36o).

Developmental type IV (4.3%) also included immature pollen that followed the embryogenic pathway and formed multicellular structures (Fig. 37). The cell significantly increased in size and cytoplasmic strands appeared before the first mitosis (Fig. 37a-d).

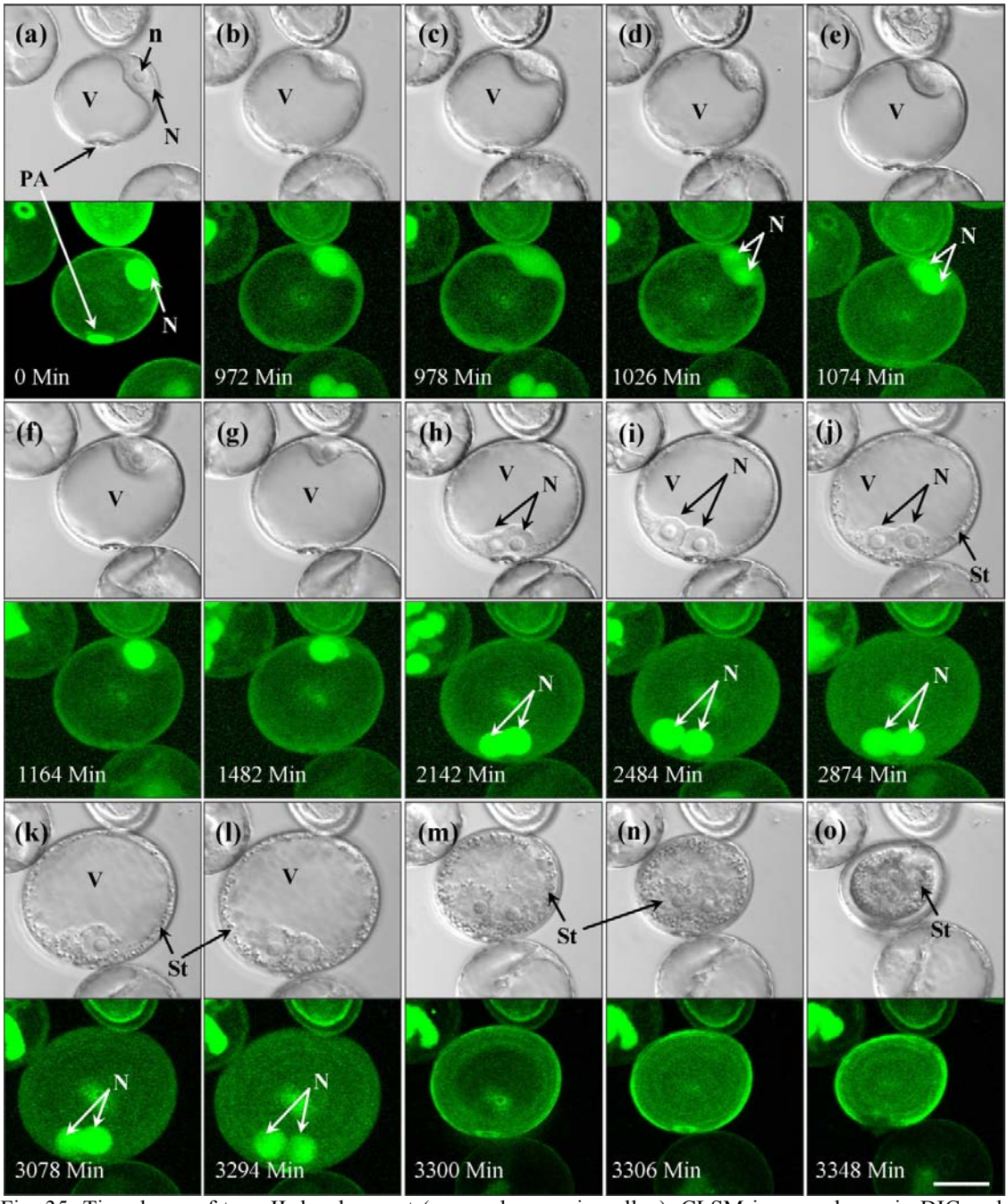

Fig. 35: Time lapse of type II development (non-embryogenic pollen). CLSM images shown in DIC and nuclear GFP fluorescence. (a) Uni-nucleate pollen with large vacuole and nucleus, containing a single nucleolus, opposite to the pollen aperture. (b, c) Size of pollen increased during the first mitosis. Note the absence of cytoplasmic strands (DIC). (d, e) Daughter nuclei are in close vicinity (GFP) and no discernable cell wall (DIC) could be observed. (f-i) The two nuclei remain side by side while they translocate to the site of the pollen aperture; cytoplasm remains peripheral and no evidence for a discernable new cell wall. (j-l) From day three onwards, the cell accumulates starch. (m-o) Cell death indicated by a decrease in cell size and disappearance of the GFP fluorescence ending with the collapse of the cytoplasm. N, nucleus; PA, pollen aparature; V, vacuole Bar=20 µm

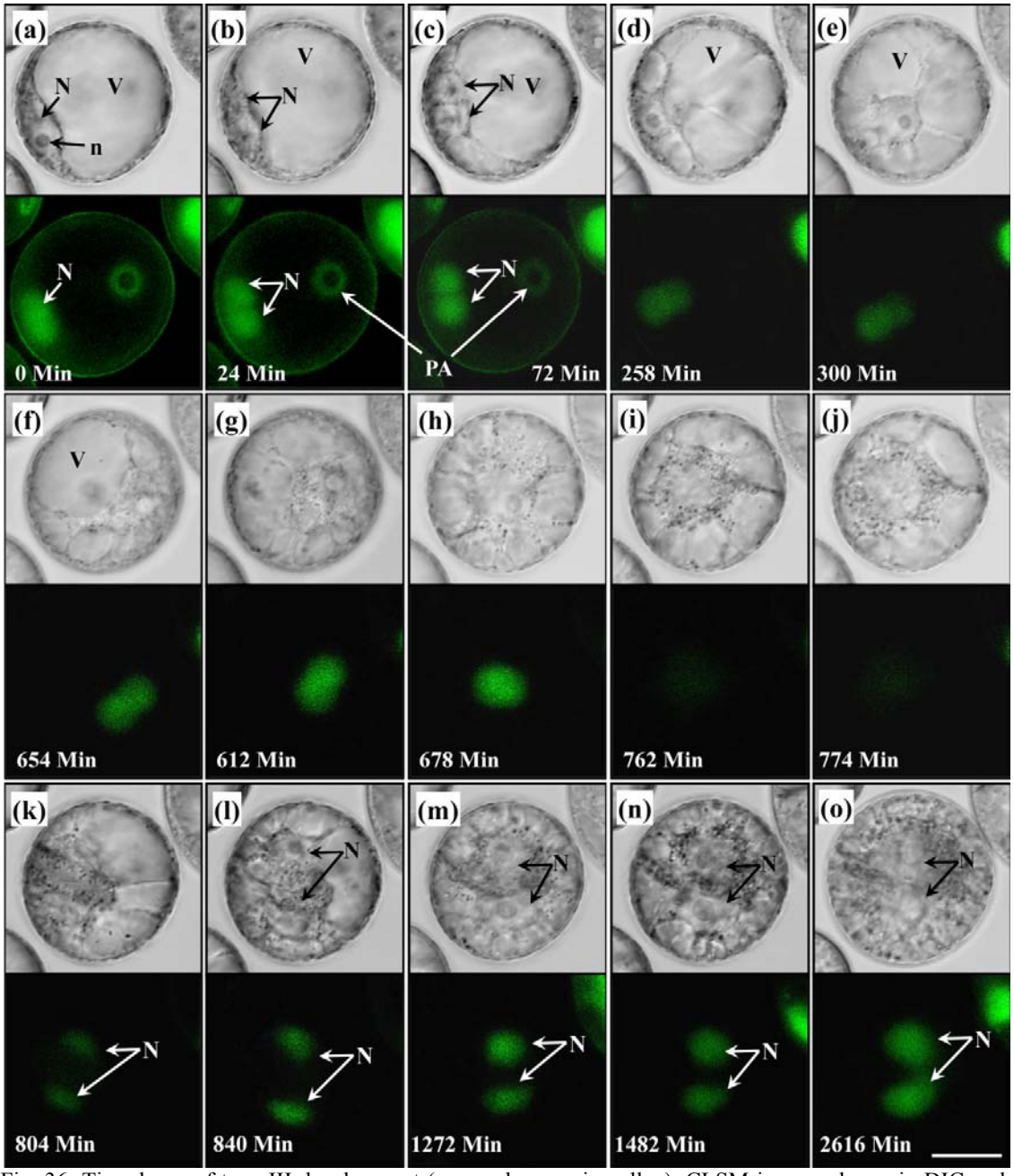

Fig. 36: Time lapse of type III development (non-embryogenic pollen). CLSM images shown in DIC and nuclear GFP fluorescence. After the initial symmetric division the pollen remains stationary bi-cellular, showing no further divisions but gradually accumulating starch while remaining viable during the time of the experiments (14 days). (a) Uni-nucleate pollen with a large vacuole and nucleus lying opposite to the pollen aperture. (b, c) Symmetrical cell division. (d, f) Cell with two similarly sized daughter nuclei and large vacuoles. (g-o) Increase of the cytoplasmic volume and starch accumulation. N, Nucleus; n, nucleolus; PA, pollen aperture; V, vacuoles. Bar = 20 µm.

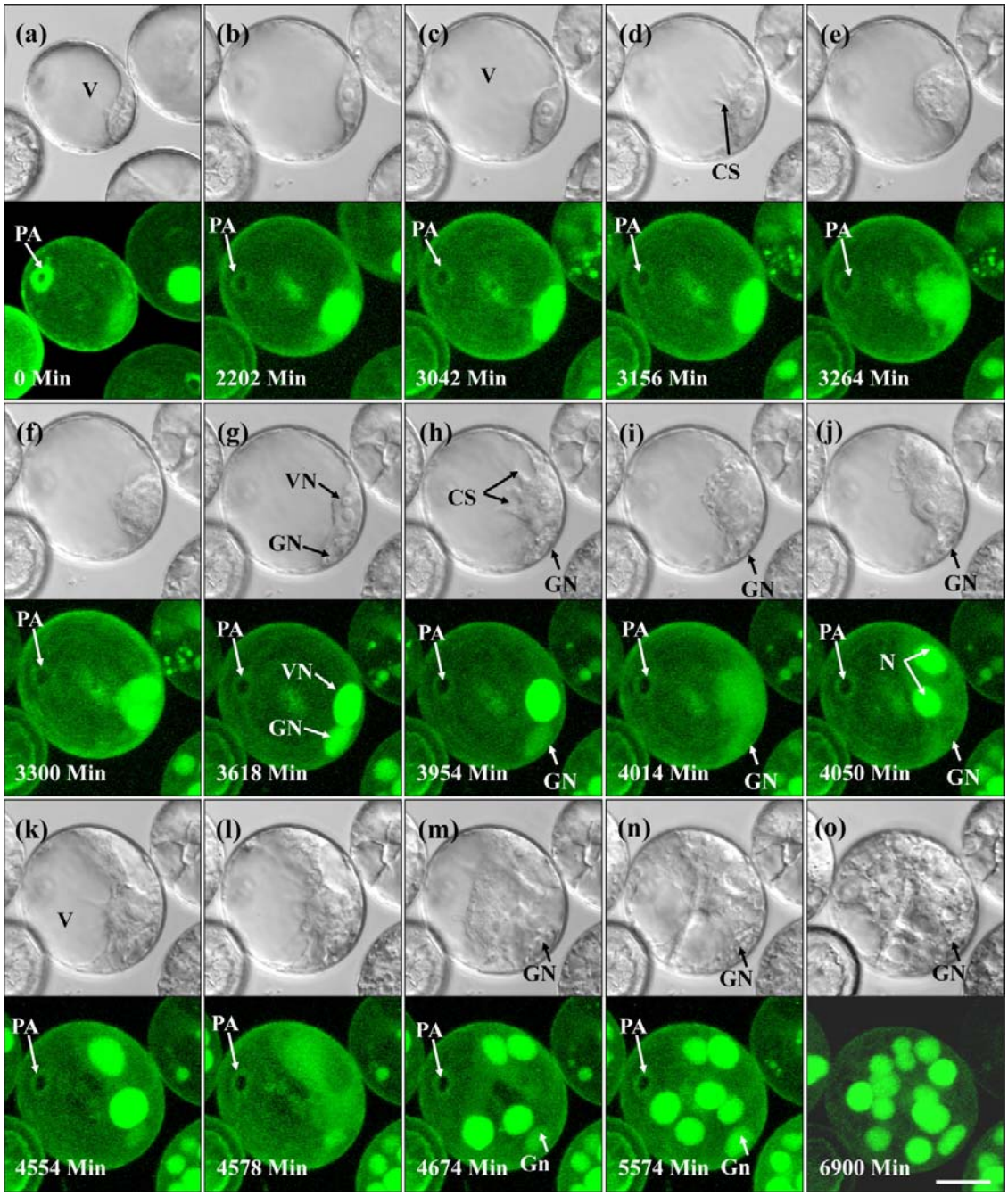

Fig. 37: Time lapse of type IV development (embryogenic pollen). CLSM images shown in DIC and nuclear GFP fluorescence. (a) Uni-nucleate pollen with a large vacuole and a thin layer of peripheral cytoplasm. (b-g) Increase in cell size and appearance of cytoplasmic strands prior to mitosis. (h, i) Large spherical vegetative nucleus and smaller ellipsoid generative nucleus after mitosis. (i-p) Vegetative nucleus performs series of synchronized mitotic events; note that the generative cell has remained stationary and did not show any mitotic activity. CS, cytoplasmic strands; GN, generative nucleus; PA, pollen aperture; V, vacuole; VN, vegetative nucleus. Bar = 20 µm.

The nucleus remained positioned at the opposite side of the pollen aperture and did not move prior to mitosis (Fig. 37a-g). This first mitosis was followed by an asymmetric division and resulted in a large vegetative cell and a much smaller generative cell (Fig. 37h). The vegetative nucleus showed very active movements and the appearance of cytoplasmic strands indicated another coming mitosis (Fig. 37i). This time the two daughter nuclei were similar in shape and size (Fig. 37k, l). Both underwent synchronized divisions leading to the formation of a multicellular structure that showed no sign of starch formation (Fig. 37l-p). Compared to the type I development, multicellular structure formation in type IV was delayed by a few days. The generative cell remained stationary at the opposite side of the pollen aperture and though remaining viable as indicated by the GFP fluorescence. There were no further structural changes (Fig. 37h-p). It must be noted that in other cases the generative cell degenerated after 7 to 10 days in culture.

Developmental type V (7.1%) again was very similar to developmental type II in terms of cell size, thin-layered cytoplasm, starch accumulation and eventual cell degeneration, except for the fact that here the first pollen mitosis was asymmetric.

Cells of developmental type VI (2.8%) underwent an asymmetric mitosis resulting in a small generative and large vegetative cell (Fig. 38a, b). The initial developmental steps of these cells thus were similar to those described for type IV (Fig. 38a-c). However, the generative cell was larger than usual (Figs. 37g; 38c) and the generative nucleus itself remained spherical and not ellipsoid. After the first mitosis, the vegetative nucleus of the type VI cells started its symmetric and synchronized divisions (Fig. 38c-h). However, for the first time it was recorded that independent of the vegetative cell, the presumed generative cell also displayed mitotic activity which was both symmetric and synchronized (Fig. 38e-h). The cells produced by the mitotic activity of the generative cell remained within the original boundaries of the generative cell as defined after the first asymmetric division (Fig. 38c). Interestingly, nuclei derived from the generative cell remained substantially smaller than those derived from the vegetative cell (Fig. 38f-h).

Developmental type VII (1.4%) represented a highly vacuolated immature pollen which failed to undergo the first pollen mitosis (Fig. 39). This pollen showed a clear increase in nuclear size over time (Fig. 39c-e). The cell itself, however, did not show any increase in size and did not accumulate starch during the time of observation. Cytoplasmic strands

did not emerge when the GFP signal disappeared from the nucleus (Fig. 39e, f). Shortly thereafter, the nucleus disintegrated into micronuclei as indicated by the bright GFP fluorescence in these spherical structures (Fig. 39g-h). Despite the fragmentation of the nucleus, the cell remained alive until the observations were conducted.Developmental type VIII (2.8%) represents pollen with exceptionally large size (around 60 µm) after

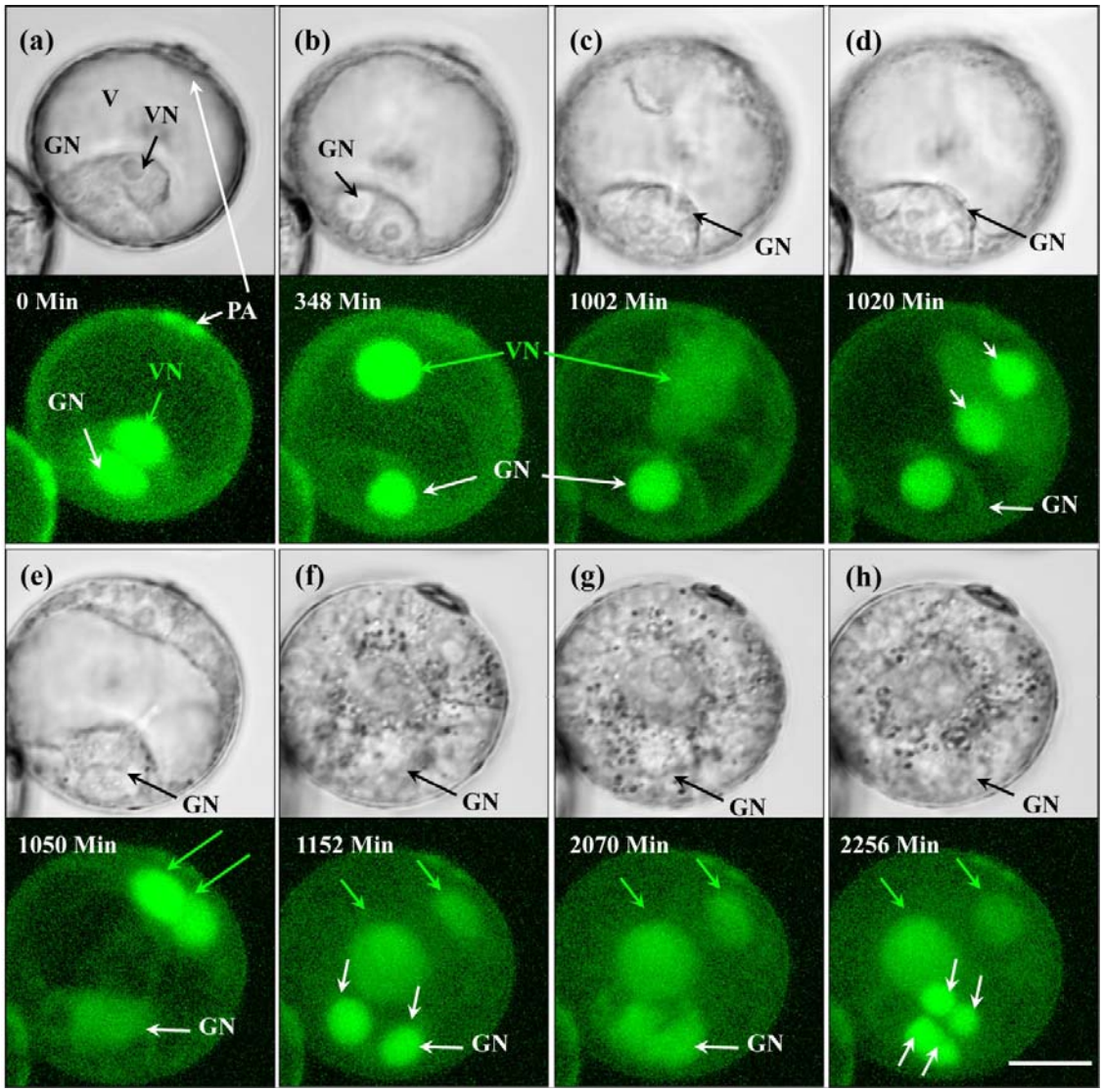

Fig. 38: Time lapse of type VI development (generative cell). CLSM images shown in DIC and nuclear GFP fluorescence. (a, b) An asymmetric division results in a large vegetative and a small generative cell. (c, d) First mitosis of the vegetative nucleus, the spherical generative nucleus remains fixed opposite to the pollen aperture. (e, f) First mitosis of the generative cell. (g, h) Second symmetric and synchronized mitosis of the nuclei stemming from the generative cell. Note that the nuclei derived from the generative nucleus are much smaller than those derived from the vegetative nucleus. CS, cytoplasmic strands; GN, generative nucleus; PA, pollen aparature; V, vacuole; VN, vegetative nucleus. Bar = 20 µm.

stress treatment. Those cells did not show any development and collapsed within the first 2 to 3 days of culture in KBP medium. Immature pollen of developmental type IX (20%) resembled vacuolated uni-nucleate pollen. However, they did not display any embryogenic activity or morphological changes. Pollen of this type died during observation or survived until the observation was conducted.

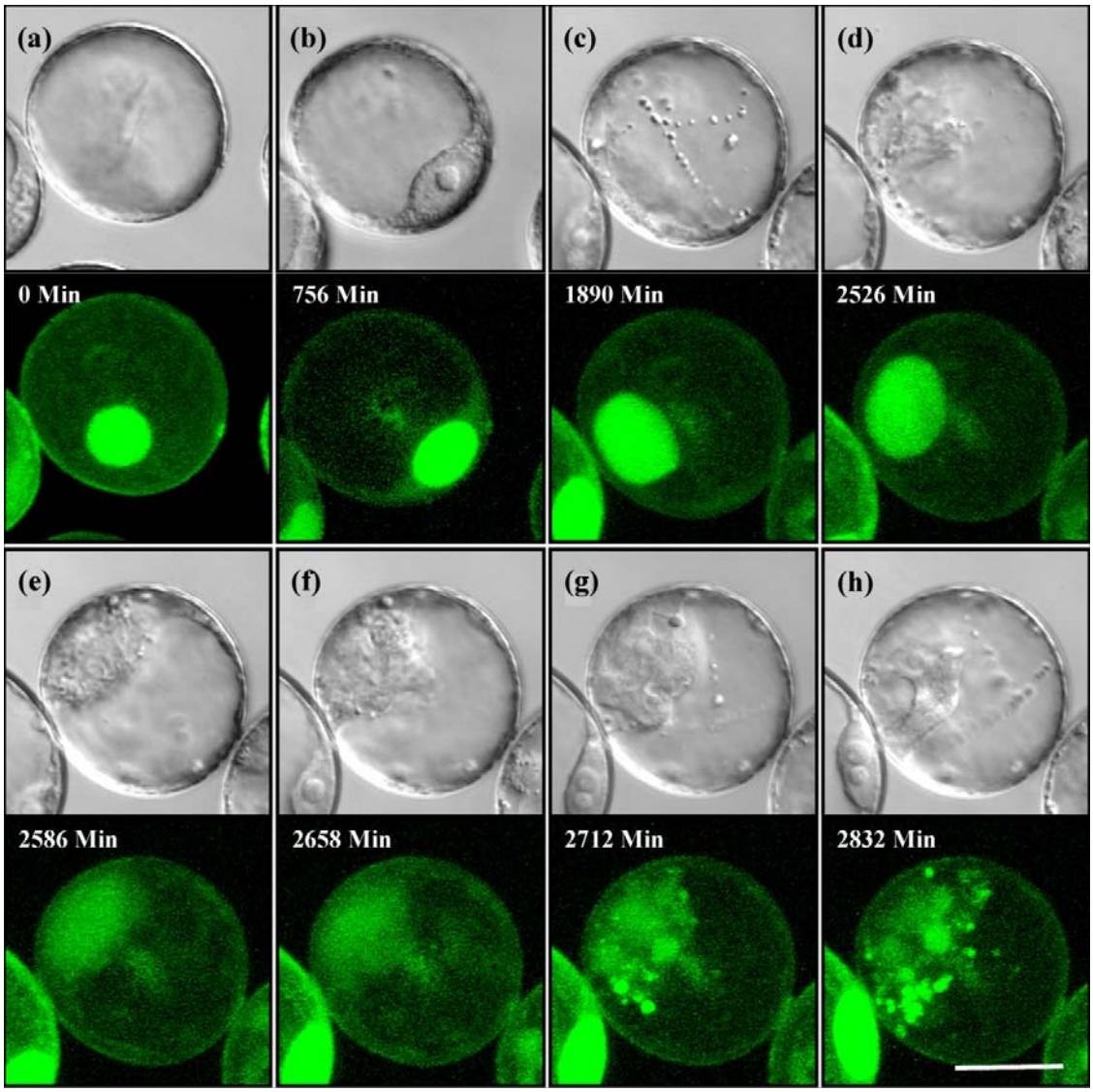

Fig. 39: Time lapse of type VII development (non-embryogenic pollen). CLSM images shown in DIC and nuclear GFP fluorescence. The pollen nucleus fails to divide and disintegrates into multiple micronuclei. (a, b) Highly vacuolated uni-nucleate pollen. (c, d) Gradual increase in nuclear size. (e, f) Blurred GFP signal of the nucleus. (g, h) Fragmentation of the nucleus into several micronuclei as indicated by a fragmented GFP signal. Bar = 20 µm.

3.4.3.3. Mechanism of spontaneous genome doubling during pollen embryogenesis

Live-cell imaging experiments of the transgenic line with nuclear GFP accumulation gave further insights into the process of spontaneous genome duplication. After the first light microscopical observations of fixed and DAPI-stained pollen, live-cell imaging did not only confirm the hypothesis of nuclear fusion, but also allowed to pursue this important process over time and in more detail. First of all, nuclear fusion was found to be a common process, i.e. in some 40% of the multicellular structures investigated nuclear fusion was observed. Secondly, nuclear fusion was not linked to a certain stage but was documented at any stages of development (Figs. 40-44). A third interesting observation was that secondary and tertiary nuclear fusions were frequently observed which result in polyploid cells (Fig. 40).

Live cell imaging experiments showed that in the immediate aftermath of the fusion process, the new nucleus often was somehow irregular and ellipsoidal in shape (Figs. 40, 43) rather than near-spherical as nuclei that had not undergone a fusion. Similarly, elongated nuclei were also observed by electron microscopy (Fig. 43). The early phase of this process involved the close alignment of two nuclei (Fig. 42). Here the two nuclei were in extreme close proximity leaving only a thin layer of cytoplasm between them. At some points, however, the nuclear envelopes were in direct contact and eventually fused (Fig. 42). Although difficult to prove beyond any doubt, there were several indications that this elongated nucleus was the result of a multiple nuclear fusion process. First, pollen grains containing a nucleus with elongated shape did not show any spherically shaped nuclei. Moreover, elongated nuclei featured a median invagination and an unusual distribution of heterochromatin which appeared to be absent from the vicinity of this median constriction (Fig. 43).

The fusion product of two spherical nuclei, at least initially, is elongated. The results presented here could identify nuclear regions with double bi-layer membranes structures (cytoplasmic pocket) with structural characteristics similar to the cytoplasm (Fig. 43). Through screening for such elongated nuclei, specimens were found with a narrow heterochromatin-free zone running through the middle of the nucleus (Fig. 43).

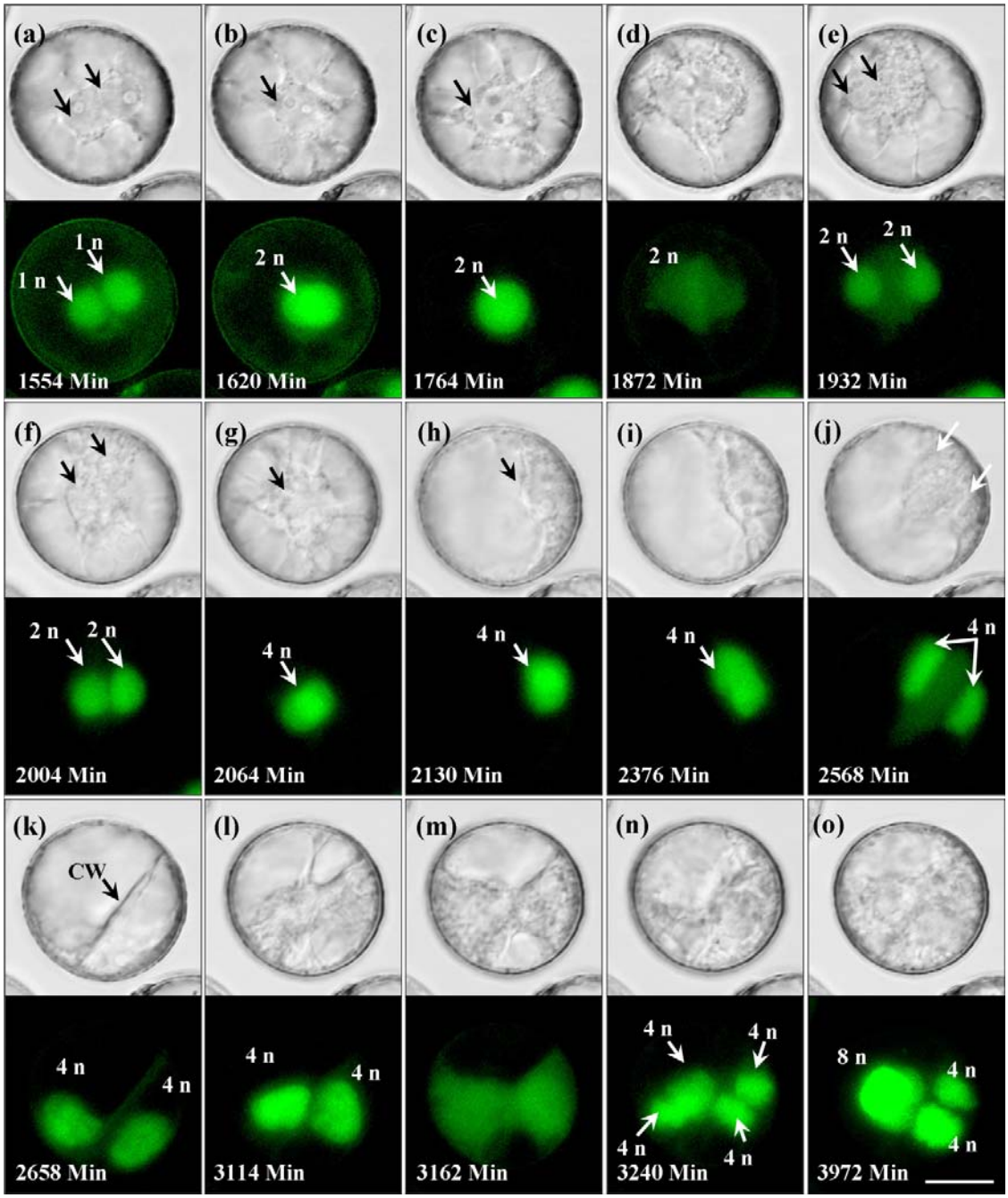

Fig. 40: Time-lapse imaging of spontaneous genome doubling during pollen embryogenesis. CLSM images shown in DIC and nuclear fluorescence. (a) Two haploid nuclei after the first pollen mitosis. (b, c) Nuclei adhere to each other and eventually fuse to form a diploid nucleus. (d-f) Second mitosis producing two diploid sister nuclei. (g-i) The two diploid nuclei adhere to each other and fuse producing a tetraploid nucleus. (j, k) Third mitosis resulted in two tetraploid nuclei. (l-o) Fourth synchronized mitosis resulting in four tetraploid nuclei, two of which later fuse to create a single octaploid nucleus. Bar = 20 µm.

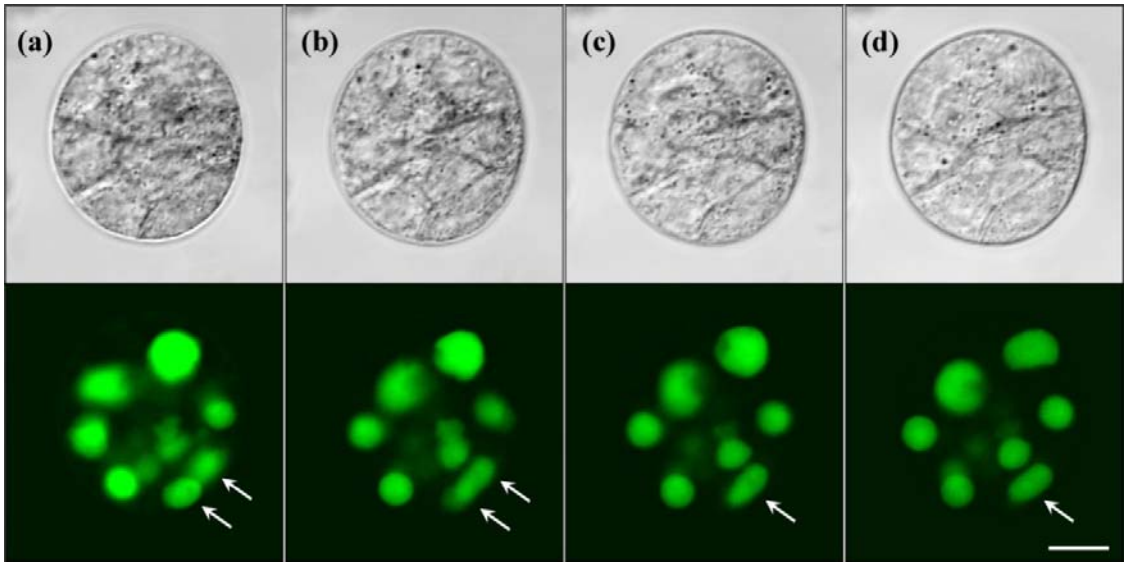

Fig. 41: Time-laps of nuclear fusion during late multicellular structure. CLSM images shown in DIC and nuclear fluorescence of nuclear fusion of a multicellular structure after 8 days in culture. (a) Arrows indicate two seperate nuclei. (b) Same two nuclei start to adhere. (c, d) Fusion resulting in a single elongated diploid nucleus. Bar = 20 µm.

The gained information from live cell imaging and TEM showed that mitosis is not always followed by cell wall formation (Fig. 40; 41). It was also found that cell wall formation and nuclear fusion occurred at any developmental stage of pollen embryogenesis, which explained the chimeric ploidy structures of the cells within one multicellular structure (Fig. 44).

Among the many hundreds of pollen investigated in the present study (either fixed or live cell material), only a single case was found, where the incomplete pollen wall (exine and intine) formation after meiotic cytokinesis, resulted in two immature pollen cells within the enclosure of a single pollen wall (Fig. 45).

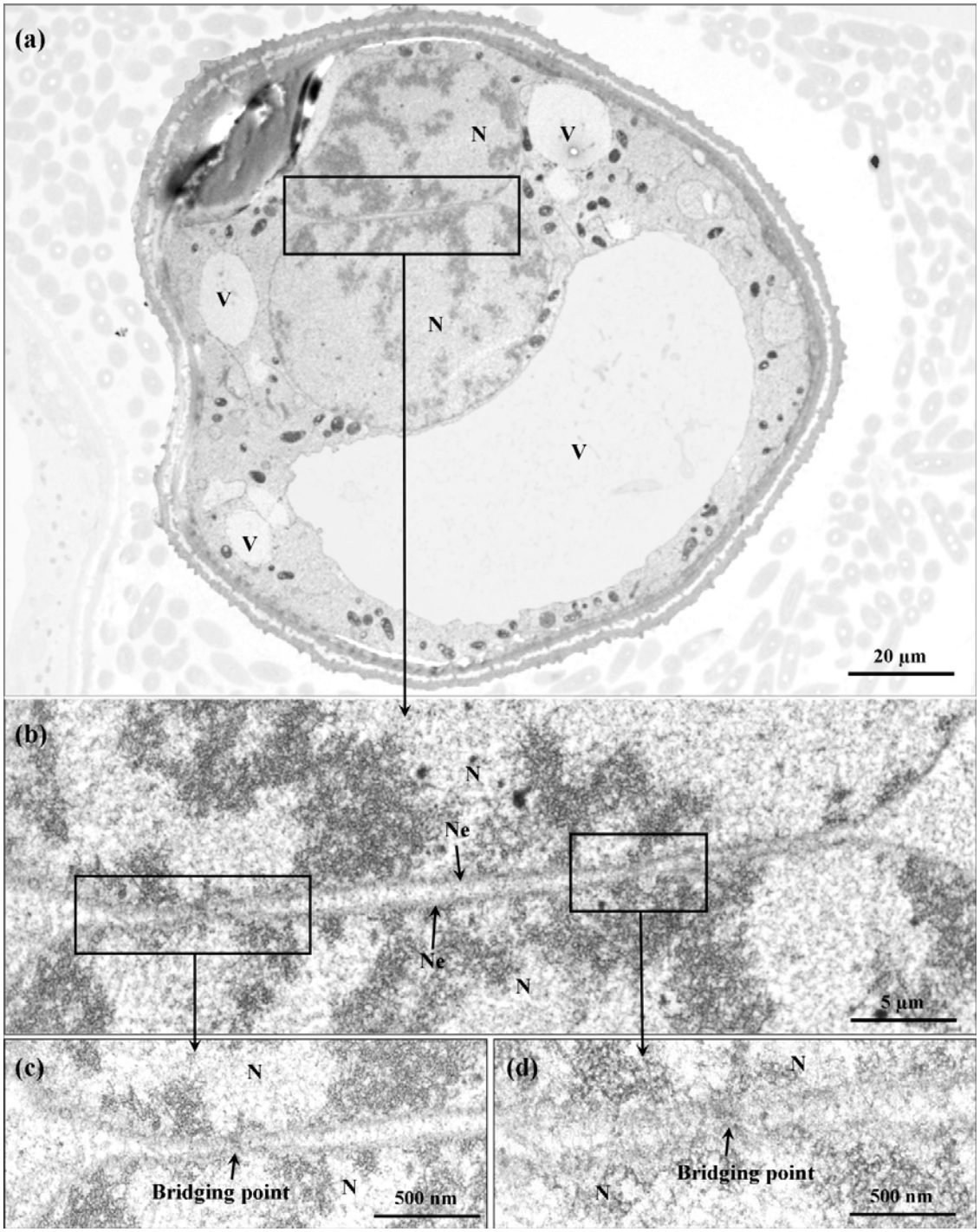

Fig. 42: TEM micrographs of nuclear fusion at early stage of pollen embryogenesis. (a, b) Induced immature pollen with two nuclei in very close vicinity. (c, d) Detailed images shows the absence of a cell wall and the close proximity of the nuclear envelopes. Arrows indicate regions of possible membrane fusion. N, nucleus; Ne, Nuclear envelop; V, Vacuole.

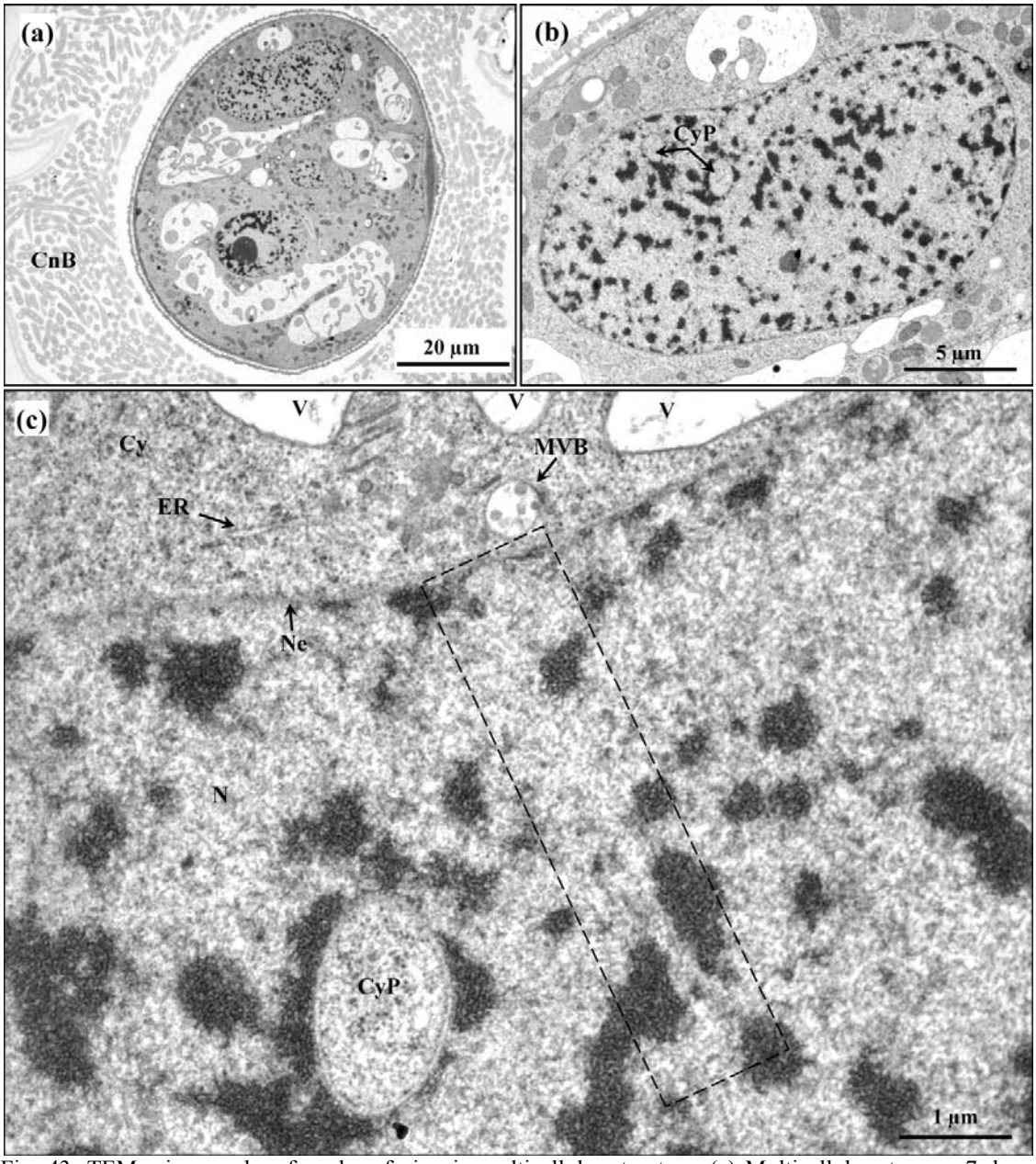

Fig. 43: TEM micrographs of nuclear fusion in multicellular structure. (a) Multicellular strucure 7 days after initiation of pollen embryogenesis. (b) Elongated nucleus with clear median constriction. (c) Detail of nucleus cytoplasmic pockets and a narrow median band (dashed box) marking the site of fusion. CnB, cyanobacteria; CyP, cytoplasmic pocket; ER, endoplasmic reticulum; MVB, multi vesicles body; Ne, Nuclear envelope; V, vacuole.

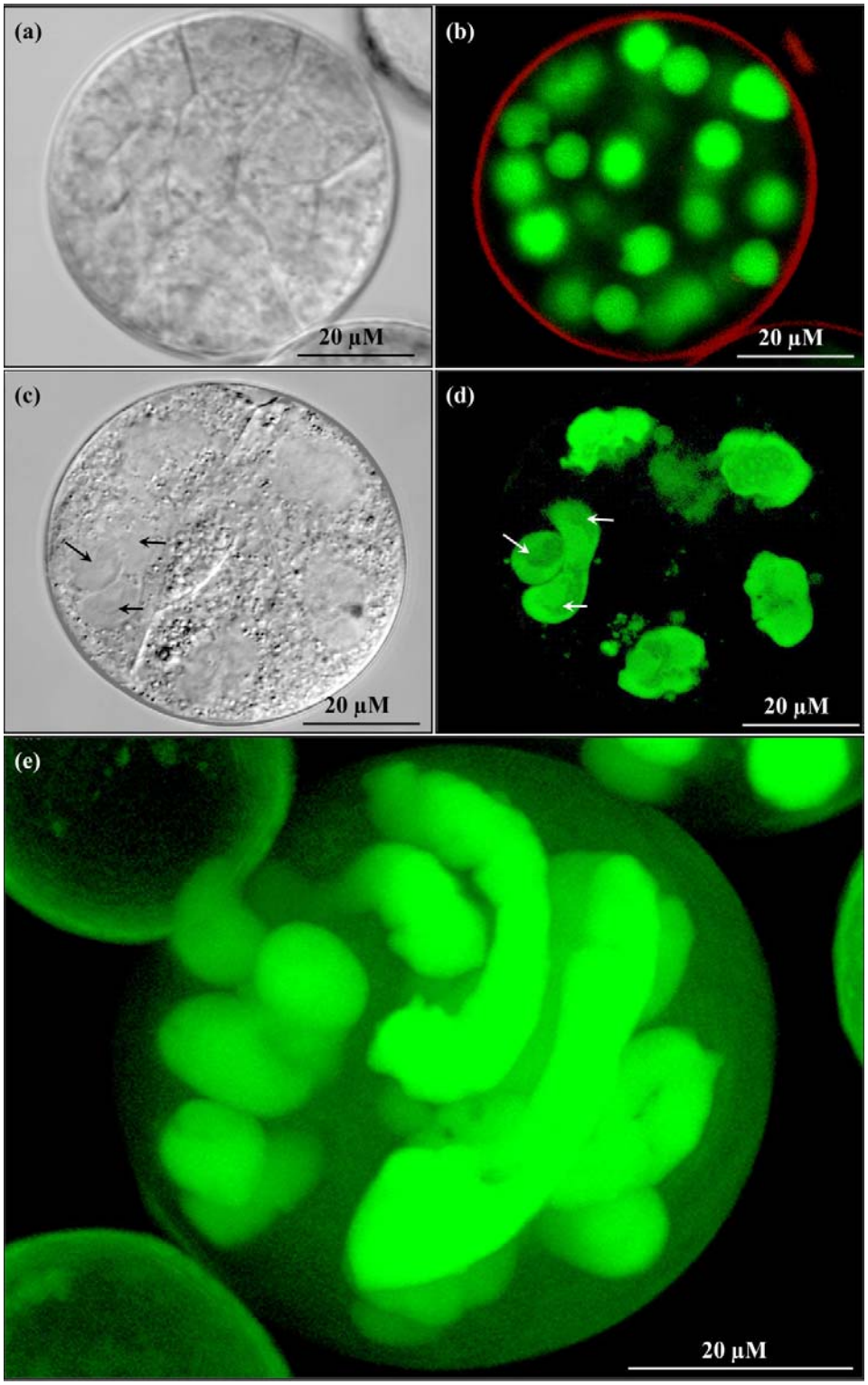

Fig. 44: Variable ploidy level in multicellular structure. (a, c) DIC images. (b, d, e) GFP signal. (a, b) Haploid multicellular structure with cell walls and round nuclei. (c, d) Chimeric polyploid multicellular structure with irregular shaped nuclei often not separated by cell walls. Note the difference of nuclear sizes. Arrows refer to a possible treble fusion (e) multicellular structure with highly polyploid nuclei next to small spherical, likely haploid, nuclei.

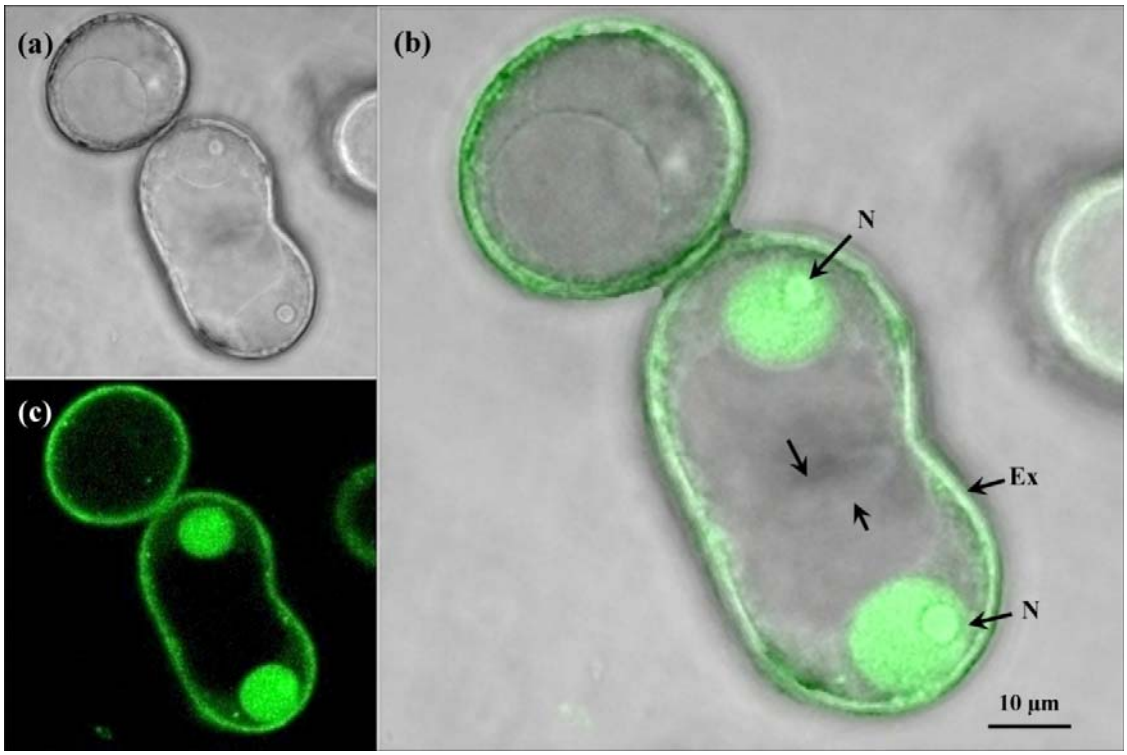

Fig. 45: Incomplete cytokinesis in freshly isolated immature pollen. Nuclear GFP labeling (a, c) shows karyokinesis to be completed. Under DIC (b) the plasma membranes (arrows) of two cells can be distinguished enclosed by a continuous cell wall.

4. Discussion

Despite the great importance of pollen embryogenesis for reproduction biology, only little information is available about the mechanisms underlying this process. Transformation from gametogenesis to embryogenesis of a pollen grain is a complex process accompanied by various structural changes. To characterise and identify the new subcellular arrangements committed to the induction of pollen embryogenesis, a comparative analysis of the structure and the ultrastructure of both pollen types need to be undertaken. As a major prerequisite, genuine ultrastructural preservation of intercellular organles was achieved using a new protocol of HPF. In addition, the new results delivered from live-cell imaging experiments provide a better understanding of pollen embryogenesis.

4.1. Sample preparation for structural and ultrastructural studies

To preserve cells under physiological conditions as close to its native state, the fixation (killing) has to act as fast as possible. To avoid extraction of cellular components or dislocation of moleculesfixation has to be as fast as possible (Pitol et al., 2007). Classical chemical fixation with aldehydes and/or osmium tetroxide, is the most widespread used technique for biological specimens. However, chemical fixation is based on a selective crosslinking of molecules and always directly related to the diffusion rate of the fixative into the specific tissue. In complex or well protected tissues or cells like pollen, diffusion will be slow, often causing uncontrollable osmotic effects and structural distortions which can be visible even at the light microscopic level (Heumann, 1992; Kääb et al., 1999; Kenako and Walter, 1995; Lool et al., 2005; Mersey and McCully, 1978). CCF was clearly inadequate for our high demands on ultrastructural preservation (see Figs. 10). Still, for some light microscopic studies using semi-thin sections (see e.g. Fig. 14 b, e, h, k, n, q), these results were acceptable and the straightforward handling and the reliability of the method an advantage albeit the protocol duration is rather long.

In the present study microwave-assisted chemical fixation turned of to be a valuable improvement of the available protocols for chemical fixation. The employment of this method clearly improved the preservation of pollen structures when compared with CCF

(Fig. 10g-h). The generation of electromagnetic waves by radiation increases an effective diffusion of the fluids in and out of cells. Therefore the duration of chemical fixatives such as formaldehyde and glutaraldehyde is reduced to a few minutes while, significantly improves the quality of fine structures (Heumann, 1992; Leong and Leong, 1997). Therefore microwave-assisted fixation was applied for light microscopical studies on semi-thin sections of immature pollen and anthers to determine the different stages of pollen embryogenesis development. In addition, DAPI staining of pollen was also facilitated by microwave treatment. Even though, MWCF was inadequate for pollen ultrastructural preservation (see Fig. 11).

Most rapid fixation of biological samples can be achieved by cryofixation, which can be achieved by plunge freezing, slam freezing or HPF. Protocols for following freeze substitution may include the addition of fixatives. These, however, are added at low temperatures which does allow their infiltration but suppresses their cross linking reactions. The warming up of well infiltrated specimens thus results in an even fixation avoiding diffusion gradient-related artifacts (Crang & Klomparens, 1988; Echlin, 1992). HPF is capable of immobilizing cellular contents within milliseconds (Moor, 1987) and the rapid freezing process vitrifies cellular water without the formation of ice crystals. To carry out ultrastructural analyses at high structural resolution, it was essential to establish a protocol for HPF of immature pollen. The HPF of pelleted immature pollen loaded into a nitrocellulose capillary as suggested by Hohenberg et al. (1994), resulted in a very poor preservation of ultrastructure (Fig. 8). During loading, the immature pollen tended to stick to the capillary, so that a substantial part of the volume was taken up by the liquid phase and leads to ice crystal formation during HPF (Fig. 8). To reduce the volume of the liquid and to increase the efficiency of capillary loading with immature pollen, it was decided to mix the pellet of the pollen with a pellet of cyanobacterial cells which are very small (5 μm) and have been used previously for HPF (Agarwal et al., 2009). Indeed the loading of a mixture of immature pollen and cyanobacteria as filler in the capillaries resulted in a more densely filled capillary and reduced the volume of the intercellular free liquid to a minimum (Fig. 8c, d). For immature pollen, cyanobacteria constituted a better filler than the cryoprotectant 1-hexadecene. Therefore there is a lower risk of chemical interaction with the specimen which could alter its physiology. Surrounding the

nitrocellulose tubes with yeast paste effectively excluded air bubbles and enabled good adherence and contact with the aluminum platelet inner surface (Fig. 3d, e). The density of the cyanobacteria also accelerated the freezing rate, since both, i.e. cyanobactria and yeast, conducted heat well and suppressed the formation of large ice crystals. As the use of cyanobacteria as a filler substantially improved the preservation of immature pollen ultrastructure (Daghma et al., 2011), the established HPF protocol was used subsequently for all samples in the comparative ultrastructural analysis. The protocol of sample preparation presented here may be useful not only in the fixation of cell suspensions but also of complex tissues like anthers.

4.2. Increasing induction efficiency of pollen embryogenesis

Abiotic stress plays an important role in the induction of pollen embryogenesis (Duckan and Heberle-Bors, 1976; Heberle-Bors and Reinert, 1981). To increase the number of pollen entering embryogenesis, most protocols developed for mono- and dicotyledonous plants include low or high temperature treatments that are either applied prior to the culture in rich medium or in the beginning of pollen culture. The initial standard protocol also relied on stressing isolated immature pollen in SMB1 medium for 2 days at 24^{o}C in the dark before transfer to KBP as described by Kumlehn and Lörz (1999) and Corronado et al. (2005). This protocol was used for all fixation experiments. However, the efficiency was not satisfactory, especially for the live cell imaging experiments, where highly efficient cultures are essential. To increase the proportion of proliferating pollen, a new protocol has been established that includes the treatment of isolated anthers followed by an additional treatment of the isolated immature pollen (APT4 treatment).

The APT4 treatment was discribed by a relatively low sub-population of isolated immature pollen, but those that did survive eventually produced the highest percentage of multicellular structures (Fig. 28; 29). In contrast to this, most immature pollen survived the SMB1 treatment, yet the frequency of proliferating pollen was the lowest of all treatments tested. The APT24 treatment, however, resulted in the lowest number of surviving immature pollen, though the percentage of multicellular structures - albeit smaller than that after APT4 treatment - was still higher than that after SMB1 (Fig. 28; 29). Similar results of combining different stress treatment in several cereal crops were

obtained in numerous studies (Touraev et al., 1996a; b; Hoekstra et al., 1997; Caredda et al., 2000; Kasha et al., 2001; Li and Devaux, 2001; Wojnarowiez et al., 2002; Jacquard et al., 2003; Cistué et al., 2003; Davies, 2003) where starvation stress was combined with low temperature for 3 to 5 days. In maize pollen embryogenesis, the efficiency was improved by the application of a cold treatment (7°C) of the tassels (Barnabás, 2003) or by treatment at 14°C in a medium containing mannitol (Nageli et al., 1999; Obert et al., 2000; Zheng et al., 2003). Zheng et al. (2002; 2003) and Liu et al. (2002a; b) used the combination of growth regulators and a short cold pre-treatment of spikes and/or starvation stress in mannitol with satisfactory results. In conclude, the APT4 yielded the highest amount of embryogenic pollen. Thus, the APT4 treatment was used predominantly during the current study for live cell imaging experiments of transgenic pollen.

4.3. Live-cell imaging and ultrastructure of cultured pollen

To date only a handful of papers has described the tracking of individually cultured cereal pollen, i.e. Indrianto et al. (2001) for wheat, Bolik and Koop (1991), Kumlehn and Lörz (1999), Maraschin et al. (2005) and Maraschin et al. (2008) for barley. The information in these pioneering papers derived from observations with time intervals of up to several hours. Though sufficient to identify general trends, these long time intervals are not suited to document the finer details of the multiple structural events characterizing pollen embryogenesis. In contrast, the current study presents for the first time a complete monitoring of pollen embryogenesis from the vacuolated immature uni-nucleate barley pollen until the development of multicellular structures at a temporal resolution of three minutes or less. This was achieved not only by DIC imaging for up to 30 days non-stop, but as well using pollen of a transgenic barley line constitutive expressing the viral SV40 nuclear localization protein tagged with GFP. The fluorescence microscopic studies allowed a hitherto un-obtained detailed investigation of the nuclear behavior during the important stages of this process.

4.3.1. Heterogeneity of immature pollen cultures

Cultures of immature pollen isolated from anthers always show a certain variability in their developmental state. This is most apparent when the progress of the individual pollen is monitored over time. Under current culture conditions an average of 58.6% of all pollen analyzed, eventually formed multicellular structure (Table 5). This proved to be very well in line with observations of Maraschin et al. (2005). They found 47.1 ± 3.3% of all tracked pollen to develop multicellular structures. They also identified three main types of cultured pollen. The type I or embryonic pathway represented 10.9 ± 4.2 of all pollen tracked and was characterized. Type II pollen, representing 36.2 ± 2.4% of the total population, formed multicellular structures which, however, did not rupture the exine and did not form embryos. The majority of the observed pollen (52.9 ± 4.5%), however, belonged to the type III, which did not displayed embryogenic division and died soon after start of the culture. By contrast, in the live-cell imaging experiments of the present study, it has been possible to distinguish 9 different types of cultured pollen with two main types undergoing cell proliferation entailing the formation of multicellular structures (Table 5).

Indrianto et al. (2001) in wheat and Maraschin et al. (2005) in barley showed that multicellular structures were only obtained from immature pollen that were enlarged after stress treatment. In the present study, 54.3% of the pollen grains underwent cell proliferation without initially increasing their size upon stress treatment (type I). Only 4.3% increased their size after one day of culture in KBP medium, but not as an immediate response to the inductive treatment (type IV). The latter pollen grains showed a remarkable delay of the first mitosis by which proliferation was initiated. Interestingly, most pollen grains that showed a clear increase in size shortly after the stress treatment (8.4%), eventually accumulated starch and failed to undergo embryogenic development (types II, V and VIII). In addition, it was observed that multicellular structures can be produced after symmetric or asymmetric first pollen mitosis (Fig. 34; 37). In the latter case however, cell proliferation is initiated by a symmetric cell division of the vegetative cell. However, the asymmetric division occurred during the culture and those pollen were at uni-nucleate stage after the inductive treatment (Fig. 37). As the cultures of immature pollen were highly heterogenic**,** it turned out to be necessary to follow the fate of

individual pollen grains, in order to unambiguously identify those pollen types that are competent for embryogenic development. In addition, the heterogeneity in the pollen population also limited the gain of conclusive results from transcriptomics or metabolomics analyses.

4.3.2. Embryogenesis and the first pollen mitosis

From the current live-cell imaging data it was found that after pollen isolation, embryogenesis was only induced in uni-nucleate pollen. In contrast, the bi-nucleate pollen (present at the time of isolation) died or followed the gametophytic pathway. But it is still necessary to conduct further experiments to elucidate the role of developmental stage at the beginning of the induction treatment. Touraev et al. (2001) showed that the induction of pollen embryogenesis is only possible at early developmental stages. In most cases, successful induction of embryogenesis in uni-nucleate pollen started with a symmetric mitosis, forming two identical cells (Table 5). Occasionally, however, the induced uni-nucleate cell would first undergo an asymmetric mitosis, forming a vegetative and generative cell, before in a later stage the vegetative cell would divide again to start embryogenesis.

The role of the type of the first pollen mitosis to embryogenesis efficiency has not yet been discussed in the literature. In the current study, it was found that 61.4% of total monitored cells underwent a first pollen mitosis symmetrically (type I, II and III), but 11.5% of them did not follow embryogenic development (type II and III) and 54.3% formed multicellular structure (type I). On the other hand, out of total monitored cells, 14.2% (types IV, V and VI) underwent a first pollen mitosis asymmetrically and only 4.3% of them followed an embryogenic pathway ending up with multicellular structures (type IV).

Sunderland and Evans (1980) in barley and Hu and Kasha (1999) in wheat described embryogenesis starting with a first symmetric division or first asymmetric division as the A-pathway and B-pathway respectively. Furthermore, an additional C-pathway was proposed, in which vegetative and generative nuclei first fused before entering embryogenic divisions. Examples of this latter pathway were not found in the present cultures.

Nuclear architecture changes in response to nuclear activity (Risueño and Medina, 1986; Raska, 1995; Dundr and Mistelli, 2001). In bi-cellular pollen, the generative and vegetative nuclei are structurally different as a result of a differential cell activity and cell cycle progression (McCormick, 1993; González-Melendi et al., 2000). The ultrastructural study performed here showed that, as in other species, the activity of the vegetative cell is reflected by its nucleus, being highly euchromatic and containing an active nucleolus, whereas the generative nucleus showed features of low transcriptional activity, including extensive patches of heterochromatin and a compact nucleolus (Risueño et al., 1988). The two nuclei resulting from the symmetric division had a similar pattern of chromatin distribution, with strongly euchromatic chromatin being predominant and small patches of heterochromatin confined to the nuclear periphery (Fig. 19) (Testillano et al., 2000; 2005).

Early multicellular structures had nuclei with a nuclear organization and a dense cytoplasm with small vacuoles, features which seem typical for cells during proliferation (Risueño and Moreno Díaz de la Espina, 1979; Risueño et al., 1982). The current results show the importance of the first mitosis for the future pathway but also revealed that it could not be a key trigger of pollen embryogenesis since both symmetric and asymmetric mitosis could eventually contribute to pollen embryogenesis.

4.3.3. The generative cell in pollen embryogenesis

In the classic definition, the generative cell is a cell of the male gametophyte (pollen grain) in flowering plants that divides to give rise directly or indirectly to sperm cells. Generative cells are therefore often regarded as uniform structures. Current observations, however, showed that the generative cells, arising after the inductive treatment of uni-nucleate pollen cultures, form a highly heterogeneous population, greatly differing in size and behaviour (Fig. 16, 38). Furthermore, all generative cells formed after the inductive treatment remained attached to the exine (Fig. 16; 37; 38), i.e. they never became fully enveloped by the vegetative cell as in unaffected gametophytic development.

Some generative cells died during the culture, others divided producing structures resembling sperm cells (Fig. 38). Some generative cells were many times bigger than the normal specimens, though also here the nuclei were significantly smaller than in the

vegetative cells (Fig. 35). Interestingly, these large generative cells could enter several rounds of divisions while still retaining their small sized nuclei (Fig. 38). This underlines that the transformation into a generative cell was a gradual process. The fact that dividing generative cells retained a small nuclear size indicates, that the developmental stage at which the generative cell was arrested, is rather stable and may somehow be transferred in subsequent divisions. It would be interesting to investigate if the development into a generative cell follows a fixed pattern of genes being silenced or activated in a well defined order.

4.3.4. Mechanism of spontaneous genome doubling

Spontaneous doubling can occur through somatic diploidization or formation of unreduced gametes due to promeiosis. Pollen embryogenesis has the potential for spontaneous genome doubling during the first divisions of cultured pollen, thus producing completely doubled and fertile plants (Castillo et al., 2009). The frequency of doubling varies depending on the species. Averages of 70–90% have been reported in barley, 25% to 70% in bread wheat, 50–60% in rice, 50–90% in rye (Maluszynski et al., 2003), 20% in maize (Martin and Widholm, 1996) and 70% in durum wheat (Cistue et al., 2006). Nuclear fusion, after a symmetric and/or an asymmetric division, has been proposed to be the main mechanism of spontaneous doubling after mannitol or cold stress treatment to incuce pollen embryogenesis in barley (Kasha et al., 2001) or maize (Testillano et al., 2004).

The first fusion of two interphasic nuclei during pollen embryogenesis was observed in barley (Chen et al., 1984a; b). Later on, fusion events have also been reported in pollen embryogenic systems of maize (Testillano et al., 2004), barley (Kasha et al., 2001), wheat (Hu and Kasha, 1999) and tomato (Seguí-Simarro and Nuez, 2007). Nuclear fusion in barley pollen embryogenesis was also deduced from studies on squashed DAPI-stained specimens and densitometry measurements after Feulgen staining (Kasha et al., 2001).

3D imaging of DAPI-stained fixed embryogenic pollen in the current study showed haploid nuclei to be spherical with a single nucleolus, while diploid nuclei were often elongated or of irregular shape with two or more nucleoli (Fig. 15). These observations resemble those of Baroux et al. (2004) and González-Melendi et al. (2005).

Combining the information from live-cell imaging using DIC and GFP-labelling with TEM studies, it was found that mitosis is not always followed by cell wall formation (Fig. 40; 42; 43; 44). The multinuclear cells thus arising allow daughter nuclei to fuse. In all cultures observed, nuclear fusion has been the only means of genome doubling recorded. It was further found that cell wall formation and nuclear fusion during pollen embryogenesis occurred randomly, which explains the chimeric ploidy level of inividual multicellular structures (Fig. 40; 44). Mitotic divisions without cell wall formation are not uncommon in plants. This process gives rise to the bi-nucleate tapetal cells, the female gametophyte and the coenocytic endosperm (Olsen, 2004). Endoreplication has been described in hypocotyls, leaf epidermis, root tips, trichomes and endosperm, and is normally associated with growth increase and higher metabolic capacity (Edgar and Orr-Weaver 2001). González-Melendi et al. (2005) showed that when nuclei coexist within the same cytoplasm their envelopes could fuse. However, the same authors also argued, that the lack of a cell wall separating two nuclei is not sufficient to explain nuclear fusion since they were absent under situations of altered cytokinesis. Expression of an inactive variant of NPK1, a MAPK involved in the signaling of cell plate synthesis, resulted in the generation of multinucleate cells with incomplete cell plates in cultured BY-2 tobacco cells (Nishihama et al., 2001). In proliferative cells, a treatment with caffeine, a blocking agent of cytokinesis, also produces bi-nucleate cells without the formation of a cell wall (López-Sáez et al., 1966; Risueño et al., 1968).

Chromosome number can also be doubled by anti-mitotic agents that inhibit spindle formation (Levan, 1938; Morejohn and Fosket, 1984; Bartels and Hilton, 1973). Nevertheless, chemical induction of genome doubling *in vivo* and *in vitro* depends on a compromise between toxicity and genome doubling efficiency. Anti-microtubule herbicides have been successfully applied *in vitro* in rapeseed (Zhao and Simmonds, 1995), wheat (Hansen and Andersen, 1998) and onion (Grzebelus and Adamus, 2004) as a less toxic alternative to colchicine.

In the current study, nuclear fusion in embryogenic pollen cultures was only observed after a preceding symmetric division. Interestingly, not fully differentiated generative cells were occasionally also capable of entering cycles of symmetric divisions (Fig. 38). However, there always remained a clear physical barrier between vegetative and

generative cells and a fusion between their nuclei was never observed. This is indirectly supported by the current observation that in cases where generative cells were able to undergo symmetric divisions, all daughter nuclei retained the size of the original mother cell's nucleus, suggesting a sustained level of differentiation (Fig. 38).

Pioneering studies proposed two possible ways of nuclear fusion: i) fusion of mitotic nuclei and ii) fusion of nuclei at interphase. Fusion of mitotic nuclei was proposed by Sunderland et al. (1974) as an explanation for genome duplication in embryogenic *Datura* pollen grains by means of a synchronous entry into mitosis of both vegetative and generative nuclei. According to Sunderland et al. (1974) and Sunderland and Evans (1980), chromosomes from both nuclei intermixed and then segregated together through a common mitotic spindle. Seguí-Simarro and Nuez (2008) showed that fusion of interphasic nuclei consisted of a normally-occurring karyokinesis and nuclear reassembly, followed by a disrupted cytokinesis that allows daughter nuclei to coalesce within the same cytoplasm and finally fuse into a single, larger nucleus with twice the chromosome number of the original nucleus. Lee and Chen (1987) reported on the fusion of generative and vegetative nuclei in cultures of barley pollen after the degradation of the separating cell wall. Kasha (2005) proposed that the generative cell wall, after detachment from the intine, may enter a process of callose dissolution and fragmentation, or that the assembly of the generative wall occasionally may be defective from the start.

Very intriguing is the report by Chen et al. (1984b) on the fusion of the nuclei of two pollen. This remarkable phenomenon was considered a result of incomplete cell wall formation during postmeiotic cytokinesis, allowing the microspores to stay physically connected. Recently, this hypothesis was confirmed in tomato meiocytes induced to proliferate into a callus (Seguí-Simarro and Nuez, 2007) and may explain the generation of non-DH individuals. Among the many hundreds of pollen followed in the present study, only a single case was found where incomplete cell wall formation after meiotic cytokinesis led to two immature pollen cells being enclosed by a single wall (Fig. 45).

The *in vitro* culture conditions are an important source of stress and may promote cell proliferation as well as cause defects in normal cytokinesis (d'Amato, 1989). Heat treatment, widely used in species such as rapeseed (Custers et al., 1994), wheat (Touraev et al., 1996b), tobacco (Touraev et al., 1996a), eggplant (Dumas de Vaulx and

Chambonnet, 1982) or pepper (Dumas de Vaulx et al., 1981) to destabilize microtubules and actin filaments (Hause et al., 1993; Simmonds and Keller, 1999; Gervais et al., 2000), which can disrupt cytokinesis or cell plate formation (Risueño et al., 1968; Yasuhara et al., 1993; Valster et al., 1997; Gimenez-Abian et al., 1998). Though disruption or absence of cytokinesis is considered a prerequisite for nuclear fusion, this fact alone may be not sufficient for the completion of the fusion process. There are many examples of multinucleate cells, both naturally (tapetum, nuclear endosperm, meiocytes) or experimentally-induced (Risueño et al., 1968; Nishihama et al., 2001; Park and Twell, 2001), in which nuclear fusion never takes place. Clearly, a multinuclear condition does not automatically lead to nuclear fusion. This means that additional hitherto unknown factors must exist, that stimulate the fusion of nuclear envelopes (Chen et al., 1984b; González-Melendi et al., 2005; Seguí-Simarro and Nuez, 2007). One of these factors may be the actin cytoskeleton, which according to Shim et al. (2006), plays a role in the nuclear approach.

The live-cell data presented here showed that nuclear fusion is both random and rapid, two features that make it difficult to study the ultrastructural features of the phenomenon. Despite extensive electron microscopic studies, only a few nuclei were found, that had been immobilized the process of fusion (Fig. 42). Evidence of fusion, however, can ocasionally be retained for some time after the fusion process itself. The fusion product of two spherical nuclei, at least initially, is elongated. When screening for such elongated nuclei, a specimen was found with a narrow heterochromatin-free zone running through the middle of the nucleus and small cytoplasmic pockets (Fig. 43), which are considered to be a typical by-product of nuclear fusion (Jensen, 1964; Schulz and Jensen, 1973). The same authors also found that nuclear fusions in female gametophytes started with the fusion of the endoplasmic reticulum network surrounding the outer membrane of the fusing nuclei. Similar observations were described for the *in vitro* induced karyogamy upon fusion of egg and sperm cell protoplasts of maize (Fowke et al., 1977; Faure et al., 1993).

The question remains under what condition do nuclear membranes fuse. By T-DNA mutant analysis, two genes potentially involved in polar and egg nuclear fusion, *GFA2* and *NFD1*, have been characterized (Christensen et al., 2002; Portereiko et al., 2006).

Since both genes code for mitochondrial proteins, Portereiko et al. (2006) suggested a role for mitochondria in nuclear fusion, also supported by the association between defective mitochondria and non-fused nuclei.

An alternative possibility, also deduced from the work of Portereiko et al. (2006), relates to a role of the *nfd1* mutation in altering the lipid composition of nuclear membranes. *NFD1* has a homolog in yeast (*MRPL49*) which is involved in phosphatidylcholine (PC) biosynthesis (Hancock et al., 2006). PC is the main lipid constituent of the plant nuclear envelope (Philipp et al., 1976) and is known to stabilize lipid membranes so, that in PC-rich membranes fusion is inhibited (Duzgunes et al., 1981). Thus, changes in the functional properties of the nuclear envelope may depend on changes in its phospholipid composition.

Since the lipid composition of nuclear membranes can be remodeled by regulating the genes responsible for phospholipid biosynthesis (Santos- Rosa et al., 2005), Portereiko et al. (2006) proposed that inhibition of karyogamy in *nfd1* mutants may be due to the alteration of PC ratios in nuclear membranes. It is tempting to speculate that nuclear fusion during pollen-derived embryos could be related to an altered lipid metabolism. They explained that in mating yeasts, nuclear membranes fuse in a sequential manner of pollen embryogenesis. It may be worth to search for plant homologs of the yeast Kar2p, Kar4p, Kar5p, Kar8p, Prm2p and Prm3p proteins which play a role in the nuclear membrane fusion processes in yeast (Lahav et al., 2007). Because of their function in homotypic membrane fusions, like vesicle-vesicle or vesicle- cell plate during plant cytokinesis, SNARE proteins may also be involved (Jürgens, 2005).

In conclusion, it was shown that nuclear fusion as a mechanism of genome doubling, can occur at any developmental stage during pollen embryogenesis, if cell wall formation has failed. The question remains to be answered whether nuclear fusion is the only mechanism of barley pollen embryogenesis or if other mechanisms are also involved.

4.3.5. Sub-cellular Changes during pollen embryogenesis

Early structural markers, that differentiate between the gametophytic and the embryogenic pathways, can be used to define the processes that are activated and/or repressed when the immature pollen change their developmental program in response to a

induction treatment (Testillano et al., 2000; Seguí et al., 2003, 2005; Ramírez et al., 2004). The analyses reported here define rearrangements in specific subcellular compartments, i.e. amyloplasts, vacuoles, cell walls and nuclei.

In the present study, live-cell imaging showed that 7.1% of the pollen grains whose first mitosis was symmetric (type II, III) and a further 2.8% whose first mitosis was asymmetric (type V) did not form multicellular structures and accumulated starch as of the first days of observations (Fig. 35; 37). Ultrastructural observations showed that starch accumulation and amyloplast formation already took place before the first gametophytic mitosis (Fig. 18a, b). In contrast to this, pollen that formed multicellular structures never accumulated starch until the late stages of multicellular structures (Figs. 19; 23a, b). This makes early stage starch accumulation a histological marker for pollen gametogenesis.

In agreement to this, embryogenic pollen always contained proplastids (Fig. 24a, c, e), while gametophytic pollen were characterized by more differentiated, starch grain-containing plastids (Fig. 24b, d). These observations confirmed earlier studies by Nitsch (1969), Nitsch and Nitsch (1970), Norreel (1970) McCormick (1993) and Sangwan and Sangwan (1987). The differentiation of proplastids into amyloplasts is a typical feature of pollen development in many species (Franchi at al., 1996; Pacini, 1996).

In contrast to this, Wojnarowiez et al. (2004) reported the presence of small starch grains during early embryogenesis in barley. In agreement to this, Indrianto et al. (2001) and Testillano et al. (2002) found early starch accumulation in pollen embryogenesis in cereals and considered it a specific feature of monocot species. However these authors did not track or monitor starch-accumulating pollen till the formation of surely multicellular structure or embryogenic calli/embryos.

Vicente et al. (1991) found starch deposition being absent not only after induction by starvation, as in tobacco, but also after induction by a heat treatment, as in rapeseed (Seguí et al., 2003). Therefore, the lack of starch is not a consequence of sucrose fasting. On the other hand, Dickinson and Willson (1983) showed that the absence of starch from cultured pollen may reflect low levels of free carbohydrate and the transfer to carbohydrate-rich medium may stimulate starch synthesis. In potato Říhová and Tupý

(1999) found, that in presence of sucrose, most first mitoses were asymmetric and most pollen entered the gametophytic pathway.

Sangwan and Sangwan (1987) screened a large number of different species for pollen embryogenesis response. In the non-embryogenic pollen species, starch accumulated throughout pollen development with a peak during pollen maturation. In contrast, the pollen grains of all studied species that were well amenable to pollen embryogenesis, had similar plastid types in common. They had proplastids from tetrad to the early bicellular stage, while the species that appeared recalcitrant to pollen embryogenesis consistently had amyloplasts. Sangwan and Sangwan (1987) reported also that during pollen embryogenesis in *Datura,* proplastids first developed into amyloplasts before transforming into chloroplasts in the cotyledons and leaves. It was shown that it is difficult to induce re-differentiation *in vitro* or to induce organ formation from starch-rich somatic tissue such as cotyledons, endosperm and tubers, while it is relatively easy from the tissues possessing proplastids (meristem) and chloroplasts (leaves, stems etc.) (Murashige, 1974; Holdgate, 1977; Sangwan, 1981; Harney, 1982; De Fossard, 1985; Tetu et al., 1987). Similar results were found in *Capsicum annuum* L. (Gonzalez-Melendi et al., 1995), *Brassica napus* (Zaki and Dickinson, 1990; Satputl et al., 2005), *Hordeum vulgare* (Maraschin et al., 2005), *Olea europaea* L. (Solís et al., 2008) and *Triticum aestivum* (Indrianto et al., 2001).

It is therefore concluded that the induction of pollen embryogenesis is only effective prior to the appearance of amyloplasts, which is an early sign of irreversible gametogenesis. Reports claiming the presence of starch granules to be a typical feature of embryogenic pollen may be misinterpretations caused by the heterogeneity of the pollen culture along with the unavailability of cell-tracking data (see 4.3.1). However, if amyloplast formation should be unambiguously associated with pollen embryogenesis in some cases, this might reflect the effect of the particular culture conditions on pollen development.

Both gametophytic and embryogenic pollen contained a large central vacuole which remained present at least until the early bi-nucleate stage. Vacuolation of the cytoplasm remains an important event in the plant embryogenesis (Barnabas et al., 1987; Lyndon 1990; Liu et al., 1996). In the gametophytic pollen, however, the central vacuole breaks up in multiple smaller ones after the first mitosis. During pollen maturation these

vacuoles eventually disappear as was also observed by Yamamoto et al. (2003) and Regan and Moffatt (1990).

Yamamoto et al. (2003) described that in young microspores of *Arabidopsis thaliana,* which started the formation of the exine, vacuoles looked like those of somatic cells. In microspores, a large vacuole arose through fusion of pre-existing vacuoles during the formation of the intine. In the young pollen grains, a large vacuole was divided into small vacuoles after the first mitosis, while somatic type vacuoles disappeared after the second mitosis. The membrane-bound structures were characterized by fine fibrillar substances that appeared in mature pollen grains just before germination. Those structures were considered as storage vacuoles, which developbed into lysosomal structures containing acid phosphatases (characteristic for lytic vacuoles) in pollen grains. The current study could not identify these structures in embryogenic barley pollen. Regan and Moffatt (1990) reported that, according to neutral red staining, vacuoles disappeared during the first or second gametophytic mitotic divisions. The appearance of a large vacuole prior to generative cell formation (as a result of pollen mitosis I) and its degradation into many small vacuoles are generally observed in pollen of various plant species (Yamamoto et al., 2003).

Beside a different level of vacuolerization, gamtophytic pollen was also characterized by a higher density of ribosomes, rough endoplasmic reticulum and organelles like mitochondria and dictyosomes as compared to embryogenic pollen, this reflects a respective difference in the metabolic activity during the two pollen developmental pathways.

The results obtained within this thesis, could be also important for other economically important cereals like members of the *Triticeae* tribe as well as for maize, sorghum and rice. As a future benefit, the technology established here, will eventually allow for an in-depth analysis of the phenomenon of pollen embryogenesis in follow-up research on the role of key genes involved in this process. The gain of comprehensive information on the initial mechanisms of pollen embryogenesis may then stimulate a knowledge-based establishment of the commercially useful haploid technology in numerous plant species. The manifold opportunities to utilise pollen embryogenesis should result in a sustainable contribution of this thesis to future advances in fundamental and applied plant research as

well as in crop improvement. In addition, the newly established methods of fixation and live-cell imaging will also be useful to study other types of developmental or physiological processes in plants.

Summary

An important aspect in the genetic improvement of crop plants is the production of genetically homogeneous plants by doubled haploid technology. Methods for the generation of pollen-derived doubled haploid plants have been described for many plant species. Despite this progress, the doubled haploid technology has not yet been successfully transferred to some major crop species due to problems including general recalcitrance, genotype dependence and albinism, and a lack of knowledge about the underlying processes still hampers directed experimental approaches. The current study conducted a comparative structural analysis of embryogenic and gametophytic pollen to understand initial mechanisms of pollen embryogenesis using the experimental model species barley. To identify structural markers and features of the embryogenic pathway, new sample preparation protocols were established for live-cell imaging, basic histological and high-resolution ultrastructural analysis of embryogenic and gametophytic pollen.

In a first step, the efficiency of induction of pollen embryogenesis was improved. By combining cold and starvation stress treatments successively administered to anthers and isolated pollen, this new protocol increased the formation of multicellular structures by 50 per cent. For an optimal ultrastructural preservation HPF protocol was tailored established by the use of cyanobacteria as a filler, which highly improved the cryofixation. Live-cell imaging of cultured pollen required the construction of a custom-made-chamber for optimum growth conditions combining high quality optical imaging with easy access to the cultures during the observations. In this context, the generation of transgenic lines constitutively accumulating GFP exclusively in the cell nuclei proved to be highly valuable, as it enabled the unprecedented monitoring of the nuclei during the complete process of pollen embryogenesis.

Live-cell imaging experiments revealed that the first embryogenic division was either symmetric, with two nuclei or cells of the same shape and size being formed, or asymmetric, where a vegetative and generative cell is created as the result of a regular pollen mitosis I. The first step of proliferation towards embryogenic development occurred in the first case directly from the pollen, whereas upon asymmetric division of the pollen, the vegetative cell proved to be the cellular origin of pollen embryogenesis.

Embryogenic development was far more frequent after a symmetric first pollen mitosis than upon asymmetric division. The generative cell usually did not show any embryogenic response, though it was very rarely found to perform one or two successive mitotic divisions.

Of the pollen examined, 7.1% whose first mitosis was symmetric and a further 2.8% whose first mitosis was asymmetric did not form multicellular structures, yet continuously accumulated starch from the first days of observations onwards. Ultrastructural observations showed that uni-nucleate pollen and embryogenic pollen contained proplastids. On the other hand, both gametophytic and non-embryogenic pollen contained differentiated amyloplasts. Gametophytic and embryogenic pollen had a large central vacuole in common, which persisted beyond the first pollen mitosis. In pollen embryogenesis however, this central vacuole was gradually replaced by many small ones, while during the gametophytic pathway, the large central vacuole gradually ceased its size to eventually disappear in fully mature pollen grains. As compared to embryogenic pollen, gametophytic pollen had a higher density of ribosomes, rough endoplasmic reticulum, and organelles such as mitochondria and dictyosomes.

Ultrastructural and live-cell imaging results identified nuclear fusion as the crucial mechanism of genome doubling during barley pollen embryogenesis. Whether other mechanisms of genome doubling play an additional, minor role could not be determined in this study.

The gained comprehensive information, within this thesis, on the initial mechanisms of pollen embryogenesis will then stimulate knowledge-based establishment for other recalcitrant genotypes or economically important crop plants like members of the *Triticeae* and *Paniceae* tribes. The technology established here will eventually allow more detailed studies of the phenomenon of pollen embryogenesis and may allow the identification of key genes of this process. In addition, the newly established methods of pollen fixation and live-cell imaging will also be useful to study other types of developmental or physiological processes in plants.

6. Zusammenfassung

Ein wichtiger gentechnologischer Aspekt zur Verbesserung von Kulturpflanzen ist die Erzeugung genetisch identischer Pflanzen unter Verwendung der Doppelhaploiden-Technik. Ungeachtet der technologischen Fortschritte konnte die erfolgreiche kommerzielle Etablierung der Technologie für viele agrarwirtschaftlich relevante Kulturpflanzen bis zum heutigen Zeitpunkt nicht zufriedenstellend realisiert werden. Genotypabhängigkeit, Albinobildung und ungenügende Kenntnisse über die zugrunde liegenden Mechanismen erschweren hierbei molekularbiologische Versuchsansätze. Zielsetzung der vergleichenden strukturellen Untersuchungen der gametophytischen und embryogenen Pollenentwicklung in Gerste, ist ein besseres Verständnis der initialen Mechanismen der Pollen-Embryogenese (POEM).

Eine wichtige Voraussetzung für die Identifizierung und Charakterisierung der strukturellen Merkmale mittels Histologie, Ultrastrukturanalyse und *Live Cell Imaging*, ist die Etablierung spezifischer Präparations- und Versuchsprotokolle, sowie eine effiziente Induzierbarkeit der POEM in isolierten Pollen. Zur Initierung der POEM in Pollenkulturen führte die kombinierte Anwendung von Kälte- und Hungerstress zur Steigerung der Bildungsrate multizellulärer Strukturen auf 50 Prozent. Die Aufklärung struktureller Mechanismen dynamischer Prozesse mittels Ultrastrukturanalysen erfordert eine möglichst schnelle Fixierung unter möglichst physiologischen Bedingungen. Die Etablierung eines Protokolls zum Hochdruckgefrieren der Pollenkulturen/Antheren unter der Verwendung von Cyanobakterien als "Filler" führte hierbei zu einer signifikanten Verbesserung der strukturellen Auflösung. Das *Live Cell Imaging* von Zellkulturen über lange Zeiträume bedingt eine Reihe von Anforderungen an den Versuchsaufbau. Zur Gewährleistung optimaler Wachstumsbedingungen bei gleichzeitiger stabiler Positionierung der zu untersuchenden Zellen unter möglichst optimalen optischen Bedingungen, wurde eine spezielle Probenkammer entwickelt. Für Langzeitbeobachtungen zum Verhalten des Zellkerns während der embryogenen Entwicklung, wurden transgene Pflanzen mit einer konstitutiven Akkumulation von GFP im Zellkern generiert. Somit konnte zum ersten Mal die gesamte Entwicklung des Pollen während der Pollenembryogenese visualisiert werden. Die erste embryogene Teilung kann hierbei zum einen symmetrisch, mit zwei Kernen oder Zellen gleicher Form und

Größe, oder assymetrisch erfolgen, wobei im Zuge der Mitose I eine generative und eine vegetative Zelle entstehen. Der erste Proliferationsschritt der embryogenen Entwicklung geht somit im ersten Fall direkt von dem Pollen aus, während die vegetative Zelle der assymetrischen Teilung ihren zellulären Ursprung in der POEM hat. Hierbei wurde eine embryogene Entwicklung weitaus häufiger nach der ersten symmetrischen Pollenmitose beobachtet. Die generative Zelle zeigt üblicherweise keine embryogene Entwicklung. Nur in ganz seltenen Fällen konnte eine oder zwei aufeinander folgende mitotische Teilungen beobachtet werden.

Es konnte gezeigt werden, dass 7,1% des Pollens, dessen erste Mitose symmetrisch erfolgt ist und weitere 2,8% mit asymmetrischer Teilung entwickeln sich nicht zu multizellulären Strukturen, akkumulieren jedoch vom ersten Tag an kontinuierlich Stärke. Ultrastrukturanalysen zeigen dass einkernige und embryogene Pollen Proplastiden oder undifferenzierte Plastiden mit 1-3 Thylakoiden besitzen, während gametophytischer und nicht-embryogener Pollen differenzierte Amyloplasten mit großen Stärkekörnern aufweisen. Bis zur ersten Pollenmitose besitzen gametophytischer und embryogener Pollen eine große zentrale Vakuole. Während diese sich bei erstem im Laufe der Entwicklung gleichmäßig verkleinert und im reifen Stadium letztendlich verschwindet, wird die zentrale Vakuole des embryogenen Pollens durch viele kleine ersetzt. Im Vergleich zum embryogenen weist der gametophytische Pollen eine deutlich höhere Anzahl an Ribosomen, endoplasmatischem Retikulum, Mitochondrien oder Dictyosomen auf.

Die Ergebnisse der Ultrastrukturanalysen und des *Live Cell Imaging* zeigen, dass es sich bei der Kernfusion um den entscheidenden Mechanismus zur Genomverdoppelung während der POEM handelt. Die funktionelle Rolle weitere Mechanismen kann bis zum heutigen Zeitpunkt nicht ausgeschlossen werden. Die umfangreichen neuen Kenntnisse zur Initiation der POEM in Gerste könnten zur Etablierung Doppelhaploider in weiteren Genotypen als auch in wirtschaftlich relevanten Kulturpflanzen wie anderen Mitgliedern der *Triticeae* oder *Paniceae* führen und somit weiterführende Studien der Mechanismen und der Identifizierung beteiligter Gene zu ermöglichen. Darüber hinaus sind die neu entwickelten bzw. optimierten zellbiologischen Methoden in vielen Bereichen der Pflanzenforschung anwendbar.

7. Litirature

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8. Publications and proceedings related to this thesis

Publication

Daghma DS, Kumlehn J, Melzer M (2011) The use of cyanobacteria as filler in nitrocellulose capillaries improves ultrastructural preservation of immature barley pollen upon high pressure freezing. Journal of Microscopy doi: 10.1111/j.1365-2818.2011.03509.x 2011

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Daghma DS (2010) Structural and ultrastructural study of barley pollen embryogenesis. The 12th International EMBL PhD Student Symposium, from science fiction to science fact: what's next? 21-23 October EMBL, Heidelberg, Germany

Daghma DS (2010) Embryogenesis of barley pollen -From live cell imaging to ultrastructural analysis-**.** Plant Science Student Conference June 15th - June 18th IPK Gatersleben, Germany

Daghma DS (2009) Structural aspects of immature pollen undergoing Pollen Embryogenesis (POEM) in *Hordeum vulgare* L**.** Plant Science Student Conference 23rd - 26th June IPB Halle, Leibniz Institue of Plant Biochemistry**,** Germany

Posters presentations

Götz H, **Daghma DS,** Melzer M, Kumlehn J **(**2011) Illuminating early pollen embryogenesis: Time-lapse livecell imaging facilitated by nucleus-specific GFP accumulation - GABI Status Meeting 2011, Potsdam, Germany, 15.-17.03

Daghma DS, Götz H, Rutten T, Kumlehn J, M Melzer (2011) GABI-POEM - Structural Study of Initial Mechanisms of Pollen Embryogenesis - GABI Status Meeting 2011, Potsdam, Germany, 15.-17.03

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Daghma DS, Hoffie K, Rutten T, Wiesner M, Melzer M (2010) High Pressure Freezing of Immature Pollen Using Cyanobacteria as 'Filler' for Loading Nitrocellulose Capillaries: - 17th International Microscopy Congress, Rui de Janeiro, Brasil, 19.-24.09

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Declaration

Herewith I declare that I independently wrote the following doctoral thesis using no other than the sources those which are listed. The principles “Verantwortung in der Wissenschaft” (Responsibility in Science), recommended by the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, were observed.

Gatersleben, 17.07.2011

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9. Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit von mir bisher weder der Naturwissenschaftlichen Fakultät I - Biowissenschaften der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.

Ich erkläre ferner, dass ich diese Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel und Literatur angefertigt habe.

Gatersleben, den ..... ----------

Diaa Eldin Sayed Daghma

11. Curriculum Vitae

Personal Data

- **Family Name** : Daghma
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Education

- **B. Sc.:** In Field Crops, Plant Breeding, Faculty of Agriculture, Cairo Univ. Egypt, Grade Good at Year 1999
- **M. Sc.:** 2007, In Plant Physiology Dpt. Topic, physiological studies of on wheat double haploid plants in response to different abiotic stresses, Faculty of Agriculture, Cairo Univ. Egypt
- **Ph. D.:** From 2008 to 2011 working on Ph. D. in Leibniz Inistitute of Plant Genetics and Crop Plant Research (IPK), Physiology and Cell Biology Dept., Structural Cell Biology, Correnstr 3, 06466, Gatersleben, Germany

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Work Experience

- **From 2000 to 2003**
  Working in biotechnology laboratory on doubled haploid lines production of wheat via male gamete culture and traditional breeding in Wheat Crop Research Dept., Field Crops Research Institute, ARC of Egypt

- **From 2003 to 2004**
  Attending training program about improvement of wheat pollen culture in Plan Reproduvtive Biology Group, Dept. of Physiology and Cell Biology, Leibniz Inistitute of plant genetics and crop plant research (IPK), Germany

- **From 2004 2007**
  Working in the National Gene Bank and Genetic Resources of Egypt as Genetic Resources specialist

- **Scientific activities:**

- Participating in the organization of the first conference of the National Gene Bank and Genetic Resources, 2005
- Attending: biovision conference Alexandria, 2006
- Attending: Seed Bank Management (Short Training Course) in ICARDA, Syria, (2007)
- Attending Winterschool 2010 Jan 17th-22th, Advanced Microscopy 2 ECTS Credits, module 2: Life Cell Microscopy, 17-22/1/ 2010 in Zürich, Swizerland