Chromatin modifications during pollen development and pollen embryogenesis in barley

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List of abbreviations

°C	degree Celsius
%	percent
µ	micro
µg	microgram
µl	microliter
µM	micromolar
5-AZA	5-azacytidine
BAP	6-benzylaminopurine
BCP	bicellular pollen
BSA	bovine serum albumin
cm	centimeter
CENH3	centromeric histone 3
DAPI	4,6-diamidin-2-phenylindol
DH	doubled haploid
DMSO	dimethyl sulfur oxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
FA	formaldehyde
FDA	fluorescein diacetate
g	gram
GFP	green fluorescence protein
h	hour/s
H3K9ac	histone H3 lysine 9 acetylation
H3K4me2	histone H3 lysine 4 dimethylation
H3K9me2	histone H3 lysine 9 dimethylation
H3K27me3	histone H3 lysine 27 trimethylation
KBP	Kumlehn's Barley Pollen (medium)
LSM	laser scanning microscope
M	molar

MΩ	megaohm
mg	miligram
min	minute/s
ml	millilitre
mm	millimeter
MTSB	microtubule stabilizing buffer
n	number of biological replicates
NC	nitrocellulose
nm	nanometer
ON	over night
PB	phosphate buffer
PEG	polyethylene glycol
pH	power of hydrogen
PM I	pollen mitosis one
PM II	pollen mitosis two
RNA	ribonucleic acid
RNAP II	RNA polymerase two
RT	room temperature
sec	second/s
SMB1	Starvation Medium Barley, version 1
sRNA	small ribo nucleic acid
SSC	saline-sodium citrate
SV	Simian Virus 40
TCP	tetracellular pollen
TE	transposable elements
TSA	Trichostatin A
UCP	unicellular pollen
W/m2	watts per square meter
v/v	volume-to-volume
x*g*	times gravitation force

1 INTRODUCTION

1.1 Haploids and plant breeding

The ability of plants to produce embryos from cells other than the zygote is referred to as totipotency. In some plants this form of embryogenesis may occur spontaneously, in others it may be induced in vitro. In case of somatic embryogenesis embryos arise from somatic tissue generating plants of the same ploidy and genetic composition as the parent plant (Gaj, 2001, 2004; George et al., 2008; Zimmerman, 1993). In gametophytic embryogenesis the embryos arise from haploid cells which after chromosome doubling yield plants that are homozygous for each locus (Bohanec, 2009; Reynolds, 1997; Seguí-Simarro, 2010). These doubled haploids (DH) are genetically pure inbred plants, produced in a single generation where traditional breeding techniques typically require multiple generations of inbreeding to stabilize desired traits. The DHs are not only homozygous at all loci, but in addition, since the founder cells are the product of meiosis, a population of double haploid plants represents a collection of meiotically recombinant but genetically fixed individuals. Consequently, this genetic variation is embodied in populations of entirely homozygous DH lines obtained from embryogenic pollen cultures (Kumlehn, 2014). DH plants have become a cornerstone in plant breeding programs (Forster et al., 2007; Germanà, 2006). Due to the ease with which male gametophytes can be harvested and isolated in large quantities as compared to the female gametophyte, embryo induction of the male gametophyte is the most common form of DH production.

1.2 Pollen gametogenesis

Male reproductive development in flowering plants is a complex series of events that culminates in the production of highly specialized male gametes (sperm cells). Unlike animals, flowering plants do not segregate a germline early in development. Instead male reproductive structures are differentiated late in post-embryonic development directly from established somatic cell lineages (Feng and Dickinson, 2007; Dickinson and Grant-Downton, 2009). In a further difference to animals the products of meiosis do not directly differentiate into gametes. In the male reproductive cell lineages each of the four haploid cells undergoes two further mitotic divisions

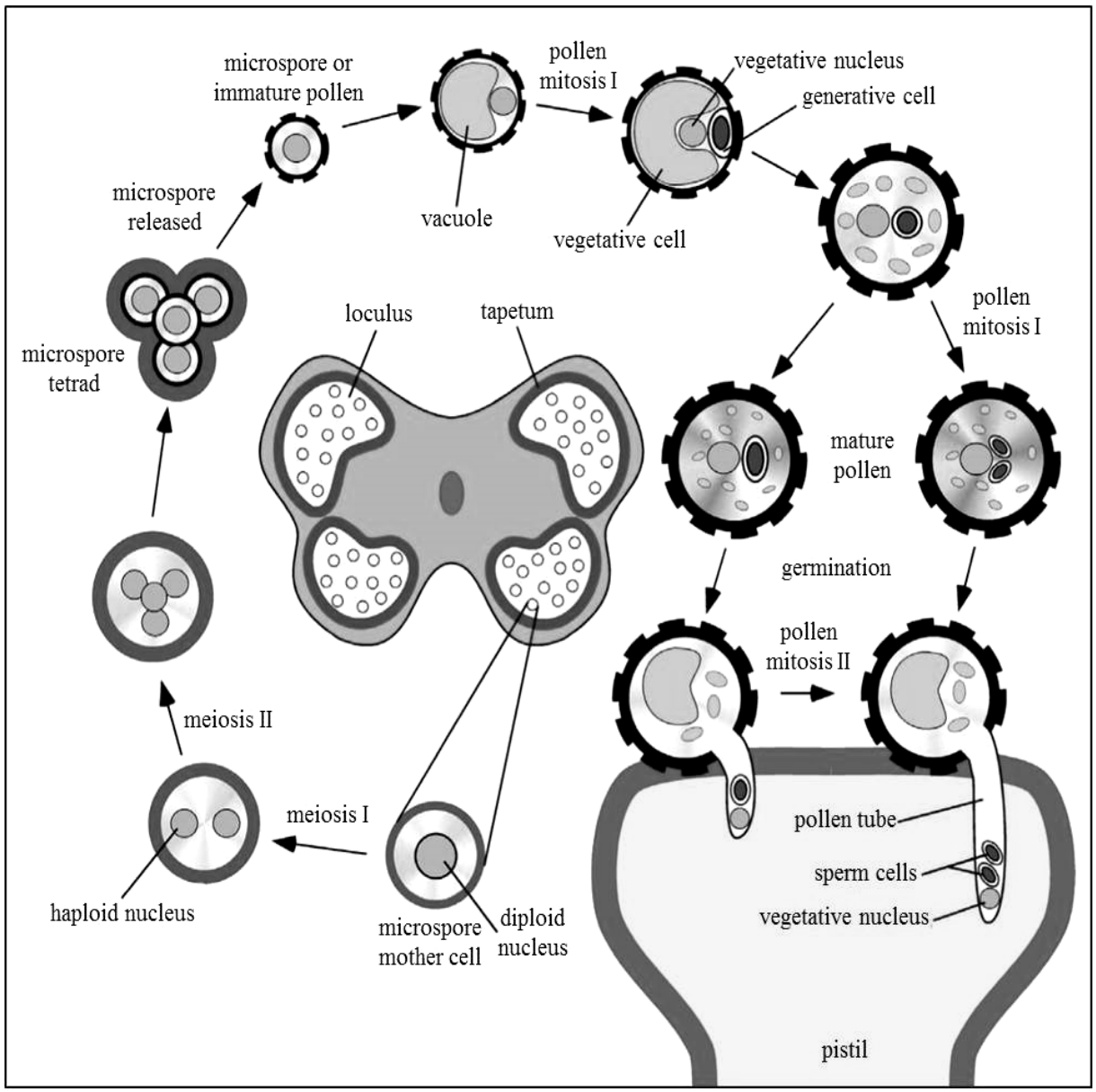


Fig. 1: Schematic illustration of pollen development. Diploid pollen mother cell undergoes meiosis to produce a tetrad of haploid microspores or immature pollen. First pollen mitosis typically is asymmetric, resulting in a small generative cell residing within a large vegetative cell. Second pollen mitosis, yielding the two sperm cells, can happen before anthesis or later within the pollen tube. (modified after Honys et al. 2006).

to produce the mature pollen (Berger and Twell, 2011; Twell, 2011). The first of these mitotic processes, PM I is highly asymmetric leading to a large vegetative cell and a small generative cell (Fig. 1). While the large vegetative cell ceases further cell divisions and becomes an

accessory cell, the smaller generative cell becomes suspended inside the vegetative cell and after PM II will further divide and differentiate into two sperm cells. Depending on the species, PM II can take place inside the anther or during pollen germination (Reynolds, 1997).

1.3 Embryogenic pollen development

The mechanisms underlying the transformation of a gametophytic pollen into an embryogenic pollen are still largely unknown which is unfortunate since efficient induction of POEM and DH plant regeneration are sought after qualities (Germanà, 2006). The ability to form haploid embryos is species and genotype dependent; which makes that induction protocols have to be defined on a case by case scenario. Studies on the model plants *Brassica napus* and tobacco for dicots, and barley and wheat for monocots have revealed that pollen induction is a process in which several routes can lead to the same endpoint. This uncertainty surrounding pollen embryogenesis is reflected in many proposals for the pathways leading to pollen embryogenesis (Fig. 2) (Sunderland and Evans, 1980; Raghavan, 1986; Hu and Kasha, 1999). These often include normal division of the unicellular pollen (pathway I in Fig. 1), division of the vegetative cell only (pathway II in Fig. 2), in the generative cell only (pathway III in Fig. 2), involving both cells (pathway IV in Fig. 2), or initiated by nuclear fusion after pollen mitosis 1 (PM I) (pathway V in Fig. 2).

Though multiple pathways can exist in the same culture (Custers et al., 1994; Kasha et al., 2001; Daghma et al., 2014), it seems that embryogenic development most commonly starts by pathway I, i.e. symmetric division of the unicellular pollen (Zaki and Dickinson, 1991; Indrianto et al., 2001; Pulido et al., 2005; Sunderland and Wicks, 1971) or pathway II, by division of the vegetative cell accompanied by degeneration of the generative cell (Reynolds, 1993; Sunderland, 1974; Daghma et al., 2014). The pathway III embryo formation based on divisions of the generative cell has been suggested for *Hyoscyamus niger* (Rhagavan, 1978; Reynolds, 1985) but is an otherwise very rare phenomenon. Multicellular structures comprising both vegetative-like and generative-like nuclei, under pathway IV, have been reported from several species including rapeseed (Fan et al., 1988), soybean (Kaltchuk-Santos et al., 1997), wheat (Reynolds, 1993; Szakács and Barnabás, 1988), barley (González and Jouve, 2005) and pepper (González-Melendi

et al., 1996; Kim et al., 2004). However, recent studies put in question that truly generative cells are involved (Daghma, 2011).

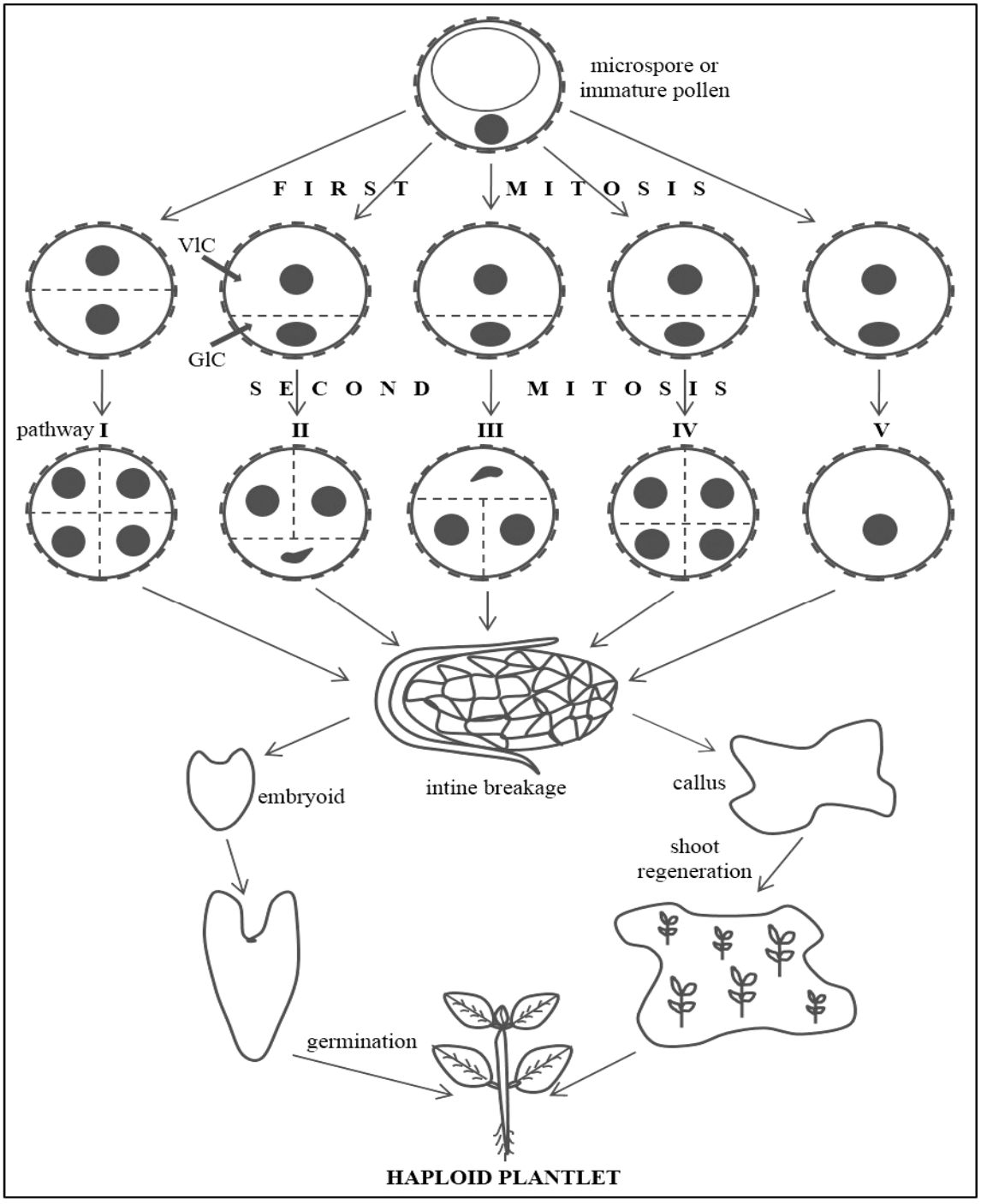


Fig. 2: Schematic illustration of different pathways of pollen embryogenesis (modified after Sarita Pujari, Plant Tissues: yourarticlelibrary.com). VlC = vegetative-like cell, GlC = generative-like cell.

1.3.1 Stress and embryogenesis

The central element in all induction protocols is the stress treatment. This can be temperature, nutrient supply, osmotic stress, either alone or in combination (reviewed by Islam and Tuteja, 2012; Shariatpanahi et al., 2006). In most species, pollen is most responsive for treatments inducing embryogenic development around PM I (Bhowmik et al., 2011; Ramírez et al., 2001). At this stage, pollen are highly vacuolated and have a peripherally located nucleus. Induction protocols always lead to heterogeneity of responses in culture. Pollen may arrest, become embryogenic or continue the gametophytic pathway. A first sign of successful induction can be the relocation of the nucleus to the center of the cell with cytoplasmic strands radiating creating a typical star-like morphology (Gervais et al., 2000; Maraschin et al., 2005; Daghma et al., 2014). This indicates a disturbance of the cytoskeletal organization and indeed, application of chemicals that disrupt the cytoskeleton can trigger embryo formation in the absence of stress treatment (Gervais et al., 2000; Soriano et al., 2008; Szakács and Barnabás, 1995; Zaki and Dickinson, 1991; Zhao et al., 1996). Chemical compounds like 2-hydroxynicotinic acid, benqotriazole-5-carboxylic acid, violuric acid monohydrate and n-butanol have also been found to trigger microspore embryogenesis (Zheng at al., 2001; Soriano et al., 2008). Also the use of specific inhibitors like Buthionine sulfoximine (reducing glutathione) and Trichostatin A (inhibitor of histone deacetylase) can enhanced embryogenic development and even improved embryo quality (Satsolla et al., 2008; Li et al., 2014).

1.3.2 Whole genome doubling

Truely haploid plants are typically sterile, since they contain only one set of chromosomes and are thus hardly able to produce gametes with a balanced set of chromosomes after meiosis. Chromosome doubling, which occurs either spontaneously in culture or after the application of chemicals such as colchicine, restores the ploidy level and yields fertile homozygous diploid plants which can be used as genetically fixed lines in breeding programmes (Castillo et al., 2009). Gonzalez-Melendi et al., (2005) used DAPI staining to proof that spontaneous genome duplication in embryogenic barley pollen cultures happens by means of nuclear fusion, a fact that was confirmed by time-lapse studies by Daghma et al. (2014). These studies showed that diploidization events are common place and occur during all stages of growth of haploid plants.

This form of diploidization process is made possible by incomplete cell wall formation which is a phenomenon often occuring during the early stages of embryogenic pollen development (Daghma, 2011). Incomplete cell wall formation or cell wall degradation has also been considered responsible for the reported fusion between the vegetative and the generative nucleus (Vazart, 1971; Dunwell and Sunderland, 1976; Lee and Chen, 1987). These claims, however, do not hold up to scrutiny as they are based on micrographs of modest quality. Superior fixation methods have shown that aberrations in the cell wall of the generative cell are most likely artifacts caused by chemical fixation protocols (Cresti et al., 1987).

1.4 Determinants of cell fate during development

1.4.1 Asymmetric cell division and cell polarity

Asymmetric cell division is a universal strategy of eukaryotes to generate the diverse cell types that are necessary for patterning and proliferation. The development of pollen in flowering plants is an extreme example in which division asymmetry controls the functional specialization and differentiation of two cells which are essential for fertilization. When immature pollen is artificially induced to undergo symmetric division using microtubule inhibitors the ability to form generative and vegetative cells is lost. This shows that asymmetry in PM I is critical for the functional pollen (Twell et al., 1998). Although microtubule destabilizing agents have also been used to induce pollen embryogenesis (Zaki and Dickinson, 1990; Telmer et al., 1993; Telmer et al., 1995), division symmetry of PM I is not fundamental to the initiation of embryogenic development. Pollen embryogenesis may start after both symmetric and asymmetric PM I as was convincingly shown in time lapse recordings of induced barley pollen by Daghma et al., (2014). Furthermore, symmetric division by itself not necessarily leads to embryogenesis as shown by the presence of pollen specific markers in both daughter cells arising after symmetric PM I in *Arabidopsis* pollen (Eady et al., 1995; Touraev et al., 1995). This raises the question as to what fundamental factors govern destiny of the pollen cells. Cell fate after PM I has been suggested to be imposed by cell fate determinants which are hypothesized to be distributed in a gradient within the unicellular pollen (Twell et al., 1998). Therefore, to generate two unequal cells with different cell fate PM I needs to be orchestrated in strict relation to the distribution of these

determinants. Within PM I one can distinguish three distinct processes: (I) relocation of the central nucleus to a position near the pollen wall, which is followed by (II) mitosis along a predetermined division axis, and ends with a (III) cytokinesis resulting in two unequal sized cells.

1.4.2 Nuclear migration

The first step in PM I is the relocation of the central nucleus to a position near the pollen wall. Nuclear migration indicates polarity which is probably laid down during tetrad formation (Twell et al., 1998). Isolated unicellular pollen can undergo normal asymmetric division in vitro (Eady et al., 1995) showing the stable lay-out of this polarity. The key mechanism behind polarization in animals cells are subcellular localizations of RNAs and/or proteins (Roegiers and Jan, 2004). Cellular polarization and subsequent asymmetric division is a widespread phenomenon in plants (Heidstra, 2007). It is not well understood, however, how pollen acquire this polarity and by what factors this polarity is translated into the cytoskeleton to ensure an asymmetric division, and what signals determine the identity of the daughter cells. It is beyond doubt, however, that the cytoskeleton plays a main role in this process.

Several of the mutations that disturb nuclear migration and asymmetric division have turned out to be microtubule related. These include the *gemini pollen1* (*gem1*) mutations in GEM1, a plant member of the MAP215 family of microtubule-associated proteins (Park et al., 1998; Gard et al., 2004), the redundant γ-tubulin proteins, TUBG1 and TUBG2 (Pastuglia et al., 2006) and the γ-tubulin targeting factor NEDD1 (Zeng et al., 2009). Microspores deficient in these proteins can produce equal daughter cells due to miss-oriented and disorganized spindles thus showing that the first nuclear positioning step is microtubule-dependent (Twell et al., 2002).

1.4.3 Spindle formation

After positioning of the nucleus near the pollen wall after meiosis a spindle is formed. Orientation and position of the spindle determine the division plane in eukaryotes. In somatic plant cells the pre-prophase band of peripheral microtubules indicates the position of the future division plane and orientation of the mitotic spindle and phragmoplast (Ambrose and Cyr, 2008).

Since pollen lack a pre-prophase band (Otegui and Staehelin, 2000), other mechanisms must be at work to determine the plane of division. The spindle at PM I is always asymmetric, being short and blunt at the side of the generative cell and elongate and sharp at the side of the vegetative cell (Geitler, 1935; Brumfield, 1941; Heslop-Harrison, 1968; Banaei et al., 2012). Construction of the spindle seems to start at the vegetative cell pole (Terasaka and Niitsu, 1990, 1995). Little is known about the regulators of asymmetric microspore division. Although factors that disturb the cytoskeletal dependent nuclear migration almost invariable also disrupt spindle formation, spindle formation and orientation seems under the control of a different set of mechanisms. Evidence for this is provided by *scp* mutations which do not affect nuclear migration but disturb the orientation of the mitotic spindle (Chen and McCorwick, 1996; Oh et al., 2010). SCP is a microspore-specific nuclear protein belonging to the LBD/ASL family of proteins with DNA binding activity (Maier and Hochholder, 2010). Since it is transiently expressed before nuclear migration but does not affect nuclear migration, SCP is thought to be a transcription factor that controls the expression of genes with functions in mitotic division and orientation. The LBD/ASL protein family is only found in plants which suggest the existence of plant-specific or even microspore-specific mechanisms regulating asymmetric cell division (Oh et al., 2010).

1.4.4 Cytokinesis

In the last step the asymmetric PM I is sealed by the cytokinesis. This starts with a crescent shaped phragmoplast formed in the mid zone of the spindle which through centrifugal expansion assigns different volumes to the designated generative and vegetative cells. The *two-in-one* (*tio*) mutations in the plant orthologue of the *FUSED* Ser/Thr protein kinase gene block cytokinesis, but they do not affect nuclear migration or the orientation of the mitotic spindle during PM I (Oh et al., 2005; Oh et al., 2012). As a result of the unsuccessful or incomplete cytokinesis two daughter nuclei of unequal size end up in the same cytoplasm (Lee et al., 2007; Oh et al., 2012).

1.4.5 Cell fate determination

Two models have been proposed to account for the different development of generative and vegetative cell after the asymmetric division. Both are based on the premise that development into a vegetative cell is the default pathway and relies on the presence of a polar distribution of induction factors. In the passive repression model the generative cell is formed in an environment lacking factors that induce vegetative cell development. In the active repression model, the generative cell is formed in an environment enriched in factors repressing vegetative cell development (Eady et al., 1995). Early evidence comes from observations made by Lacour (1949) who reported that the generative nucleus develops in a region relative free of RNA while the vegetative nucleus remained in a RNA-rich environment. The role of chromatin condensation is not completely clear. According to chromosome studies during PM I in species of several genera, chromatin structures of vegetative cell and generative cell are already different before the enclosure of the chromosomes within the daughter nuclei (Terasaka, 1982; Terasaka and Tanaka, 1974). This has been seen as proof of the presence of factors enriched in the domain of the generative cell that induce chromatin condensing. In this view the limited chromatin dispersal and a concomitant repression of gene expression may thus determine the fate of the generative cell. Putative candidates for such chromatin condensation factors could be histones. However, reports on the different histone contents between generative cell and vegetative cell of *Lilium* (Jalouzet, 1969; Sheridan, 1973; Ueda and Tanaka, 1995a, 1995b) did not focus on the stages immediately after PM I.

In recent work on rye pollen the asymmetry of the spindle was shown to cause supernumerous B chromosomes to be differently distributed in generative and vegetative cells (Banaei et al., 2012). A possible presence of biased DNA segregation mechanisms during PM I could also serve as cell fate determining factors. Asymmetric or nonrandom chromatid segregation is a well studied phenomenon in mammalian stem cell division (Rocheteau et al., 2012). Also known as the immortal DNA strand hypothesis (Cairns, 2006), it suggests that in stem cell division one daughter cell contains only the old DNA while the other carries newly synthesized chromosomes. Cells carrying the old DNA maintain an undifferentiated state and preserve the stem cell compartment within the organ, whereas cells containing the new DNA acquire specialized functions. Early proof for the occurrence of non-random chromosome segregations in

plants come from radioactive pulse chase experiments on dividing root tips cells of *Vicia faba* and *Triticum boeoticum* (Lark, 1967).

Given the role of the phragmoplast in cell fate determination, the nuclear/cytoplasmic ratio may also play a role. Terasaka and Nitsu (1987) found a strong correlation between cell size and chromatin dispersion at PM I and the topological constraint may help in the process leading to differentiation of the generative cell.

1.5 Pollen epigenetics

Pollen experiences a conspicuous reprogramming of the vegetative and generative cell. Throughout pollen development the overall number of genes expressed decreases but at the same time pollen specific transcripts increase (Honys and Twell, 2004). Thus while a pollen specific machinery is activated, somatic genes are down-regulated. After PM I vegetative and generative cells activate distinct transcriptional programmes (Borges et al., 2008). Though all major components of the core cell cycle machinery are expressed there is a reduced expression during pollen maturation which is associated with the exit of the vegetative cell from the cell cycle and the low transcriptional activity of the generative cell (Honys and Twell, 2004; Pina et al., 2005). Concomitant the vegetative cell becomes enriched in transcripts related to pollen germination and pollen tube growth while the transcriptome of the generative cell, which undergoes DNA replication, shows an emphasis on DNA repair, cell cycle transition and protein degradation (Borges et al., 2008; Pina et al., 2005).

The cellular events that give rise to male and female gametophytes are under strict epigenetic control. Epigenetic events play a pivotal role during the cell cycle, cell dedifferentiation and developmental reprogramming (Koukalova et al., 2005; Desvoyes et al., 2010). The dynamic changes in small non-coding RNAs, DNA methylation and histone modifications through which gene expression is modulated are part of the complex epigenetic resetting during pollen development.

1.5.1 Role of small RNAs

Small RNA pathways are very active in pollen development especially in the sperm cells (Honys and Twell, 2004; Le Trionnair et al., 2011; Borgess et al., 2008; Borges et al., 2011) confirming that pollen development is under strict epigenetic control. In both dicots and monocots the small RNA diversity increases during stamen development (Lu et al., 2005; Nobuta et al., 2007). The significant overlap in the population of microRNAs found in the pollen from rice and *Arabidopsis* (Grant-Downton et al., 2009a; Grant-Downton et al., 2009b; Borges et al., 2011) indicate highly conserved roles for microRNAs in pollen function between distantly related plant species (Wei et al., 2011; Peng et al., 2012).

Studies on *Arabidopsis* have shown that normally silenced transposable elements (TE) become transcribed during meiosis (Chen et al., 2010; Yang et al., 2011). This indirect proof for a partial release of epigenetic repression during meiosis does not last as in young pollen TE transcripts become less abundant (Honys and Twell, 2004). It thus seems that epigenetic silencing is restored after meiosis. As an indirect result of chromatin decondensation vegetative cells are enriched in miRNAs transcripts (Slotkin et al., 2009; Schoft et al., 2011). Also in the more condensed nuclei of the generative cells TE silencing mechanisms are well established and may be involved in chromatin remodeling and resetting of epigenetic markers (Slotkin et al., 2009). Particular genes involved in small RNA activity and DNA methylation are highly enriched in sperm cells (Borges et al., 2008; Slotkin et al., 2009). Late in pollen development the chromatin remodeling factor DECREASE IN DNA METHYLATION 1 (DDM1) becomes down-regulated in the vegetative nucleus with a concomitant increase in transcripts of retrotransposons (Dickinson and Grant-Downton, 2009). At the same time TE derived siRNAs accumulate in the sperm cells where the corresponding TE loci are highly methylated and transciptionally silenced (Slotkin et al., 2009). This suggests a cytoplasmic communication between vegetative cell and generative cell (Slotkin et al., 2009; McCue et al., 2011) that allows the translocation of siRNAs generated in the vegetative nucleus to the sperm cells where they enforce the silencing of TEs by RNA-dependent DNA methylation. Similar silencing mechanisms probably exist long before PM II. Slotkin et al. (2009) observed that expression of a transgenic sperm-specific green fluorescent protein (GFP) was down-regulated by an artificial miRNA expressed in the vegetative cell under the LAT52 promoter. Since the latter is expressed from the unicellular stage of pollen

development onward (Eady et al., 1995), this raises the possibility of vegetative cell siRNAs silencing TEs in the generative cell. By targeting DNA methylation, RNAi pathways in plants also play a role in inherited epigenetic variation (Bernstein and Allis, 2005; Matzke and Birchler, 2005; Wassenegger, 2005).

1.5.2 DNA-methylation

DNA methylation is one of the best described chromatin modification that contributes to epigenetic regulation of gene expression. The most common examples are modifications of TEs. Imprinted genes tend to be flanked by TEs, whose methylation can influence gene expression (Radford et al., 2011). The general picture is that in plants, in contrast to animals, epigenetic modification can be inherited, a process which involves DNA methylation (Becker et al., 2011; Cubas et al., 1999; Martienssen and Baron, 1994; Schmitz et al., 2011; Li et al., 2014). On a genome wide level, DNA methylation is controlled by the composition of the nucleosomes and associated histone modifications (Chodavarapu et al., 2010). In *Arabidopsis* disruption of the chromatin remodeling enzymes KYP, SUVH5, and SUVH6 cause a decrease in cytosine methylation and lowering of H3K9me2 levels, leading to a transcriptional reactivation of heterochromatic transposons (Ebbs and Bender, 2006).

Small noncoding RNA production and DNA methylation pathways are upregulated in sperm cells compared with vegetative cells (Borges et al., 2008). DNA methylation plays a major role in silencing transposable elements which is important for genome integrity (Zilberman, 2008; Zilberman et al. 2007). The majority of DNA methylation occurs on cytosines in symmetrical CG sites and depends on the maintenance DNA methyltransferase 1 (MET1) (Cokus et al. 2008, Zilberman et al. 2007) which also in pollen is required to silence transcription from several loci (Julien et al., 2006; Julien et al., 2008). Despite its role in regulating gene activation, DNA methylation and especially the CG methylation, does not seem to play an essential role in the differentiation of the vegetative or generative cell. There is no reprogramming of DNA methylation in male gametes and *met1* mutant pollen do not show obvious defects in the vegetative cell or the germline (Julien and Berger, 2010; Saze, 2008).

1.5.3 Histones and histone modifications

Within the cell DNA is packaged into chromatin whose fundamental unit is the nucleosome, an octamer of the four core histones (H3, H4, H2A, H2B) around which 147 base pairs of DNA are wrapped (for review Kornberg and Lorch, 1999). The core histones are predominantly globular except for their N-terminal tails which can be covalently modified (Kouzarides, 2007). There are at least eight distinct types of posttranslational modifications known (Table 1) of which acetylation, methylation, and phosphorylation are best studied.

Table 1: Overview of different classes of histones modifications (Kouzarides 2007).

Chromatin Modifications	Residues Modified	Functions
Acetylation	**K**-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	**K**-me1 **K**-me2 **K**-me3	Transcription, Repair
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me2s	Transcription
Phosphorylation	**S**-ph **T**-ph	Transcription, Repair, Condensation
Ubiquitylation	**K**-ub	Transcription, Repair
Sumoylation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deimination	**R > Cit**	Transcription
Proline Isomerization	**P-cis > P-trans**	Transcription

This vast array of modifications gives enormous potential for functional responses by which chromatin structure, and ultimately gene transcription, can be influenced in reply to diverse exogenous and endogenous stimuli including stress, pathogen attack, temperature, light, and hormones (Berger, 2007; Chen and Tian, 2007; Anzola et al., 2010). Histone modifications are also major mechanisms for epigenetic regulation of remodelling higher-order chromatin structure and controlling gene expression during development and differentiation (Kuo, 1998; Li, 2002; Vanhaecke, 2004; Cedar and Bergman, 2009). Modifications on histones are dynamic and can appear or disappear within minutes of a stimulus arriving at the cell surface (Cheung et al., 2000). The basic function of histone modifications is to disrupt the contacts between nucleosomes in order to ‘‘unravel’’ chromatin. The second function is the recruitment of non-histone proteins.

Of all the known modifications, acetylation has the most potential to unfold chromatin since it neutralizes the basic charge of the lysine. As a consequence acetylation is almost invariably associated with activation of transcription whereas deacetylation correlates with transcriptional repression (Hebbes et al., 1988). Classically H3K27 methylation is implicated in silent chromatin and H3K4 methylation is involved in active chromatin (Zhang et al., 2007; Li et al., 2007). However, context may be everything as it seems that any given modification has the potential to activate or repress under different conditions (Bannister and Kouzarides, 2005). For example, methylation at H3K9 may be negative in the promoter and positive in the coding region (Vakoc et al., 2005). Dissimilar translational activity in vegetative and germline cells is also reflected by different histone modifications (Sano and Tanaka 2010, Houben et al. 2011).

Not only by means of histone modifications but also by expressing different histone variants cells can alter the chromatin consistency. The *Arabidopsis* genome for example comprises 15 histone three related genes among which histone H3.3 is associated with transcriptionally active genes in euchromatin and H3.1 is linked to silent genes in heterochromatin (Henikoff and Ahmad, 2005; Corpet and Almouzni, 2009). Whereas in somatic cells nine variants of histone H3 are present only three each are expressed in the vegetative and generative cell (Okada et al., 2005; Ingoufff et al., 2007, 2010). The vegetative cell carries the variants HTR5, HTR8 and the unusual HTR14, whereas the sperm cells express HTR5, CENH3, and the male-germline specific HTR10 thus distinguishing the male gamete chromatin from the non-gametic tissues (Ingouff and Berger, 2010). Upon fertilization, the histone H3 variants contributed by male and female gametes are completely removed from the zygote nucleus and replaced by histones H3.1 and H3.3 variants synthesized de novo (Ingouff et al., 2007).

1.5.3.1 Histone acetyl transferases and histone deacetylases

Differential core histone acetylation is the best-understood chromatin remodeling mechanism (Verbsky and Richards, 2001).The acetylation and deacetylation of histones controls gene expression by changing the accessibility of DNA to DNA-binding transcription factors (Kuo and Allis, 1998; Kuo et al., 2000). This is accomplished with the assistance of histone acetyl transferases (HAT), which acetylate the lysine residues in core histones leading to a less compact

and more transcriptionally active chromatin, and, on the converse, the actions of histone deacetylases (HDAC), which remove the acetyl groups from the lysine residues leading to the formation of a condensed and transcriptionally silenced chromatin (Shahbazian and Grunstein, 2007). HATs and HDACs occur in multiprotein complexes and may act either site-specific through interaction with sequence-specific DNA binding factors, or in a broad manner across large genomic areas.

At least four distinct families of HATs and three families of HDACs have been identified (Sterner and Berger, 2000; for review Pandey et al., 2002). For the HATs these include: I) the GNAT-MYST family which have sequence motifs shared with enzymes that acetylate non-histone proteins, II) the p300/CREB binding protein (CBP) co-activator family which in in animals are thought to regulate genes for cell cycle control, differentiation and apoptosis and III) the family related to mammalian TAFII250, the largest of the TATA binding protein-associated factors (TAFs) within the transcription factor complex TFIID. These three families are widespread in all eukaryotes, and homologous proteins are also involved in non-HAT reactions in prokaryotes and archaea. Mammals have a fourth HAT family that includes nuclear receptor coactivators such as steroid receptor coactivator (SRC-1) and ACTR, a thyroid hormone and retinoic acid coactivator that is not found in plants, fungi or lower animals. The major groups of HDACs include: I) the RPD3/HDA1 super family, II) the Silent Information Regulator 2 (SIR2) family, and III) the HD2 family. Members of RPD3/HDA1 are present in all eukaryotes with homologous proteins in bacteria and Archaea that lack histones (Leipe and Landsman, 1997). The large SIR2 family, which has no structural similarity to other HDACs is also found in all kingdoms, including bacteria (Frye, 2000). The HD2-type HDACs, on the other hand, are plant specific (Wu et al., 2000).

Since studies on interferon stimulated genes revealed that HDACs may also act as coactivators of transcription (Sakamoto et al., 2004) a more complex picture has emerged. Active genes are bound by both HATs and HDACs, whereas inactive genes are not bound by HDACs (Wang and Dymock, 2009). Inactive genes that were primed for activation by H3K4 methylation were transiently bound by both HATs and HDACs (Wang and Dymock, 2009). HDAC expression and activity are intimately associated with neoplasias (Minucci et al., 2001; Minucci and Pelicci, 2006). In solid tumors, mutations in HATs (Iyer et al., 2004) and overexpression of HDAC-

associated proteins lead to hyperactivity of HDAC. Consequently, HDAC inhibitors were established antitumor agents long before their target HDAC molecules had been identified (Minucci and Pelicci, 2006; Botrugno et al., 2009).

1.5.3.2 Histone acetylation and histone methylation during the cell cycle

Acetylation of histones is associated with 'open' chromatin configuration and gene transcription (reviewed by Eberharter and Becker, 2002). The transition from interphase to mitosis is accompanied by dramatic changes in chromosome structure and function. Mitosis involves condensation of chromosomes and the formation of repressive chromatin associated with inhibition of transcriptional activity operated by all three RNA polymerases (Gottesfeld and Forbes, 1997). Chromosome condensation is associated with substantial reduction in histone acetylation. Li et al. (2005) found that while methylation of histone H3 at lysine 4 (H3K4me2) and 9 (H3K9me2) remained unchanged during all phases of the tobacco cell cycle, the acetylation of histones H4 and H3 was dramatically reduced during mitosis in a stage-specific manner; histone H3 remained acetylated up to metaphase but was deacetylated at anaphase and telophase. Deacetylation of H4 during the interphase to metaphase transition was also observed in barley cell cultures and maize root tips (Wako et al., 2002; Wako et al., 2005; Yang et al., 2010). Preventing histone deacetylation by treatment with TSA led to accumulation of tobacco protoplasts at metaphase–anaphase. Similar effects were observed in pea cell culture (Murphy et al., 2000). Histone hypoacetylation is often required to prepare the histone template for histone methyltransferases (HMTs) that act at different lysine residues (Eberharter & Becker, 2002). Yang et al. (2010) found that H4 hyperacetylation and DNA hypomethylation led to cell cycle arrest at metaphase associated with H3K9me2 hypomethylation in maize, and that Histone H4 hyperacetylation caused by TSA correlates with a decrease in H3K9me2 and DNA methylation.

1.5.4 Interaction between histone acetylation, methylation and DNA methylation

Mutual reinforcing actions between histone acetylation, histone methylation and DNA methylation occur during plant mitosis (Yang et al., 2010). In maize cold stress can induce DNA demethylation (Steward et al., 2002). DNA methylation can control methylation of histone H3K9

and heterochromatin assembly in *Arabidopsis* (Bernatavichute et al., 2008; Tariq et al., 2003). Hyperacetylation of histones on the other hand can affect DNA methylation levels (Selker, 1998; Chiurazzi et al., 1999; Laherty et al., 1997). Thus, multiple modifications of histones and DNA may co-function in various combinations in response to environmental stresses. The correlation between different histone modifications is particularly clear for the two histone modifications involved in gene activation, histone H3 lysine 9 acetylation (H3K9ac) and H3 lysine 4 methylation (H3K4me) (Zhang et al., 2004; Nightingale et al., 2006a, 2006b). This interaction, in which the extent of histone H3 acetylation determines both the abundance and the degree of H3K4 methylation, plays a major role in the epigenetic response to histone deacetylase inhibitors (Nightingale et al., 2006b).

1.6 Histone deacetylase inhibitors

Generally speaking HDAC inhibitors (HDACis) are natural or synthetic molecules that inhibit the activities of HDACs. By interfering with the removal of acetyl groups from histones HDACis can result in hyperacetylation of histones, thereby altering the ability of DNA transcription factors to access the DNA molecules inside chromatin thus affecting gene expression (Thiagalingam et al., 2003; Dokmanovic et al., 2007; Kretsovali et al., 2012). Genome-wide analyses of gene expression changes upon HDACi administration have revealed that approximately equal numbers of genes are induced and repressed (Xu et al., 2007). The genes affected are highly dependent on the cell type (Haberland et al., 2009). HDACis have a long history of use in psychiatry and neurology as mood stabilizers and anti-epileptics. They are also studied as a treatment for diseases and cancer therapies (Hahnen et al., 2008; Haberland et al., 2009).

Mammalian studies have shown that the events that govern stem cell differentiation and somatic cell reprogramming to pluripotency are mainly epigenetic (Hochedlinger and Plath, 2009). HDACis are indeed able to potentiate both stem cell differentiation as well as reprogramming somatic cells into pluripotency. Apparently HDACis exert an antidifferentiation effect when low doses are applied on cells that have started differentiation (Lee et al., 2004; Hayashi et al., 2008),

whereas higher doses applied on undifferentiated cells provoke differentiations (McCool et al., 2007; Karantzali et al., 2008).

Since HDACis not only have a broad spectrum of epigenetic activities but also multiple effects on non-histone effector molecules, it is difficult to identify exact how these drugs work. In addition to mRNA profiling, analysis of miRNA expression changes that follow HDACi may reveal mechanisms why these reagents have so specific effects on different cell differentiation backgrounds.

1.6.1 Trichostatin A

Trichostatin A (TSA), one of the most potent HDAC inhibitors available, was initially isolated in the 1970s in a screen for antifungal compounds from the soil bacterium *Streptomyces hygroscopicus* (Tsuji et al., 1976). Soon a much wider potential of this drug became apparent. Research during the 1980s showed that TSA can arrest the mammalian cell cycle and induces differentiation of tumor cells (Yoshida et al., 1987). In following years it was found that TSA causes accumulation of highly acetylated histone molecules in mammalian cells that leads to chromatin decondensation and modulation of gene transcription/expression (Yoshida et al., 1990; Bui et al., 2010). TSA selectively inhibits the group I and II mammalian histone deacetylase (HDAC), but not group III HDACs (VanHaeke et al., 2004). Studies on tumor cells have shown that TSA inhibits the eukaryotic cell cycle during the beginning of the growth stage rendering TSA some potential as an anti-cancer drug (Drummond et al., 2005; Shankar & Srivastava, 2008). In NIH 3T3 cells, it induced reversion of oncogenic ras-transformed cells to a normal morphology (Futamura et al., 1995). TSA and other HDAC inhibitors induces differentiation in carcinoma cells, normal cells and neoplastic cells and in this process both up-regulate and down-regulates the expression of genes (Nagy et al., 1997; Leoni et al., 2002; Rahman et al., 2003).

1.6.2 Histone acetylation in plants and the effects of TSA

The complex modulation of plant histone acetylation is highlighted by the large complement of 12 HAC genes and 18 HDA genes in the *Arabidopsis* genome (Dangle et al., 2001). Overall, plants display a higher diversification of HAT and HDAC as compared to animals and fungi (for

review Pandey et al., 2002) and in the HD2 gene family possess a plant specific family of HDACs (Demetriou et al., 2009) This may reflect fundamental differences in the way chromatin controls gene expression in these three major kingdoms of eukaryotes, and suggests that plants have developed unique mechanisms of gene regulation to accommodate their responses to changing environmental factors.

Epigenetic modification contributes to tissue-specific gene expression (Kuo, 1998) and thus play an important role in the control of plant and seed development (Verbsky and Richards, 2001; Reyes et al., 2002; Reyes, 2006; Zhang and Ogas, 2009). Plant specific HDACs are involved in the regulation of embryogenesis by suppressing embryogenic properties after germination (Wu et al., 2000; Zhou et al., 2004). In *Arabidopsis*, LEAFY COTYLEDON (LEC) genes are expressed during the embryonic stage and must be repressed to allow germination (Braybrook and Harada, 2008). Tanaka et al. (2008) found that treatment of germinating *Arabidopsis* seeds with TSA not only inhibited growth but also induced the expression of embryo-specific transcription factors LEC1, ABSCISIC ACID INSENSITIVE3 (ABI3) and FUS3. Similar results were reported by Uddenberg et al. (2011) using germinating Norway spruce embryos. When Xu et al. (2005) treated *Arabidopsis* roots with TSA they noticed it affected the cellular patterning of the root epidermis by altering the expression of patterning genes leading to root hair development in non-hair positions. The same authors also observed that the effects of TSA were rapid, reversible and concentration dependent.

1.6.3 Role of histone deacetylase inhibitors in pollen embryogenesis

Induction of embryogenesis is a two stage process. In the first step cells reverse their state of differentiation and acquire pluripotency, followed by the re-entry into the cell cycle (Grafi, 2004). Given the long known potential of TSA to reverse cell identity, this drug and similar acetyl transferase inhibitors are tools in waiting to be applied in induction protocols for pollen embryogenesis. First work on this topic by Li et al., (2014) has convincingly shown that TSA may not only enhance existing protocols as in case of Brassica, but also help to break the lock on hitherto un-inducible species like *Arabidopsis*.

1.7 Stress and chromatin modifications

In order to survive under variable environmental conditions, sessile organisms like plants have developed a wide array of mechanisms to protect, adapt or minimize stress influences. In general, stress has a negative effect on normal plant development (Boyko and Kovalchuk, 2008) for which reason stress prevention and adaptation are intensively studied. Upon sensing environmental changes plant cells respond by altered gene expression. Stress-induced gene expression is often directly associated and depending upon chromatin modifications (Barrett et al., 1994; Hazzalin et al. 1996, Manzanero et al., 2002; Sokol et al., 2007). Once the stress is relieved most of these stress-induced modifications are reset to the basal level, while others remain (Chinnusamy& Zhu 2009 for review). Such epigenetic stress memory may help plants to cope with recurrent stress sconditions more effectively. The ability to rapidly and reversibly alter the epigenetic status could be a key component in the flexibility of plant responses to the environment (Luo et al., 2012).

1.8 Aims and scope of the thesis

Pollen embryogenesis requires the reprogramming of cells. This is traditionally achieved by subjecting immature highly vacuolated pollen to stress conditions. However, as a promotional factor, stress is a rather imprecise means with massive effects on the overall cellular constitution. Furthermore, not always does stress lead to the desired results and many species have resisted all efforts to induce embryogenesis. This recalcitrance is attributed to species-specific gametophytic pathways and/or species-specific epigenetic profiles. Without proper information on such factors, resetting of pollen identity remains a troublesome undertaking.

The aim of the present thesis was to fill some of the gaps in our knowledge on resetting of gametophytic pollen development to embryogenic pollen development with an approach to chromatin modifications study.The work was framed within the timeline most decisive for pollen embryogenesis, i.e. from late unicellular pollen to mature pollen just before anthesis. Using DAPI staining a histological analysis of pollen development was performed based on nuclear morphology. In the dicot model species tobacco and the monocot model species barley the

events surrounding PM I were examined in detail. Size and fluorescence measurements were undertaken to give insights into cellular differentiation events.

The morphological part is followed by extensive immunological studies on epigenetic remodeling. After a thorough investigation of gametophytic pollen development in barley and tobacco, the studies were extended to the events leading to embryogenic development in barley. Special attention has been paid to the epigenetic status of barley pollen undergoing embryogenesis after a first asymmetric PM I, to elucidate the role of the generative cell.

Inhibitor experiments, initially used to validate the dynamic redistribution of histone modifications, were extended to test the potential of the deacetylase inhibitor TSA to bolster induction efficiency of embryogenesis. For this, epigenetic profiling helped to select optimal time points for application.

The larger perspective provided by this study is to contribute knowledge allowing to improve double haploid technologies. By enhancing embryogenic pollen development more double haploid plants can be produced to accelerate the breeding of crop plants and thus improve future agricultural food production.

2 MATERIAL AND METHODS

2.1 Plant material

2.1.1 Barley (Hordeum vulgare L.) cv. Igri

Seeds from 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.

For root collection, barley seeds were surface sterilized with 20% and 10% commercial bleach for 20 min each and washed 3 times five min with water. Seed were germinated in 50 ml culture glass tubes (Sigma, Steinheim, Germany) half filled with water. Wet cotton plugs were inserted just above the waterlevel and sterile seeds were carefully inserted at the base of the cotton plug. After covering with a cap tubes were kept at room temperature on a bench. After five days when roots were on average 2-3 cm in length, the apical 1 cm was removed and processed for PEG embedding.

2.1.2 Transgenic barley expressing SV40-NLS: GFP

Transgenic barley expressing GFP under control of the viral SV40 promotor with nuclear localization signal were grown under the conditions described for non-transgenic plants. Generation of these transgenics is described in detail by Daghma et al. (2014).

2.1.3 Tobacco (Nicotiana tabacum L.) cv. Samsun-NN

Seeds were germinated in a growth chamber (20/22 °C day/night, 16 h light cycle), and cultivated in a climate controlled glasshouse (18/24 °C day/night, 16 h light cycle). Artificial illumination was provided as described for barley.

2.1.4 Tobacco (Nicotiana tabacum L.) cv. Petit Havana SR1 suspension culture

Tobacco cell suspension culture was initiated from a root-derived callus according to Maliga et al. (1973).

2.2 Nutrient media

All culture media were prepared with autoclaved macronutrients and sterile-filtered stock solutions (micronutrients, vitamins, NaFeEDTA), pH was adjusted with NaOH and HCl solutions. Before use, media were sterilized once more by filtration through a Nalgene 0.2 µm filter (Rochester, USA). Unless stated otherwise all chemicals were from Sigma Chemical Company, all solutions were prepared using purified Milli-Q water (Millipore) with a resistance of 18 MΩ.cm.

2.2.1 Barley starvation medium

Starvation Medium Barley 1 (SMB 1) used to stress immature barley pollen was according to Coronado et al. (2005) comprising 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 $ZnSO_4.7H_2O$, 0.5 mM $Na_2MoO_4.2H_2O$, 0.1 mM $CuSO_4.5H_2O$, 0.1 mM $CoCl_2.6H2O$, 1 mM KI), 4 µM benzyladenine (BA) and 2 mM morpholinoethanesulfonic acid, pH adjusted to 5.5.

2.2.2 Tobacco starvation medium

Tobacco microspore were isolated and cultured in Medium B (Kyo and Harada, 1986). The ingredients of this osmotically well balanced medium are: mannitol (0.3 M), KCL (20 mM M), $MgCl_2$ (1 mM), $CaCl_2$ (1 mM) and KHP (1 mM) with pH adjusted to 6.8.

2.2.3 Pollen embryogenesis medium

Kumlehn Barley Pollen medium (KBP, Kumlehn et al., 2006) in which immature pollen are incubated after 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, Steinheim, Germany) with pH adjusted to 5.9. KBP was used in both barley and tobacco pollen cultures.

2.2.4 Tobacco cell culture medium

Tobacco cell suspensions were grown in Linsmaier-Skoog medium (Linsmaier and Skoog, 1965) supplemented with 30 g L-1 sucrose, 2 mg L-1 α-naphtalene acetic acid (NAA) and 0.3 mg L-1 kinetin. Medium was renewed on a weekly basis and cells were grown in the dark, as described by Lippmann et al. (2009).

2.3 Isolation and culture of pollen

2.3.1 Pollen development

The different stages of pollen development were based on nuclear morphology determined after staining with 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes®, Goettingen, Germany). For this anthers at different stages of development were collected and pollen isolated on a cover slip in a droplet of extraction buffer (50 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, 10% DMSO, 0.1% Tween 20, pH 7.0) (Traas et al., 1987) supplemented with 1 mg/ml DAPI. After removing large tissue samples a coverslip was placed and sealed with fixogum. Samples were examined on the same day.

2.3.2 Staging of anthers

For staging of anthers, plant material of a wide developmental range was collected between 7 and 8 a.m. Barley anthers were harvested from the central half of a spike. In case of multiday experiments plants were kept in the greenhouse. Pollen were isolated and stained immediately after harvesting. Alternatively, whole anthers were fixated with 3% formaldehyde in 50 mM phosphate buffer pH 7.0 and kept in fixative until used for pollen isolation. Control experiments showed that storage in formaldehyde had no measurable effect on pollen nuclear morphology. For each anther the developmental status of the first 250 normal looking (i.e. nuclei containing)

pollen was analysed. By assigning ascending values of 1 to 6 to the individual developmental stages, the stage of an anther was determined as the sum of these values divided by 250.

In case of barley pollen suspensions, when binucleate pollen were present, the first 20 pollen in which the two nuclei were of dissimilar size, next to the first 20 pollen in which the nuclei were of similar size, were recorded by LSM 510 using a 40x water objective with zoom 3.0. For comparative analysis all recordings were made with similar settings for pinhole, gain and digital offset. Under- and over-exposure were avoided, optical sections were made at the level of the widest diameter of a nucleus. Surface area and relative fluorescence intensity of single optical sections was determined with ZEN 2009 software (Carl Zeiss, Jena Gemany). If the surface area of the nuclei of a binucleate pollen did not differ by more than 10% these nuclei were considered equal sized.

2.3.3 Isolation of immature pollen, culturing and induction of pollen embryogenesis

2.3.3.1 Barley

Highly vacuolated uninucleate pollen is most amenable to induction of embryogenesis (Dhagma, 2011). This stage occurs immediately before PM I. Phenotypic observations of tillers have shown that when awns of cv. 'Igri' emerge for 3-5 mm beyond the flag leafs (Fig. 3a), the corresponding spike is highly enriched in pollen of this particular stage (Dhagma, 2011). After this, correlation was confirmed for the present plant material. Spikes were harvested based on this criterium and anthers collected from the central half of a spike.

Isolation and culturing of immature pollen was based on Kumlehn et al. (2006) with minor modifications. After harvesting tillers were surface sterilized by spraying with 70% ethanol for 15 sec. Spikes were removed from the flag leaf sheath and awns were cut off and subsequently dried on sterilized tissue paper. All further steps were carried out under aseptic conditions in a laminar flow. Anthers were collected from a total of 8 to 10 spikes and distributed in 35 mm Petridishes at a density of 50-60 anthers/dish (Fig. 3b). After addition of 2 ml of 0.4 M mannitol, dishes were incubated in the dark at 25 °C for 24 h before transfer to 4 °C for another 24 h. On the third day anthers were collected and transferred into a 25 ml tube (Sarstedt, Nümbrecht, Germany) containing 20 ml 0.4 M mannitol and two magnetic stirring bars (Fig. 3c). The tube

was placed three times for 3 min on a magnetic stirrer running at maximum speed allowing the irregular stirring of the magnetic bars to thresh immature pollen from the anthers (Fig 3d). Afterwards, the suspension (Fig 3e) was filtered through a 100 µm nylon mesh (Fig. f). The collected solution was transferred into 50 ml Falcon tubes and centrifuged at 4 °C at 100 *g* in a swing-out rotor for 10 min. Excess liquid was carefully pipetted off and the pellet re-suspended in 2 ml ice-cold 0.55 M maltose. The suspension was transferred into a 15 ml Falcon tube and carefully overlaid with 3 ml solution of 0.4 M ice-cold mannitol (Fig. 3g). Density gradient centrifugation at 4 °C at 100 *g* in a swing-out rotor for 10 min resulted in a pellet of debris and dead microspores while the highly vacuolated immature pollen accumulated at the maltose-mannitol interphase (Fig. 3h). The population of highly vacuolated pollen was collected and transferred into a 50 ml tube. Final volume was adjusted to 20 ml with 0.4 M mannitol. Small aliquots were taken to assess total number of pollen using a haemocytometer (Paul Marienfeld,

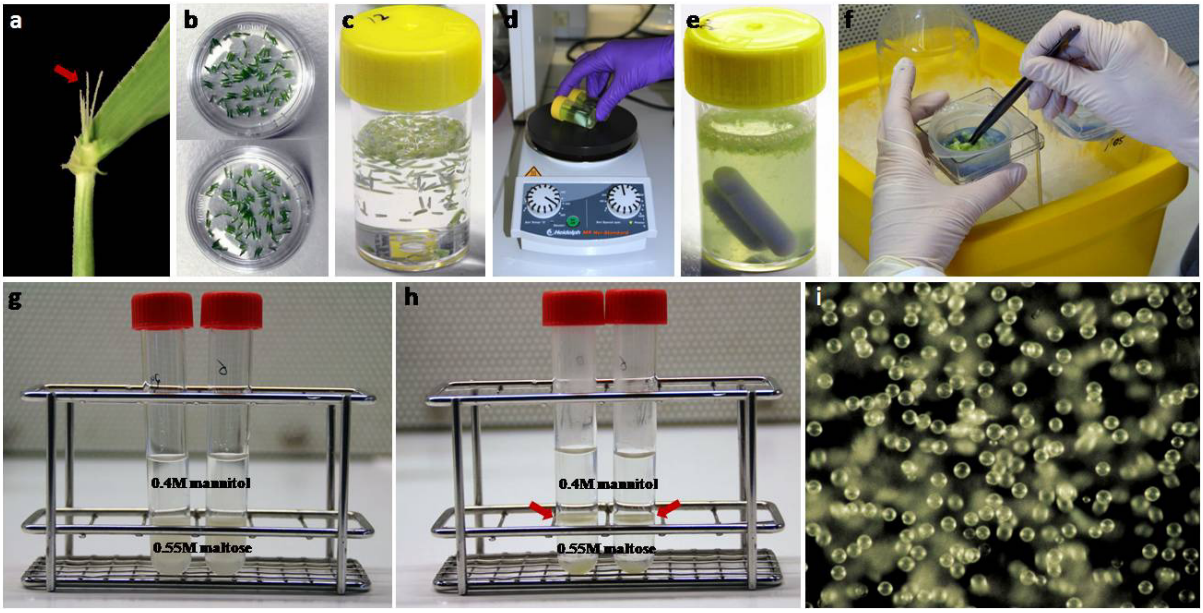


Fig. 3: Isolation and culture of immature pollen. (a) Tiller with spike at proper developmental stage (arrow pointing at awns emerging beyond the flag leaf sheath. (b) Isolated anthers in 35 mm Petridish with 0.4 M mannitol. (c) 25 ml tube with two magnetic stirrer bars before blending. (d) Manual blending on magnetic stirrer. (e) Anther suspension after blending. (f) Removal of large debris by filtering over 100 µm mesh nylon filter. (g) Pollen suspension in 0.55 M maltose solution overlaid with 0.4 M mannitol before centrifugation. (h) And after centrifugation showing accumulation of highly vacuolated immature pollen at the interface (arrows). (i) Purified highly vacuolated immature pollen in starvation medium. (image i reprinted with permission from Daghma (2011)).

Lauda-Königshofen, Germany). After centrifugation, excess medium was discarded and population density adjusted to 2x105 pollen/ml by addition of SMB1 (Fig. 3i). Incubation in SMB1 medium was carried out for 24 h at 25 ºC in the dark. After this time SMB1 was replaced with the same amount of KBP induction medium (Daghma, 2011). Since the heavily vacuolated micropspores do not settle, SMB1 was carefully pipetted off using a cut-off 1 ml blue tip covered with 30 µm nylon filter. Care was taken to avoid letting cells dry out. Incubation was continued at 25 ºC in the dark without further changes.

2.3.3.2 Tobacco

To obtain uninucleate and early binucleate pollen flower buds measuring 11-13 mm from base to tip were selected. Flower buds were surface sterilized by suspension into 70% ethanol for 30 sec after which they were transferred to a sterile laminar flow and dried on filter paper. All subsequent steps were carried out under aseptic conditions.

Flower buds were cut open base to tip with a sharp tweezer and anthers gently squeezed out. Anthers were transferred to 90 mm Petri dishes (25 anthers/dish) containing 5 ml of medium B. To wash away possible trace amounts of ethanol the Petri dishes were gently agitated for about one min. Following anthers were transferred into a Waring blender (Eberbach, Ann Arbor, MI, USA) pre-cooled to 4 °C in a refrigerator containing 20 ml fresh medium B. Anthers were homogenized by blending twice for 30 sec at 'high' speed. After the homogenate was poured through a 40 µm nylon filter (Wilson, Nottingham, UK) the Waring blender was rinsed twice with 10 ml medium B which was also poured through the 40 µm nylon filter. Pollen suspensions were transferred into a 50 ml tube and centrifuged for 3 min at 200 *g* and 22 °C. Since pollen sediment faster than anther debris, centrifugation results in two distinct pellet layers: a basal whitish pellet on top of which a clear greenish layer is visible. The supernatant and the top green layer which contains anther wall debris, are removed. The whitish pellet enriched in microspores was resuspended in 5 ml medium B and centrifuged again. This procedure has to repeat several times until there is no greenish pellet layer present anymore. Final volume was increased to 20 ml with medium B. A haemocytometer was used to determine total pollen number (see 2.3.3.1). After centrifugation, supernatant was discarded and population density of pollen adjusted to $5x10^5$ per ml with medium B. Aliquotes of 1 ml pollen suspension were distributed in 35mm

Petri dishes. After incubation for 7 days at 32 ºC in the dark, the medium B was pipetted off through a cut-off 1ml blue pipette tip covered with 30 µm nylon filter and replaced by KBP medium (Daghma, 2011).

Pollen cultures were incubated at 25 ºC in the dark. To facilitate induction each Petri dish culture was supplemented with five longitudinally cut wheat (*Triticum aestivum* L.) pistils as a feeder. To this purpose ovaries were collected from tillers in which spikes were 3-4 cm exposed beyond the flag leaf sheath. After spikes had been surface sterilized with 70% ethanol in a laminar flow, ovaries were isolated, cut longitudinal and incubated in KBP medium for 2 to 3 days at 10 ^{o}C in dark before transfer to the tobacco cultures.

2.4 Chemical fixation of anther, pollen and root

For immunolabeling plant material was fixed with 3% formaldehyde in microtubule stabilizing buffer (MTSB) (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 0.4% PEG 6000, 0.5% Triton X100, pH 6.9). The normal fixation protocol was 1-2 h at RT for cell and pollen cultures and overnight in a fridge at 8 °C for larger tissues. Before fixation pollen were concentrated in 2 ml Eppendorf tubes by careful pipetting off culture medium through a 30 µm nylon filter since centrifugation was found to result in a significant portion of damaged pollen. In case of anthers, the tips of both ends were cut to assure proper infiltration of the fixative.

2.5 Resin embedding of anther, pollen and root

2.5.1 Technovit 7100

The protocol for embedding in technovit 7100 resin (Heraeus Kulzer GmbH, Wehrheim, Germany) is shown in table 2.

The resin is based on HEMA (2-Hydroxyethylmethacrylate) and is widely used in histological and immunological studies. It has a low viscosity, low toxicity and is easy to polymerize at room temperature. After addition of hardener II Technovit 7100 polymerizes within min. The blocks were allowed to harden overnight before trimming and sectioning.

Table 2: Protocol for embedding in Technovit 7100

			Temp	Time
Fixation:	3.0% FA in MTSB pH 6.9		RT	2h*
Washing:	MTSB		RT	15’
	Aqua dest.		RT	15’
Dehydration:	30%	Ethanol	RT	15’
	50%	Ethanol	RT	15’
	70%	Ethanol	RT	20’
	90%	Ethanol	RT	20’
	100%	Ethanol	RT	30’
	100%	Ethanol	RT	30’
Infiltration:	50%	Technovit 7100	RT	4h
	100 %	Technovit 7100 + Hardner I	RT	ON
Polymerisation:	100 %	Technovit 7100 + Hardner I	RT	ON
Embedding mould:	LR-White capsules			
RT: Room Temrerature; ON: Over Night			*alternative: 8°C for 16 h	

2.5.2 PEG 1500

Embedding in PEG 1500 resin (Sigma, Steinheim, Germany) was performed according to Houben et al. (2011) with slight modification as shown in table 3.

To avoid premature solidification of the PEG solutions, all solutions and infiltration steps were carried out in an oven set at the appropriate temperatures. Embedding and positioning of probes was performed on a bench using pre-warmed equipment stored on heating plates. Probes were placed in pre-heated embedding moulds on a heating plate set at 52 °C. After positioning the moulds were transferred to the bench and the PEG allowed to solidify at RT. Evenly solidified PEG 1500 is weakly transparent and garantees optimal sectioning quality. Poor solidification is recognizable by large whitish intransparent areas. If these were present, the process of liquification and solification was repeated until the blocks were of good quality. After removal from the moulds, PEG blocks (Fig. 4a) were kept at RT in a cartboard box. Storage in a fridge is not advisable as in a cold and moist environment PEG blocks tend to “sweat”.

Table 3: Protocol for embedding in PEG 1500

			Temp	Time
Fixation:	3.0% FA in MTSB pH 6.9		RT	2h*
Washing:	MTSB		RT	15’
	Aqua dest.		RT	15’
Dehydration:	30%	Ethanol	RT	15’
	50%	Ethanol	RT	15’
	70%	Ethanol	RT	20’
	90%	Ethanol	RT	20’
	100%	Ethanol	RT	30’
	100%	Ethanol	RT	30’
Infiltration:	25%	PEG 1500	46 °C	60’
	50%	PEG 1500	48 °C	60’
	75%	PEG 1500	50 °C	90’
	100%	PEG 1500	52 °C	120’
Embedding:	Flat mould		RT	30’
RT: Room Temrerature; ON: Over Night			*alternative: 8 °C for 16 h	

2.5.2.1 Resin embedding of embryogenic pollen

For immunolabeling studies fixated pollen was embedded in the highest possible concentration. Provided that the 100% PEG 1500 can be kept from solidifying, it is possible to centrifuge pollen into a pellet. However, because of the gelatinous nature of PEG 1500, such blocks cannot be removed from a mould without seriously damaging this pellet. An alternative was mixing a pollen pellet with warm 3% agarose. This way agarose blocks with high concentrations of pollen could be obtained and embedded in PEG. However, after section and upon removal of PEG pollen the remaining thin sheet of agarose with entrapped pollen proved difficult to handle. The agarose sheets were often folded, did not stick well to slides and entrapped pollen was often washed out during labeling steps. The problems were solved when pelleted pollen (100 *g*, 5 min) were mixed 1:1 with 3% aqueous alginate (Roth, Karlsruhe, Germany). This suspension was pipetted with a 3 ml plastic pipet from a height of 20 cm into a beaker with 500 ml of 0.05 M $CaCl_2$ (cross linking solution) causing the alginate to solidify. The resulting alginate beads were found to effectively entrap pollen and remain stable during all subsequent embedding steps.

2.6 Trimming and sectioning of resin blocks

2.6.1 Technovit 7100

After polymerization and hardening, blocks were trimmed with a Leica EM TRIM (Leica Microsystems, Bensheim, Germany) device. Sides were trimmed at an angle of 45° eventually resulting in a trapezoid surface area. Semithin sections of 1-10 µm were cut on a Leica Ultracut ultramicrotome (Leica Microsystems, Bensheim, Germany) using a 45° histo diamond knife (Diatome, Weinheim, Switzerland) for sections up to 5 µm and a glass knife for 10 µm sections. Sections were collected on poly-L-lysine coated slides (Thermo Scientific, Braunschweig, Germany) and incubated for 5 min on a heating plate set at 80 °C.

2.6.2 PEG 1500

PEG blocks containing embedded material were cut to shape with a razor blade. Trimmed blocks were mounted on a PEG 1500 filled specimen frame using a hot spatula to melt the resin. PEG was allowed to solidify for 30 min at RT (Fig. 4a), after which the specimen frame with block was attached into the specimen holder (Fig. 4b) of a LEICA RM2165 microtome (Fig. 4c) (Leica, Wetzlar, Germany). Set at continuous and uniform motion, semi-thin sections with a thickness of 12 µm were prepared using Disposable Microtome Blades 819 (Leica, Wetzlar, Germany). Under ideal conditions long ribbons of sections were obtained and stored in flat cardboard boxes until use. Unfortunately, sections more often would roll up. However, when placed on a droplet of buffer, these PEG rolls completely unrolled. After having confirmed that the sectioned tissues showed no obvious loss in quality, rolled sections were collected and stored in 1.5 ml Eppendorf tubes until use.

2.7 Immunolabeling of anther, pollen and root

After it was discovcered that Technovit 7100 sections did not stick very well to glass surfaces while the resin proved difficult to remove, this embedding method was considered unsuitable for

labeling of thickly sectioned pollen. From here on immunolabeling was carried out on PEG sections only.

2.7.1 Immobilization of sectioned material

Houben et al. (2011) described the attachment of section-ribbons on poly-L-lysine coated glass slides which were then submerged in PBS, under an angle, dissolving the PEG and simultaneously making the sections stick unto the coated glass surface. However, even under optimal conditions the vast majority of material is lost, the absence of boundaries hinders immunolabeling, and rolled sections cannot be used this way. Problems were overcome with the introduction of Teflon-coated 8-well slides (Fig. 4d) (Thermo Scientific, Portsmouth, Va., USA). Large (50 µl) droplets of liquid and sections floating within, are contained by the Teflon. All solutions were carefully removed by 20 µl syringe allowing labeling steps to be carried out on large free moving sections.

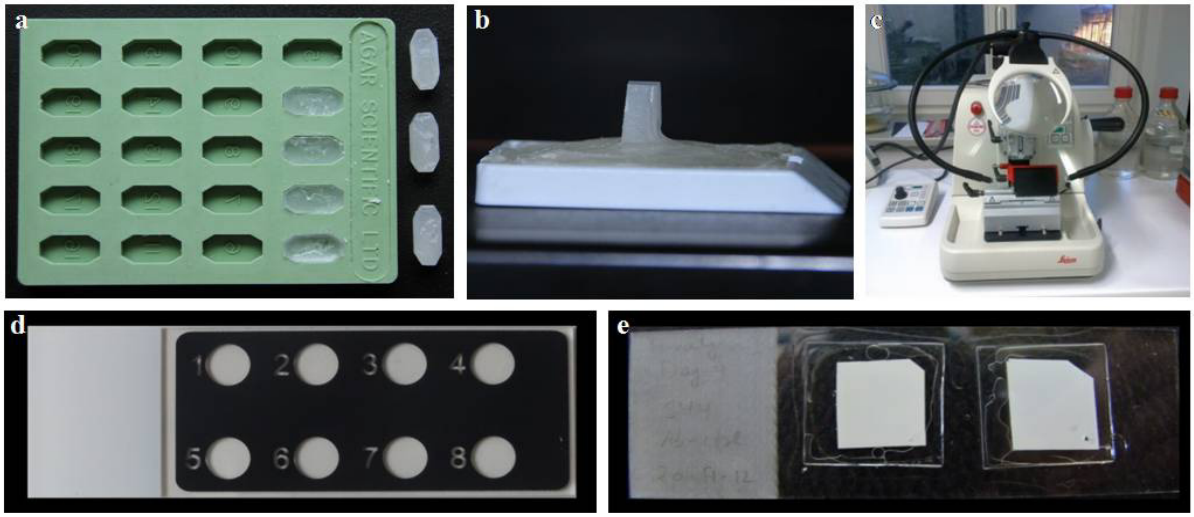


Fig. 4: Embedding, sectioning and immunostaining of plant tissue. (a) PEG blocks containing embedded tissues. (b) PEG block (arrow) immobilized on specimen frame. (c) Leica RM2165 microtome. (d) Labeling on 8-well poly L-lysin coated slide. (e) Labeling on nitrocellulose membrane pads.

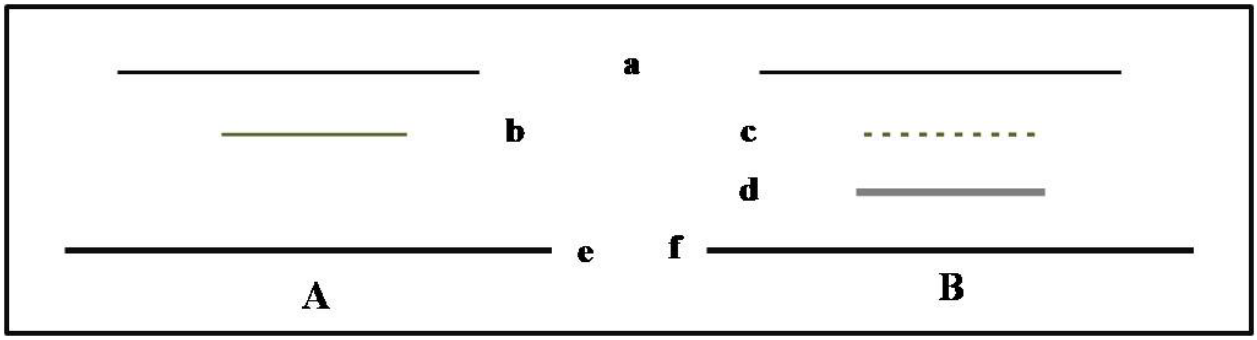


Fig. 5: Schematic diagram of immunolabeling setup. (A) Immunolabeling using 8 welled poly L-lysin coated slide for large samples. (B) Immunolabeling using plain slide with nitorocellulose membrane for small samples. (a) Cover slip. (b) Large sample. (c) Small sample. (d) Nitrocellulose membrane. (e) 8 welled slide poly L-lysin coated slide. (f) Plain slide.

In case of sectioned pollen, however, immobilization of sections prior to labeling was essential. Attempt to immobilize pollen sections on poly-L-lysine coated surfaces by centrifugation or by drying were unsuccessful. Overall, poly-L-lysine slides, whether purchased or self-made, made for poor immobilization surfaces. Eventually sectioned material was placed on a droplet of 50 mM phosphate buffer pH 7.0 pipetted onto 6 x 6 mm pads of 0.45 µm nitrocellulose membrane (NC) (Fig. 4e) (Serva, Heidelberg, Germany) positioned on top of a 50 mm vacuum filter with pore size of 0.45 µm (Nalgene, New York, USA). Under mild vacuum filtration more phosphate buffer pH 7.0 was pipetted onto the sections to dissolve the PEG and alginate and simultaneously immobilize the individual pollen sections onto the nitrocellulose membrane. Following, pads of nitrocellulose paper were placed on a piece of parafilm and processed for immunolabeling as described below (2.7.2).

2.7.2 Immunolabeling on 8-well slides

Immunolabeling was performed according to Houben et al. (2011) using 8-well poly-L lysine coated slides kept in a wet chamber. Positions 3 and 7 were used for antibody labelling while positions 2 and 6 served as a control (incubation without primary antibody). Polyethylene glycol sections were placed on a droplet of 50 mM phosphate buffer (PB), pH 7.0 to dissolve the embedding medium and allow the sectioned material to settle on the slide. Excess liquid was

carefully taken off using a 20 µl syringe. After washing with PB, sections were treated with freshly prepared 0.1 M NH_4Cl in PB for 5 min to quench eventally remaining free aldehyde residues. Following sections were washed with washing buffer (0.1% BSA and 0.05% Tween 20 in PB) and blocked with 5% BSA in PB for 30 min at room temperature before incubation with the following primary anti rabbit antibodies:

- anti-histone H3K4me2 (Upstate, Cat. No. 07-030; dilution 1: 200)
- anti-histone H3K9ac (Upstate, Cat. No. 06-942; dilution 1: 200)
- anti-histone H3K9me2 (Upstate, Cat. No. 07-441; dilution 1: 200)
- anti-histone H3K27me3 (Upstate, Cat. No. 07-449; dilution 1: 200)
- anti-RNA polymerase II CDC phospho Ser5 (Active Motif, Cat. No. 39233; dilution 1: 200)
- anti-rice CENH3 (Pineda antibody service, Berlin, Germany; dilution 1: 500)

Per well 20 µl of primary antibody was added and slides were incubated overnight at 8 °C. After probes were transferred to RT they were washed three times for 5 min with washing buffer and incubated with the corresponding secondary antibody goat anti-rabbit Alexa fluor 488 (MoBiTec, Cat. No. A-11008; dilution 1: 200) for 60 min at 37 °C. After three final 5 min washes with washing buffer, the sections were mounted in antifade (70% (v/v) glycerol, 30 mM Tris pH 9.0, 2.5% (w/v) n-propylgallate) supplemented with 1 µg/ml DAPI and a coverslip placed. Normally slides were examined immediately yet they could also be stored at 8 °C for several months without loss in staining quality. Every labeling was repeated at least 3 times on sections derived from a similar number of independent embedding events. Only nuclei which DAPI staining showed to be intact, were used for evaluation. Before being considered valid, specific labelling patterns had to be regularly present in all experiments analysed, and not in the controls.

2.7.3 Immunolabeling on nitrocellulose pads

Labeling for sections attached to nitrocellulose pads was carried out on parafilm involving the same steps as described above. However, after labelling the results had to be examined within

one week as reactions with the nitrocellulose underground could strongly diminished contrast between background and specific fluorescence after that time.

2.7.4 Immunolabeling for 5-methylcytidine

5-methylcytidine immunolabeling was performed according to Castiglione et al. (2002). Chromosomal DNA was denatured by immersing NC membranes carrying sectioned microspores for 2 min in 70% (v/v) formamide (in 2x saline-sodium citrate (SSC) buffer) at 70 °C. Membranes were dehydrated in ice cold 70% and 100% ethanol for 5 min each, and air dried. After blocking at 37 °C (5% (w/v) BSA, 0.1% Triton X-100 in PBS), membranes were incubated with 20 µl mouse monoclonal 5-methylcytidine antibody (Eurogentec, BI-MECY-0100, diluted 1:100 in PBS containing 1% BSA) for overnight at 8 °C. After three washings with PBS for 5 min, membranes were incubated with 20 µL secondary goat anti-mouse Alexa fluor 488 (MoBiTec, Cat. No. A-11001; diluted 1:200 in PBS with 1% BSA) for 1 h at 37 °C in the dark. After two final washing with PBS for 10 min NC membranes were placed on slides counterstained with 1 µl/ml DAPI in antifade solution and covered with cover slips.

2.7.5 Fluorescence microscopy

Immunofluorescence was analyzed in a LSM 510 META confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). DAPI was visualized with a 364 nm laser line of an Enterprise II UV laser in combination with a 385 nm longpass filter. Alexa 488 was detected with a 488 nm laser line from an argon-krypton laser in combination with a 505–550 nm bandpass filter. Z-stacks and photospectrometric unmixing with the Lambda detector were employed when necessary. Recordings of DAPI stained pollen were occasionally also made with an Andor spinning disc microscope (Andor technologies, Belfast, Ireland), and the LSM 780 confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.8 Inhibitor experiments

2.8.1 Effects of TSA and 5-AZA on chromatin modifications

Dynamics of histone modifications during induction of barley pollen embryogenesis were investigated with 5-azacytidine (5-AZA) (Sigma, Steinheim, Germany), an analog of cytosine which inhibits DNA-methylation, and Trichostatin A (TSA) (Sigma, Steinheim, Germany) which inhibits histone deacetylases. To this purpose highly vacuolated immature pollen isolated after stress treatment (2.3.3.1.) were resuspended in SMB1 supplemented with 40 µM 5-AZA (stock 10 mM in DMSO) or with 0.5 µM -1.6 µM TSA (stock 3.3 mM in DMSO). Samples were initially collected at 24 h intervals and prepared for immunolabeling as described before. However, after it had been established by fluorescein diacetate (FDA) staining (Molecular Probes, USA; final concentration 4 µM; stock: 12 mM in acetone) that viability of pollen cultures rapidly decline after 48 h or more in the presence of either 5-AZA or TSA, maximum exposure time to these inhibitors was restricted to 24 h.

2.8.2 Effect of TSA on embryogenic induction efficiency

To investigate the potential of TSA in optimizing induction of embryogenesis in barley pollen, immature pollen at different stages of the induction process were exposed to 0.5 µM TSA in DMSO for 24 h after which the pollen were transferred to medium without TSA. For this experiment the pollen induction protocol according to Daghma (2011) (see 2.3.3.1.) was replaced by the protocol described by Kumlehn and Lörz (1999) and Coronado et al. (2005). In the latter protocol anther treatment in mannitol is followed by two days of starvation stress treatment of isolated microspores in SMB1 before transfer into KBP medium. Incubation was carried out at 25 °C in the dark. Treatments with TSA were initiated at t = 0 h (directly after harvesting of anthers), t = 48 h (directly after isolation and transfer of pollen into SMB1), t = 72 h (after one day in SMB1) and at t = 96 h (directly after transfer of pollen into KBP medium). After this pollen culture was continued for a total of 8 weeks at 25 °C. For controls pollen were exposed to DMSO without TSA. To allow exposure to TSA when pollen were still confined within anthers anther tips were removed and the anthers themselves cut into three parts. Over a time-span of 10

days 20 µl aliquots were taken at 24 h intervals. Viability was checked by staining with FDA, developmental status was analysed by staining with DAPI in extraction buffer.

2.9 Setup of live cell imaging experiments

Live-cell imaging chambers were based on 2-wells Chambered Coverglasses (Thermo Scientific-Cat. No. 155380, USA). Construction of these chambers, loading with immature highly vacuolated barley pollen and maintenance of culture conditions were as described in detail by Daghma (2011) and Daghma et al. (2012). Induced immature tobacco culture samples (1 ml) were pipetted into the well of an unmodified 2-wells chambered coverglass In contrast to the immature barley pollen, immature tobacco pollen are not highly vacuolated and lack the tendancy to float thus easily settle in the culture medium used. Once settled, tobacco pollen did not require immobilization to perform time-lapse recording.

Time-lapse experiments were carried out in a darkened room which was kept close to 25 °C. Time-lapse recordings of embryogenic transgenic barley pollen with 3 min intervals was initially conducted on a Zeiss LSM 510 META microscope using a 20x objective, zoom factor 2, 1024 x 1024 pixel image size and ZEN 2009 software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Owing to the inavailability of GFP expressing tobacco plants in our laboratories, time-lapse recordings of non-transgenic embryogenic tobacco pollen cultures only involved bright-field recordings using a 633 nm laser line in combination with a 20x objective, zoom factor 2, and 1024 x 1024 pixel image size. Z-stacks with a step size of 3 µm were aquired every three min.

2.9.1 Live cell imaging of embryogenic pollen

Using time-lapse recording of embryogenic transgenic barley pollen Daghma et al. (2014) were able to record spontaneous genome duplication. One of the initial aims of the present study was to use fluorescence to measure the ploidy of individual nuclei and the effect of culture conditions on diploidisation events. The original plan envisaged the use of transgenic barley with a stable expression of CENH3: GFP. As these were not available at that time, it was decided to perform

the experiments on the transgenic SV40-NLS: GFP lines already used by Daghma (2011). After confirming these plants still expressed GFP (Fig. 6) pollen embryogenesis was induced and pollen prepared for time-lapse imaging.

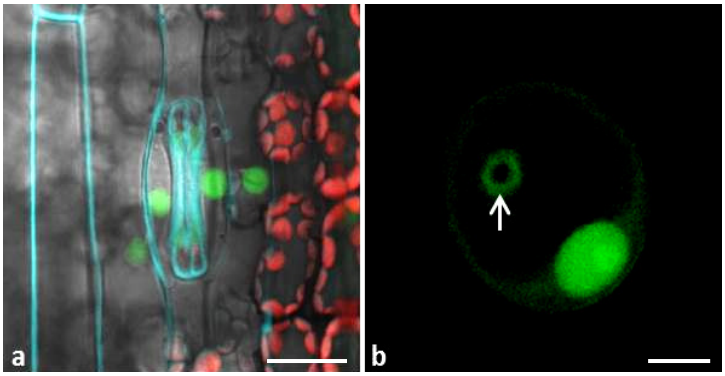


Fig. 6: Fluorescence microscopy of transgenic barley expressing *SV40-NLS: GFP*. (a) Leaf tissue with GFP signals in the nuclei of stomatal and epidermis cells, cell wall autofluorescence in cyan, chlorophyll autofluorescence in red. (b) Isolated unicellular pollen with GFP in nucleus, note the relative clear autofluorescence of the pollen wall (arrow). Bar = 10 µm.

Widefield fluorescence microscopy showed GFP fluorescence to be remarkably weak. This was confirmed by the settings needed to get an image in the LSM 510. Intensity for the 488 nm laser line had to be increased to >10%, the pinhole opened > 400 µm and the detector gain rose to near 700. In comparison, for his recordings Daghma mostly used 6% laser intensity together with pinhole and detector gain settings only 1/3 of those used here (Daghma personal communication). This shows that with successive generations, GFP expression in the transgenic plants/pollen can decrease by more than 90%. The settings needed for vizualisation of GFP in the LSM 510 made bleaching a serious problem. As in subsequent cultures of transgenic pollen fluorescence intensity waned even further, time-lapse recordings eventually were made on an Andor spinning disc (Andor Technology, Belfast, Ireland) using a 20x objective. Z-stacks were made with 0.75 µm spatial intervals (Nyquist) and a 488 nm laser line in combination with 505-530 bandpass filter. Settings for the 488 nm laser line intensity and recording speed were adjusted accordingly and Z-stacks made with 3 min intervals. Under these conditions it was possible to follow embryogenic pollen for up to 20 days. However, as a consequence of the low specific GFP fluorescence, only cells with the strongest GFP expression could be followed and autofluorescence could not be avoided.

Also in tobacco there were no plants available with a stable expression of CENH3:GFP or plain nuclear GFP. Time-lapse experiments were performed on the LSM 510 using non-transgenic embryogenic tobacco pollen cultures. For the brightfield recordings a 633 nm laser line was used in combination with a 20x objective, zoom factor 2, 1024 x 1024 pixel image size. These pilot experiments showed two things: culture conditions had to be improved since most pollen died within three days. More importantly was, however, that due to the dense granular cytoplasm nuclei were very hard to identify and their movements in time could not be followed.

2.9.2 Alternative nuclear stains for live cell imaging

Using tobacco cell cultures several water soluble fluorescent dyes were tested for their potential to stain nuclei without affecting cell viability. These dyes included DAPI (0.1 µg/ml), Hoechst 33342 (Molecular probes, USA; final: 1 µg/ml, stock: 10 mg/ml), Hoechst 33258 (Molecular Probes, USA; final: 1 µg/ml, stock: 10 mg/ml) and Draq 5 (Biostatus Limited, United Kingdom; final: 0.5 µM, stock: 0.5 mM). Hoechst stock was prepared in DMSO.

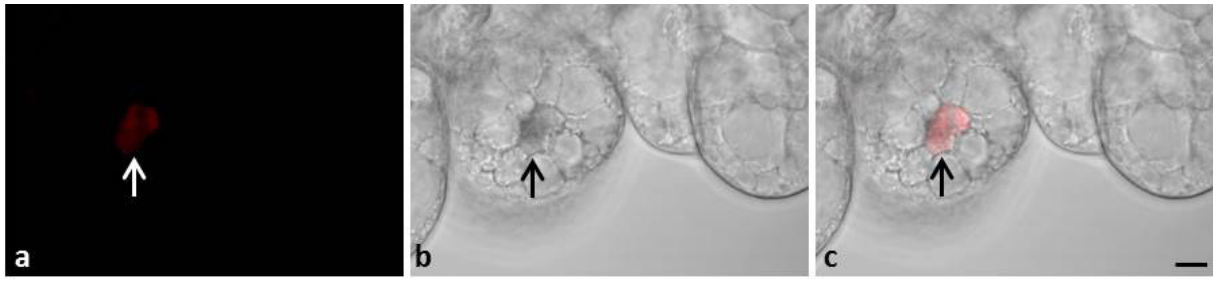


Fig. 7: Staining of tobacco cell culture with DRAQ 5. (a) Staining of nuclei by draq 5. (b) Cytoplasm has stopped streaming and has acquired a foamy appearance. (c) Merged image shows staining of nuclei in the dead cell.

For all dyes tested it appeared that nuclear staining was only observed in cells that if not yet dead, did not show any cytoplasmic movements anymore, i.e. were dying (Fig. 7a-c). Without proper transgenic lines and after alternatives proved ineffective time-lapse recording was not further pursued.

3 RESULTS

3.1 Nuclear morphology

Pollen embryogenesis forms a basis for the production of haploid and double haploid plants. However, not all species of plants are equally suited for this process. Barley pollen can be induced but the efficiency is rather low. The answer to this problem may be found in the pollen development itself. For this the dynamics of nuclear behavior during barley and tobacco pollen development were characterized using DAPI staining.

3.1.1 Barley

3.1.1.1 Nuclear dynamics during pollen development

Nuclear dynamics were followed from the stages of highly vacuolated immature pollen immediately before first pollen mitosis until the fully mature tricellular pollen at the time of anthesis. Selection of the appropriate spikes was roughly based on the length of awns visible beyond the flag leaf (Fig. 3a). Highly vacuolated pollen was characterized by a large roundish nucleus (Fig. 8a). After condensation of the chromatin the following mitotic figures were often observed opposite to the pore of the pollen (Fig. 8b, c). The sister nuclei that arise after first mitosis initially were heavily condensed and of similar size (Fig. 8d). The orientation of the chromosomes during anaphase and the localization of early sister nuclei was predominantly side by side and close to the pollen wall (Fig. 8c-e). This indicated a more or less parallel orientation of the axis of the mitotic spindle to the pollen wall during pollen PM I. Throughout the initial stages of decondensation the two nuclei often remained in close proximity and of similar size (Fig. 8e, f). Clear differences in size, which allow the distinction of vegetative (large) and generative (small) nucleus, were accompanied by a spatial separation of the nuclei (Fig. 8g). Before second pollen mitosis (PM II) the fluorescence intensity of the smaller generative nucleus became distinctly stronger in comparison to the larger vegetative nucleus (Fig. 8h, i). PM II yields two small sperm cells whose nuclei initially were roundish but eventually become spindle-shaped (Fig. 8j, k).

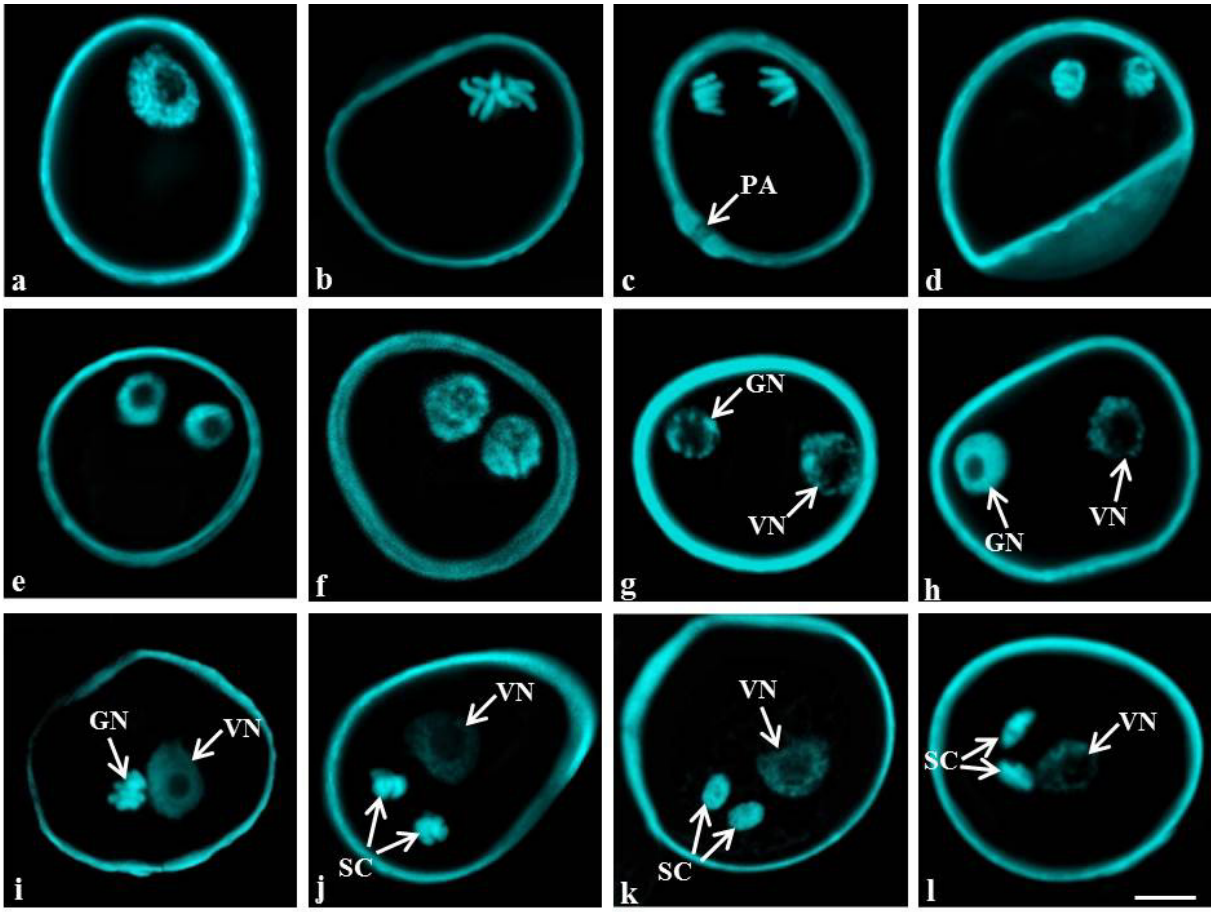
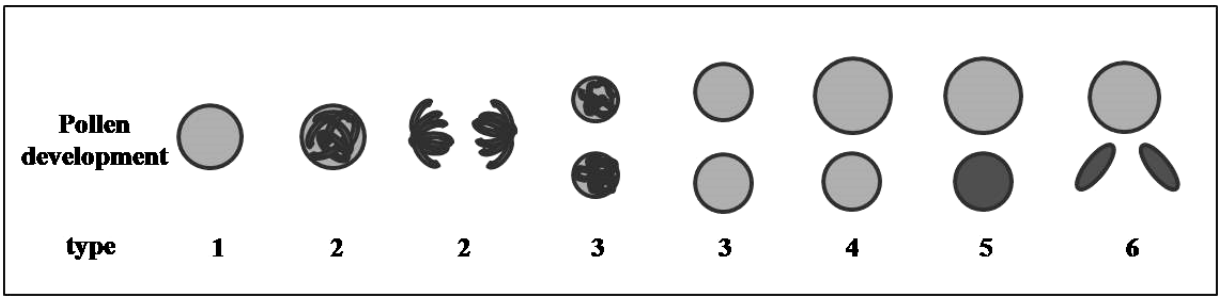


Fig. 8: Pollen development in barley from late unicellular to mature tricellular stage. (a) Unicellular pollen. (b, c) PM I opposite the pollen pore (arrow in c). (d-f) Early bicellular pollen with nuclei of similar size and similar fluorescence intensity. (g) Median bicellular pollen with different size nuclei but similar fluorescence intensity. (h, i) Late bicellular pollen with nuclei of different size and different fluorescence intensity. (j) PM II. (k, l) Early and late tricellular pollen. VN: vegetative nucleus, GN: generative nucleus, SC: sperm cells. Bar = 10 µm.

3.1.1.2 Association of pollen and anther developmental stages

DAPI studies revealed that the pollen of an individual anther can display a range of developmental stages and the order of development as proposed under 3.1.1.1. An alternative method was tried out based on population dynamics. For this, the chromatin pattern after DAPI staining was divided into six easily recognizable types, each of which was allocated a distinct value based on the presumed stage of development (Fig. 9).



Type 1	**Unicelllular** (interphase nucleus of immature unicellular pollen)
Type 2	**Pollen Mitosis I** (from prophase to telophase)
Type 3	**Early bicellular** (nuclei with similar sized)
Type 4	**Median bicellular** (nuclei with dissimilar size and similar fluorescence intensity)
Type 5	**Late bicellular** (nuclei with dissimilar size and fluorescence intensity)
Type 6	**Tricellular**

Fig. 9: Schematic diagram of proposed types of pollen development in barley used for anther staging calculations. Weak intensity of DAPI fluorescence shown in pale grey, strong fluorescence shown in dark grey.

The Type of the first 250 pollen of a single anther was determined and used to calculate a stage for this anther based on the formula:

$$\text{Anther stage} = \frac{\sum 250 \text{ pollen x type}}{250}$$

This way, anther stage can thus vary between 1 (100% unicellular) and 6 (100% tricellular). The types proposed favor the important events before and after PM I. Features of PM II, which when present were at low frequencies, were not separated in detail. A total of 80 anthers representing the transition of immature unicellular to mature tricellular pollen were analysed this way.

The graphic evaluation of anther staging based on pollen development in Fig. 10, have shown a gradual succession of different pollen conditions thus confirming the asynchronous ripening process of barley pollen development. This was best exemplified by the mitotic figures (metaphase to late anaphase) of PM I (Fig. 8b, c). Although they never accounted for more than 15% of a population, they remained present at low frequencies up to very late anther stages. The graphs also seemed to confirm that PM I initially results in two equal sized sister nuclei (type 3 pollen). Between anther stage 2 and 3 this type 3 pollen can make up to 25% of total pollen after

which their number rapidly declines. Type 4 pollen also appeared early yet in contrast to type 3 pollen, they were most numerous between anther stage 3 and 4 when they account for up to 70% of all pollen. Substantial numbers of type 5 pollen did not appear until after anther stage 3. Eventually type 5 pollen, showed no sign of imminent PM II, can make up 80-90% of all pollen before being succeeded by near to pure contents of tri-cellular pollen (type 6) (Fig. 10).

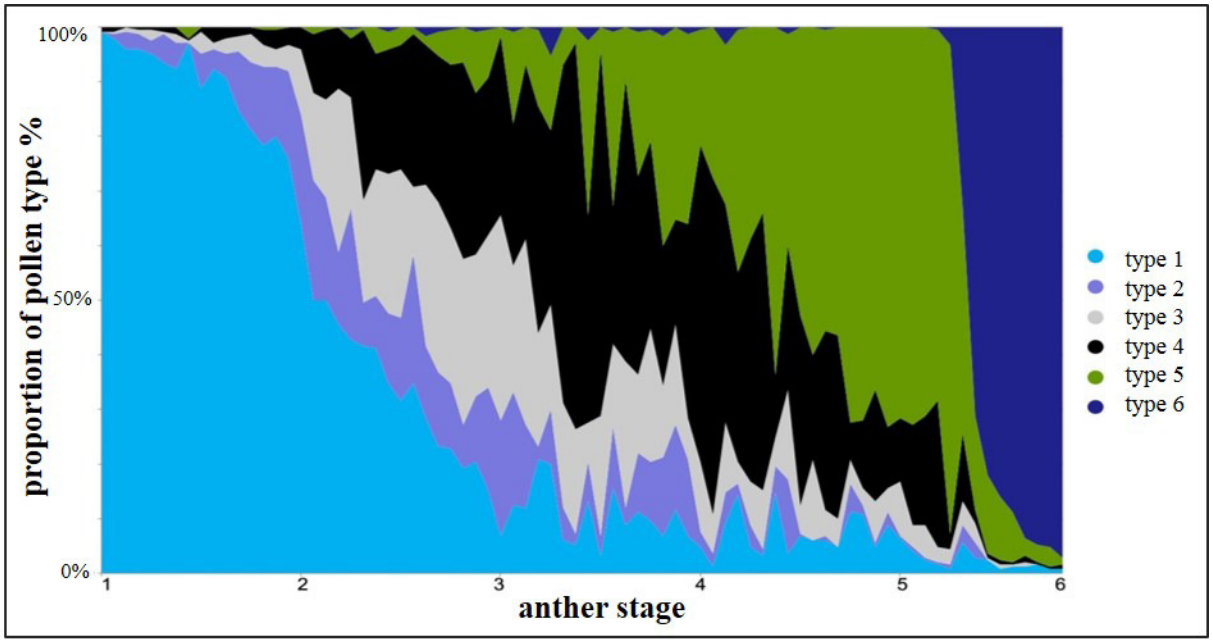


Fig. 10: Anther staging in barley based on distribution of pollen types within individual anthers.

All anthers analysed contained only a neglectable amount of dead pollen. This not only means that virtually all unicellular pollen successfully developed into mature tricellular pollen, but that all chromatin patterns identified represent stages towards this end product.

3.1.1.3 Chromatin decondensation and DNA doubling in generative nuclei

The subpopulation of pollen with nuclei of similar size (type 3 pollen) allowed some insights in the process of chromatin decondensation and DNA replication within the generative nucleus. For these three groups of bicellular pollen with similar sized nuclei were investigated and compared. Group I was derived from anthers with a predominance of type 3 pollen but low in type 4 and

beyond; group II came from anthers with a predominance of type 4 but low in type 5 or beyond; and finally group III consisting of equal sized bicellular found in anthers with a predominance of type 5 pollen (Table 4). For each groups size and relative fluorescence intensity of the nuclei were measured and plotted in graphs (Fig. 11) as described under 2.3.2.

Table 4: Division of barley anthers in three groups. Group I: Bicellular pollen population dominated by type 3 pollen. Group II: Bicellular pollen population dominated by type 4 pollen. Group III: Bicellular pollen population dominated by type 5 pollen.

Anther groups	**Type of pollen**					
	1	**2**	**3**	**4**	**5**	**6**
Group I						
anther 32	114	20	88	28		
anther 33	125	32	60	32	1	
anther 34	125	33	62	27	3	
anther 65	166	44	24	16		
GroupII						
anther 41	48	14	82	90	16	
anther 43	22	2	93	109	24	
anther 58	8	7	57	168	10	
anther 71	13	5	50	177	7	
Group III						
anther 52	6	1	15	50	178	
anther 68	15		37	48	150	
anther 75	22	6	11	28	183	
anther 76	36	9	16	28	156	5

The nuclei of type 3 pollen from group I have a fluorescence index (highest fluorescence/lowest fluorescence) close to 1 (Fig. 11) indicating that in the generative nucleus DNA replication has probably not yet started (see 4.1.2.). The rather wide range in diameter (Fig. 11) seemed to speak for variable degrees of chromatin decondensation within these nuclei, thus also in designated

generative nuclei. Support for this assumption was obtained when one compared the data on diameter with those of nuclear fluorescence. Average diameter of nuclei increased from 51.7 ± 8.3 µm² in group I (n = 71) to 59.0 ± 8.8 µm² in group II (n = 54) and finally 70.8 ± 10.8 µm² in group III (n = 60). Under the same conditions the fluorescence intensity plots first shifted in the direction of 0 before increasing in the direction of Y (Fig. 11). The fluorescence data thus indicated that decondensation (the shift towards 0) preceded DNA duplication (shift in Y).

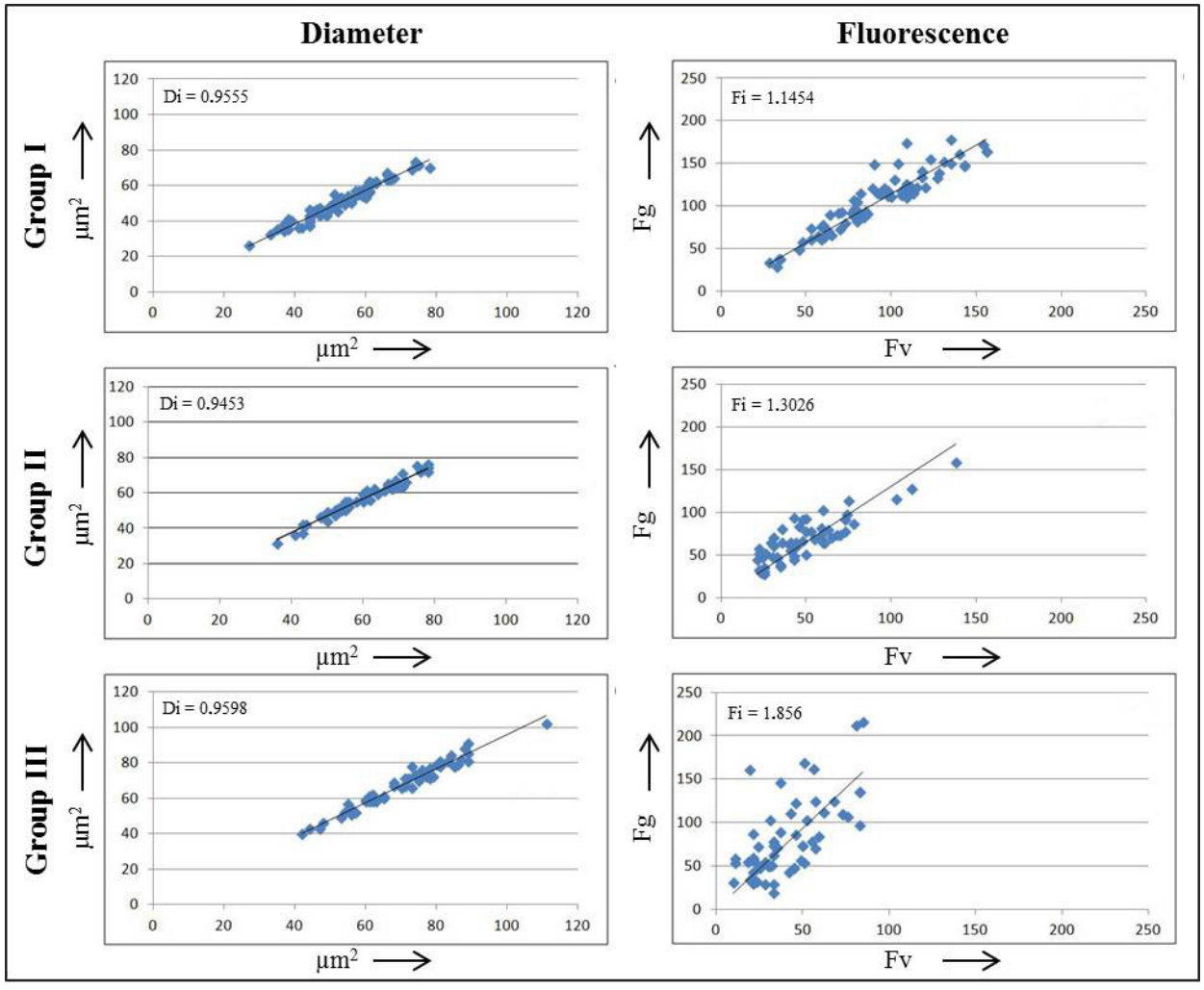


Fig. 11: Measurements of size and relative fluorescence intensity in equal sized nuclei present in populations dominated by early (Group, I), median (Group II) and late (Group III) bicellular barley pollen. Di = diameter index, and Fi = fluorescence index were based on ∑value generative nuclei/∑value vegetative nuclei. Fg = relative fluorescence generative nucleus, Fv = relative fluorescence vegetative nucleus.

Combined with the data on diameter, it appeared that the size increase from group I to group II is due to ongoing decondensation, whereas the increase between group II and group III can be attributed to DNA replication. In group II a fluorescence index of 1.3 indicated starting DNA replication while in group III a fluorescence index approaching 2 implicated a near to complete DNA replication event in the population of generative nuclei (Fig. 11).

Also within the population of pollen with clearly dissimilar generative and vegetative nuclei (type 4 and 5 in Fig. 9) both generative and vegetative nucleus showed a clear size increase between group I to group III (Table 5). For vegetative nuclei this again indicated that chromatin decondensation progresses with time.

Table 5: Diameter of generative and vegetative nuclei in barley pollen with dissimilar nuclei.

Diameter of nuclei (µm²) in bicellular with unequal nuclei		
	Generative nucleus	vegetative nucleus
Group I (n = 80)	47.9 ± 7.7	70.0 ± 11.7
Group II (n = 76)	51.4 ± 8.3	78.2 ± 9.3
Group III (n = 80)	55.1 ± 8.3	84.2 ± 15.1

3.1.1.4 Plant specific variation in anther development

To illustrate the variation in pollen developmental in barley, two plants were selected with awns showing 5 mm above the flag leaf. On three consecutive days anthers were collected at 07.00 a.m. For this the flag leaf was opened, taking care not to damage the spike, and a single floret removed. The pollen of all three anthers of a floret was analysed.

The results displayed in Fig. 12 show that populations can differ substantially between plants and even between the anthers of a single floret. These variations were most significant in the younger spikes. With time, as spikes mature, pollen populations showed increasingly similar distributions (Fig. 12). For embryogenesis, however, the early stages were essential (here: day 1 spikes).

These data underline that external morphological features as length of awns were only a rough estimation for the correct harvesting time to obtain pollen in the optimal stage for induction of embryogenesis.

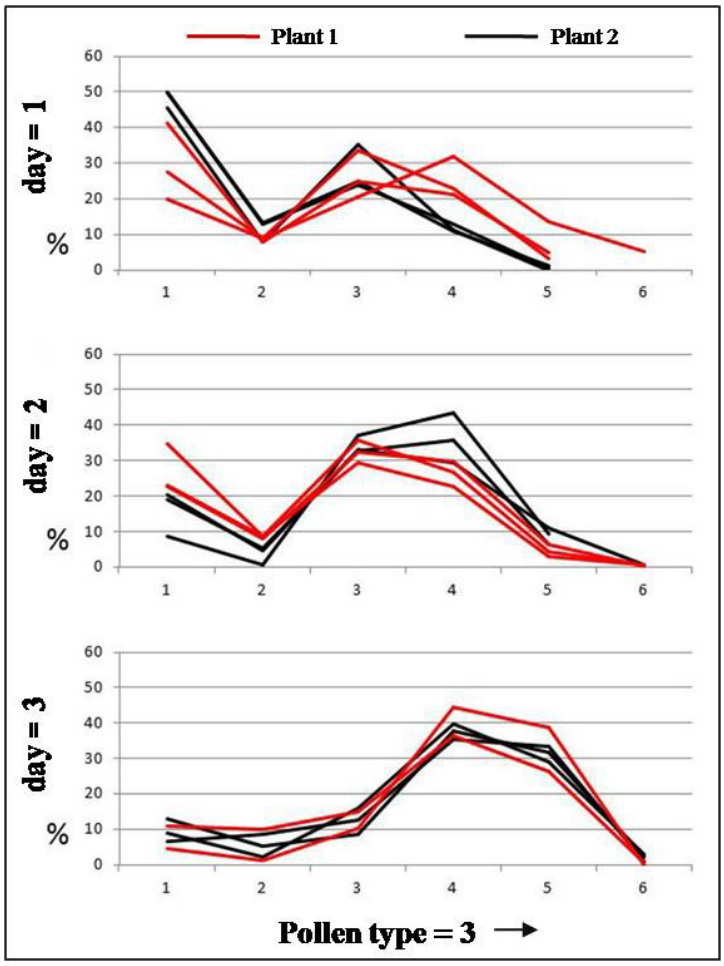


Fig. 12: Variation in barley pollen population. Single florets were harvested from the central spikes of two plants (red and black lines) on three consecutive days, starting from the moment when awns protruding 5 mm beyond the flag leaf (day = 1).

3.1.2 Tobacco

3.1.2.1 Nuclear dynamics during pollen development

Mature pollen of the dicotyledon tobacco is binucleate. Analogue to barley DAPI staining was used to identify distinct stages of development from unicellular to mature bicellular pollen (Fig. 13).

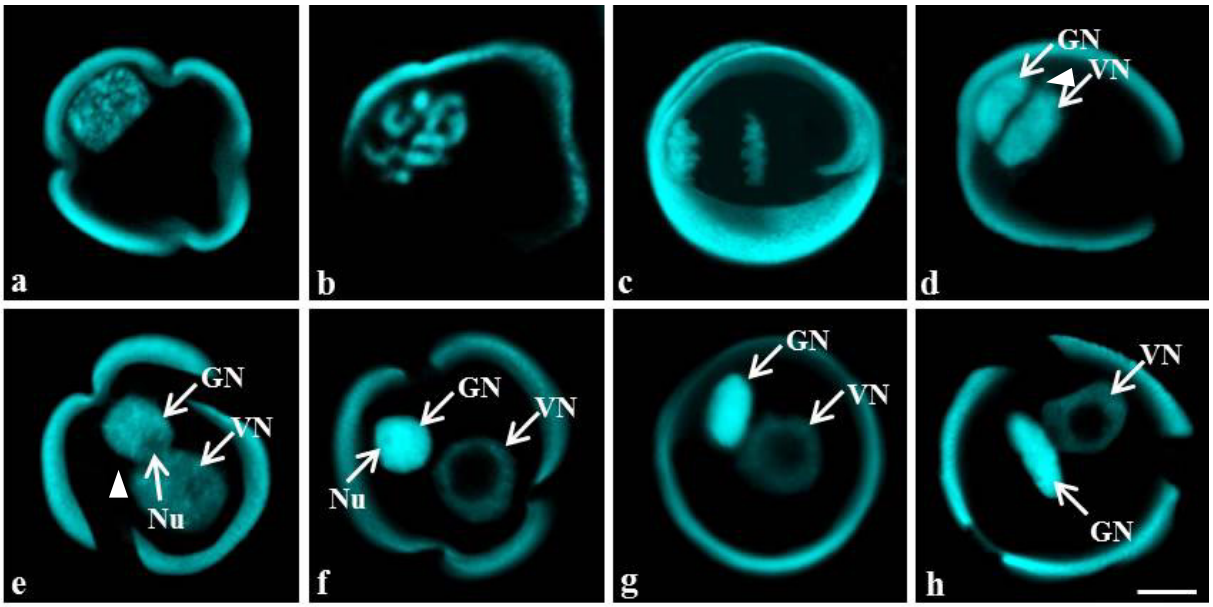


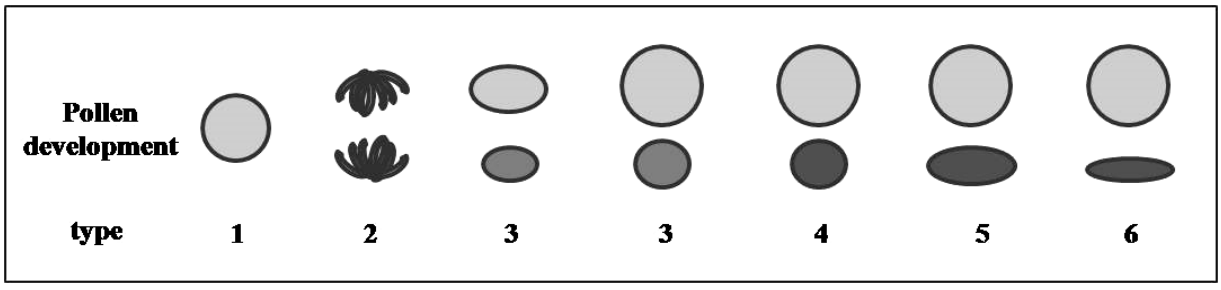
Fig. 13: Pollen development in tobacco from late uninucleate to mature bicellular stage after DAPI staining. (a) Unicellular. (b, c) PM I. (d) Two sister nuclei after mitosis different in size and fluorescence (e) Early bicellular pollen with two roundish nuclei of different size and slightly different fluorescence intensity arrowhead indicates position of nucleolus in the generative nucleus. (f) Late binuclear pollen with a strongly fluorescent generative nucleus. (g) Late bicellular pollen with elongated generative nucleus. (h) Mature pollen with a spindle shaped generative nucleus. VN: vegetative nucleus, GN: generative nucleus, Nu: nucleolus. Bar = 5 µm.

Pollen development in tobacco differed in many aspects from that observed in barley. Like in barley, the nucleus took up a position near the pollen wall before PM I (see Fig. 8a). However, whereas in barley the spindle apparatus was orientated parallel to the pollen wall (see Fig. 8b-c), in tobacco the axis of the spindle apparatus was orientated perpendicular to the pollen wall (Fig. 13b, c). After PM I in barley vegetative and generative nuclei were exist side by side at the pollen wall (Fig. 13d-f) and remained of similar size for some time afterwards. In tobacco, PM I

resulted in a peripheral generative nucleus covered by a more central vegetative nucleus (Fig. 13d). The initially ellipsoid nuclei differed in size and fluorescence intensity from the moment mitosis has ended making it always possible to distinguish the generative and vegetative nucleus (Fig. 13d). Generative and vegetative nuclei initially were ellipsoid (Fig. 13d). However, since even in populations with high incidence of PM I, ellipsoid nuclei were very rarely observed, the conclusion must be that after completion of PM I the nuclei round up rapidly into a larger vegetative nucleus and smaller, somewhat more intensely stained, generative nucleus (Fig. 13e). Whereas the vegetative nucleus remaied roundish, the generative nucleus underwent several changes. First, with the onset of DNA replication, DAPI fluorescence intensity strongly increased, a condition which with some practice can be easily identified. This phase in which the nucleolus of the generative nucleus also significantly reduces in size (Fig. 13e, f) was followed by a phase in which the generative nucleus gradually became ellipsoid and a nucleolus eventually was no longer visible (Fig. 13g, h). This last condition typified the fully mature pollen.

3.1.2.2 Association of pollen and anther developmental stages

Also for tobacco anther staging was attempted based on general DAPI staining patterns shown in Fig. 14. Due to their scarcity, very early bicellular pollen with ellipsoid nuclei were not assigned a type of their own. Similar to the situation in barley, the latest phases of development were a bit compromised due to the fact that all elongating generative nuclei were grouped into a single type of “late bicellular”.



Type 1	**Unicellular** (interphase nucleus of immature pollen)
Type 2	**First pollen mitosis**

Type 3	**Very early to early bicellular** (initially with ellipsoid, later with roundish nuclei slightly differing in size and fluorescence intensity)
Type 4	**Median bicellular** (strongly increased fluorescence in generative nucleus)
Type 5	**Late bicellular** (elongating generative nucleus)
Type 6	**Mature pollen** (spindle-shaped generative nucleus)

Fig. 14: Schematic diagram of proposed types of pollen development in tobacco used for anther staging calculations. Weak intensity of DAPI fluorescence shown in pale grey, strong fluorescence shown in dark grey.

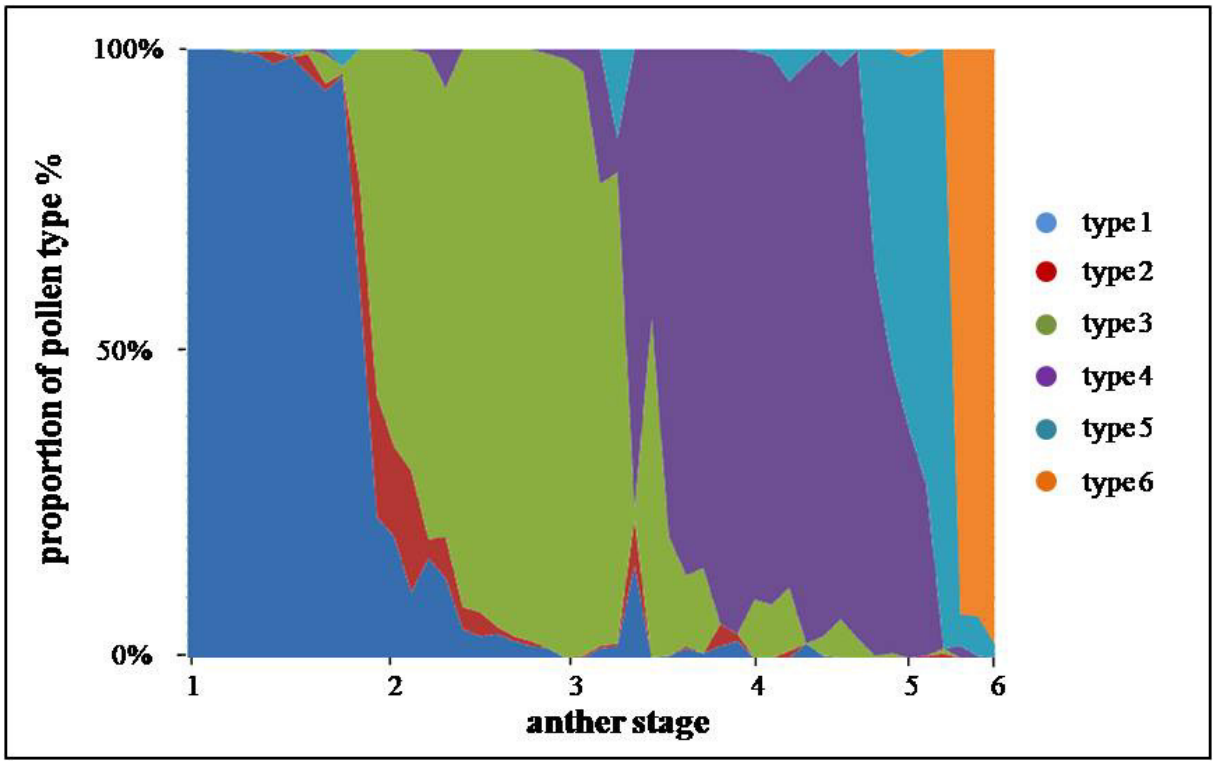
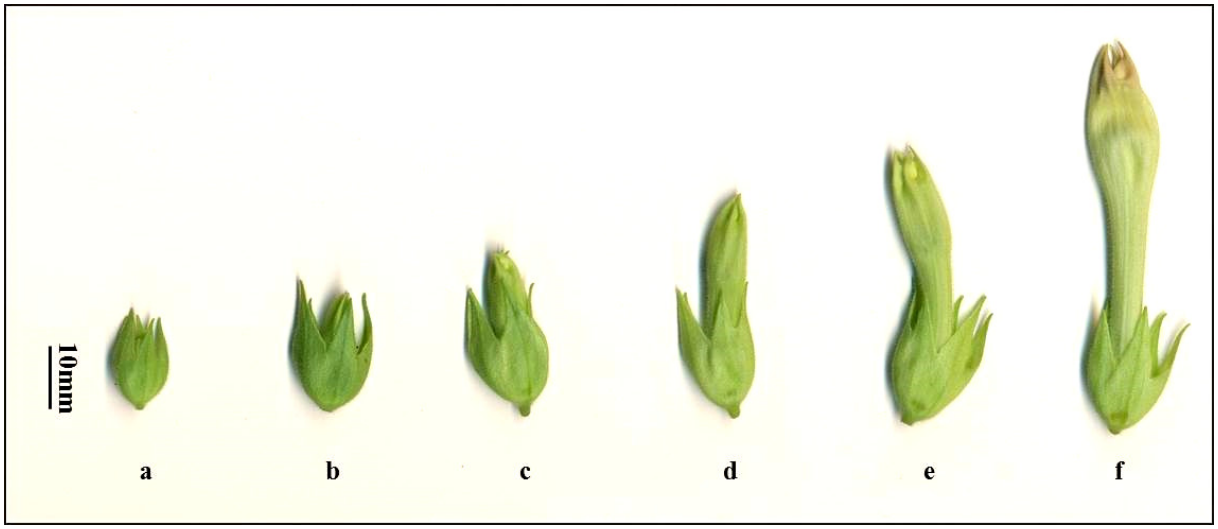


Fig. 15: Anther staging in tobacco based on distribution of pollen types within individual anthers. (X-axis)

Pollen development in tobacco was highly synchronized (Kyo and Harada, 1986). The experiments conducted here confirmed that all individual anthers of a single flower always contained near to identical pollen populations (Fig. 15). The anther staging resulted (Fig. 13) also supported well-orchestrated transitions in pollen development. The high synchronicity made that the short process of mitosis easily missed. Mitosis usually happened in the early morning

(around 7 a.m.), yet occasionally also several hours later. Size of the flowers, measured from flower base to tip of the petals (Fig. 16), can be used as a rough guideline to pollen developmental stage. Up to 13 mm in length, all pollen was uninucleate but already at 14-15 mm populations ranged from completely unicellular to predominantly late bicellular (Fig. 16).



Types of pollen development	Flowers					
	a	**b**	**c**	**d**	**e**	**F**
Type 1	250	3				
Type 2			1		1	
Type 3		1				
Type 4		48				
Type 5		197	248	215	11	3
Type 6		1	1	35	238	247

Fig. 16: Flower bud morphology and pollen population in tobacco. (a) 13 mm long flower buds contain late unicellular pollen only, (b) at 15 mm pollen are predominantly early bicellular, (c) at 20 mm mainly median bicellular, (d, e) at 29 and 35 mm mainly late bicellular and (f) at 50 mm mature bicellular pollen are found. For each flower the first 250 pollen were analysed.

3.1.2.3 Spontaneous division of vegetative cells in tobacco pollen

In contrast to textbook knowledge claiming mature tobacco pollen to be bicellular (e.g. Russel & Jones 2015), DAPI staining revealed that a consistent 1-2% of the pollen in early and median bicellular pollen populations were tri-cellular. Observations based on DAPI staining showed that the additional mitosis only involved the vegetative cell and seemed to take place soon after PM I. The ultimate fate of this type of pollen was uncertain. The fact that tricellular pollen were only observed in immature and in mature pollen populations suggested that this aberrant pathway may not be viable. The phenomenon of tricellular pollen indicated an intrinsic capacity of the vegetative cell to divide. However, it remained unclear whether the resultant daughter cells were comparable to those occuring at the onset of pollen embryogenic development.

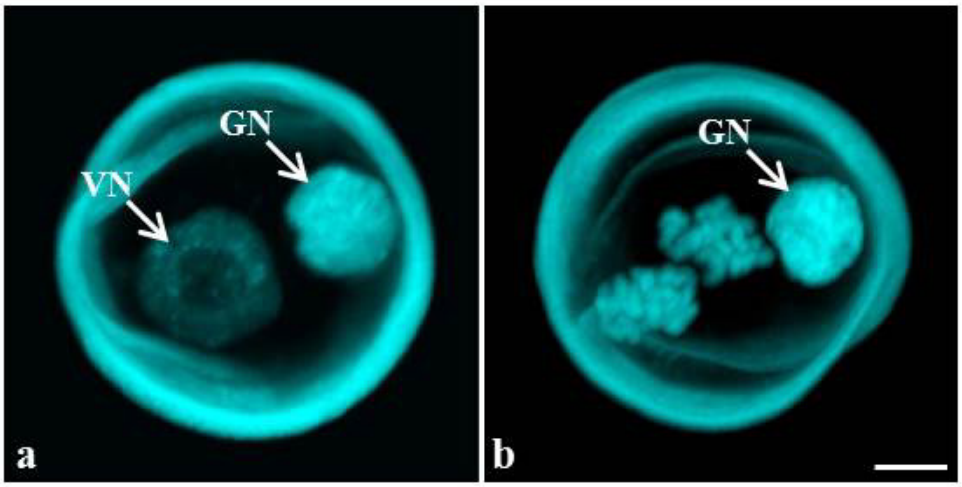


Fig. 17: Spontaneous embryogenesis in tobacco pollen visualized by DAPI staining. (a) Typical early bicellular stage with roundish nuclei. (b) Dividing vegetative nucleus at anaphase. GN = generative nucleus, VN = vegetative nucleus. Bar = 5 µm.

3.2 Chromatin alterations during pollen development

Pollen development was immunologically investigated from the late unicellular until the mature tricellular stage. Based on DAPI staining (Fig. 8), five basic developmental stages were identified: (1) late unicellular, (2) PM I, (3) early bicellular in which vegetative and generative

nuclei are morphologically identical, (4) late bicellular up until early PM II in which the vegetative nucleus was larger and less heavily stained than the generative nucleus, (5) tricellular pollen.

The selection of antibodies used is listed in table 6. Distribution of phosphorylated RNAP II was used as a measure of transcriptional activity of a nucleus whereas the label for CENH3 was considered to shed light on the activity of the centromeres and hence indirectly on the ability of a cell to undergo mitosis. The remaining four antibodies were directed against histone modifications. Interpretation of labeling results was as described under 2.7.2.

Table 6: Antibodies used for immunolabeling and function of the corresponding antigenes.

Antibody	**Function**
anti-RNAP II	Essential for transcriptional activity (Kronberg, 1999)
anti-CENH3	Central component of centromeres (Talbert et al., 2002)
anti-H3K9me2	Required for silencing transposons and repetitive sequences (Jackson et al., 2002; Malagnac et al. 2002)
anti-H3K27me3	Involved in transcriptional gene silencing (Roudier et al., 2009; Zhang et al. 2009)
anti-H3K4me2	Involved in transcriptional gene activation (Zhang et al., 2009)
anti-H3K9ac	Involved in transcriptional gene activation (Struhl, 1998)

3.2.1 Barley

3.2.1.1 Distribution of RNA polymerase II

RNAP II was present throughout the nucleoplasm of unicellular pollen. It is not part of the chromatin which is clearly shown in pro-metaphase when label for RNAP II was excluded from the condensed chromosomes (arrows in Fig. 18a, b). Unclear is what happened to this nuclear RNAP II after disruption of the nuclear envelope. A presumed redistribution throughout the

cytoplasm during mitosis could not be confirmed as the diffuse cytoplasmic background labeling did not differ from that found in the controls (results not shown).

With the reformation of the nuclear envelope after PM I, label for RNAP II reappeared in both sister nuclei (Fig. 18e, f), suggesting that the designated vegetative and generative nucleus display significant transcriptional activity at this stage. RNAP II signal disappeared from the generative nucleus only in late bicellular and tricellular pollen, while it remained present in the vegetative nucleus (Fig. 18g-j).

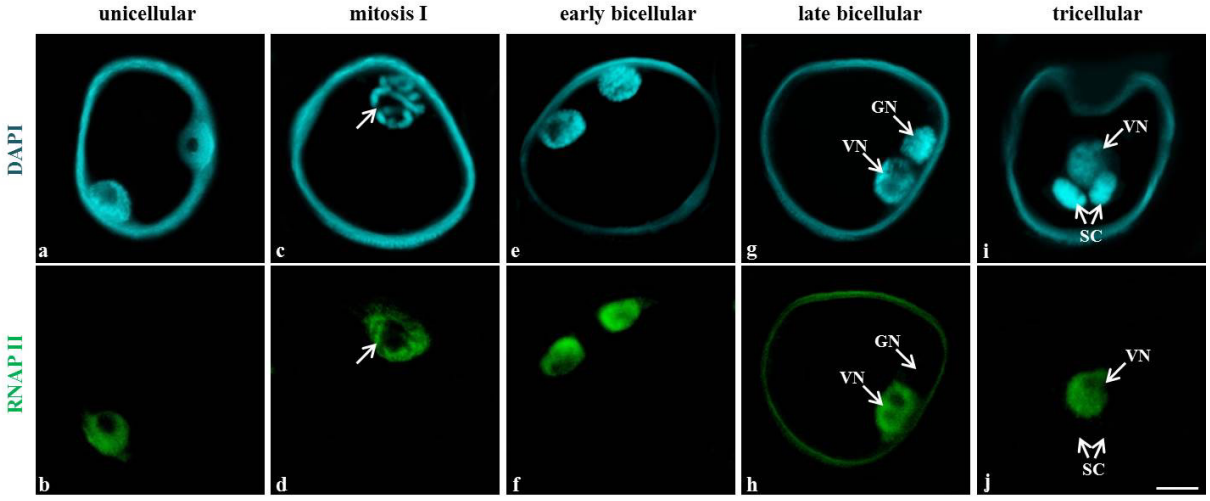


Fig. 18: Immunolocalization of RNAP II during barley pollen development. (a, b) Strong signals in nucleus of unicellular pollen. (c-f) Signals of RNAP II in the nucleoplasm of prophase and in the nuclei of early bicellular stage. (g-j) RNAP II restricted to the vegetative nucleus in late bicellular and tricellular pollen. DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus, SC = sperm cells. Bar = 10 µm.

3.2.1.2 Distribution of centromeric histone H3

As an integral part of the plant chromosome the centromere-specific histone 3-variant (CENH3) is essential for mitotic activity. Clear labeling was found in unicellular and mitotic pollen (Fig. 19a-d). In early bicellular pollen strong CENH3 signals were observed in both nuclei (Fig. 19e, f). However, despite the fact that according to DAPI staining the two nuclei of this early bicellular pollen appeared similar in size and fluorescence intensity, CENH3 signals were always

more aggregated in one nucleus and more dispersed in the other nucleus (arrows in Fig. 19e, f). In late bicellular and tricellular pollen label for CENH3 remained very clear in the generative cell and sperm cells respectively (Fig. 19g-j). However, in the vegetative nucleus CENH3 signals were always much weaker and often hardly detectable at all (Fig. 19g-j).

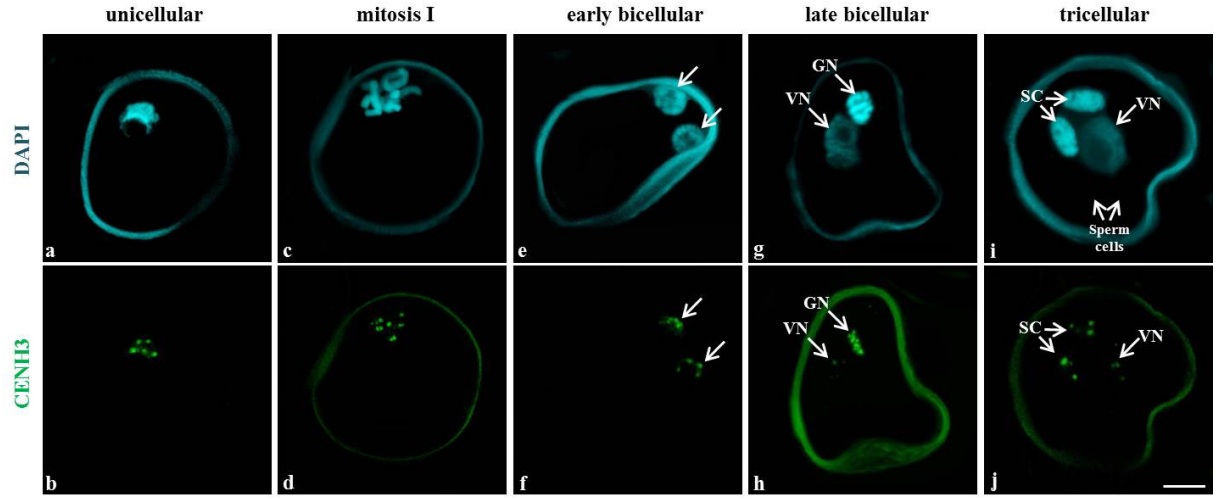


Fig. 19: Immunolabeling of CENH3 during barley pollen development. (a-d) CENH3 during interphase and prophase of unicellular pollen. (e, f) After PM I signals are clustered in one nucleus and more widely distributed in the other nucleus (arrows). (g-j) Strong CENH3 labeling in the generative nucleus of late bicellular pollen and in sperm cells accompanied by weak fluorescence in the vegetative nucleus. DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus, SC = sperm cells. Bar = 10 µm.

3.2.1.3 Distribution of histone H3 dimethylated at position lysine 9

H3K9me2 was ubiquitously present in all nuclei during all stages of pollen development and also persisted in chromosomes during all phases of the cell cycle including mitosis (Fig. 20a-j). In bicellular pollen label intensity in vegetative and generative nuclei was always similar, even in late bicellular stages when the generative nucleus stained much stronger for DAPI (Fig. 20g-h). Based on the near to identical microscope settings with which the fluorescence was recorded, it seems that absolute label intensity remained more or less constant during pollen development.

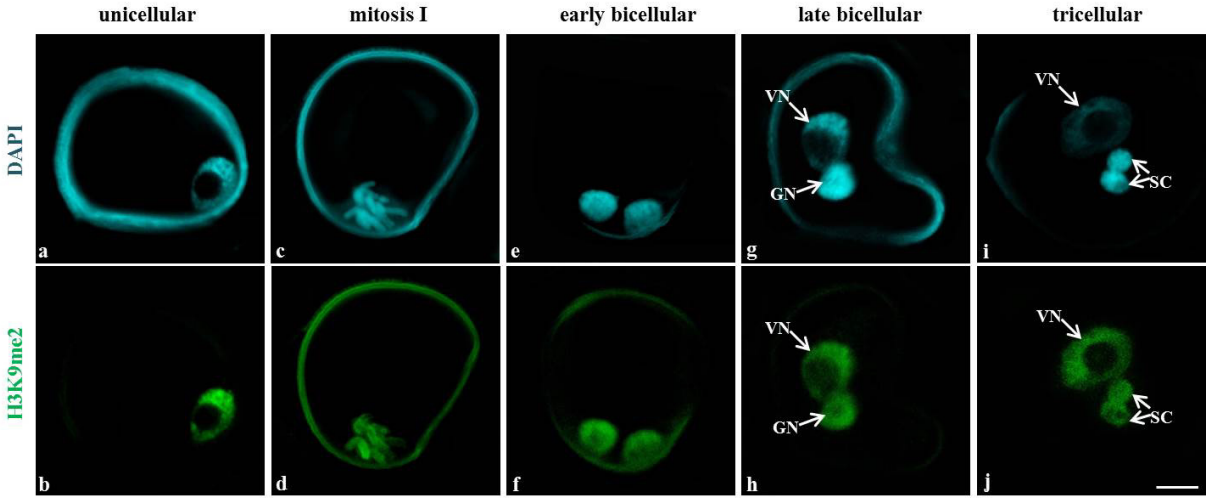


Fig. 20: Immunolabeling of H3K9me2 during barley pollen development. (a-j) H3K9me2 is present in all nuclei throughout all stages of pollen development showing no obvious temporal or spatial change in distribution. DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus, SC = sperm cells. Bar = 10 µm.

3.2.1.4 Distribution of histone H3 dimethylated at position lysine 4

Although H3K4me2 label was found throughout the nucleus, the distribution was not uniform with interphase nuclei in particular showing local accumulations (Fig. 21a, b). This uneven distribution of H3K4me2 was most apparent in the condensed chromosomes, where label was strongest in what appeared to be the subtelomeric regions (Fig. 21c, d). With time, however, labeling of the vegetative nucleus became weaker and in the late bicellular and tricellular stages, the generative nucleus and sperm cell nuclei were distinctly stronger labeled than the vegetative nucleus (Fig. 21g-j).

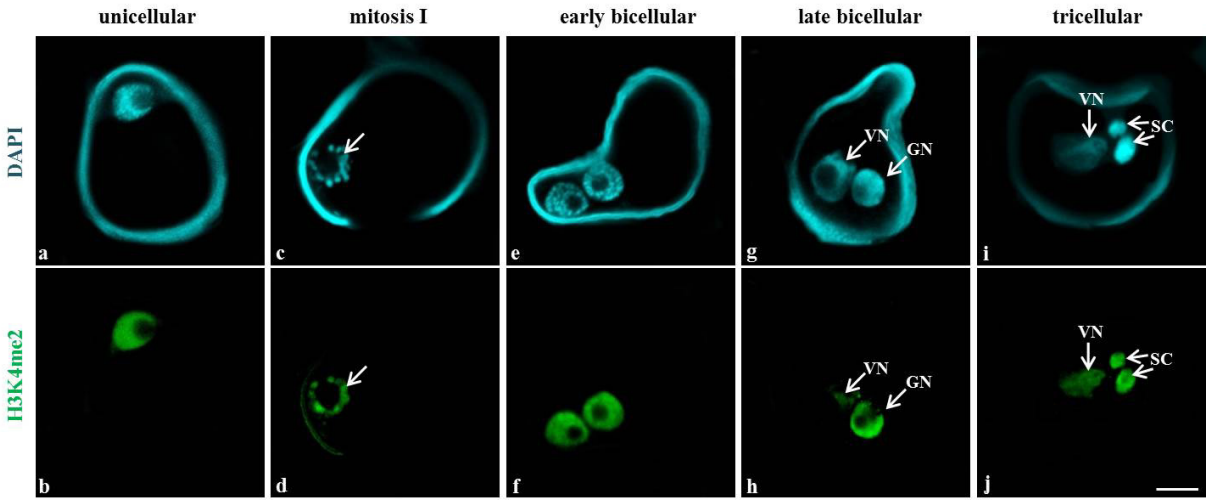


Fig. 21: Immunolabeling of H3K4me2 during barley pollen development. (a, b) H3K4me2 in the nucleoplasm of unicellular pollen. (c, d) Labeling of subtelomeric domains during mitosis I. (e, f) Equally strong labeling in nuclei of early bicellular pollen. (g-j) Weak labeling of vegetative nucleus in late bicellular and tricellular stages compared to strong labeling of generative nucleus and sperm cell nuclei. DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus, SC = sperm cells. Bar = 10 µm.

3.2.1.5 Distribution of histone H3 trimethylated at position lysine 27

H3K27me3 was always localized in spot-like patterns that were predominantly present in the nuclear periphery (Fig. 22a-j). Although at some stages, like just before PM I, the spots appeared rather evenly distributed (Fig. 22a, b), in general the H3K27me3 patches were concentrated in specific areas of the nucleus. The mitotic figures of PM I revealed that signals for H3K27me3 were particularly strong on or near subtelomeric regions (Fig. 22c, d and Fig. 23d-f).

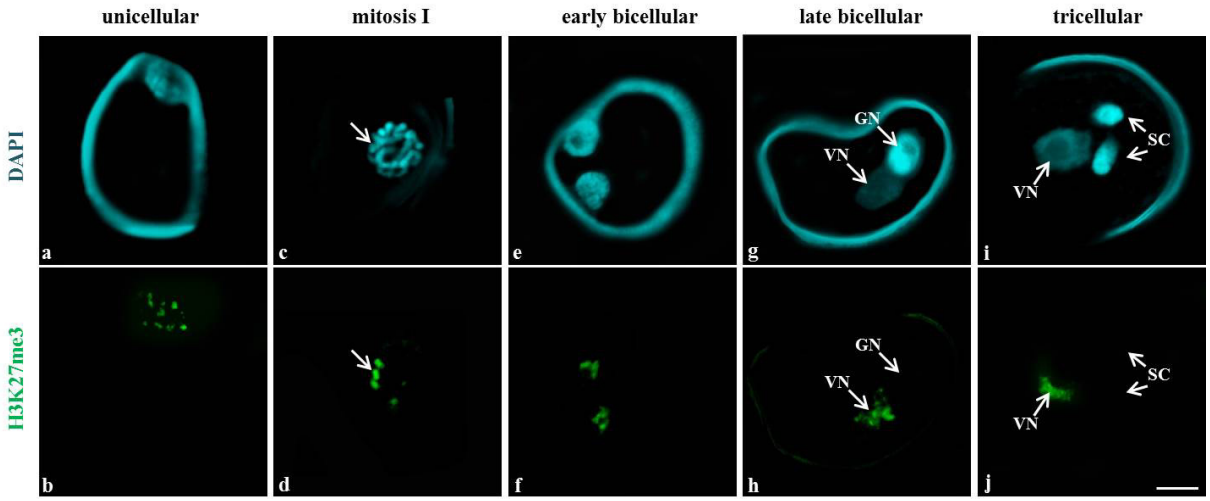


Fig. 22: Immunolabeling of H3K27me3 during barley pollen development. (a-d) Spot-like pattern of H3K27me3 in interphase and prophase nucleus of unicellular pollen (arrow in d indicate subtelomeric domains). (e, f) H3K27me3 signal in both nuclei of early bicellular stage. (g-j) In late and tricellular pollen label is restricted to the vegetative nucleus. DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus, SC = sperm cells. Bar = 10 µm.

In comparison to H3K4me2, the chromosomal distribution of the H3K27me3 signals seemed even more restricted to specific domains (Fig. 23a-c and 23d-f). In the early bicellular stage, generative and vegetative nucleus stained equally well for H3K27me3 and, in contrast to the results for CENH3, displayed similar distribution patterns (compare Fig. 19 and 22). Labeling of the vegetative nucleus remained strong during pollen maturation (Fig. 22g, h), in contrast to this labeling of the generative nucleus was absent or very weak in the late bicellular stage (Fig. 22g, h) and H3K27me3 signals were absent from sperm cell nuclei (Fig. 22i, j).

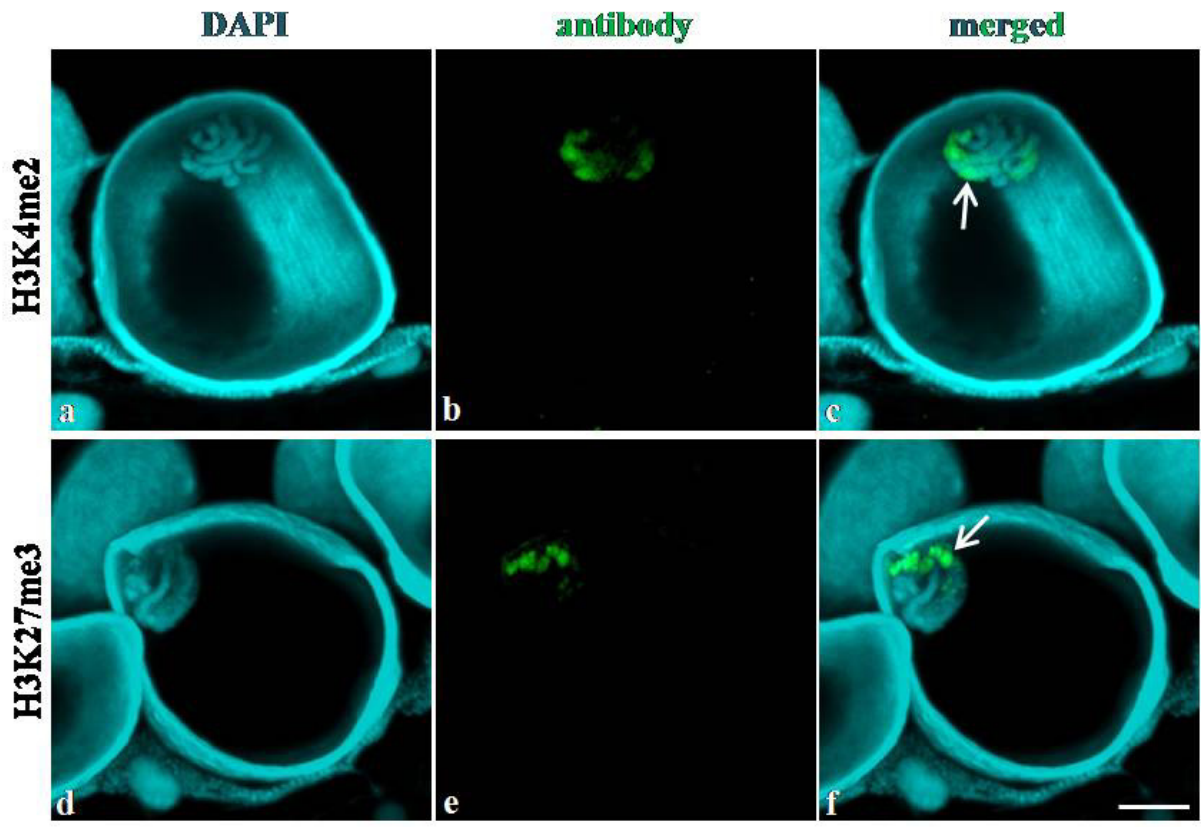


Fig. 23: Immunolabeling of H3K4me2 and H3K27me3 in barley pollen prophase. Distribution pattern of H3K4me2 (a-c) is more diffuse than that of H3K27me3 (d-f). DAPI in blue, antibody labeling in green. Bar = 10 µm

3.2.1.6 Distribution of histone H3 acetylated at position lysine 9

H3K9ac was clearly present in the interphase nucleus of the uninucleate pollen (Fig. 24a, b). By contrast, signals seemed to disappear from mitotic figures (Fig. 24c, d) and were also not found in the condensed nuclei present immediately after PM I (Fig. 24e, f). As the initially compact chromatin of generative and vegetative nuclei became more decondensed signals for H3K9ac first reappeared in the cytoplasm of both generative and vegetative cell (Fig. 24g, h). This cytoplasmic component remained present in all subsequent developmental stages. Based on DAPI staining patterns, H3K9ac signals first entered the vegetative nucleus before eventually

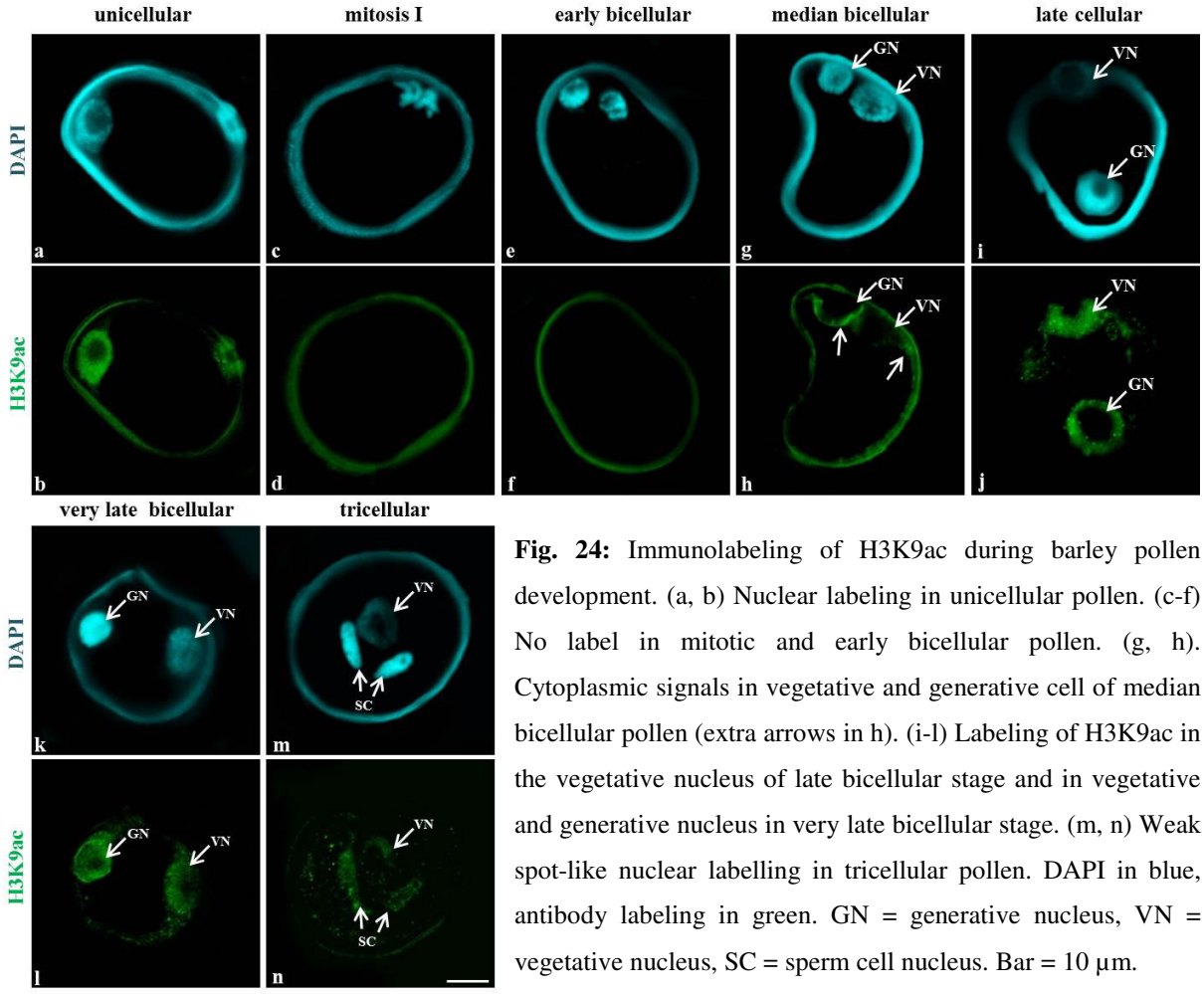


Fig. 24: Immunolabeling of H3K9ac during barley pollen development. (a, b) Nuclear labeling in unicellular pollen. (c-f) No label in mitotic and early bicellular pollen. (g, h). Cytoplasmic signals in vegetative and generative cell of median bicellular pollen (extra arrows in h). (i-l) Labeling of H3K9ac in the vegetative nucleus of late bicellular stage and in vegetative and generative nucleus in very late bicellular stage. (m, n) Weak spot-like nuclear labelling in tricellular pollen. DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus, SC = sperm cell nucleus. Bar = 10 µm.

also generative nucleus became labeled (Fig. 24i, j). At least in case of generative nucleus labeling seemed to develop from the periphery inwards (Fig. 24k, l). At the tricellular stage, weak spotty signals were found in vegetative nucleus and in sperm cell nuclei (Fig. 24m, n).

3.2.2 Tobacco

According to the manufacturers datasheets the antibodies used in the barley should have a broad reactivity across species. In contrast to the canonical histones which are among the most conserved eukaryotic proteins, CENH3's are rapidly evolving (Masonbrink et al. 2014, Hirsch et

al. 2008). For these reasons the anti-CENH3 antibody, generated against CENH3 from rice (*O. sativa*) was not used in the immunolabeling experiments on tobacco pollen. Pollen development in tobacco differed in many aspects from that of barley (see section 3.1. and 3.2.). For immunological studies the following developmental stages were considered based on DAPI staining (Fig. 13): (1) unicellular, (2) early-bicellular with less condensed generative nucleus, (3) median bi-cellular with highly condensed generative nucleus, (4) late bicellular stages characterized by a spindle shaped generative nucleus.

3.2.2.1 Distribution of RNA polymerase II

RNAP II was found in the nucleus of unicellular pollen (Fig. 25a, b) and in the vegetative and generative nucleus of early and median bicellular pollen (Fig. 25c-f). In late bicellular pollen an irregular spot-like labeling was present distributed over nuclei and cytoplasm alike (Fig. 25g, h).

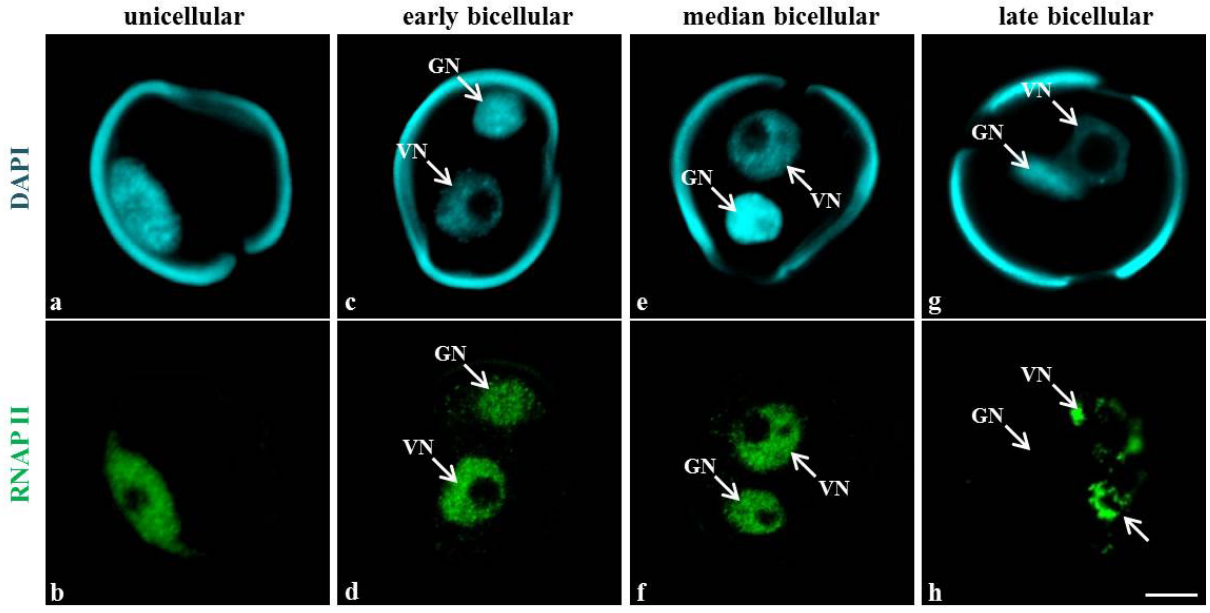


Fig. 25: Immunolabeling of RNAP II during tobacco pollen development. (a-f) RNAP II present in nuclei of unicellular, early and median bicellular pollen. (g, h) Irregular labeling pattern in late bicellular pollen (extra arrow). DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus. Bar = 5 µm.

3.2.2.2 Distribution of histone H3 dimethylated at position lysine 9

Label for H3K9me2 was granular and confined to nuclei of the unicellular (Fig. 26a-b), early bicellular (Fig. 26c-d) and median bicellular pollen (Fig. 26e-f). In the median bicellular stage nuclear signals were rather weak (Fig. 26e-f) while in late bicellular stage fluorescence signals were absent altogether (Fig. 26g-h).

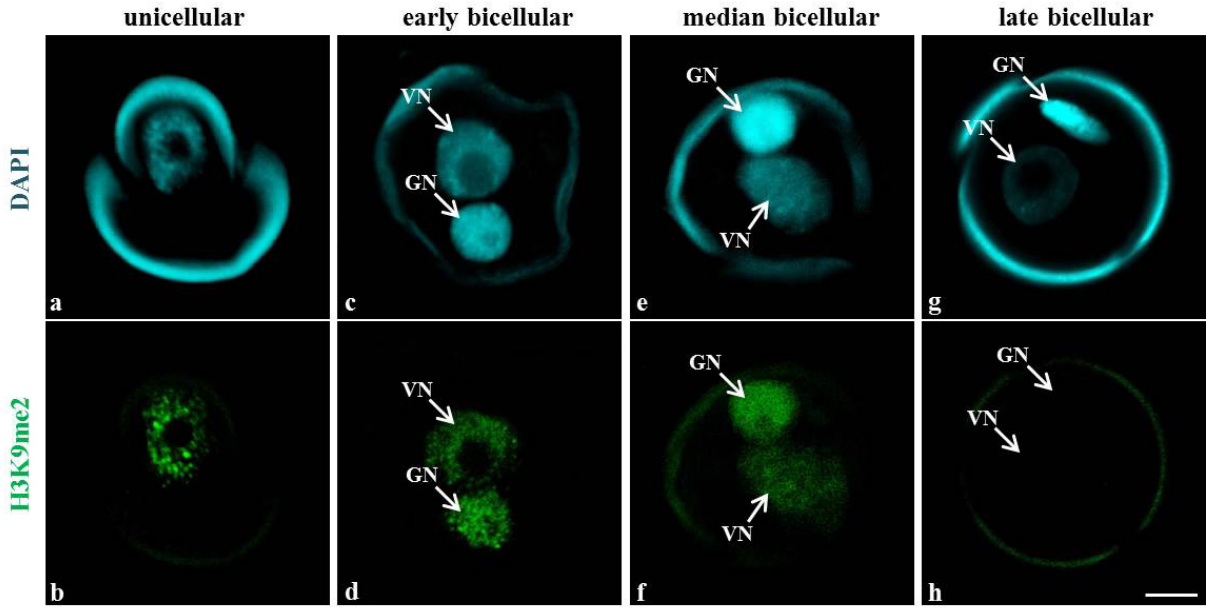


Fig. 26: Immunolabeling of H3K9me2 during tobacco pollen development. (a-f) Nuclear signals for H3K9me2 in unicellular, early bicellular and median bicellular pollen. (g, h) Label is absent from late bicellular pollen. DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus. Bar = 5 µm.

3.2.2.3 Distribution of histone H3 dimethylated at position lysine 4

Labeling for H3K4me2 was strong and uniform in the nucleus of unicellular pollen (Fig. 27a, b) while in the early binuclear pollen it was strong in the generative nucleus but much weaker in the vegetative nucleus (Fig. 27c, d). In the median bicellular stage the generative nucleus displayed a weak labeled center surrounded by a stronger labeled periphery; the outline of the now smooth labeled vegetative nucleus was difficult to determine due to the presence of widespread cytoplasmic signals (Fig. 27e, f). In late bicellular pollen labeling was absent from both nuclei

and seemed restricted to numerous, mainly peripheral, organelle-like structures within the cytoplasm of the vegetative cell and probably of the generative cell as well (Fig. 27g, h).

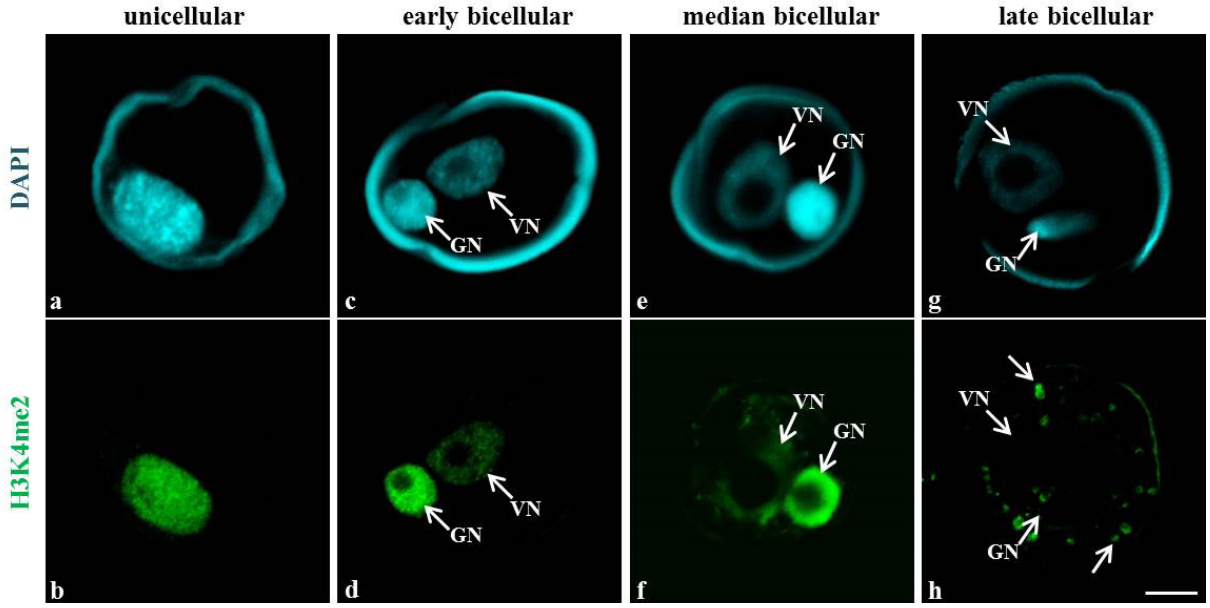


Fig. 27: Immunolabeling of H3K4me2 during tobacco pollen development. (a, b) Strong nuclear labeling in unicellular pollen. (c, d) Early bicellular pollen with generative nucleus stronger labeled than vegetative nucleus. (e, f) Clear indication of cytoplasmic label in median bicellular pollen. (g, h) Label in late bicellular pollen seems restricted to organelle-like structures in the peripheral cytoplasm of vegetative and possible generative cell (extra arrows). DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus. Bar = 5 µm.

3.2.2.4 Distribution of histone H3 trimethylated at position lysine 27

Strong fluorescence signals for H3K27me3 were restricted to the cytoplasm of uninucleate pollen (Fig. 28a-b). In early and median bicellular pollen label was only found in the cytoplasm of vegetative and generative cells (Fig. 28c-f). Despite the hitherto strong labeling, no signals were found in late bicellular pollen (Fig. 28g-h).

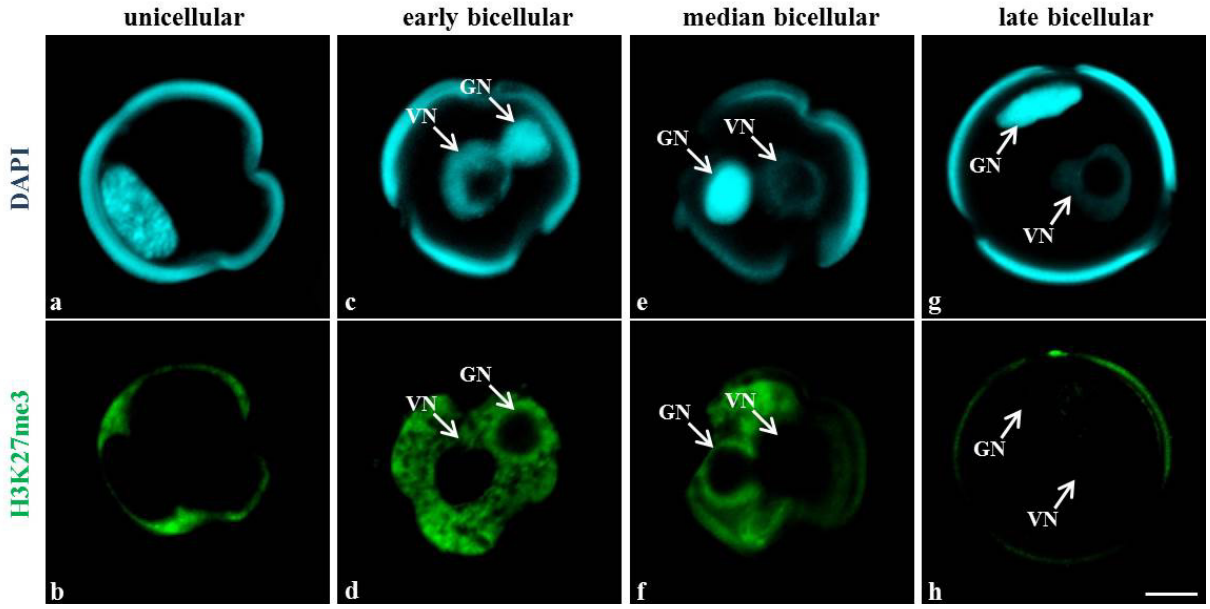


Fig 28: Immunolabeling of H3K27me3 during tobacco pollen development. (a-f) Until median bicellular stage signals are restricted to the cytoplasm. (g, h) Label is absent from late bicellular pollen. DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus. Bar = 5 µm.

3.2.2.5 Distribution of histone H3 acetylated at position lysine 9

Labeling pattern for H3K9ac was very dynamic. While in unicellular pollen there was a weak labeling of the cytoplasm and a much stronger label in the nucleus (Fig. 29a, b), in the early bicellular stage pollen clear fluorescence signals were found in the generative and vegetative nucleus and in the cytoplasm of at least the vegetative cell (Fig. 29c, d). In the following median bicellular stage the generative nucleus was strongly stained while labeling of the vegetative nucleus was much weaker and cytoplasmic labeling could not be identified with certainty (Fig. 29e, f). This situation had changed again in late bicellular pollen where there was no labeling of the generative and vegetative nucleus while at the same time there were clear signals present in the peripheral cytoplasm of the vegetative and the generative cell (Fig. 29g, h).

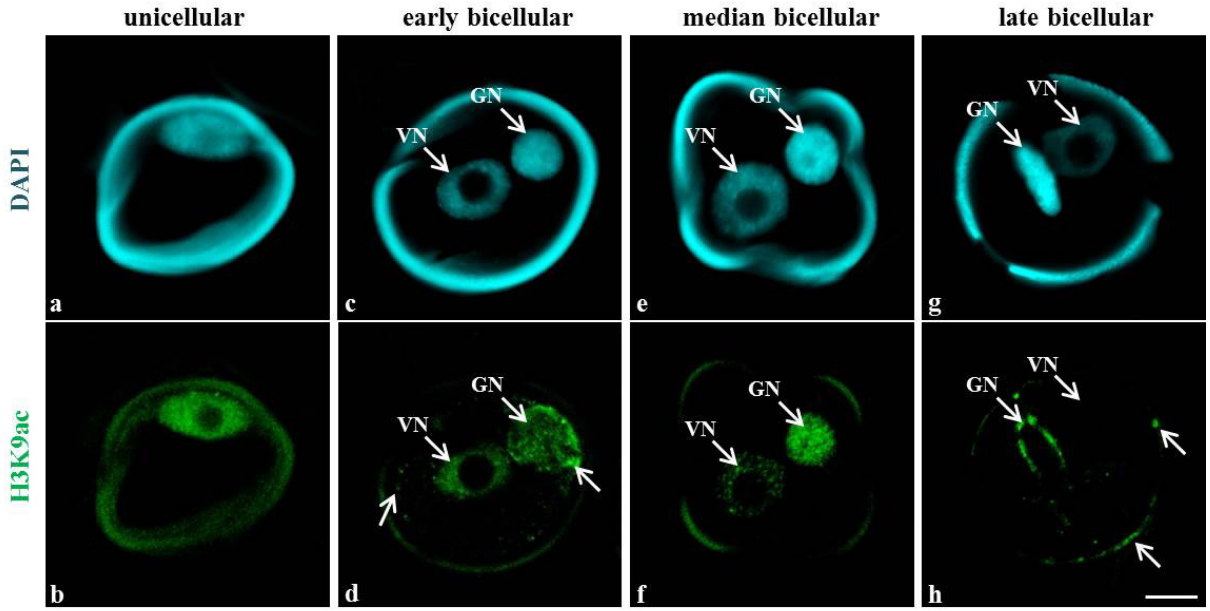


Fig. 29: Immunosignals of H3K9ac during tobacco pollen development. (a, b) H3K9ac present in nucleus and cytoplasm of unicellular pollen. (c, d) Vegetative and generative nucleus of early bicellular pollen label equally strong, cytoplasmic label has become spotty. (e, f) Median bicellular pollen shows weak label in vegetative and strong label in generativenucleus, cytoplasmic signals absent. (g, h) Late bicellular pollen with label confined to cytoplasm of generative and vegetative cell. DAPI in blue, antibody labeling in green. GN = generative nucleus, VN = vegetative nucleus. Bar = 5 µm.

3.2.3 The differences between tobacco and barley pollen labeling patterns

Two features distinguished the labeling results of the tobacco pollen from barley. The first observation was that the clarity of labeling strongly diminished with age. This phenomenon was already seen in the labeling of barley pollen, but in tobacco pollen it was more extreme. Up until median bicellular stages decent labeling patterns were obtained but in late bicellular stages labeling patterns deteriorated to such a degree that for several antibodies no labeling was observed anymore. Most striking in comparison to the results for barley, however, was the strong cytoplasmic component in most labeling patterns. Since there were not much information on accumulations of modified histones within the cytoplasm, this raised the question about the reliability of such staining. For an indirect assessment, antibodies were tested on transverse sections of barley roots made at or near the elongation zone. This way the general labeling

pattern of barley pollen could be compared with that of barley cells belonging to a number of different tissues.

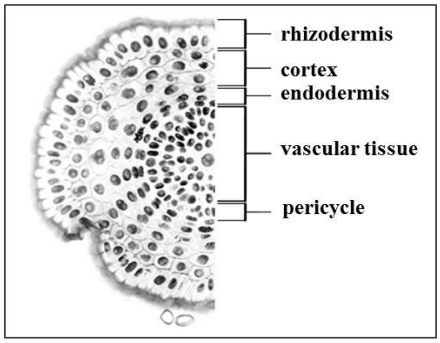


Fig. 30: Transverse section through the elongation zone of barley root. (doi:10.1371/journal.pone.0069204.g001)

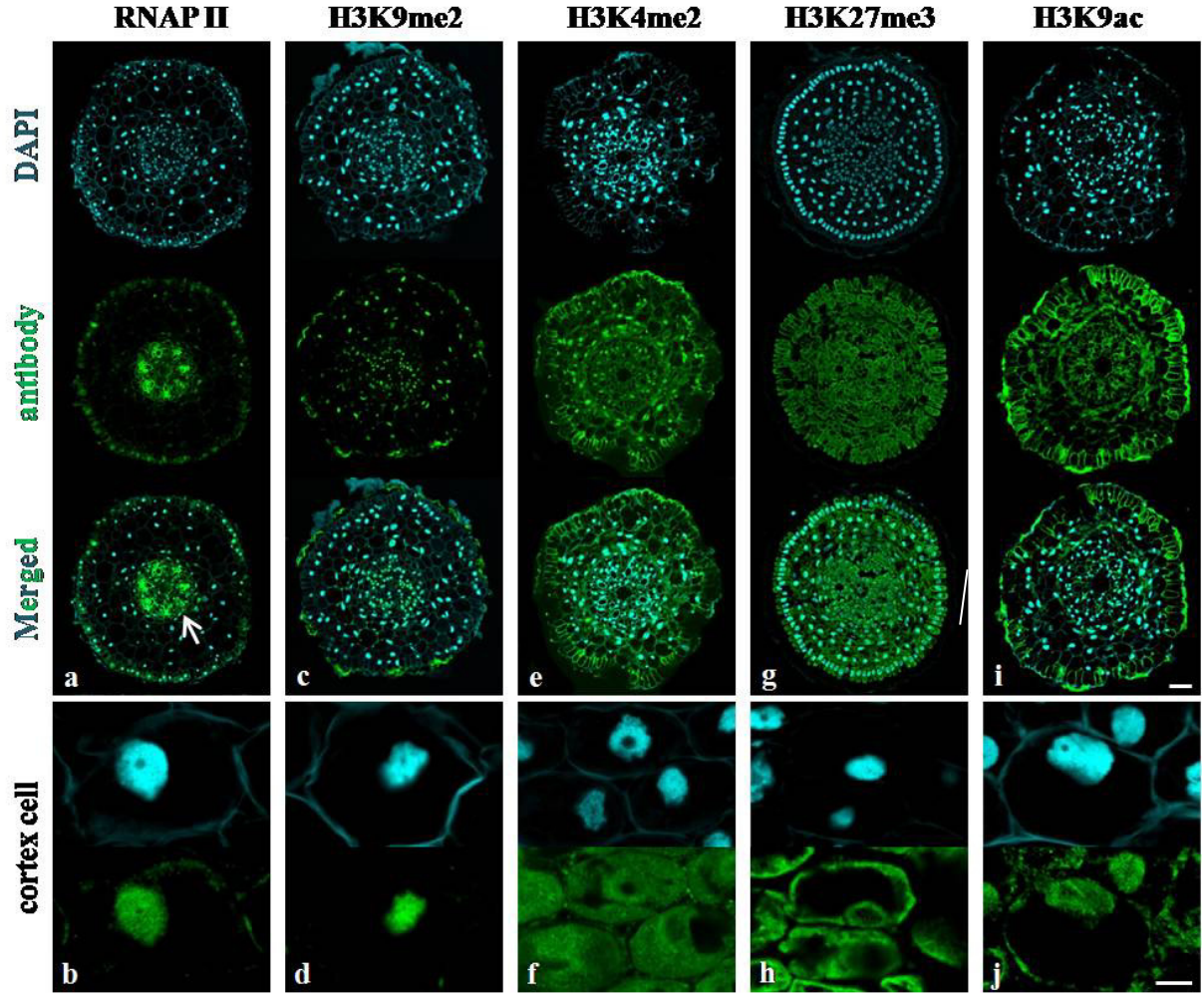


Fig. 31: Immunostaining of barley root transverse section and detailed view of cortex cell. DAPI in blue, antibody labeling in green. Bar = 50µm (root section), 10 µm (cortex cell)

Label for RNAP II was predominantly found in nuclei of cortex cells (Fig. 31a) and particularly strong in the vascular tissue (arrow in Fig. 31a). The histone modification H3K9me2 was found in the nuclei of all tissues with the exception of the rhizodermis (Fig. 31c, d). Signals for H3K4me2 (Fig. 31e, f), H3K27me3 (Fig. 31g, h) and H3K9ac (Fig. 31i, j) were present in the cytoplasm and nuclei of all root tissues. The results proved that cytoplasmic labeling patterns were common in many barley tissues. From this follows that the cytoplasmic profiles in tobacco pollen should be looked upon as a testimony of a different developmental program as compared to barley.

3.3 Barley pollen embryogenic development

3.3.1 Changing cell and nuclear size during pollen embryogenesis

Induction of pollen embryogenesis in barley consists of several distinct steps (Fig. 32). Pollen stages identified during embryogenesis are given in Fig. 33. Pollen was qualified as dead when DAPI staining showed a nucleus to be absent or clearly deformed.

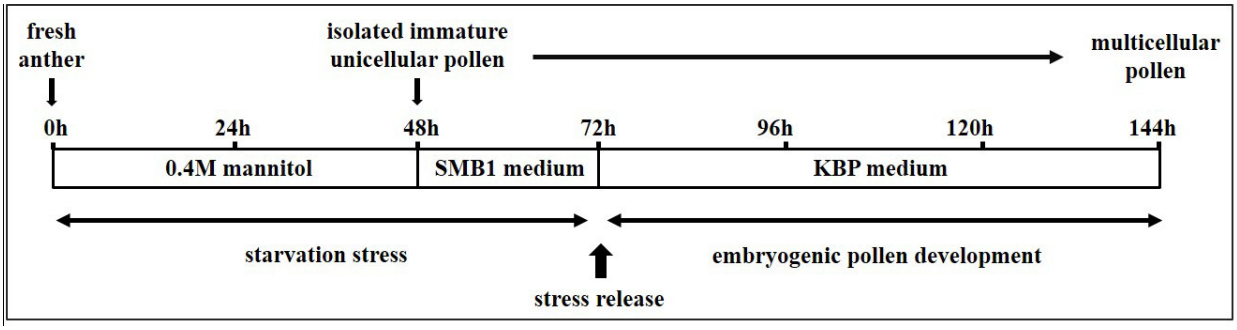


Fig. 32: Time scale of induction of embryogenesis in barley pollen according to Daghma (2011).

The transformation of the unicellular immature pollen into a multicellular structure has taken place within the boundaries of the original pollen wall. Although the pollen wall allowed for some flexibility and embryogenic pollen did increase significantly in size during embryogenic development (Fig. 34a), size increase of the pollen did not hold pace with mitotic activity. As a

consequence embryogenic pollen development was accompanied with a progressive reduction in average cell size (Fig. 34b, blue line). When average cell volume and average nuclear volume were plotted against total number of nuclei/cells within an embryogenic pollen, two trends become apparent. The first was that after an initial sharp decrease in volume, the graphs seemed to become more horizontal suggesting the existence of minimal threshold values for both nuclear and cellular volumes. The second trend was even more intriguing as it indeed seemed that in the confined space of an embryogenic pollen, cellular and nuclear volume were intimately linked in a fixed ratio of approximately 10:1 (Fig. 34b).

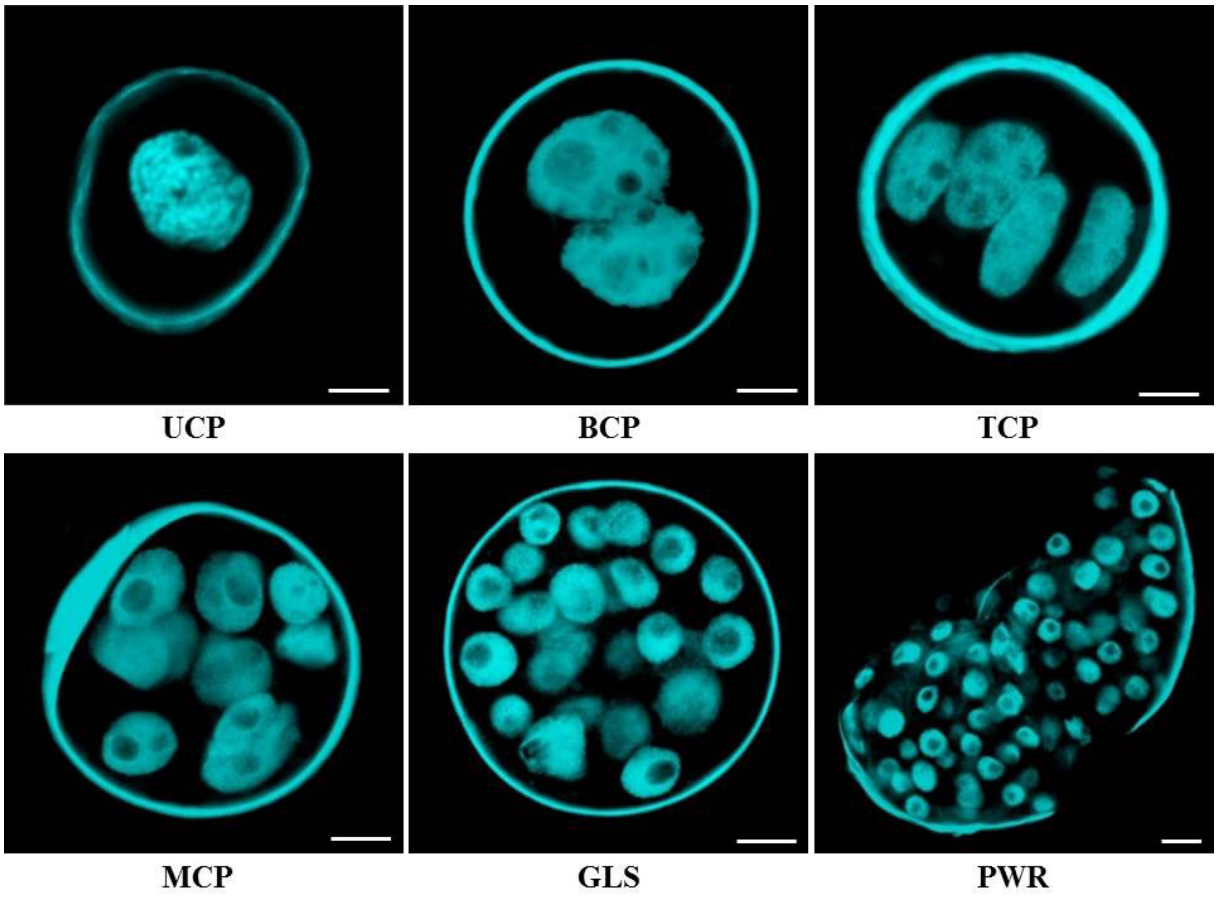


Fig. 33: Development of embryogenic barley pollen in culture after DAPI staining. UCP = Uni-cellular pollen, BCP = Bi-cellular pollen, TCP = Tetra-cellular pollen, MCP = Multi-cellular pollen with up to 12 nuclei, GLS = Globular structure with pollen wall still intact, PWR = Pollen wall ruptured. Bar = 10 µm.

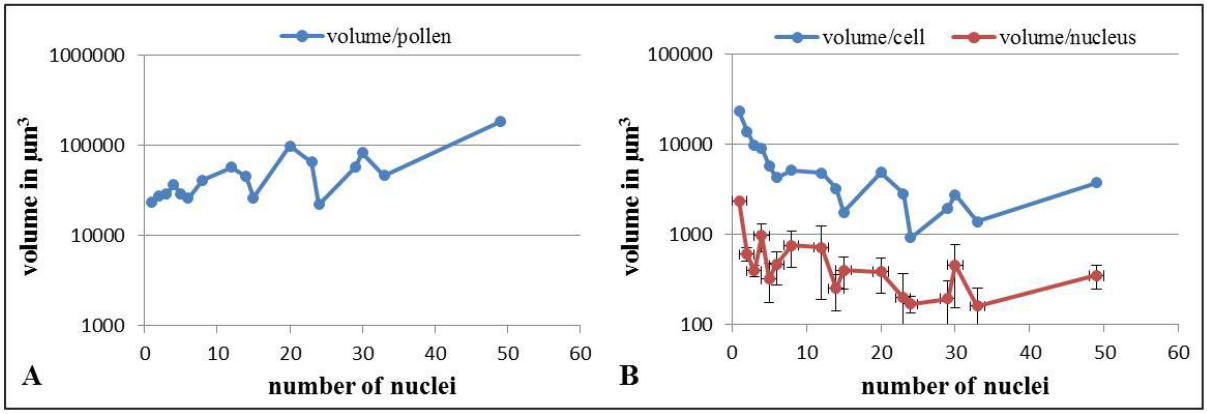


Fig. 34: Volume measurements on embryogenic pollen. (A) Volume of embryogenic pollen in relation to cell number. (B) Volume of cells and nuclei in embryogenic pollen in relation to cell number. Cell number in multicellular embryogenic pollen was considered identical to the number of nuclei seen after DAPI staining.

3.3.2 Chromatin modifications during pollen embryogenesis

The immunological analysis of nuclear dynamics during barley pollen embryogenesis was investigated with the same antibodies as employed for the pollen development study (3.2.). In each experiment the t = 0 h pollen were analysed as well, always rendering results typical for late unicellular pollen, i.e. centromere labeling by CENH3 and uniform nuclear labeling for RNAP II, H3K9me2, H3K4me2, and H3K9ac and a rather punctuate nuclear label pattern for H3K27me3 (Fig. 35, t = 0 h, see also 3.2.).

After anthers had been kept for 24 h (t = 24 h) in mannitol medium at 25 °C pollen labeling results for CENH3 and H3K9me2 remained unchanged (Fig. 35) yet intensities and distribution patterns of the other chromatin modifications had altered. Signals for RNAP II had become distinctly weaker as compared to before the start of starvation (Fig. 35) and in case of H3K9ac (Fig. 35) and H3K27me3 (Fig. 35) labeling was almost exclusively restricted to the cytoplasm with only weak traces left in the nucleus. Most striking, however, was the complete absence of H3K4me2 signals (Fig. 35).

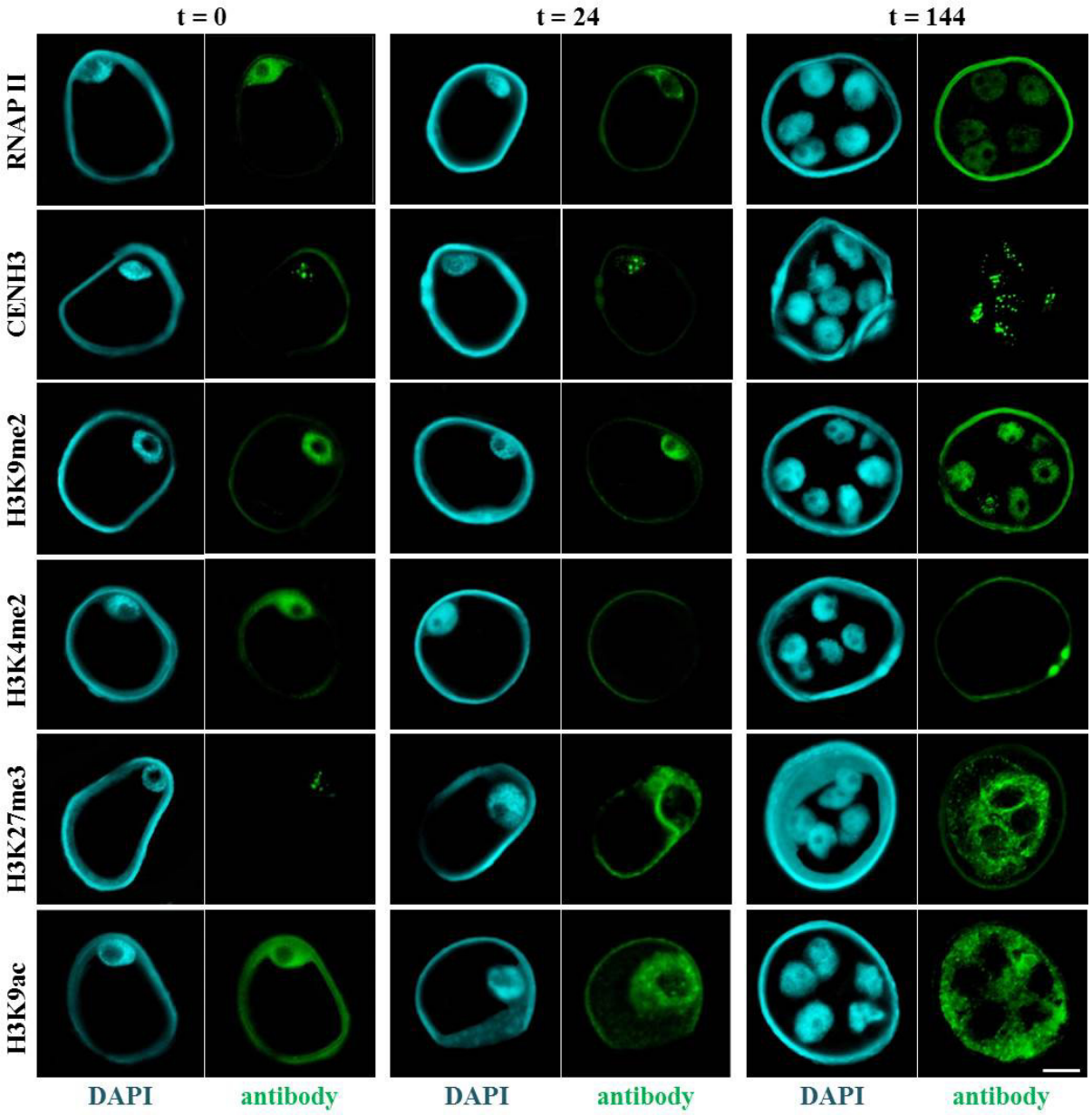


Fig. 35: Immunostaining of barley pollen during induction of embryogenesis. Pollen were analysed immediately before anther stress treatment (t = 0 h), after 24 h of anther stress treatment (t = 24 h) and three days after stress release (t = 144 h). Embryogenesis results in a weakening of RNAP II labeling, disappearance of H3K4me2, while label for H3K27me3 and H3K9ac becomes restricted to the cytoplasm. Note that all changes occur within 24 h after the start of induction. DAPI in blue, antibody labeling in green. Bar = 10 µm.

With intervals of 24 h chromatin modifications were followed for another 96 h. Although during this time highly vacuolated uninucleate pollen were isolated (t = 48 h), stress released (t = 72 h)

and eventually developed into multicellular structures (t = 144 h), distribution and label intensity of the chromatin modifications investigated did not change any further (Fig. 35). Thus, within 24 h of starvation treatment and while still within the anther, immature microspores acquired a distinctly different and stable reorganization of several important histone modifications. These changes were accompanied by a weaker labeling for RNAP II (Fig. 35) indicating an overall change in transcriptional activity.

For a more precise timetable of events, the epigenetic constitution of pollen was also investigated after 6, 12 and 18 h of starvation treatment. The results, however, were not conclusive. At all timepoints a mixture of embryogenic and gametogenic patterns was found (results not shown). Maybe due to biological variations between the samples there was no clear linear increase of embryogenic profiles with time. After further experiments were equally inconclusive, it was decided to not continue these investigations.

A second question that could not be answered with certainty was whether there is a hierarchy of events in the epigenetic transformation from gametogenic into embryogenic profiles. However, the fact that at all early timepoints examined there was a clear relocation of H3K9ac signals towards the cytoplasm, may indicate that the redistribution of H3K9ac preceded the relocations of the other histone modifications.

3.3.3 Chromatin alterations after first asymmetric mitosis during pollen embryogenesis

A clear sign of successful induction of embryogenesis is a symmetric PM I. In part of the cultured microspores, however, PM I was asymmetric yielding a large vegetative-like cell and a small generative-like cell. These cases were considered to comprise both unsuccessful and delayed inductions (Daghma, 2011). Previous studies including work from our group have revealed that after an asymmetric first division only the larger cell may undergo embryogenesis; the smaller of the two cells usually does not show mitotic activity anymore and eventually succumbs (Sunderland and Wicks, 1971; Daghma et al., 2014).

In PEG sections pollen that underwent asymmetric divisions can immediately be recognized after DAPI staining by the presence of a large weakly fluorescent nucleus accompanied by a small

strongly fluorescent one (Fig. 36B). In the asymmetric divisions identified this way distribution of chromatin modifications within the larger nucleus (Fig. 36B) appeared identical to those found in normal pollen embryogenesis, i.e. in the nuclei after a symmetric first mitosis (Fig. 36A).

Evaluation of the labeling results for the smaller cell was more complicated because precise boundaries between the large and small cellular unit are hard to establish, making it difficult to determine absence or presence of labeling within the cytoplasm of the generative-like cell. Focusing therefore on nuclear labeling patterns we found that similar to the large nucleus, the small generative-like nucleus labels positive for RNAP II and does not stain for H3K27me3, H3K9ac and H3K4me2 (Fig. 36B). However, in contrast to the larger nucleus, the smaller nucleus consistently stained negative for CENH3 and H3K9me2 (Fig. 36B).

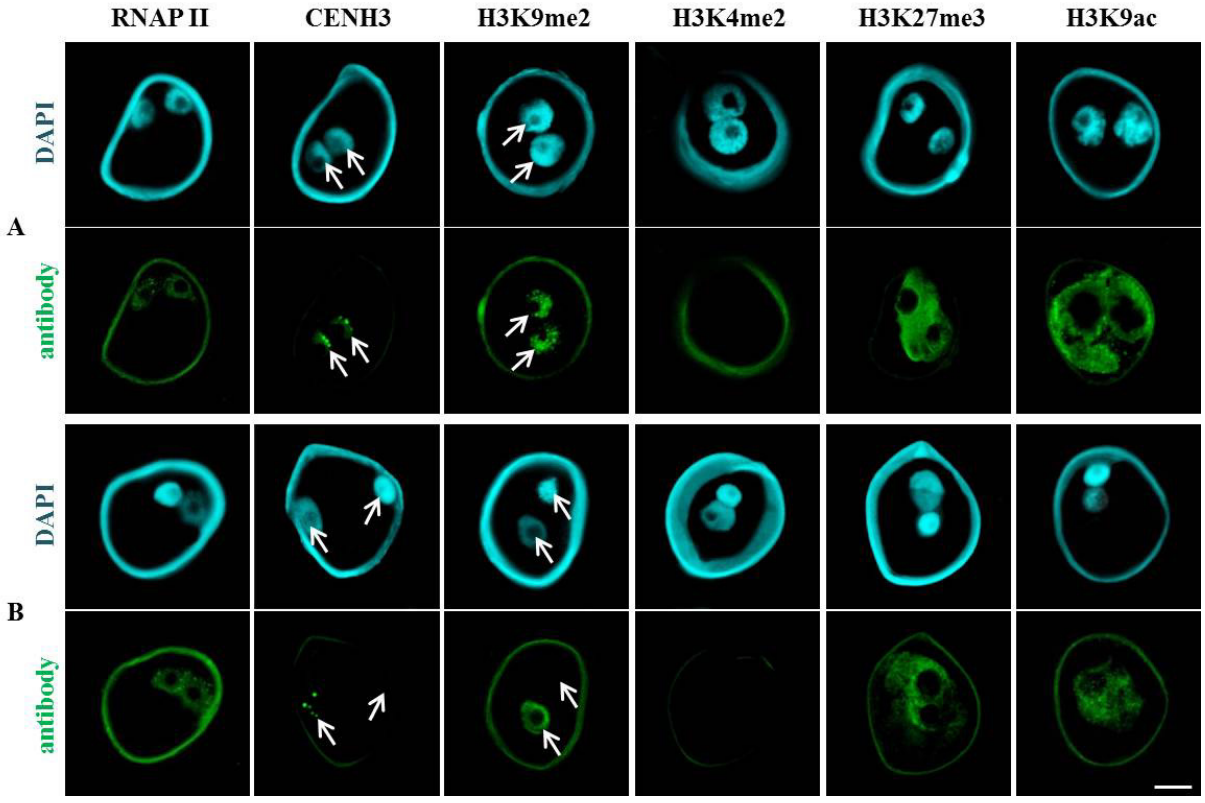


Fig. 36: Chromatin alterations in induced pollen after symmetric and asymmetric PM I. (A) Symmetric division yields nuclei of similar morphology and similar chromatin labeling patterns. (B) Asymmetric division yields two nuclei of unequal size, with chromatin labeling pattern of the large nucleus similar to that found in embryogenic

pollen while the smaller nucleus does not label for CENH3 and H3K9me2. DAPI in blue, antibody labeling in green. Bar = 10 µm.

3.3.4 Effect of inhibitor treatment on chromatin modification in embryogenic pollen

To investigate the dynamics of chromatin modifications in more detail, immature microspores were treated with specific inhibitors viz the DNA-methylation inhibitor 5-Azacytidine (5-AZA) and the histone deacetylase inhibitor Trichostatin A (TSA). In a first series of experiments the overall effect of inhibitors in DMSO and of DMSO without inhibitors on viability of embryogenic pollen cultures was investigated to establish optimum exposure times. For this two concentrations of inhibitors in DMSO were compared with the same concentrations of DMSO without inhibitors and controls without these additives. Inhibitors were added after the transfer of pollen into KBP medium i.e. immediately after stress release, and viability of pollen was checked at 24 h intervals (for details see 2.8.1.).

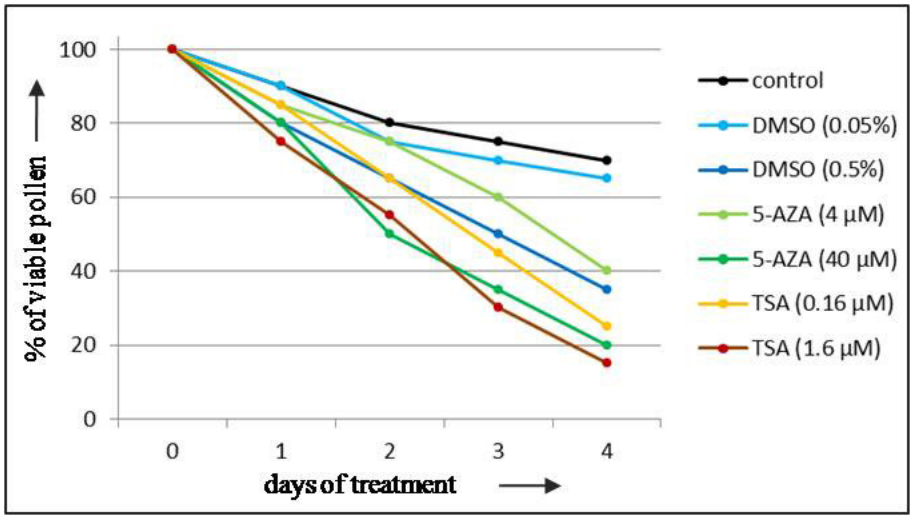


Fig. 37: Effect of TSA, 5-AZA and DMSO on viability of embryogenic pollen culture.

The graphs in Fig. 37 show that prolonged exposure to even low concentrations of TSA or 5-AZA seem to have a very detrimental effect on pollen survival rate. DMSO may play an important role in this effect since it will increase the permeability of cell membranes thus

possibly enhancing the effect of TSA and 5-AZA. On its own 0.05% DMSO had no noticeable effect, though when used at 0.5% the survival rate of pollen after 4 days of exposure was virtually similar regardless of whether TSA or 5-AZA was included or not. However, the graphs also show that treatment for only 24 h had no significant effect on viability. For an analysis of the effect of TSA and 5-AZA on embryogenic pollen, inhibition experiments were started after transfer of pollen into KBP medium (t = 72 h) and exposure time limited to 24 h.

3.3.4.1 Chromatin alterations after inhibition of DNA methylation by 5-Azacytidine

Immunological analysis of embryogenic pollen after 24 h exposure to 40 µm of 5-AZA failed to show a difference in intensity and distribution of DNA methylation signals before and after this treatment (Fig. 38). Distribution patterns of CENH3, H3K4me2, H3K9me2 and H3K27me3 were also not affected by this treatment (Fig. 39B). However, RNAP II signals appeared no longer present (Fig. 39B) and in case of H3K9ac a clear nuclear labeling was observed while cytoplasmic labeling was strongly reduced and restricted to the periphery of the cytoplasm (Fig. 39B). Similar data were obtained when pollen were exposed to 4 µM 5-AZA while the results after exposure to 0.05% DMSO were identical to those found in the controls (results not shown).

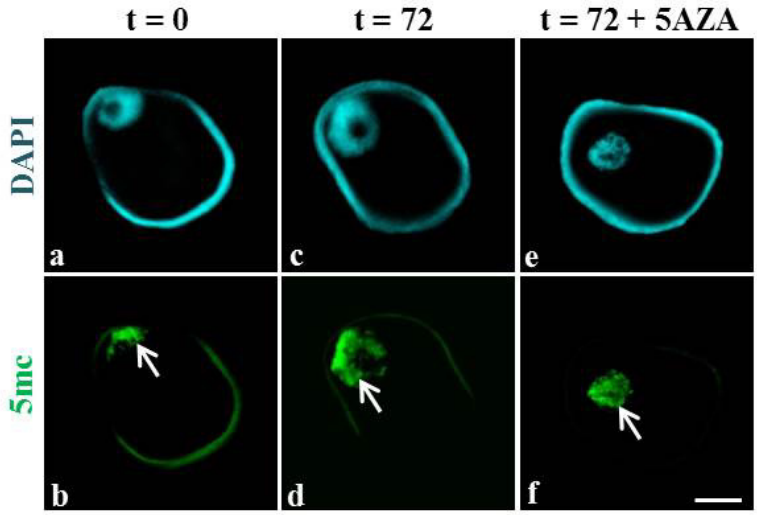


Fig. 38: Immunolabeling for methylated DNA (5 mc) in induced pollen of barley. Nuclear label of 5 mc before anther stress treatment (a, b) after stress release (c, d), andafter inhibition of DNA methylation by 5AZA (e, f). DAPI in blue, antibody labeling in green. Bar = 10µm.

3.3.4.2 Chromatin alterations after inhibition of histone deacetylase by Trichostatin A

In embryogenic pollen treated for 24 h with 1.6 µM TSA, labeling of H3K9ac was no longer in the cytoplasm but again found restricted to the nucleus (Fig. 39C). TSA treatment also changed the labeling patterns of H3K27me3 and H3K4me2 (Fig. 39C).

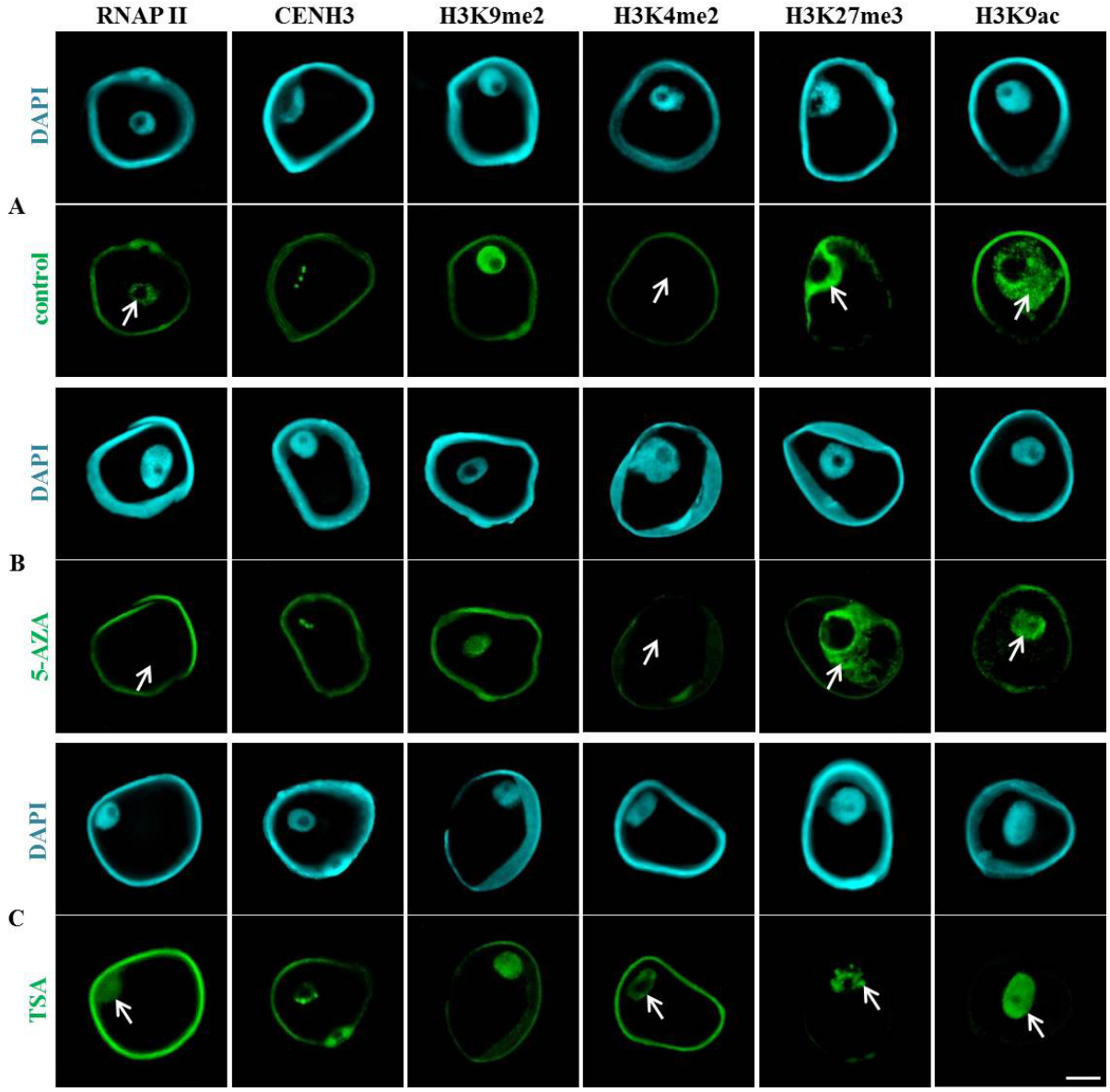


Fig. 39: Effect of DNA methylation inhibitor 5AZA and histone deacetylase inhibitor TSA on chromatin modifications in isolated embryogenic pollen of barley 24 h after stress release. (A) without inhibitors, (B) after 24 h exposure to 5AZA, (C) after 24 h exposure to TSA. DAPI in blue,antibody labeling in green. Bar = 10 µm.

As with H3K9ac, signals for H3K27me3 again displayed a nuclear localization often with spot like aggregations reminiscent of the subtelomeric distributions found in young untreated pollen (Fig. 39C, see Pandey et al., 2013). Remarkable also the reappearance of clear H3K4me2 signals in the nucleus (Fig. 39C) the more so as this histone modification had completely disappeared from the pollen within 24 h after the start of mannitol treatment. Labeling patterns of CENH3 and H3K9me2 remained unaffected while also RNAP II retained its relatively weak labeling intensity (Fig. 39C).

3.4 Trichostatin A as an enhancer for pollen embryogenesis

The deacetylase inhibitor TSA has the ability to alter cell fate (see 1.4). Recently, Li et al. (2014) reported on a greatly improved efficiency of pollen embryogenesis in rapeseed (*Brassica napus*) when pollen cultures were exposed to TSA for 20 hours. The same effect was also obtained in *Arabidopsis*, a species highly obstinate to embryogenic induction. While in rapeseed pollen treatment was started 20 h after isolation of the pollen, in case of *Arabidopsis* pollen were still in anther with treatment starting immediately after harvesting (Li et al., 2014).

Following these results, the potential of TSA for boosting the induction of barley pollen cultures was also tested. In accordance to the methods used by Li et al. (2014), barley pollen was exposed to 0.5 µM TSA for 24 h. Test studies performed for the initial inhibitor experiments described under 3.4.2 had already indicated that neither concentration nor duration should have a strong effect on pollen viability. To allow for an immediate application in barley induction protocols used in other groups, the procedure for pollen induction was slightly adjusted by extending the anther incubation period of isolated microspores in SMB1 from 24 h to 48 h before transfer into KBP at t = 96 h (Fig. 41). For a more comprehensive overview of the possible effect of TSA, the treatment of barley pollen was started at various time-points during the process. These 24 h treatments started immediately after harvesting of anthers (t = 0 h), immediately after pollen transfer into SMB1 medium (t = 48 h), after one day in SMB1 medium (t = 72 h) and after transfer of pollen into KBP medium (t = 96 h) (Fig. 41). For optimal exposure to TSA in DMSO

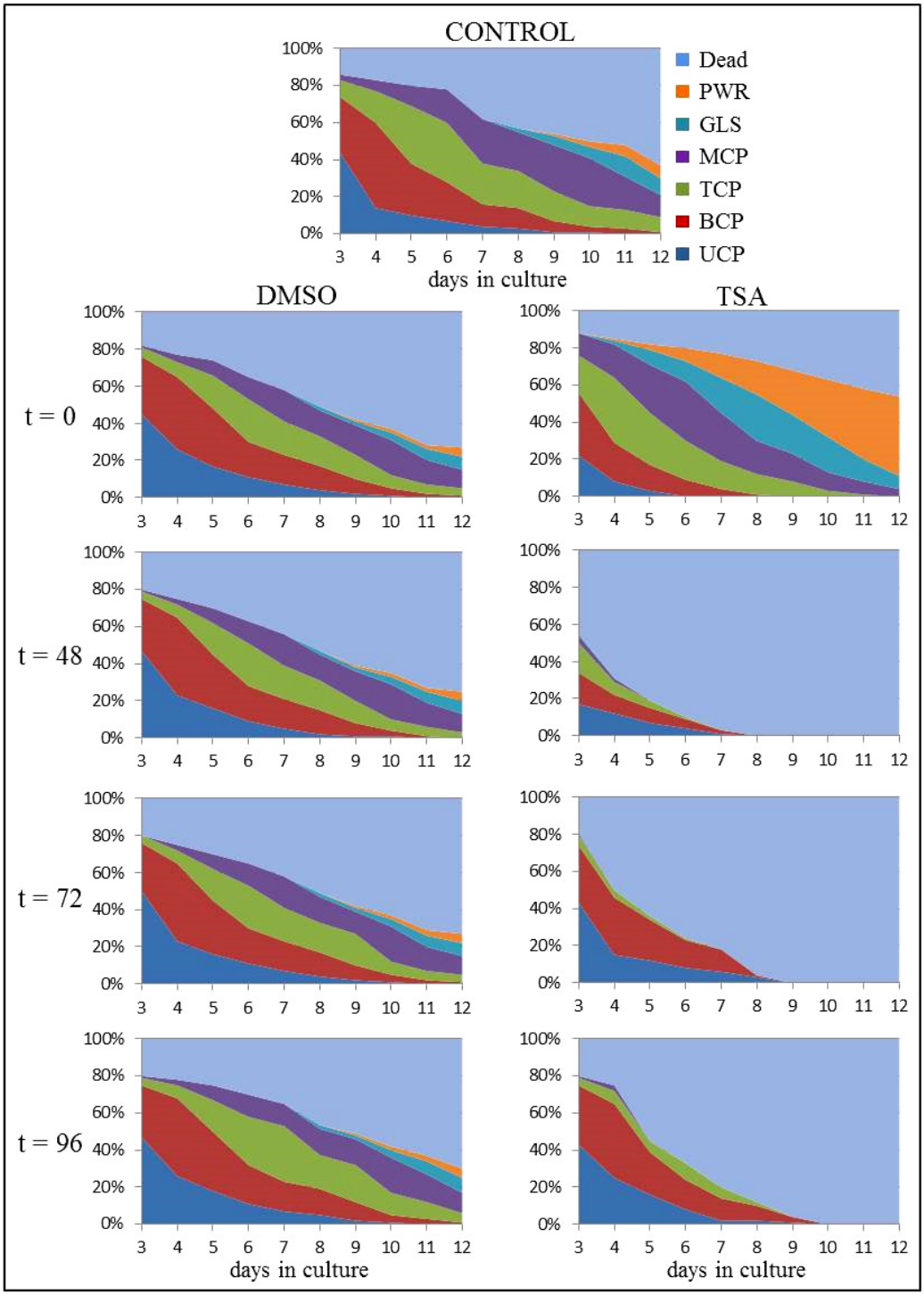


Fig. 40: Effect of TSA treatment regimes on the proportion of different pollen types over time in barley. Control without DMSO or inhibitor. Treatment with TSA in DMSO or DMSO alone was initiated after anther harvesting (t = 0), after isolation and transfer of pollen into SMB1 medium (t = 48 h), after one day in SMB1 medium (t = 72 h), and after transfer of pollen into KBP medium (t = 96 h). Starting at t =72 h (end of day 3), cultures were analysed on a daily basis until 12 days after the start of the induction protocol. (t = start of treatment)

and DMSO alone anthers were cut into three pieces. Starting at t = 72 h the composition of pollen cultures was analysed on a daily basis for a total of 10 days as described under 2.8.2.

The effects of TSA exposure on barley pollen embryogenesis are summarized in Fig. 40. The control (Fig. 40) showed that it is quite normal for a substantial amount of pollen to die within these first 12 days in culture. The pollen still alive at the end of this time were mainly embryogenic including multicellular and globular structures next to cellular aggregates that had broken the exine wall of the original pollen. This breakage of the exine wall was first observed after 9 days in culture (Fig. 40).

The populations exposed to DMSO showed results rather similar to those found in the controls, regardless of the time point at which DMSO was added (Fig. 40). With an overall survival rate of 25-35% DMSO treated cultures faired only slightly worse than the control with 40% survival rate. Also in the DMSO treated pollen exine breakage was first observed after 9 days in culture (Fig. 40).

The response of pollen to TSA appeared to be very strong and extremely dependent on the time-point of treatment. The most radical changes were seen when exposure was directly after selection and transfer of the anthers into starvation medium equals t = 0 h (Fig. 40). Under these conditions overall survival rate at the end of day 12 was distinctly higher than found in any control population (Fig. 41). Most of these pollen were clearly embryogenic with many showing exine breakage (Fig. 40). This last phenomenon marked the most clear-cut difference with the controls where exine breakage after at the end of 12 days in culture accounted for a rather stable 5% of the pollen involved. In contrast to this early TSA-treated pollen displayed a staggering 40% exine breakage rate at this time. Similarly remarkable was the fact that first samples of exine breakage were already present after 4 days in culture, compared to after 9 days in the controls (Fig. 40). If, however, TSA treatment was initiated on a later time in the pollen induction process, results were radically different. Whether TSA was added after pollen isolation (t = 48 h), after 24 (t = 72 h) in SMB1 medium or after the transfer into KBP medium (t = 96 h), all pollen died within 6 days after the start of exposure to TSA (Fig. 40).

Pollen cultures that were maintained for much longer times showed that early TSA-treatment resulted in a high number of small callus-like structures being remarkable uniform in size (Fig. 41).

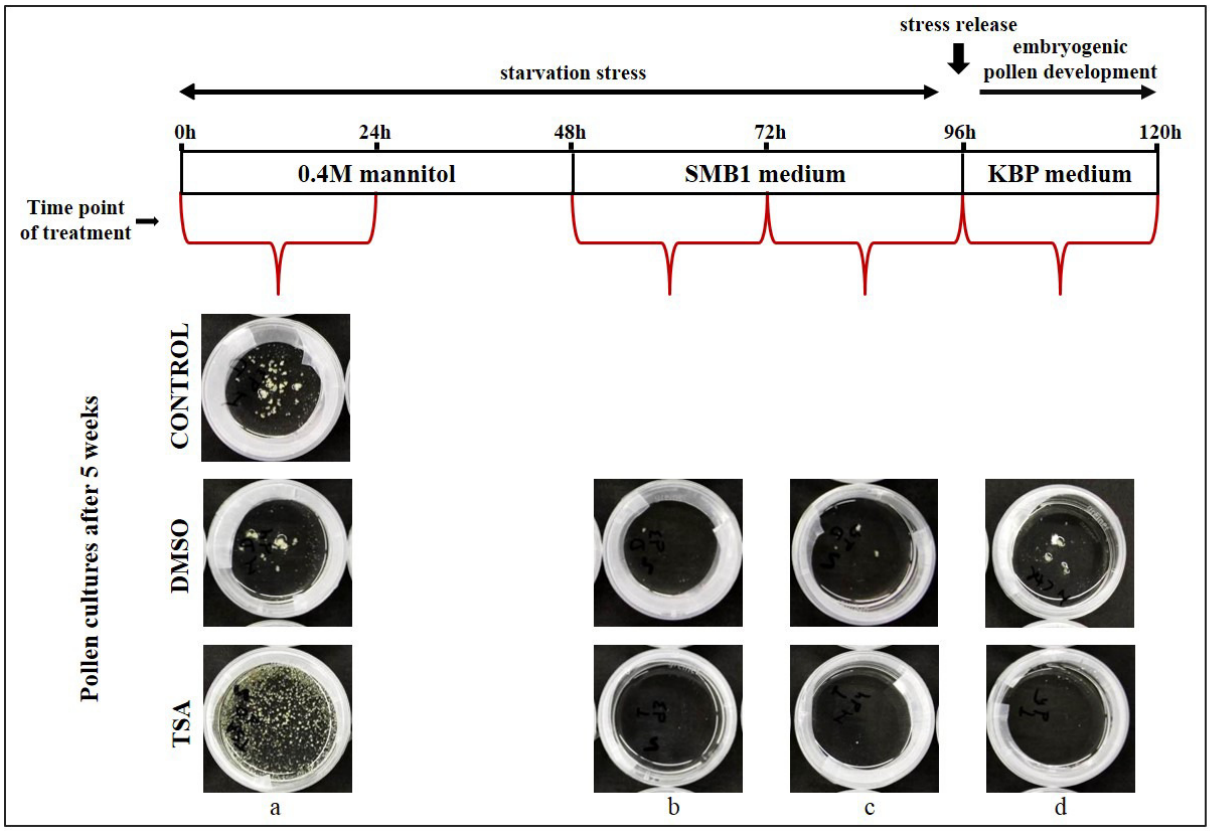


Fig. 41: Five weeks old embryogenic barley pollen cultures showing the effect of 24 h treatment of TSA at different time points (red brackets). (a) Treatment of anthers in mannitol with TSA and DMSO at t = 0. (b) Treatment of isolated pollen in SMB1 at t = 48 h. (c) Treatment of pollen after 24 h of isolation in SMB1 at t = 72. (d) Treatment of pollen at the start of induction in KPB medium at t = 96 h. Control is without DMSO or TSA.

This indicated that a correct timing of TSA application can not only improve the efficiency of induction of pollen embryogenesis but also enhance and synchronize embryogenic development.

4 DISCUSSION

Male gametogenesis begins with the differentiation of the pollen mother cell from somatic cells in the adult plant. Through meiosis the pollen mother cell gives rise to four haploid immature pollen termed microspores, which undergo a first mitotic division producing a bicellular pollen grain consisting of one vegetative and one generative cell, followed by a second mitotic division of the generative cell generating two sperm cells. The final product is a terminally differentiated organism, yet for some time after the meiotic division of the pollen mother cell, the young haploid pollen remains developmentally labile. Application of stress, usually in the form of temperature or nutrient deprivation (Dickinson, 1994), can divert pollen toward excess cell proliferation that, under artificial conditions, can entail embryogenic pattern formation and the eventual production of doubled haploid plants (Hoekstra el al., 1992). Despite its huge importance for modern day agriculture the field of pollen embryogenesis is remarkably poor on fundamental research. As underlying mechanisms are not well understood, it has proven difficult to break the deadlock in species recalcitrant to pollen embryogenesis, among which various important crop plants (Riley, 1974). A better understanding of embryogenesis requires a two-pronged approach. For one embryogenesis should be dissected in its individual processes. These include arrest and reversal of the state of differentiation, the acquisition of totipotency and re-entry into the cell cycle (Grafi et al., 2004). On the other hand those stages of the pollen development that can be induced have to be investigated more closely. The molecular mechanisms of pollen development have been studied extensively and recent years have seen a wealth of new information on the role of epigenetics (see 1.3.). Still, even now interest often by-passes the stages prior to and immediately after PM I, stages most amenable to induction of embryogenesis. Remarkable meager is the information on histone modifications despite reports that implicate highly dynamic changes during this time in pollen development (Sano and Tanaka, 2010; Houben et al., 2011). The aim of this thesis was to extend our knowledge on pollen embryogenesis by investigating histone modifications in normally developing (gametogenic) and embryogenic pollen. In the course of this approach, results highlighted the promising role of acetylase inhibitors for increasing induction efficiency.

4.1 Nuclear morphology in pollen development

Besides the genotype the most important factor determining the efficiency of embryogenic induction is the developmental stage of the pollen (Heberle-Bors, 1985, 1989). Generally, unicellular or early bicellular pollen are most amenable for induction. In many species, however, the transition from the immature unicellular pollen to the mature bi- or tricellular pollen is highly asynchronous (Vasil, 1967; Ostrolucka, 1983; Wang et al., 2009), representing a potentially yield-limiting factor. For the monocot barley and the dicot tobacco a profile of pollen development was compiled from the late unicellular stage onwards based on nuclear morphology after DAPI staining (Fig. 8 and Fig. 13). The results revealed several hitherto poorly described features providing valuable background information for the immunological studies.

4.1.1 Pollen development in barley

Barley belongs to those species in which pollen development is asynchronous. Not only do pollen mature earlier in the central part of a barley spike, different stages of development are also found within a single anther and between the anthers of a single flower.

Intriguingly the two sister nuclei arising after PM I appear to remain similar in size and of similar fluorescence intensity for some time making an early identification of generative and vegetative nucleus after DAPI staining often impossible. Based on this nuclear morphology one could argue that after the asymmetric PM I, cellular identity of designated generative and vegetative cells initially may remain similar for a certain period of time. This statement seems contradicted by the studies of Terasaka and Tanaka (1974) and Terasaka (1982) who reported clear differences between vegetative and generative chromatin even before the end of karyokinesis. The explanation lies in the asymmetric spindle which is much shorter on the side of the generative cell. When the chromosomes move apart this asymmetry ensures that the chromosomes of the generative cell converge stronger than those of the vegetative cell. Using rye Banaei et al. (2012) showed that as a result the centromeres in the generative cells are initially less dispersed than those of the vegetative cell. Similar observations were made for the CENH3 markings in early bicellular barley pollen (Fig. 19). Evidently, the DAPI staining used in

this study, though easier to perform as compared to the aceto orcein staining used by Terasaka and Tanaka (1974) and Terasaka (1982), gives fewer details concerning chromosome patterning. Although nuclear spatial positioning may affect gene activity (Misteli, 2004) distributions of epigenetic modifications like H3K27me3 did not show any noticeable difference between the nuclei of early bicellular pollen (Fig. 22). The fate of generative-like cells after induction of embryogenesis, discussed in 4.4., indicates that the differential distribution of CENH3 probably plays no, or only an auxiliary role in the process of pollen differentiation.

After PM I the vegetative nucleus continuously increases in size indicating ongoing chromatin decondensation (Fig. 8). Evaluation of bicellular pollen with nuclei of similar size revealed that also generative nuclei initially undergo similar style decondensation as the vegetative nucleus (Fig. 8). Size increase in late anther stage generative nuclei was the result of DNA replication prior to PM II (Fig. 8). Diameter measurements of nuclei (Fig. 11) showed that doubling of the DNA content is accompanied by an average volume increase of approximately just 30%. Accordingly the newly synthesized DNA is stored in a more highly condensed form, thus contributing to the heterochromatic character of the generative nucleus towards PM II (Mascarenhas, 1993). It remains unclear what, if any, role the initial partial and variable degree of chromatin decondensation within the generative nucleus has. Although decondensation greatly enhances the replication of DNA, condensed DNA is also replicated (Nagl, 1977). The DNA replication machinery is based on transient interactions of replication enzymes with a stable DNA bound core consisting of the so-called processivity factor PCNA (Schermelleh et al., 2007; Gorisch et al., 2008). Assembly of new PCNA occurs at sites adjacent to the existing ones showing that replication of a genomic region facilitates the loading of new replication factors at neighboring sites, possibly by opening the chromatin conformation (Sporbert et al., 2002; Sadoni et al., 2004). This model, in which replication, once started, can rapidly spread across whole genomes, Chagin et al. (2010) refer to as the domino model. As more and more of the chromatin starts replication eventually also heterochromatin becomes replicated. The role of chromatin modifications in this process is still unclear although some studies suggests they may play a role in facilitating replication of heterochromatic regions (Collins et al., 2002; Quivy et al., 2008).

4.1.2 Pollen development in tobacco

Tobacco, in contrast to barley, shows a higher degree of synchronicity in its pollen development (Fig. 15). The most compelling difference between barley and tobacco pollen development is the different orientation of the spindle. Based on the organization of the mitotic chromosomes the spindle in tobacco is always formed along the direction of nuclear migration (Fig. 42) whereas in barley the spindle orientation is perpendicular to the nuclear migration (Fig. 42). Also a careful re-examination of time-lapse recordings showing asymmetric PM I in barley (Daghma 2011, 2014) revealed the plane of division to be parallel to the pollen wall. As a result early daughter nuclei are always lying on top of each other in tobacco and side by side in barley (Fig. 42). This, so it seems, has important implications for the differentiation of the generative nucleus. In tobacco the daughter nuclei exist along an axis of polarity and are therefore exposed to different factors (Twell, 1998). The phragmoplast formed perpendicular to the axis of polarity guarantees the enclosure of the generative nucleus in a small fraction of polarized cytoplasm. This would explain why even with DAPI staining it was always possible to distinguish generative and vegetative nuclei. Along this path in tobacco the designated generative cell may assume different cell identity very early on which may explain why in contrast to barley, induction of embryogenesis in tobacco apparently only involves the vegetative cell (Sunderland and Wicks, 1971; Bhojwani et al., 1973; Žárský et al., 1992). In barley, on the other hand, daughter nuclei after PM I may initially end up in cytoplasm of similar constitution. Only after completion of the phragmoplast and spatial separation of the nuclei, differential development may start. This may explain why daughter nuclei in barley remain similar for some time after PM I.

Although the data showed that the orientation of the spindle can vary, with possible consequences for the developmental program, there is hardly any literature on this topic. Most studies have indeed found spindle orientation to be along the axis of nuclear migration (see e.g. Heslop-Harisson, 1968; Eady et al., 1995; Twell et al., 1998). The consistency of this mechanism, however, was already challenged by Dunwell and Sunderland (1976b) who found spindle orientation in the dicot species *Datura innoxia* to vary between parallel and oblique to the pollen wall. This raises questions as to whether spindle orientation is fixed or flexible,

species dependent, and most important, whether or not it plays a role at all in pollen development.

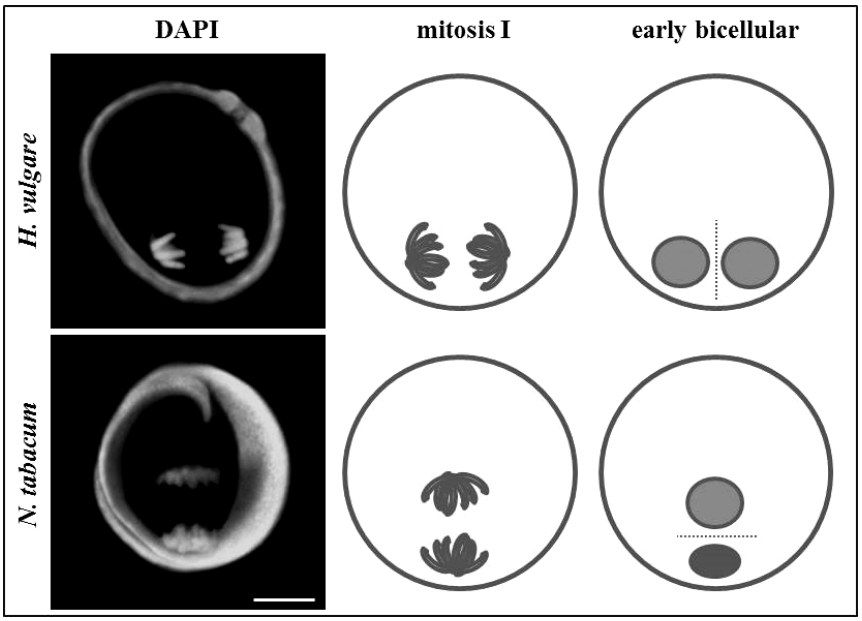


Fig. 42: Different orientation of PM I between barley and tobacco as shown by DAPI staining and schematic diagram. Dotted line shows position of phragmoplast. Bar = 10µm.

Spontaneous mitosis of the vegetative cell was never observed in barley but is a remarkably common feature in tobacco found in 1 to 2 % of all early bicellular pollen (Fig. 17). Many researchers must have come across this facet of tobacco pollen devleopment which makes it hard to understand why it is so rarely explicitly mentioned. Kyo and Harada (1986) reserve this phenomenon a few words hidden in the "material and methods" section. This spontaneous mitosis of the vegetative cell challenges the validity of generally held believes surrounding pollen development, viz the fixed identity of the vegetative cell and its arrest in G0 (Bino et al., 1990, Žárský et al., 1992). Pulse chase experiments performed in the 1960s and 70s made Hsemann (1973) concluding that, although with some delay, also vegetative cells may start DNA replication which, depending on the plant species, may or may not be completed.

Epigenetic imprinting after meiosis makes that unicellular pollen assume a vegetative cell identity by default (Eady et al. 1995). Asymmetric PM I enclosing one daughter nucleus into a small portion of polarized cytoplasm triggering a secondary imprinting event leading to the formation of the final male germ line. Identity of the male germ line therefore is the cumulative effect of a primary and secondary imprinting step. Differences in barley and tobacco pollen development may be the result of an inverse relationship between these two imprinting steps. Spindle orientation (Fig. 42) suggested that tobacco possesses a more efficient mechanism for asymmetric PM I, and thus secondary imprinting, than barley. At the same time the epigenetic profile of unicellular pollen (see below) suggested a stronger primary imprinting event in barley. One may hypothesize that due to an efficient PM I, tobacco can afford a weaker imprinting of vegetative cell identity. The latter may therefore retain many somatic traits leading to the high incidence of spontaneous embryogenesis observed in early bicellular pollen populations.

4.2 Chromatin modifications

4.2.1 Chromatin alterations during barley pollen development

Pollen gametogenesis is a classic example of the dynamic relationship between nuclear differentiation and histone alterations. The chromatin modifications followed during pollen development reflected the gradual differentiation of the unicellular into the late bicellular pollen. Most obvious were the changes in transcriptional activity which previous studies found to be much lower in the generative nucleus compared to the vegetative nucleus (Saito et al., 1997; Borg et al., 2009). The RNAP II labeling confirmed this condition but also showed that in the early bicellular pollen, both nuclei appear equally active in transcription (Fig. 18). Evidently, transcriptional activity of the generative nucleus is suppressed during its development.

After PM I the vegetative nucleus became arrested in G0 and terminally differentiated into a helper cell that does not participate in reproduction (Tanaka, 1997). Without the need for mitotic activity in *Arabidopsis* centromeric chromatin of the vegetative nucleus became disrupted before PM II (Ingouff et al., 2007; Schoft et al., 2009). In rye, however, centromeres remain intact as conspicuous CENH3 signals were found in both generative and vegetative nuclei after PM I and

PM II (Houben et al., 2011). The same authors also found no differences in condensation degree between the centromeric regions of vegetative and generative nuclei. In barley, CENH3 signals were equally strong in vegetative and generative nuclei of early bicellular pollen yet in late bicellular and tricellular pollen the vegetative nucleus labeled consistently weaker (Fig. 19). This suggests that for some time after PM I vegetative cells retain intact centromeres and that their progressive instability and disruption over time may follow a species dependent pattern.

Cellular differentiation is usually accompanied with an increase of repressive heterochromatin, which results in a reduction of transcriptional activity (Loring et al., 2001, Yadav et al., 2013). The distribution and label intensity of H3K9me2, one of the most prominent markers for transcriptional suppression (Liu, 2010; Vaillant and Paszowski, 2007) was commonly present in all stages of development without any local or temporal changes in abundancy. Similar observations were reported by Lienert et al. (2011) who suggested that H3K9me2 may serve comparable functions in all cell types. Cellular differentiation may require local rather than large-scale changes in transcriptional activity and tissue-specific regulated genes may thus carry a distinct chromatin signature, opposite to ubiquitously expressed genes.

One such candidate for local control of gene expression is H3K27me3 which is a major silencing mechanism in plants (Zhang et al., 2007) and thought to direct gene expression in the vegetative nucleus (Okada et al., 2006; Sano and Tanaka, 2010). This histone modification is primarily involved in the developmental repression of endogenous genes (Schubert et al., 2005; Lindroth et al., 2004) although recent studies have shown that H3K27me3 can also promote gene expression indirectly by repressing miRNA genes (Lafos et al., 2011). This last function may explain why in the course of pollen development H3K27me3 signals disappeared from the transcriptionally inactive generative nucleus but remained in the active vegetative nucleus (Fig. 22). In the latter H3K27me3 signals were almost restricted to subtelomeric domains. Generally, these regions are gene-poor and heterochromatic in character (Fuchs et al., 2006; Vaquero-Sedas et al., 2010) but also a source of non-coding RNAs (Schoeftner and Blasco, 2009) underlining a possible role in controlling miRNA expression.

H3K4 methylated markers are predominantly associated with transcriptionally active genes (van dijk et al., 2010). In *Arabidopsis*, approximately two-thirds of all *Arabidopsis* genes at any single

developmental stage contain at least one type of H3K4 methylation suggesting that H3K4 methylation is required for the normal expression of a large number of genes in plants (Zhang, 2009). In human cells H3K4me2 has been found involved in tissue specific gene regulation (Pekowska et al., 2010). In plants H3K4me2 and H3K27me3 often co-localize (Zhang, 2009). In agreement with this both H3K4me2 and H3K27me3 signals were stronger in subtelomeric regions (Fig. 21 and Fig. 22). However, the possibility of H3K27 and H3K4 acting as bivalent switches to turn on/off their associated genes during development (Lan et al., 2007; Swigut and Wysocka, 2007) seems unlikely. Towards late binuclear stage H3K27me3 signals remain in the vegetative nucleus but disappear from the generative nucleus whereas H3K4me2 remains in the generative but becomes substantially weaker in the vegetative nucleus. Restriction of H3K4me2 signals to the generative nucleus has also been found in lily (Okada et al., 2006; Sano and Tanaka, 2010), *Arabidopsis* (Cartagena et al., 2008), oak (Ribeiro et al., 2009) and rye (Houben et al., 2011). The predominance of H3K4me2 in the generative nucleus at a time when the latter is transcriptionally largely inactive hints at a role in suppression rather than activation of transcription. In erythroid development a subset of transcriptionally silent genes was identified characterized by containing H3K4Me2 but not H3K4me3 (Orford et al., 2008). Also in rice transcriptional activity was positively correlated with the ratio of H3K4me3/H3K4me2 revealing that genes with predominantly H3K4me3 were actively transcribed, whereas genes with predominantly H3K4me2 were transcribed at moderate levels (Li et al., 2008). Labeling for H3K9ac, invariably correlated with transcriptional activation (Pfluger & Wagner, 2007), was present in interphase nuclei of uninucleate pollen but absent from mitotic and early bicellular pollen (Fig. 24). This coincides with observations of Li et al. (2005) reporting a significant reduction in the acetylation level of H3K9/14 in tobacco protoplasts during mitosis. This is an additional indication that at least for some time after PM I, there is no significant difference between the transcriptional activity of vegetative and generative nuclei. With time acetylated histones reappear in the cytoplasm first before being found again in the nuclei (Fig. 24). The cytoplasmic pool of H3K9ac may be under the control of type B histone acetyltransferases. This group of acetyltransferases, originally defined as cytoplasmic enzymes, acetylate newly synthesized histones (Kuo & Allis, 1998). In maize meristematic cells, type B activity coincided with DNA replication (Lechner et al., 2000). Nuclear acetylation reappeared in later binuclear

stages, first in the vegetative nucleus followed by the generative nucleus as well. Acetylation of the nuclear histones may be the work of type A histone acetyltransferases (Carrozza et al., 2003) and may correlate with an increase in transcriptional activity in the vegetative nucleus. Acetylation in the generative cell is unlikely to be a sign of transcriptional activity. Zhou et al. (2010) found that the positive regulatory effect of H3K9ac can be attenuated by a combination of repressive marks like H3K27me3. It may also be that histone acetylation in the generative cell plays a role in histone replacement during cell maturation (Christensen & Dixon, 1982).

4.2.2 Chromatin alterations during tobacco pollen development

Analogous to barley also in tobacco RNAP II was initially present in the nuclei of generative and vegetative cells before it eventually disappeared from the generative nucleus (Fig. 27). Similarly label for H3K9me2 was equally strong in both cells before it could no longer be detected in late bicellular stages (Fig. 28), and also H3K4me2 was present in nuclei of generative and vegetative cells with strongest signals in the former (Fig. 29). Other labeling results were in clear contrast to barley. At the median bicellular stage H3K4me2 showed an additional cytoplasmic signal while marks for H3K27me3 were exclusively cytoplasmic at all stages of development (Fig. 30). Distribution of H3K9ac was almost opposite to that found in barley: initially equally strong in the nuclei of generative and vegetative cells, before becoming stronger in the generative nucleus only to end up restricted to the cytoplasm of both cells (Fig. 31).

Evidently pollen gametogenesis in barley and tobacco are under different epigenetic control. The labeling patterns of H3K27me3 and H3K9ac seem to suggest a more stringent repression of gene activity in the barley pollen from unicellular stages onwards. A possible reason may be found in PM I. In tobacco the nucleus of the designated generative cell is pinched between the pollen wall and the nucleus of the vegetative cell with the phragmoplast effectively encapsulating the generative nucleus in a small volume of polarized cytoplasm. Through its different orientation the spindle in barley executes this process in a probably less efficient manner. In this view effective isolation of the generative nucleus in cytoplasm rich in inducing factors may reduce the need for early pre-determinative epigenetic imprinting. Due to such low level imprinting,

immature pollen identity may remain close to that of normal somatic cells which could explain why in tobacco vegetative cells regularly undergo spontaneous mitosis (see 3.1.2.2.) which in barley they never do.

4.2.3 Histone reshuffling during barley pollen embryogenesis

Similar to embryogenic stem cells, plant stem cell maintenance involves the active suppression of differentiation by transcriptional repression (Loring et al., 2001, Yadav et al., 2013). This corresponds with the significant changes in epigenetic modifications observed during the induction of pollen embryogenesis. With the exception of CENH3 and H3K9me2, all other modifications investigated showed clear changes in intensity or distribution (Fig. 43). In agreement with reports on fast cellular responses on stress, immature pollen acquired their new epigenetic patterns within 24 h under stress. Once obtained, the epigenetic patterns remained during the subsequent transfer into induction medium and development of multicellular structures. This agrees with studies on *Arabidopsis* embryogenesis showing that expression profiles within the developing embryo do not change significantly until the globular stage has been reached (Xiang et al., 2011).

The weak label for RNAP II (Fig. 35) and the near to absence of H3K9ac signals from the nuclei (Fig. 35) suggest that embryogenic pollen display a reduced transcriptional activity (Berger, 2002; Nightingale et al., 1998; Hnilicova et al., 2011). Fluorescence signals for H3K4me2 were rapidly and completely lost from microspores under starvation stress (Fig. 35). Similar results were also found in rice under hypoxia stress (Tsuji et al., 2006) a condition which many plants react to with metabolic adaptations (for review Drew, 1997; Liao and Lin, 2001). Since during pollen gametogenesis H3K4me2 signals also became weaker in the nucleus of the metabolic active vegetative cell and stronger in the nucleus of the metabolic inactive sperm cells (Pandey et al., 2013) it can be speculated that H3K4me2 may play a role in the regulation of complex metabolic processes. Like H3K4me2 the modification H3K27me3 is also associated with gene repression (Schubert et al., 2005) and may be directly involved in the repression of cell cycle genes (Blais et al., 2007). This would explain why during gametogenesis H3K27me3 was

restricted to the metabolic active but mitotic inactive vegetative nucleus (3.2.5. see also Pandey et al., 2013) while during embryogenesis it completely disappeared from the, now mitotic active, nuclei (Fig. 43).

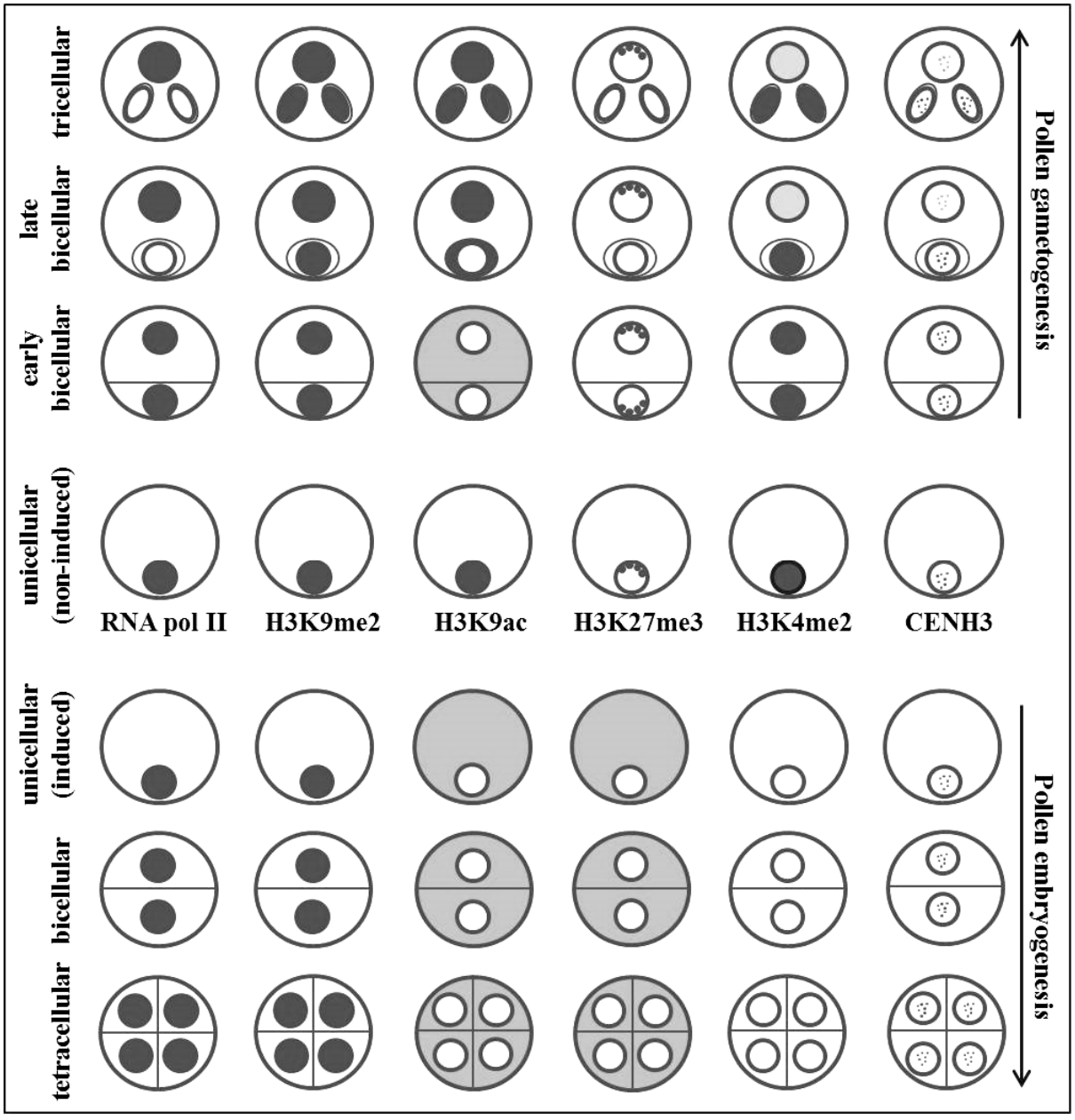


Fig. 43: Schematic overview of chromatin modifications during barley pollen gametogensis and pollen embryogenesis. Weak intensity of DAPI fluorescence shown in pale grey, strong fluorescence shown in dark grey.

4.2.4 Cytoplasmic histones

One of the most conspicuous effects of reprogramming gametophytic pollen into embryogenic cells is the accumulation of the histone modifications H3K9ac and H3K27me3 in the cytoplasm of embryogenic pollen (Fig. 35). This may indicate altered synthesis and/or turnover rates of histones in respectively cytoplasm and nucleus. Studying epigenetic modifications in the root apical region, Braszewska et al., (2013) found cytoplasmic distribution of modified histones to be highly tissue specific i.e. related to cell developmental status. Embryogenesis causes a dedifferentiation of immature pollen and triggers a sustained mitotic activity (Pechan and Smykal, 2001). During replication new histones are synthesized and transported to the nucleus where together with the new DNA they assemble into chromatin (Krude, 1995). High levels of histone synthesis occur as DNA replication begins (Wu et al., 1981). Indeed, animal embryonic systems often contain large cytoplasmic histone pools (Woodland and Adamson, 1977; Laskey et al., 1977) which are used during phases of rapid DNA replication. The cytoplasmic localization of H3K9ac and H3K27me3 may thus be representative of an overall accumulation of histones within the cytoplasm of the embryogenic pollen in response to ongoing replication. This seems especially evident for histone H3K9ac. The enzymes responsible for histone acetylation belong to the group of histone acetyltransferases (HAT). HATs are divided into the nuclear HAT A, which acetylate histones present in nucleosomes, and the cytoplasmic HAT B which are specific for free histones. Whereas HAT A enzymes are responsible for histone acetylation patterns that correlate with transcriptionally active chromatin, the cytoplasmic acetylation mediated by HAT B plays an important role in the assembly of newly synthesized histones into chromatin during DNA replication (Brownell and Allis 1996; Parthun et al., 1996). Following assembly into chromatin the cytoplasmically acetylated histones are rapidly deacetylated (Ruiz-Carrillo et al., 1975). Thus while histone H3K9ac found in the cytoplasm of embryogenic pollen may supply sustained cycles of mitosis, the absence from the nucleus may be indicative of an overall gene suppression during embryogenesis (Borkird et al., 1998).

4.2.5 Histone replacement and reduced label intensity

The use of PEG sections warrants excellent accessibility to antigens. In barley but especially in tobacco, fluorescence intensity after labeling for methylated histone modifications weakened as pollen approached maturation (3.2., 3.3.). This indicates a decline in specific histone modifications which is probably the direct result of a replacement of the original complement of histone 3 variants in the vegetative and generative nucleus as has been described for *Arabidopsis* (Okada et al., 2005; Ingoufff et al., 2007; Ingouff et al., 2010). The incorporation of new histone 3 variants may be important for keeping the generative nucleus in a gene silencing heterochromatic condition and preventing the vegetative nucleus from entering a new cell cycle. For this the new histone variants may not need the epigenetic modifications found in the histones they replace. Labeling for H3K9ac reflected the dual role of this modification. When present in the nucleus H3K9ac is associated with facilitating gene activity while cytoplasmic H3K9ac plays a role in transport and incorporation of newly synthesized histones into the nucleus (Brownell and Allis 1996, Parthun et al. 1996). Especially in tobacco nuclear signals were no longer visible when pollen neared maturity. This indicates a deacetylation or, as suggested here, an exchange of acetylated histones within the nucleus with time (Fig. 31). Signals for cytoplasmic H3K9ac followed a different pattern. In both barley and tobacco cytoplasmic signals for H3K9ac were strongest in the generative cell, particularly at stages which, according to DAPI staining, involved DNA duplication (Fig. 24 and Fig. 31). Accordingly, in barley the cytoplasmic signals for acetylated histone 3 rapidly declined after PM II (Fig. 24).

4.3 To be or not to be a generative cell

Conflicting reports exist as to whether or not the generative cell arising after an asymmetric PM I can participate in embryogenesis (Constantin et al., 1981; Malik et al., 2007). Given that development into a vegetative cell is the default pathway, one would expect that induction favours the vegetative cell since it is under less epigenetic control. Furthermore induction is most successful when applied just before to just after PM I (Bhowmik et al., 2011; Ramírez et al., 2001). Induction of late bicellular pollen is rarely successful and if, requires rather extreme

conditions (Binarova et al., 1997). This proves that progressive differentiation, like that of the generative cell, reduces the susceptibility to induction. Although examples of generative cells taking part in embryogenesis have been reported for several species, the work by Daghma (2011) raises questions about their true identity. Time-lapse recording of embryogenic pollen of barley revealed that so-called generative cells undergoing embryogenic divisions were rare and did not fit the morphological definition. Instead of being small and detached from the pollen wall, so-called embryogenic generative-like cells were much larger in volume and retained their position at the pollen wall (Daghma, 2011). They seemed to be the product of an asymmetric division going astray. Apparently these were cases where the nucleus had migrated to the pollen wall after which maybe the mitotic spindle but most certainly the formation of the phragmoplast had been faulty resulting in a less extreme asymmetric division leaving the generative-like cell in a much larger cytoplasmic environment. In the work for this thesis the majority of induced unicellular pollen also went through a first symmetric division. If this first division was asymmetric, the smaller cell usually was the size of a normal generative cell, rarely larger, and always remained attached to the pollen wall.

Immunological studies of embryogenic pollen that had experienced a first asymmetric division resulting in a large vegetative cell and very small non-detached generative cell revealed that only the large vegetative-like cell displayed to have chromatin modification patterns identical to those found in normal embryogenic cells (Fig. 36).

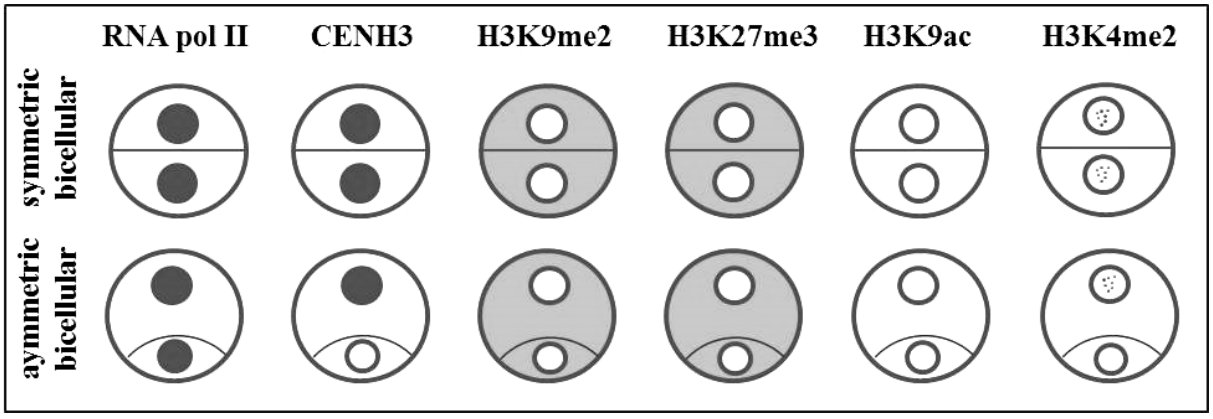


Fig. 44: Schematic overview of chromatin modifications in embryogenic pollen after symmetric and asymmetric mitosis. Weak intensity fluorescence shown in pale grey, strong fluorescence shown in dark grey.

Although the labelling profile found in the nuclei of the small generative-like cells was clearly not that of a normal embryogenic cell, it was definitively also not that of a generative cell (Fig. 44).

As discussed under 4.2.1 and 4.2.2, the proper organization of nuclear migration, spindle and phragmoplast formation only serves to bring the two nuclei in areas appropriate for the correct cell fate determination by factors that are still unknown. The observation that under conditions of induction small generative-like cells do not automatically assume generative cell properties even when confined to small polar areas indicates that the distribution of these factors does not depend on intact cytoskeletal organization. Although cell-fate depends on the proper organization of cytoskeletal driven events it is not caused by them. Similarly, induction of embryogenesis is a process that in essence is independent of the cytoskeletal organization. This is most evidently proven by the fact that embryogenesis does not require a symmetric PM I (Daghma et al., 2014). The main role of stress therefore is to alter the identity of the pollen nucleus and for this it has to be applied at a time when this pollen identity is most labile, i.e. around PM I. The induction factor stress results in a broad spectrum of responses (reviewed by Islam and Tuteja, 2012; Shariatpanahi et al., 2006) and its application concurs with the preparation of several distinct ultrastructural reorganizations like nuclear migration and spindle orientation. It is more than likely that stress will affect these processes. The extent of the stress effects will depend on the progressional state of PM I. Diversification of the stress response will be further enhanced by the moderate synchronization of barley pollen development (see Fig. 12).

The question whether a generative cell in barley pollen can participate in embryogenic development is based on the assumption that after induction a generative cell can still be formed. The data presented here make it more likely that stress changes identity of the cells within a very short time long before mitosis. What later is identified as generative-like cells is the result of a still largely intact asymmetric cell division mechanism that allocates two embryogenic nuclei into dissimilar compartments. As measurements on multicellular embryogenic structures have shown, nuclear size is directly related to cellular size (Fig. 34). It is hypothesized that what

appears to be generative-like nuclei undergoing embryogenesis, are in fact small embryogenic nuclei undergoing embryogenesis (Fig. 45).

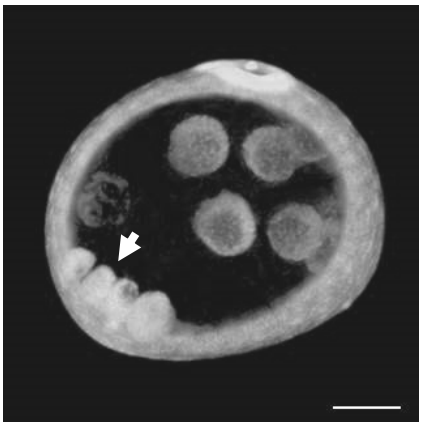


Fig. 45: Partial 3D recording of multicellular structure after DAPI staining reveals peripheral aggregation of small nuclei (arrow). Bar = 10 µm

4.4 Effect of inhibitors on chromatin modifications

DNA methylation patterns in plant cell development never undergo complete resetting as happens in mammals (Jullien & Berger, 2010). This explains the persistent level of DNA methylation found in 5-AZA treated pollen (Fig. 38). The effects caused by 5-AZA were limited to a sharp decline in RNAP II labeling and a reappearance of H3K9ac in the nucleus (compare Fig. 39 A and B). It is known that 5-AZA can suppress RNAP II recruitment (Wada et al., (2005) and induce heterochromatin hyperacetylation (Takebayashi et al., 2001). Since 5-AZA also inhibits RNA methylation and with it protein synthesis (Lee & Karon, 1976; Vesely and Cihak, 1978; Glazer et al., 1980), reduced levels of cytoplasmic acetyltransferases and nuclear deacetylases could also be responsible for the nuclear signals of H3K9ac.

Far more intriguing were the changes observed after incubation with TSA. Due to the inhibition of HAT enzymes signals for unprocessed H3K9ac disappeared form the cytoplasm and accumulated in the nucleus, an effect far more extreme than seen after treatment with 5-AZA (compare Fig. 39 A and C) . By definition, aspecific bulk acetylation of chromatin must cause an

overall opening up of nucleosomes and may thus result in a poorly controlled general gene expression (Gao et al., 2002). What made the effects induced by TSA extra special was the reappearance of the gene activity regulating histone modifications H3K27me3 and H3K4me2 within the nucleus. It thus seemed that increased gene activation went hand in hand with the triggering of gene expression control mechanisms. If it had not been for the somewhat weak signals for RNAP II, the TSA induced redistribution of epigenetic markers would have been identical to that found in gametophytic pollen (compare Fig. 39 A and C), raising the question: has TSA reset the embryogenic program into a gametophytic program again?

4.5 A role for TSA in pollen embryogenesis

For nearly 30 years now the deacetylase inhibitor TSA is well known for its ability to reverse cell fate in a variety of systems (Lee et al., 2004; Ware et al., 2009; McCool et al., 2007; Karantzali et al., 2008). Induction of pollen embryogenesis is exactly this: changing cell fate. It is therefore remarkable to realize that it took until very recently before the potential of TSA to enhance pollen embryogenesis was seriously tested (Li et al., 2014). Induction of embryogenesis is often said to be a two-step process with the first step being the change in cell fate and the second step the reactivation of the cell cycle. Barley pollen assume their new epigenetic profile within 24 h of induction while still within the anther (see 3.5.). It thus seems that no more than 24 h of stress is needed to change cell fate. TSA also changes cell fate. Therefore, for TSA to have a stimulating effect on pollen embryogenesis it was hypothesized that it must be applied immediately after harvesting of the anthers, while the uninucleate pollen still have an undisturbed gametophytic imprinting. The results proved the soundness of this reasoning (Fig. 40 and 41). Only when applied at the very start of induction TSA would enhance pollen embryogenesis. All later time points proved detrimental as here TSA would act on and reverse cell fate of already embryogenic pollen, exactly the opposite effect of intended action.

4.5.1 TSA and cell synchronisation

A remarkable feature of the TSA induced embryogenesis was the prompt and highly synchronous development of multicellular structures (Fig. 41). This may be the result of a number of mutual reinforcing factors. Treatment with TSA can arrest the cell cycle in plant cell cultures, causing an accumulation at metaphase-anaphase (Murphy et al., 2000; Li et al., 2005). This effectively results in a synchronized cell culture which upon removal of the TSA starts a synchronized development. TSA exposure may also accelerate these first cell division(s). Kemp et al. (2005) found that in human cells the pattern of DNA initiation sites within replication origin loci is altered upon treatment with TSA, in becoming more dispersive. Preferred initiation sites turned less dominant while infrequently used initiation sites in the genome became more active after treatment with TSA. When cells arrested in late G0 were released, they completed S phase more rapidly than untreated cells.

4.5.2 TSA: a new chapter in the induction of pollen embryogenesis?

Being sessile, plants have evolved sophisticated mechanisms in response to stress. The most sought after element of this response, altered gene expression (Chinnusamy et al., 2008; Chinnusamy and Zhu, 2009; Kim et al., 2010) is also at the base of nearly all induction protocols for pollen embryogenesis. Stress results in altered gene expression over a cascade of receptors and signals, in contrast to this TSA directly targets gene expression through large scale hyper-acetylation of chromatin. Stress causes a reaction but TSA imposes a reaction. As a result of massive disturbances of existing epigenetic programming TSA typically brings about a cell fate reversal between differentiation and dedifferentiation. The application of deacetylase inhibitors, however, is in no way supplementary to normal stress application. Without the need for an additional stress factor, TSA may also markedly shorten the time needed for induction. It should also be remembered that stress works best on cells with labile identity. It is conceivable that deacetylase inhibitors like TSA may be more effective when utilized in slightly more differentiated cell cultures. This brings anther harvesting time into the picture. Standard induction of barley pollen seems more effective when anthers are harvested in early morning (Dr. D. Daghma, personal communication). It is worth mentioning that the stress treatments

applied often mimic the conditions the plants are exposed to at night, i.e. lower temperatures combined with darkness. The early morning higher sensitivity hints at the existence of a circadian pattern of epigenetic modifications. In *Arabidopsis* the circadian transcription of the TOC1 gene is regulated by changes in chromatin structure which are controlled by a biological clock and involve a rhythmic pattern of histone acetylation at the TOC1 locus (Perales and Mas, 2007). The activation of mammalian clock genes is also often coupled with changes in histone acetylation (Etchegaray et al., 2003; Curtis et al., 2004; Naruse et al., 2004; Ripperger and Schibler, 2006) and recent studies have shown that the CLOCK protein, an essential component of the mammalian circadian system, is a histone acetyltransferase (Doi et al., 2006). Therefore, for optimal results, the use of deacetylase inhibitors like TSA may also require a different time of application as compared to traditional stress treatment protocols. An important aspect of TSA treatment is the potential reduction of stress. As such this application might also hold a key in tackling the problem of albino formation that is often observed in recalcitrant accessions of monocots.

It is clear, however, that with the introduction of inhibitors like TSA a door has been opened to a new area of inducing pollen embryogenesis.

5 SUMMARY

Pollen embryogenesis requires the resetting of cell identity which is usually achieved by application of stress just before or around PM I. Stress, however, is a not very specific mediator, producing a wide variety of responses that often do not yield the desired result. For that reason optimizing induction efficiency and overcoming recalcitrance to embryogenesis are major goals in present day plant breeding.

The processes that lead to embryogenesis need to be seen in the wider field of pollen development and cell fate. The central components, cellular differentiation and dedifferentiation, are inherently linked to epigenetic reprogramming. This also includes the modification of the DNA-binding histone proteins, a field of research that has been remarkably neglected in both pollen development and pollen embryogenesis. The immunological approach applied in the present study has yielded the most comprehensive overview of the epigenetic changes occurring during pollen development and pollen embryogenesis available to date.

The pattern and dynamics of several chromatin markers and histone 3 modifications during barley pollen development reflected the eventual silencing of the generative cell and the loss of mitotic potential in the vegetative cell. Focusing on the events immediately following PM I the results indicated that generative and vegetative cell retain their original identity for some time before differentiating. This confirmed observations obtained after DAPI staining which showed that size and DNA content of generative and vegetative nucleus develop identical for some time after PM I.

Comparing epigenetic histone 3 modifications during barley and tobacco pollen development, revealed the existence of species-specific variations that suggested different regulatory mechanisms of pollen development. An early indication for this was found in the orientation of the spindle in PM I which was perpendicular to the cell wall in barley but parallel in case of tobacco. It is hypothesized that the apparently more stringent epigenetic programming of the immature barley pollen as compared to tobacco is inversely related to the control of asymmetric PM I which in tobacco seems more fit to effectively confine the generative cell in a small polar area of the pollen as compared to barley.

Stress induction protocols for barley pollen embryogenesis typically run over 3-4 days (Daghma 2011). However, the present work showed that the epigenetic transformation into an embryogenic profile was completed within 24 h which indicated that cell fate reversal was attained well before PM I. The fact that after induction PM I may still be symmetric or asymmetric emphasizes that cell fate reversal and PM I underlie independent mechanisms with different sensitivities towards stress.

It is hypothesized that the cytoskeletal organization guiding PM I is far more resilliant to stress. As a consequence, while resetting the cell identity is achieved in a relatively short time, the original cell dividing machinery may be still unaltered at the time of first mitosis. This would explain why mitosis of an embryogenic cell may be accompanied by a symmetric or more or less asymmetric cell division. Furthermore, depending on the amount of cytoplasm allocated to the smaller of the two embryogenic cells, the latter may or may not undergo several rounds of mitosis until cell size becomes prohibitive. As such, this provides an elegant explanation for some of the hitherto peculiar pathways proposed for embryogenesis in barley.

Among the histone modifications analyzed here, acetylation appeared most intriguing. Newly synthesized histones become acetylated to facilitate their integration into the chromatin of the nucleus, and once incorporated, they are quickly deacetylated to prevent uncontrolled gene expression which is followed by a site-specific re-acetylation to stimulate controlled gene expression (Shahbazian and Grunstein, 2007).

The immunological results showed that the dynamics of H3K9ac went hand in hand with differentiation processes in pollen and dedifferentiation in embryogenesis. This allegedly important role in determining cell fate, identified histone acetylation as a target for altering cell identity. Indeed, TSA, one of the most potent histone deacetylase inhibitors, was found to enhance embryogenesis in barley pollen.

The introduction of TSA also highlighted an apparent weakness in plant breeding studies in terms of interdisciplinary research. With hindsight it is astounding to realize that it took nearly 30 years before the well-known ability of TSA to induce cell fate reversal in mammal cells, was also tested in the very plant cell types in which cell fate reversal is the most sought after quality.

It seems that the use of inhibitors of epigenetic modifications may be the start of a new and exciting chapter in the quest for optimizing pollen embryogenesis.

6 REFERENCES

Ambrose JC, Cyr R (2008) Mitotic spindle organization by the pre-prophase band. Molecular Plant **1:** 950–960

Anzola JM, Sieberer T, Ortbauer M, Butt H, Korbei B, Weinhofer I, Müllner AE, Luschnig C (2010) Putative *Arabidopsis* transcriptional adaptor protein (PROPORZ1) is required to modulate histone acetylation in response to auxin. Proceedings of the National Academy of Sciences, USA **107:** 10308-13

Banaei-Moghaddam AM, Schubert V, Kumke K, Weib O, Klemme S, Nagaki K, Macas J, González-Sánchez M, Heredia V, Gómez-Revilla D, González-García M, Vega JM, Puertas MJ, Andreas Houben A (2012) Nondisjunction in Favor of a Chromosome: The Mechanism of Rye B Chromosome Drive during Pollen Mitosis. The Plant Cell **24:** 4124-34

Bannister AJ, Kouzarides T (2005) Reversing histone methylation. Nature **436:** 1103–1106

Becker C, Hagmann J, Müller D, Koenig O, Stegle, Borgwardt K, Weigel D (2011) Spontaneous epigenetic variation in the *Arabidopsis thaliana* methylome. Nature **480:** 245–249

Berger F, Twell D (2011) Germline specification and function in plants. Annual Review of Plant Biology **62:** 461–484

Berger SL (2002) Histone modifications in transcriptional regulation. Current Opinion in Genetics and Development **12:** 142–8

Berger SL (2007) The complex language of chromatin regulation during transcription. Nature **447:** 407-12

Bernatavichute YV, Zhang X, Cokus S, Pellegrini M, Jacobsen SE (2008) Genome-Wide Association of Histone H3 Lysine Nine Methylation with CHG DNA Methylation in *Arabidopsis thaliana*. PLoS ONE **3:** 3156

Bernstein E, Allis CD (2005) RNA meets chromatin. Genes Development **19:** 1635-1655

Bhojwani SS, Dunwell JM, Sunderland N (1973) Protein contents of embryogenic tobacco pollen. Journal of Experimental Botany **24:** 863-871

Bhowmik P, Dirpaul J, Polowick P, Ferrie AM (2011) A high throughput *Brassica napus* microspore culture system: influence of percoll gradient separation and bud selection on embryogenesis. Plant Cell, Tissue and Organ Culture **106:** 359–362

Binarova P, Hause G, Cenklova V, Cordewener JHG, Campagne MV (1997) A short severe heat shock is required to induce embryogenesis in late bicellular pollen of Brassica napus L. Sexual Plant Reproduction **10:** 200-208

Bino R, Van Tuyl J, De Vries J (1990) Flow cytometric determination of relative nuclear DNA contents in bicellulate and tricellulate pollen. Annals of Botany **65:** 3–8

Blais A, van Oevelen, CJ, Margueron R, Acosta-Alvear D, Dynlacht BD (2007) Retinoblastoma tumor suppressor protein-dependent methylation of histone H3 lysine 27 is associated with irreversible cell cycle exit. The Journal of Cell Biology **179:** 1399-1412

Bohanec B (2009) Doubled haploids via gynogenesis. In: Touraev A, Forster BP, Jain SM (eds) Advances in haploid production in higher plants. Springer, Dordrecht pp 35–46

Borg M, Brownfield L, Twell D (2009) Male gametophyte development: a molecular perspective. Journal of Experimental Botany **60:** 1465–1478

Borges F, Gomes G, Gardner R, Moreno N, McCormick S, Feijo JA, Becker JD (2008) Comparative transcriptomics of *Arabidopsis* sperm cells. Plant Physiology **148:** 1168–1181

Borges F, Pereira PA, Slotkin RK, Martienssen RA, Becker JD (2011) MicroRNA activity in the *Arabidopsis* male germline. Journal of Experimental Botany **62:** 1611–1620

Borkird C, Choi JH, Jin ZH, Franz G, Hatzopoulos P, Chorneau R, Bonas U, Pelegri F, Sung, ZR (1988) Developmental regulation of embryonic genes in plants. Developmental Biology **85:** 6399-6403

Botrugno OA, Santoro F, Minucci S (2009) Histone deacetylaseinhibitors as a new weapon in the arsenal of differentiation therapies of cancer. Cancer Letters **280:** 134–144

Braszewska-Zalewska AJ, Wolny EA, Smialek L, Hasterok R (2013) Tissue-specific epigenetic modifications in root apical meristem cells of *Hordeum vulgare*. PLoS ONE **8:** e69204 doi: 10.1371/journal.pone.0069204

Braybrook SA, Harada JJ (2008) LECs go crazy in embryo development. Trends in Plant Science **13:** 624–630

Brownell JE, Allis CD (1996) Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. Current Opinion in Genetics and Development **6:** 176–184

Brumfield RT (1941) Asymmetrical spindles in the first microspore division of certain angiosperms. American Journal of Botany **28:** 713-722

Bui HT, Wakayama S, Kishigami S, Park KK, Kim JH, Thuan NV, Wakayama T (2010) Effect of trichostatin A on chromatin remodeling, histone modifications, DNA replication, and transcriptional activity in cloned mouse embryos. Biology of Reproduction **83:** 454–463

Cairns J (2006) Cancer and the immortal strand hypothesis. Genetics **174:** 1069–1072

Carrozza MJ, Utley RT, Workman JL, Cote, J (2003) The diverse functions of histone acetyltransferase complexes. Trends in Genetics **19:** 321–329

Cartagena JA, Matsunaga S, Seki M, Kurihara D, Yokoyama M, Shinozaki K, Fujimoto S, Azumi Y, Uchiyama S, Fukui K (2008) The *Arabidopsis* SDG4 contributes to the regulation of pollen tube growth by methylation of histone H3 lysines 4 and 36 in mature pollen. Developmental Biology **315:** 355–368

Castiglione MR, Cremonini R, Frediani M (2002) DNA methylation patterns on plant chromosomes. Caryologia **55:** 275-282

Castillo AM, Cistué L, Vallés MP, Soriano M (2009) Chromosome doubling in monocots. *In* A Touraev, BP Forster, S Mohan Jain, eds, Advances in haploid production in higher plants. Springer pp 329-338

Cedar H, Bergman Y (2009) Linking DNA methylation and histone modification: patterns and paradigms. Nature Reviews Genetics 10: 295-304

Chagin, VO, Stear JH, Cardoso MC (2010) Organization of DNA Replication. Cold Spring Harbor Perspectives in Biology **2:** a000737 doi: 10.1101/cshperspect.a000737

Chen C, Farmer AD, Langley RJ, Mudge J, Crow JA, May GD, Huntley J, Smith AG, Retzel EF (2010) Meiosis-specific gene discovery in plants: RNA-Seq applied to isolated *Arabidopsis* male meiocytes. BMC Plant Biology **10:** 280

Chen YCS, McCormick S (1996) sidecar pollen, an *Arabidopsis* thaliana male gametophytic mutant with aberrant cell divisions during pollen development. Development **122:** 3243-3253

Chen ZF, Tian L (2007) Roles of dynamic and reversible histone acetylation in plant development and polyploidy. Biochimca et Biophysica Acta **1769:** 295–307

Cheung P, Tanner KG, Cheung WL, Sassone-Corsi P, Denu JM, Allis CD (2000) Synergistic Coupling of Histone H3 Phosphorylation and Acetylation in Response to Epidermal Growth Factor Stimulation. Molecular Cell **5:** 905–915

Chinnusamy V, Gong Z, Zhu JK (2008) Abscisic acid-mediated epigenetic processes in plant development and stress responses. Journal of Integrative Plant Biology **50:** 1187–1195

Chinnusamy V, Zhu J-K (2009) Epigenetic regulation of stress responses in plants.Current Opinion Plant Biology **12:** 133–139

Chiurazzi P, Pomponi M, Pietrobono R, Bakker C, Neri G, Oostra BA (1999) Synergistic effect of histone hyperacetylation and DNA demethylation in the reactivation of the FMR1 gene. Human Molecular Genetics **8:** 2317–2323

Christensen ME, Dixon GH (1982) Hyperacetylation of histone H4 correlates with the terminal, transcriptionally inactive stages of spermatogenesis in rainbow trout. Developmental Biology **93:** 404–415

Chodavarapu RK, Feng,S. Bernatavichute,Y. Chen PY, Stroud H, Yu Y, Hetzel JA. Kuo F, Kim J, Cokus SJ, Casero D, Bernal M, Huijser P, Clark AT, Krämer U, Merchant SS,

Zhang X, Jacobsen SE, Pellegrini M (2010) Relationship between nucleosome positioning and DNA methylation. Nature **466:** 388-392

Cokus SJ, Feng S, Zhang X, Chen Z, Merriman B, Haudenschild CD, Pradhan S, Nelson SF, Pellegrini M, Jacobsen SE (2008) Shotgun bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning. Nature **452:** 215–19

Collins N, Poot RA, Kukimoto I, Garcia-Jimenez C, Dellaire G, Varga-Weisz PD (2002) An ACF1-ISWI chromatinremodeling complex is required for DNA replication through heterochromatin. Nature Genetics **32:** 627–632

Constantin MJ (1981) Pollen embryogenesis to induce, detect, and analyze mutants. Environmental Health Perspectives **37:** 27-33

Coronado MJ, Hensel G, Broeders S, Otto I, Kumlehn J (2005) Immature pollen-derived doubled haploid formation in barley cv. Golden Promise as a tool for transgene recombination. Acta Physiologica Plantarum **27:** 591–599

Corpet A, Almouzni G (2009) Making copies of chromatin: The challenge of nucleosomal organization and epigenetic information. Trends in Cell Biology **19:** 29–41

Cresti M, Lancelle SA, Hepler K (1987) Structure of the generative cell wall complex after freeze substitution in pollen tubes of *Nicotiana* and *Impatiens*. Journal of Cell Science **88:** 373-378

Cubas P C, Vincent E, Coen (1999) An epigenetic mutation responsible for natural variation in floral symmetry. Nature **401:** 157-161

Curtis AM, Seo S-b, Westgate EJ, Rudic RD, Smyth EM, Chakravarti D, FitzGerald GA, McNamara P (2004) Histone acetyltransferase-dependent chromatin remodeling and the vascular clock. Journal of Biological Chemistry **279:** 7091–7097

Custers JBM, Cordewener JHG, Nöllen Y, Dons JJM, VanLockeren Campagne MM (1994) Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Reproduction **13:** 267–271

Daghma DS (2011) Structural changes during the initiation of pollen embryogenesis in barley (*Hordeum vulgare* L.) (PhD Thesis) Martin-Luther-University Halle-Wittenberg, Halle/Salle pp 167

Daghma D, Kumlehn J, Hensel G, Rutten T, Melzer M (2012) Timelapse imaging of the initiation of pollen embryogenesis in barley (*Hordeum vulgare* L.). Journal Experimental Botany **63:** 6017–6021

Daghma DES, Hensel G, Rutten T, Melzer M, Kumlehn J (2014) Cellular dynamics during early barley pollen embryogenesis revealed by time-lapse imaging. Frontiers in Plant science doi: 10.3389/fpls.2014.00675

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 **244:** 79-84

Dangl M, Brosch G, Haas H, Loidl P, Lusser A (2001) Comparative analysis of HD2 type histone deacetylases in higher plants. Planta **213:** 280-285

Demetriou K, Kapazoglou A, Tondelli A, Francia E, Stansa MA, Bladenopoulos K, Tsaftaris AS (2009) Epigenetic chromatin modifiers in barley: I. Cloning, mapping and expression analysis of the plant specific HD2 family of histone deacetylases from barley, during seed development and after hormonal treatment. Physiolgia Plantarum **136:** 358-368

Desvoyes B, Sanchez MP, Ramirez-Parra E, Gutierrez C (2010) Impact of nucleosome dynamics and histone modifications on cell proliferation during *Arabidopsis* development. Heredity **105:** 80–91

Dickinson HG (1994) The regulation of alternation of generation in flowering plants. Biological Review **69:** 419-442

Dickinson HG, Grant-Downton R (2009) Bridging the generation gap: Flowering plant gametophytes and animal germlines reveal unexpected similarities. Biological Reviews of the Cambridge Philological Society **84:** 589–615

Doi M, Hirayama J, Sassone-Corsi P (2006) Circadian regulator CLOCK is a histone acetyltransferase. Cell **125:** 497–508

Dokmanovic M, Clarke C, Marks PA (2007) Histone deacetylase inhibitors: Overview and perspectives. Molecular Cancer Research **5:** 981–9

Drew MC (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. Annual Review of Plant Physiology and Plant Molecular Biology **48:** 223–250

Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC (2005) Clinical development of histone deacetylase inhibitors as anticancer agents. Annual Review of Pharmacology and Toxicology **45:** 495–528

Dunwell JM, Sunderland N (1976a) Pollen ultrastructure in anther cultures of Datura innoxia. II. The generative cell wall. Journal of Cell Science **22:** 481-491

Dunwell JM, Sunderland N (1976b) Pollen ultrastructure in anther cultures of *Datura innoxia*. Journal of Cell Science **32:** 493-495

Eady C, Lindsey K, Twell D (1995) The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. Plant Cell **7:** 65–74

Ebbs ML, Bender J (2006) Locus-specific control of DNA methylation by the *Arabidopsis* SUVH5 histone methyltransferase. Plant Cell **18:** 1166–1176

Eberharter A, Becker PB (2002) Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. EMBO Reports **3:** 224–229

Etchegaray J-P, Lee C, Wade PA, Reppert SM (2003) Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature **421:** 177–182

Fan Z, Armstrong KC, Keller WA (1988) Development of microspores *in vivo* and *in vitro* in *Brassica napus*. Protoplasma **147:** 191-199

Feng X, Dickinson HG (2007) Packaging the male germline in plants. Trends Genetics **23:** 503–510

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

Frye RA (2000) Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochemical and Biophysical Research Communications **273:** 793-798

Fuchs J, Demidov D, Houben A, Schubert I (2006) Chromosomal histone modification patterns – from conservation to diversity. Trends in Plant Science **11:** 199–208

Futamura M, Monden Y, Okabe T, Fujita-Yoshigaki J, Yokoyama S, Nishimura S (1995) Trichostatin A inhibits both ras-induced neurite outgrowth of PC12 cells and morphological transformation of NIH3T3 cells. Oncogene **10:** 1119-1123

Gaj MD (2001) Direct somatic embryogenesis as a rapid and efficient system for in vitro regeneration of *Arabidopsis thaliana*. Plant Cell, Tissue and Organ Culture **64:** 39–46

Gao L, Cueto MA, Asselbergs F, Atadja, P (2002) Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. Journal of Biological Chemistry **277:** 25748–25755

Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regulation **43:** 27–47

Gard DL, Becker BE, Romney SJ (2004) MAPping the eukaryotic tree of life: Structure, function and evolution of the MAP215/Dis1 family of microtubule-associated proteins. International Review of Cytology **239:** 179-272

Geitler L (1935) Beobachtungen über dies erste teilung im pollenkorn der angiospermen. Planta **24:** 361 - 386

George EF, Hall MA, De Klerk G-J (2008) Somatic embryogenesis plant propagation by tissue culture. Springer, Dordrecht pp 335–354

Germaná MA (2006) Doubled haploid production in fruit crops. Plant Cell, Tissue and Organ Culture **86:** 131–146

Gervais C, Newcomb W, Simmonds DH (2000) Rearrangement of the actin filament and microtubule cytoskeleton during induction of microspore embryogenesis in *Brasssica napus* L. cv. Topas. Protoplasma **213:** 194-202

Glazer RI, Peale AL, Beisler JA, Abbasi MM (1980) The effect of 5-azacytidine and dihydro-5-azacytidine on nuclear ribosomal RNA and poly(A)RNA synthesis in L1210 cells *in vitro*. Molecular Pharmacology **17:** 111-117

Gottesfeld JM, Forbes D (1997) Mitotic repression of the transcriptional machinery. Trends in Biochemical Science **22:** 197–202

Grafi G (2004) How cells dedifferentiate: a lesson from plants. Developmental Biology **268:** 1-6

Grant-Downton R, Hafidh S, Twell D, Dickinson HG (2009a) Small RNA pathways are present and functional in the angiosperm male gametophyte. Molecular Plant **2:** 500–512

Gonzales JM, Jouve N (2005) Microspore development during in vitro androgenesis in *Triticale*. Biologia Plantarum **4:** 23–28

González-Melendi P, Testillano P, Ahmadian P, Fadón B, Risueño M (1996) New in situ approaches to study the induction of pollen embryogenesis in *Capsicum annuum* L. European Journal of Cell Biology **69:** 373–386

Gorisch SM, Sporbert A, Stear JH, Grunewald I, Nowak D, Warbrick E, Leonhardt H, Cardoso MC (2008) Uncoupling the replication machinery: Replication fork progression in the absence of processive DNA synthesis. Cell Cycle **7:** 1983–1990

Hebbes TR, Thorne AW, Crane-Robinson C (1988) A direct link between core histone acetylation and transcriptionally active chromatin. EMBO Journal **7:** 1395–1402

Haberland M, Montgomery RL, Olson EN (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nature Reviews Genetics **10:** 32–42

Hahnen E, Hauke J, Tränkle C, Eyüpoglu IY, Wirth B, Blümcke I (2008) Histone deacetylase inhibitors: possible implications for neurodegenerative disorders. Expert Opinion Investigational Drugs **17:** 169–84

Hayashi K, Lopes SMCDS, Tang F, Surani MA (2008) Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. Cell Stem Cell **3:** 391–401

Heberle-Bors E (1985) In vitro haploid formation from pollen: a critical review. Theoretical and Applied Genetics **71:** 361–374

Heberle-Bors E (1989) Isolated pollen culture in tobacco: plant reproductive development in a nutshell. Sexual Plant Reproduction **2:** 1–10

Heidstra R (2007) Asymmetric cell division in plant development. Progress Molecular Subcellular Biololgy **45:** 1-37

Henikoff S, Ahmad K (2005) Assembly of variant histones into chromatin. Annual Review of Cell and Development Biology **21:** 133–153

Heslop-Harrison J (1968) Synchronous pollen mitosis and the formation of the generative cell in massulate orchids. Journal of Cell Science **3:** 457-466

Hirsch CD, Wu Y, Yan H, Jiang H (2009) Lineage-specific adaptive evolution of the centromeric protein CENH3 in diploid and allotetraploid *Oryza* species. Molecular Biology Evolution **26:** 2877–2885

Hnilicova J, Hozeifi S, Duskova E, Icha J, Tomankova T, Staněk D (2011) Histone deacetylase activity modulates alternative splicing. PLoS ONE **6:** e16727, doi:10.1371/journal.pone.0016727

Hochedlinger K, Plath K (2009) Epigenetic reprogramming and induced pluripotency. Development **136:** 509–523

Hoekstra S, van Zijderveld MH, Louwerse JD, Heidekamp F, van der Mark F (1992) Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Science **86:** 89-96

Honys D, Reňák D, Twell D (2006) Male gametophyte development and function. In: Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues I (eds). Global Science Books, UK pp 76-87

Honys D, Twell D (2004) Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*. Genome Biology **5:** 85

Houben A, Kumke K, Nagaki K, Hause G (2011) CENH3 distribution and differential chromatin modifications during pollen development in rye (*Secale cereale* L.) Chromosome Research **19:** 471– 480

Hsemann CU (1973) The development of pollen grains and formation of pollen tubes in higher plants: III. DNA-replication in vegetative and sperm nuclei in mature pollen grains of barley. Theoretical Applied genetics **43:** 232-24

Hu TC, Kasha KJ (1999) A cytological study of pretreatments used to improve isolated microspore cultures of wheat (*Triticum aestivum* L.) cv. Chris. Genome **42:** 432-441

Ingouff M, Berger F (2010) Histone3 variants in plants. Chromosoma **119:** 17–23

Ingouff M, Hamamura Y, Gourgues M, Higashiyama T, Berger F (2007) Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. Current Biology **17:** 1032– 1037

Ingouff M, Rademacher S, Holec S, Soljic L, Xin N, Readshaw A, Foo SH, Lahouze B, Sprunck S, Berger F (2010) Zygotic resetting of the histone 3 variant repertoire participates in epigenetic reprogramming in *Arabidopsis*. Current Biology **20:** 2137–2143

Islam SMS, Tuteja N (2012) Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. Plant Science **182:** 134-144

Iyer NG, Ozdag H, Caldas C (2004) p300/CBP and cancer. Oncogene **23:** 4225–423

Jackson JP, Lindroth AM, Cao X, Jacobsen SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature **416:** 556–560

Jullien PE, Berger F (2010) DNA methylation reprogramming during plant sexual reproduction? Trends in Genetics **26:** 394–399

Jullien PE, Kinoshita T, Ohad N, Berger F (2006) Maintenance of DNA methylation during the *Arabidopsis* life cycle is essential for parental imprinting. Plant Cell **18:** 1360–72

Jullien PE, Mosquna A, Ingouff M, Sakata T, Ohad N, Berger F (2008) Retinoblastoma and its binding partner MSI1 control imprinting in *Arabidopsis*. PLoS Biology **6:** 194

Jalouzot R (1969) Differenciation nucleaire et cytoplasmique du grain de pollen de Liliurn candidum. Experimental Cell Research **55:** 1-8

Kaltchuk-Santos E, Mariath JE, Mundstock E, Hu C, Bodanese-Zanettini MH (1997) Cytological analysis of early microspore divisions and embryo formation in cultured soybean anthers. Plant Cell, Tissue and Organ Culture **49:** 107-115

Karantzali K,Schulz H, Hummel O, Hubner N, Hatzopoulos AK, Kretsovali K (2008) Histone deacetylase inhibition accelerates the early events of stem cell differentiation: transcriptomic and epigenetic analysis. Genome Biology **9:** 65

Kasha KJ, Hu TC, Oro R, Simion E, Shim YS (2001) Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (*Hordeum vulgare* L.) microspores. Journal of Experimental Botany **52:** 1227-1238

Kemp MG, Ghosh M, Liu G, Leffak M (2005) The histone deacetylase inhibitor trichostatin A alters the pattern of DNA replication origin activity in human cells. Nucleic Acids Research **33:** 325-336

Kim J-M, To TK, Nishioka T, Seki M (2010) Chromatin regulation functions in plant abiotic stress responses. Plant Cell and Development **33:** 604

Kim M, Kim J, Yoon M, Choi D-I, Lee K-M (2004) Origin of multicellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum annuum*). Plant Cell, Tissue and Organ Culture **77:** 63–72

Kornberg RD (2009) Eukaryotic transcriptional control. Trends in Biochemical Science **24:** 46–49

Kornberg RD, Lorch Y (1999) Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell **98:** 285–294

Koukalova B, Fojtova M, Lim KY, Fulnecek J, Leitch AR, Kovarik A (2005) Dedifferentiation of tobacco cells is associated with ribosomal RNA gene hypomethylation, increased transcription, and chromatin alterations. Plant Physiology **139:** 275–286

Kouzarides T (2007) Chromatin modifications and their function. Cell **128:** 693–705

Kretsovali A, Hadjimichael C, Charmpilas N (2012) Histone deacetylase inhibitors in cell pluripotency, differentiation, and reprogramming. Stem Cell International doi:10.1155/2012/184154

Krude T (1995) Chromatin: Nucleosome assembly during DNA replication. Current Biology **5:** 1232–1234

Kumlenh J (2014) Haploid technology. Biotechnology in Agriculture and Forestry **69:** 379-392

Kumlehn J, Lörz H (1999) Momnitoring sporophytic development of indidvidual microspores of barley (*Hordeum vulgare* L.). In Anther and Pollen. In C Clement, E Pacini, J-C Audran, eds, Biology to Biotechnology. Springer, Berlin Heidlberg New York, pp 183-190

Kuo MH, Allis CD (1998) Roles of histone acetyltransferases and deacetylases in gene regulation. Bioessays **20:** 615–626

Kuo MH, vom Baur E, Struhl K, Allis CD (2000) Gcn4 activator targets Gcn5 histone acetyltransferase to specific promoters independently of transcription. Molecular Cell **6:** 1309–1320

Kyo M, Harada H (1986) Control of the developmental pathway of tobacco pollen in vitro. Planta **168:** 427-432

Lacour LF (1949) Nuclear differentiation in the pollen grain. Heredity **3:** 319–337

Laherty C, Yang W, Sun J, Davie J, Seto E, Eisenman RN (1997) Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. Cell **89:** 349–356

Lafos M, Kroll P, Hohenstatt ML, Thorpe FL, Clarenz O, Schubert D (2011) Dynamic regulation of H3K27 trimethylation during *Arabidopsis* differentiation. PLoS Genet **7:** e1002040, doi: 10.1371/journal.pgen.1002040

Lan F, Bayliss PE, Rinn JL, Whetstine JR, Wang JK, Chen S, Iwase S, Alpatov R, Issaeva I, Canaani E, Roberts TM, Chang HY, Shi Y (2007) A histone H3 lysine 27 demethylase regulates animal posterior development. Nature **449:** 689–694

Lark KG (1967) Non-random segregation of sister chromatids in *Vicia faba* and *Triticum boeoticum*. Proceedings of the National Academy of Sciences, USA **58:** 352–359

Laskey RA, Mills AD, Morris NR (1977) Assembly of SV40 chromatin in a cell-free system from Xenopus eggs. Cell **10:** 237-243

Le Trionnaire G, Grant-Downton RT, Kourmpetli S, Dickinson HG, Twell D (2011) Small RNA activity and function in angiosperm gametophytes. Journal of Experimental Botany **62:** 1601–1610

Lechner T, Lusser A, Pipal A, Brosch G, Loidl A, Goralik-Schramel M, Sendra R, Wegener S, Walton JD, Loidl P (2000) RPD3-type histone deacetylases in maize embryos. Biochemistry **39:** 1683–1692

Lee F, Chen C (1987) Nuclear fusion in cultured microspores of barley. Plant Cell Reports **6:** 191-193

Lee JH, Hart SRL, Skalnik DG (2004) Histone deacetylase activity is required for embryonic stem cell differentiation. Genesis **38:** 32–38

Lee TH, Karon, MR (1976) Inhibition of protein synthesis in 5-azacytidine treated HeLa cells. Biochemical Pharmacology **25:** 1737-1742

Lee YRJ, Li Y, Liu B (2007) Two *Arabidopsis* phragmoplast associated kinesins play a critical role in cytokinesis during male gametogenesis. Plant Cell **19:** 2595-605

Leipe DD, Landsman D (1997) Histone deacetylases, acetoin utilization proteins and acetylpolyamine amidohydrolases are members of an ancient protein superfamily. Nucleic Acids Research **25:** 3693–3697

Leoni F, Zaliani A, Bertolini G, Porro G, Pagani P, Pozzi P, Donà G, Fossati G, Sozzani S, Azam T, Bufler P, Fantuzzi G, Goncharov I, Kim SH, Pomerantz BJ,Reznikov LL, Siegmund B, Dinarello CA, Mascagni P (2002) The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. Proceeding of the National Academy of Sciences, USA **99:** 2995-3000

Li B, Carey M, Workman JL (2007) The role of chromatin during transcription. Cell **128:** 707-719

Li H, Soriano M, Cordewener J, Muiño JM, Riksen T, Fukuoka H, Angenent GC, Boutiliera K (2014) The histone deacetylase inhibitor Trichostatin A promotes totipotency in the male gametophyte. The Plant Cell **113:** 116491

Li Q, Eichten SR, Hermanson PJ, Springer NM (2014) Inheritance patterns and stability of DNA methylation variation in maize near-isogenic lines. Genetics **196:** 667-676

Li Y, Butenko Y, Grafi G (2005) Histone deacetylation is required for progression through mitosis in tobacco cells. Plant Journal **41:** 346–352

Li X, Wang X, He K, Ma Y, Su N, b Hang He, Viktor Stolc, Tongprasit W, Jin W, Jiang J, Terzaghi W, Li S, Deng XW (2008) High-resolution mapping of epigenetic modifications of the rice genome uncovers interplay between DNA methylation, histone methylation, and gene expression. Plant Cell **20:** 259– 276

Liao CT, Lin CH (2001) Physiological adaptation of crop plants to flooding stress. Proceedings of the National Science Council of ROC **25:** 148–157

Lienert F, Mohn F, Tiwari VK, Baubec T, Roloff TC, Gaidatzis D, Stadler MB, Schübeler D (2011) Genomic prevalence of heterochromatic H3K9me2 and transcription do not discriminate pluripotent from terminally differentiated cells. PLoS Genetics **7:** e1002090, doi: 10.1371/journal.pgen.1002090

Lindroth AM, Shultis D, Jasencakova Z, Fuchs J, Johnson L, Schubert D, Patnaik D, Pradhan S, Goodrich J, Schubert I, Jenuwein T, Khorasanizadeh S, Jacobsen SE (2004) Dual histone H3 methylation marks at lysines 9 and 27 required for interaction with CHROMOMETHYLASE3. EMBO Journal **23:** 4286–4296

Linsmaier EM, Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Physiologica Plantarum **18:** 100 – 127

Lippmann R, Kaspar S, Rutten T, Melzer M, Kumlehn J, Matros A, Mock HP (2009) Protein and metabolite analysis reveals permanent induction of stress defense and cell regeneration processes in a tobacco cell suspension culture. International Journal of Molecular Science **10:** 3012-3032

Liu C, Lu F, Cui X, Cao X (2010) Histone methylation in higher plants. Annual Review of Plant **Biology 61:** 395– 420

Loring JF, Porter JG, Seilhamer J, Kaser MR, Wesselschmidt A (2001) Gene expression profile of embryonic stem cells and embryonic stem cell-derived neuron. Restorative Neurology and Neuroscience **18:** 81-88

Lu C, Tej SS, Luo S, Haudenschild CD, Meyers BC, Green PJ (2005) Elucidation of the small RNA component of the transcriptome. Science **309:** 1567–1569

Majer C, Hochholdinger F (2010) Defining the boundaries: Structure and function of LOB domain proteins. Trends in Plant Science **16:** 47-52

Malagnac F, Bartee L, Bender J (2002) An *Arabidopsis* SET domain protein required for maintenance but not establishment of DNA methylation. EMBO Journal **21:** 6842–6852

Maliga P, Breznovitz A, Marton L (1973) Streptomycin-resistant plants from callus culture of haploid tobacco. Nature New Biology **244:** 29–30

Malik MR, Wang F, Dirpaul JM, Zhou N, Polowick PL, Ferrie AMR, Krochko JE (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. American Society of Plant Biologists **144:** 134–154

Maraschin SF, de Priester W, Spaink HP, Wang M (2005a) Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. Journal of Experimental Botany **56:** 1711–1726

Martienssen R, Baron A (1994) Coordinate suppression of mutations caused by Robertson's mutator transposons in maize. Genetics **136:** 1157–1170

Mascarenhas JP (1993) Molecular mechanisms of pollen tube growth and differentiation. Plant Cell **5:** 1303–1314

Masonbrink RE, Gallagher JP, Jareczek JJ, Renny-Byfield S, Grover CE, Gong L, Wendel JF (2014) CenH3 evolution in diploids and polyploids of three angiosperm genera. BMC Plant Biology **14:** 383

Matzke MA, Birchler JA (2005) RNAi-mediated pathways in the nucleus. Nature Reviews Genetics 6: 24–35

McCool KW, Xu X, Singer DB, Murdoch FE, Fritsch MK (2007) The role of histone acetylation in regulating early gene expression patterns during early embryonic stem cell differentiation. Journal of Biological Chemistry **282:** 6696–6706

McCue AD, Cresti M, Feijo JA Slotkin RK (2011) Cytoplasmic connection of sperm cells to the pollen vegetative cell nucleus: potential roles of the male germ unit revisited. Journal of Experimental Botany **62:** 1621–1631

Minucci S, Nervi C, Lo Coco F, Pelicci PG (2001) Histone deacetylases: a common molecular target for differentiation treatment of acute myeloid leukemias? Oncogene **20:** 3110–3115

Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nature Reviews Cancer **6:** 38–51

Misteli T (2004) Spatial positioning: A new dimension I genome function. Cell **119:** 153-156

Murphy JP, McAleer JP, Uglialoro A, Papile J, Weniger J, Bethelmie F, Tramontano WA (2000) Histone deacetylase inhibitors and cell proliferation in pea root meristems. Phytochemistry **55:** 11–18

Nagl W (1977) Early and late DNA replication in respectively condensed and decondensed heterochromatin of *Allium carinatum*. Protoplasma **4:** 389-407

Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL, Evans RM (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell **89:** 373-380

Naruse Y, Oh-hashi K, Iijima N, Naruse M, Yoshioka H, Tanaka M (2004) Circadian and light-induced transcription of clock gene Per1 depends on histone acetylation and deacetylation. Molecular Cell Biology **24:** 6278–6287

Nightingale KP, O'Neill LP, Turner BM (2006a) Histone modifications: signalling receptors and potential elements of a heritable epigenetic code. Current Opinion in Genetics and Development 16: 125–136

Nightingale KP, Wellinger RE, Sogo JM, Becker PB (1998) Histone acetylation facilitates RNA polymerase II transcription of the *Drosophila* hsp26 gene in chromatin. EMBO Journal **17:** 2865–2876

Nobuta K, Venu RC, Lu C, Belo A, Vemaraju K, Kulkarni K, Wang W, Pillay M, Green PJ, Wang GL, Meyers BC (2007) An expression atlas of rice mRNAs and small RNAs. Nature Biotechnology **25:** 473–477

Oh SA, Allen T, Kim GJ, Sidorova A, Borg M, Park SK, Twell D (2012) *Arabidopsis* Fused kinase and the Kinesin-12 subfamily constitute a signalling module required for phragmoplast expansion. The Plant Journal **72:** 308-319

Oh SA, Johnson A, Smertenko A, Rahman D, Park SK, Hussey PJ, Twell D (2005) A divergent cellular role for the FUSED kinase family in the plant-specific cytokinetic phragmoplast. Current Biology **15:** 2107–2111

Oh SA, Park KS, Twell D, Park SK (2010) The SIDECAR POLLEN gene encodes a microspore-specific LOB/AS2 domain protein required for the correct timing and orientation of asymmetric cell division. Plant Journal **64:** 839-850

Okada T, Endo M, Singh MB, Bhalla PL (2005) Analysis of the histone H3 gene family in *Arabidopsis* and identification of the malegamete- specific variant AtMGH3. Plant Journal **44:** 557–568

Okada T, Singh MB, Bhalla PL (2006) Histone H3 variants in male gametic cells of lily and H3 methylation in mature pollen. Plant Molecular Biology **62:** 503–512

Orford K, Kharchenko P, Lai W, Dao MC, Worhunsky DJ, Ferro A, Janzen V, Park PJ, Scadden DT (2008) Differential H3K4 methylation identifies developmentally poised hematopoietic genes. Developmental Cell **14:** 798–809

Otegui M, Staehelin LA (2000) Cytokinesis in flowering plants: more than one way to divide a cell. Current Opinion in Plant Biology **3:** 493-502

Pandey P, Houben A, Kumlehn J, Melzer M, Rutten T (2013) Natural chromatin modification during pollen gametogenesis in *Hordeum vulgare*. Cytogenet Genome Research **141:** 50–57

Pandey R, Müller A, Napoli CA, Selinger DA, Pikaard CS, Richards EJ, Bender J, Mount DW, Jorgensen RA (2002) Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Research **30:** 5036-5055

Parthun MR, Widom replica J, Gottschling DE (1996) The major cytoplasmic histone acetyltransferase in yeast: links to chromatin replication and histone metabolism. Cell **87:** 85–94

Park SK, Howden R, Twell D (1998) The *Arabidopsis thaliana* gametophytic mutation gemini pollen1 disrupts microspore polarity, division asymmetry and pollen cell fate. Development **125:** 3789-99

Pastuglia M, Azimzadeh J, Goussot M, Camilleri C, Belcram K, Evrard JL, Schmit AC, Guerche P, Boucheza D (2006) Gamma-tubulin is essential for microtubule organization and development in *Arabidopsis*. The Plant Cell **18:** 1412-1425

Pechan PM, Smykal P (2001) Androgenesis: Affecting the fate of the male gametophyte. Physiologia Plantarum **111:** 1–8

Pekowska A, Benoukraf T, Ferrier P, Spicuglia S (2010) A unique H3K4me2 profile marks tissue-specific gene regulation. Genome Research **20:** 1493–1502

Peng H, Chun J, Ai TB, Tong YA, Zhang R, Zhao MM, Chen F, Wang SH (2012) MicroRNA profiles and their control of male gametophyte development in rice. Plant Molecular Biology **80:** 85–102

Perales M, Más P (2007) A functional link between rhythmic changes in chromatin structure and the *Arabidopsis* biological clock. Plant Cell. **19:** 2111–2123

Pfluger J, Wagner D (2007) Histone modifications and dynamic regulation of genome accessibility in plants. Current Opinion in Plant Biology **10:** 645–652

Pina C, Pinto F, Feijo JA, Becker JD (2005) Gene family analysis of the *Arabidopsis* pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. Plant Physiology **138:** 744–56

Pulido A, Bakos F, Castillo A, Vallés M, Barnabas B, Olmedilla A (2005) Cytological and ultrastructural changes induced in anther and isolated-microspore cultures in barley: Fe deposits in isolated-microspore cultures. Journal of Structural Biology **149:** 170–181

Quivy JP, Ge´rard A, Cook AJL, Roche D, Almouzni G (2008) The HP1–p150/CAF-1 interaction is required for pericentric heterochromatin replication and S-phase progression in mouse cells. Nature Structural Molecular Biology **15:** 972–979

Radford EJ, Ferron SR, Ferguson-Smith AC (2011) Genomic imprinting as an adaptative model of developmental plasticity. FEBS Letter **585:** 2059–2066

Raghavan V (1978) Origin and development of pollen embryoids and pollen calluses in cultured anther segments of *Hyoscyamus niger* (henbane). American Journal of Botany **65:** 984-1002

Raghavan V (1986) Embryogenesis in angiosperms: A developmental and experimental study. Cambridge University Press, Cambridge ISBN: 9780521267717

Rahman, M, Kukita, A, Kukita, T, Shobuike, T, Nakamura, T, Kohashi, O (2003) Two histone deacetylase inhibitors, trichostatin-A and sodium butyrate, suppress differentiation into osteoclasts but not into macrophages. Blood **101:** 3451

Ramirez C, Testillano PS, Castillo AM, Valles MP, Coronado MJ, Cistue L, Risueńo MC (2001) The early microspore embryogenesis pathway in barley is accompanied by concrete ultrastructural and expression changes. International Journal of Developmental Biology **45:** 57–58

Reyes JC (2006) Chromatin modifiers that control plant development. Current Opinion in Plant Biology **9:** 21–27

Reyes JC, Hennig L, Gruissem W (2002) Chromatin-remodeling and memory factors: new regulators of plant development. Plant Physiology **130:** 1090–1101

Reynolds TL (1985) Ultrastructure of anomalous pollen devlomeni in embryogenic anther cultures of *Hyoscyamus niger*. American Journal of Botany **72:** 44-51

Reynolds TL (1993) A cytological analysis of microspores of *Triticum aestivum* (Poaceae) during normal ontogeny and induced embryogenic development. American Journal of Botany **80:** 569–576

Reynolds TL (1997) Pollen embryogenesis. Plant Molecular Biology 33:1–10

Ribeiro T, Viegas W, Morais-Cecilio L (2009) Epigenetic marks in the mature pollen of *Quercus suber* L. (Fagaceae). Sexual Plant Reproduction **22:** 1–7

Riley R (1974) The status of haploid research. In KJ Kasha, eds, Proceeding of the First International Symposium on Haploids in Higher Plants: Advances and Potential. Univ. Guelph, Guelph, Canada, pp 3-9

Ripperger JA, Schibler U (2006) Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. Nature Genetics **38:** 369–374

Rocheteau P, Gayraud-Morel B, Siegl-Cachedenier I, Blasco MA, Tajbakhsh S (2012) A subpopulation of adult skeletal muscle stem cells retains all template DNA strands after cell division. Cell **148:** 112–125

Roegiers F, Jan YN (2004) Asymmetric cell division. Current Opinion in Cell Biology **16:** 195-205

Roudier F, Teixeira FK, Colot V (2009) Chromatin indexing in *Arabidopsis*: An epigenomic tale of tails and more. Trends Genet **25:** 511–517

Ruiz-Carrillo A, Wangh LJ, Allfrey VG (1975) Processing of newly synthesized histone molecules. Science **190:** 117–128

Russell SD, Jones DS (2015) The male germline of angiosperms: repertoire of an inconspicuous but important cell lineage. Frontiers of Plant Science doi: 10.3389/fpls.2015.00173

Sadoni N, Cardoso MC, Stelzer EH, Leonhardt H, Zink D (2004) Stable chromosomal units determine the spatial and temporal organization of DNA replication. Journal of Cell Science **117:** 5353–5365

Saito C, Fujie M, Sakai A, Kuroiwa H, Kuroiwa T (1997) Changes in the extent of the condensation of nuclear chromatin and the localization of RNA during pollen development in *Nicotiana tabacum*. Cytologia **62:** 121–132

Sakamoto S, Potla R, Larner AC (2004) Histone deacetylase activity is required to recruit RNA polymerase II to the promoters of selected interferon-stimulated early response genes. Journal of Biological Chemistry **279:** 40362–40367

Sano Y, Tanaka I (2010) Distinct localization of histone H3 methylation in the vegetative nucleus of lily pollen. Cell Biology International **34:** 253–259

Satsolla C, Belmonte MF, Tahir M, Elthiti M, Khamiss K, Joosen R, Maliepaard C, Sharpe A, Gjetvaj B, Boultilier (2008) Buthionine sulfoimine (BSO)-mediated improvement in cultured embryo quality in vitro entails changes in ascorbate metabolism, meristem development and embryo maturation. Planta **228:** 255-272

Saze H (2008) Epigenetic memory transmission through mitosis and meiosis in plants. Seminar in Cell Developmental Biology **19:** 527–36

Schermelleh L, Haemmer A, Spada F, Rosing N,MeilingerD, Rothbauer U, Cardoso MC, Leonhardt H (2007) Dynamics of Dnmt1 interaction with the replication machinery and its role in postreplicative maintenance of DNA methylation. Nucleic Acids Research **35:** 4301–4312

Schmitz RJ, Schultz MD, Lewsey MG, O'Malley RC, Urich MA, Libiger O, J Schork N, R Ecker J (2011) Transgenerational epigenetic instability is a source of novel methylation variants. Science **334:** 369-373

Schoeftner S, Blasco MA (2009) A 'higher order' of telomere regulation: telomere heterochromatin and telomeric RNAs. EMBO Journal **19:** 2323–2336

Schoft VK, Chumak N, Choi Y, Hannon M, Garcia-Aguilar M, Machlicova A, Slusarza L, Mosioleka M, Parkb J-S, Parkb GT, L Fischerc R, Tamarua H (2011) Function of the DEMETER DNA glycosylase in the *Arabidopsis thaliana* male gametophyte. Proceedings of the National Academy Sciences, USA **108:** 8042–8047

Schoft VK, Chumak N, Mosiolek M, Slusarz L, Komnenovic V, Brownfield L, Twell D, Kakutani T, Tamaru H (2009) Induction of RNA-directed DNA methylation upon decondensation of constitutive heterochromatin. EMBO Reports **10:** 1015–1021

Schubert D, Clarenz, O, Goodrich J (2005) Epigenetic control of plant development by Polycombgroup proteins. Current Opinion in Plant Biology **8:** 553–561

Seguí-Simarro JM (2010) Androgenesis revisited. Botanical Review **76:** 377–404

Selker E (1998) Trichostatin A causes selective loss of DNA methylation in Neurospora. Proceedings of the National Academy of Sciences, USA **95:** 9430–9435

Shahbazian MD, Grunstein M (2007) Functions of site specific histone acetylation and deacetylation. Annual Review of Biochemistry **76:** 75–100

Shankar S, Srivastava RK (2008) Histone deacetylase inhibitors: mechanisms and clinical significance in cancer: HDAC inhibitor-induced apoptosis. Advances in Experimental Medicine and Biology **615:** 261–98

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. Plant Physiology **127:** 519–534

Sheridan WF (1973) Nonaqueous isolation of nuclei from lily pollen and an examination of their histones. Zeitschrift für Pflanzenphysiologie **68:** 450-459

Slotkin RK, Vaughn M, Borges F, Tanurdzic M, Becker JD, Feijo JA, Martienssen RA (2009) Epigenetic reprogramming and small RNA silencing of transposable elements in pollen. Cell **136:** 461–472

Soriano M, Cistue´ L, Castillo A (2008) Enhanced induction of microspore embryogenesis after n-butanol treatment in wheat (*Triticum aestivum* L.) anther culture. Plant Cell Reports **27:** 805–811

Sporbert A, Gahl A, Ankerhold R, Leonhardt H, Cardoso MC (2002) DNA polymerase clamp shows little turnover at established replication sites but sequential de novo assembly at adjacent origin clusters. Molecular Cell **10:** 1355–1365

Sterner DE, Berger SL (2000) Acetylation of histones and transcription-related factors. Microbiology and Molecular Biology Review **64:** 435-459

Steward N, Ito M, Yamaguchi Y, Koizumi N, Sano H (2002) Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress. Journal of Biological Chemistry **277:** 37741–37746

Struhl K (1998) Histone acetylation and transcriptional regulatory mechanisms. Genes Development **12:** 599– 606

Sunderland N (1974) Anther culture as a means of haploid induction, *In* KJ Kasha, eds, Haploids in Higher Plants: Advances and Potential. University of Guelph, Guelph, pp 91-122

Sunderland N, Evans LJ (1980) Multicellular pollen formation in cultured barley anthers. II. The A, B and C Pathways. Journal of Experimental Botany **31:** 501-514

Sunderland N, Wicks FM (1971) Embryoid formation in pollen grains of *Nicotiana tabacum*. Journal of Experimental Botany **22:** 213-226

Swigut T, Wysocka J (2007) H3K27 demethylases, at long last. Cell **131:** 29–32

Szakács È, Barnabás B (1988) Cytological aspects of in vitro androgenesis in wheat (*Triticum aestivum* L.) using fluorescent microscopy. Sexual Plant Reproduction **1:** 217–222

Takebayashi S, Nakao M, Fujita N, Sado T, Tanaka M, Taguchi H, Okumura K (2001) 5-Aza-2'-deoxycytidine induces histone hyperacetylation of mouse centromeric heterochromatin by a mechanism independent of DNA demethylation. Biochemical and Biophysical Research Communications **9:** 921-6

Talbert PB, Masuelli R, Tyagi AP, Comai L, Henikoff S (2002) Centromeric localization and adaptive evolution of an *Arabidopsis* histone H3 variant. Plant Cell **14:** 1053–1066

Tanaka I (1997) Differentiation of generative and vegetative cells in angiosperm pollen. Sexual Plant Reproduction **10:** 1–7

Tanaka M, Kikuchi A, Kamada H (2008) The *Arabidopsis* histone deacetylases HDA6 and HDA19 contribute to the repression of embryonic properties after germination. Plant Physiology **146:** 149–161

Tariq M, Saze H, Probst A, Lichota J, Habu Y, Paszkowski J (2003) Erasure of CpG methylation in *Arabidopsis* alters patterns of histone H3 methylation in heterochromatin. Proceedings of the National Academy of Sciences, USA **100:** 8823–8827

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

Telmer CA, Newcomb W, Simmonds DH (1995) Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. Protoplasma **185:** 106-112

Terasaka O (1982) Nuclear differentiation of male gametophytes in gymnosperms. Cytologia **47:** 27-46

Terasaka O, Niitsu T (1987) Unequal cell division and chromatin differentiation in pollen grain cells I. Centrifugal, cold and caffeine treatments. Botanical Magazine Tokyo **100:** 205-216

Terasaka O, Niitsu T (1990) Unequal cell division and chromatin differentiation in pollen grain cells. Botanical Magazine Tokyo **103:** 133–142

Terasaka O, Niitsu T (1995) The mitotic apparatus during unequal microspore division observed by a confocal laser scanning microscope. Protoplasma **189:** 187–193

Terasaka O, Tanaka R (1974) Cytological studies on the nuclear differentiation in microspore division of some angiosperms. Botanical Magazine Tokyo **87:** 209-217

Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF (2003) Histone Deacetylases: Unique players in shaping the epigenetic histone code. Annals of the New York Academy of Sciences **983:** 84–100

Touraev A, Fink CS, Stoger E, Heberle-Bors E (1995) Pollen Selection: A transgenic reconstruction approach. Proceedings of the National Academy of Sciences, USA pp 12165-12169

Traas JA, Doonan JH, Rawlins DJ, Watts J, Lloyd CW (1987) An actin network is present in the cytoplasm throughout the cell cycle of carrot cells and associates with the dividing nucleus. Journal of Cell Biology **105:** 387–395

Tsuji N, Kobayashi M, Nagashima K, Wakisaka Y, Koizumi KJ (1976) A new antifungal antibiotic, trichostatin. Antibiotics. **29:** 1-6

Tsuji H, Saika H, Tsutsumi N, Hirai A, Nakazono M (2006) Dynamic and reversible changes in histone H3-Lys4 methylation and H3 acetylation occurring at submergence inducible genes in rice. Plant Cell Physiology **47:** 995–1003

Twell D (2011) Male gametogenesis and germline specification in flowering plants. Sexual Plant Reproduction **24:** 149–160

Twell D, Park SK, Hawkins TJ, Schubert D, Schmidt R, Smertenko A, Hussey PJ (2002) MOR1/GEM1 plays an essential role in the plant-specific cytokinetic phragmoplast. Natural Cell Biololy **4:** 711-4

Twell D, Park SK, Lalanne E (1998) Asymmetric division and cell-fate determination in developing pollen. Trends in Plant Science **3:** 305-10

Uddenberg, D, Valladares , S, Abrahamsson, M, Fredrik Sundström, J, Sundås-Larsson, A, von Arnold, S (2011) Embryogenic potential and expression of embryogenesis-related genes in conifers are affected by treatment with a histone deacetylase inhibitor. Planta **234:** 527–539

Ueda K, Tanaka I (1995a) The appearance of male gamete-specific histones gH2B and gH3 during pollen development in *Lillium longiflorum*. Developmental Biology **169:** 210-217

Ueda K, Tanaka I (1995b) Male gametic nucleus-specific H2B and H3 histones, designated gH2B and gH3, in *Lillium longiflorum*. Planta **197:** 289-295

Vaillant I, Paszkowski, J (2007) Role of histone and DNA methylation in gene regulation. Current Opinion Plant Biology **10:** 528–533

Vakoc CR, Mandat SA, Olenchock BA, Blobel GA (2005) Histone H3 lysine 9 methylation and HP1_ are associated with transcription elongation through mammalian chromatin. Molecular Cell **19:** 381-391

van Dijk K, Ding Y, Malkaram S, Riethoven JJM, Liu R, Yang J, Laczko P, Chen H, Xia Y, Ladunga I, Avramova Z, Fromm M (2010) Dynamic changes in genome-wide histone H3 lysine 4 methylation patterns in response to dehydration stress in *Arabidopsis thaliana*. BMC Plant Biology **10:** 238

Vanhaecke T, Papeleu P, Elaut G Rogiers V (2004) Trichostatin A-like hydroxamate histone deacetylase inhibitors as therapeutic agents: toxicological point of view. Current Medicinal Chemistry **11:** 1629-1643

Vaquero-Sedas MI, Gamez-Arjona FM, Vega-Palas MA (2010) *Arabidopsis thaliana* telomeres exhibit euchromatic features. Nucleic Acids Research **39:** 2007–2017

Vasil IK (1976) Physiology and cytology of anther development. Biological Reviews **42:** 327-373

Vazart B (1971) Premiere division haploide et formation de la cellule génératrice dans le pollen de Tabac. Anna University A.R.E.R.S. **9:** 179-187

Verbsky ML, Richards EJ (2001) Chromatin remodeling in plants. Current Opinion in Plant Biology **4:** 494–500

Veselý J, Čihák A (1978) 5-AZACYTIDINE: Mechanism of action and biological effects in mammalian cells. Pharmacology and Therapeutics **2:** 813-840

Wada H, Kagoshima M, Ito K, Barnes PJ, Adcock IM (2005) 5-Azacytidine suppresses RNA polymerase II recruitment to the SLPI gene. Biochemical and Biophysical Research Communications **331:** 93-99

Wako T, Fukuda M, Furushima-Shimogawara R, Belyaev ND, Fukui K (2002) Cell cycle-dependent and lysine residue-specific dynamic changes of histone H4 acetylation in barley. Plant Molecular Biology **49:** 645-653

Wako T, Murakami Y, Fukui K (2005) Comprehensive analysis of dynamics of histone H4 acetylation in mitotic barley cells. Genes & Genetic Systems **80:** 269-276

Wang H, Dymock BW (2009) New patented histone deacetylase inhibitors. Expert Opinion on Therapeutic Patents **19:** 1727–1757

Wang J, Kang, X-J, Wei Q, Wang S-D (2009) Pollen development and floral morphology of Populus pseudo-simonii. Forestry Studies in China **11:** 99-104

Ware CB, Wang L, Mecham BH, Shen L, Nelson AM, Bar M, Lamba DA, Dauphin DS, Buckingham B, Askari B, Lim R, Tewari M, Gartler SM, Issa JP, Pavlidis P, Duan Z, Blau CA (2009) Histone deacetylase inhibition elicits an evolutionarily conserved selfrenewal program in embryonic stem cells. Cell Stem Cell **4:** 359–369

Wassenegger M (2005) The role of the RNAi machinery in heterochromatin formation. **122:** 13-6

Wei LQ, Yan LF, Wang T (2011) Deep sequencing on genome-wide scale reveals the unique composition and expression patterns of microRNAs in developing pollen of *Oryza sativa*. Genome Biology **12:** 53

Woodland HR, Adamson ED (1977) The synthesis and storage of histones during the oogenesis of *Xenopus laevis*. Developmental Biology **57:** 118-135

Wu K, Tian L, Malik K, Brown D, Miki B (2000) Functional analysis of HD2 histone deacetylase homologues in *Arabidopsis thaliana*. Plant Journal **22:** 19–27

Wu RS, Kohn KW, Bonner WM (1981) Metabolism of ubiquitinated histones The Journal of Biological Chemistry **256:** 5916-5920

Xiang D, Venglat P, Tibiche C, Yang H, Risseeuw E, Cao Y, Babic V, Cloutier M, Keller W., Wang E, Selvaraj G, Datla R (2011) Genome-wide analysis reveals gene expression and metabolic network dynamics during embryo development in *Arabidopsis*. Plant Physiology **156:** 346-56

Xu CR, Liu C, Wang YL, Li LC, Chen WQ, Xu ZH, Bai S-N (2005) Histone acetylation affects expression of cellular patterning genes in the *Arabidopsis* root epidermis. Proceedings of The National Academy of Sciences, USA **102:** 14469–14474

Yadav RK, Perales M, Gruel J, Ohno C, Heisler M, Girke T, Jönsson H, Reddy GV (2013) Plant stem cell maintenace involves direct transcriptional repression of differentiation program. Molecular System Biology **9:** 654

Yang JS, Lai EC (2011) Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. Molecular Cell **43:** 892–903

Yang F, Zhang L, Li J, Huang J, Wen R, Ma L, Zhou D, Li L (2010) Trichostatin A and 5-azacytidine both cause an increase in global histone H4 acetylation and a decrease in global DNA and H 3 K 9 methylation during mitosis in maize. BMC Plant Biology **10:** 178–189

Yoshida M, Kijima M, Akita M, Beppu T (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. Journal of Biological Chemistry **265:** 17174-17179

Yoshida M, Nomura S, Beppu T (1987) Effects of trichostatins on differentiation of murine erythroleukemia cells. Cancer Research **47:** 3688-3691

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

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

Zeng CJT, Lee YRJ, Liu B (2009) The WD40 repeat protein NEDD1 functions in microtubule organization during cell Division in *Arabidopsis thaliana*. Plant Cell **21:** 1129–1140

Žárský V, Garrido D, Říhová L, Tupý J, Vicente, O, Heberle-Bors E (1992) Derepression of the cell cycle by starvation is involved in the induction of tobacco pollen embryogenesis. Sexual Plant Reproduction **5:** 189-194

Zhang H, Ogas J (2009) An epigenetic perspective on developmental regulation of seed genes. Molecular Plant **2:** 610–627

Zhang K, Siino JS, Jones PR, Yau PM, Bradbury EM (2004) A mass spectrometric "Western blot" to evaluate the correlations between histone methylation and histone acetylation. Proteomics **4:** 3765–3775

Zhang X, Bernatavichute YV, Cokus S, Pellegrini M, Jacobsen SE (2009) Genome-wide analysis of mono-, di- and trimethylation of histone H3 lysine 4 in *Arabidopsis thaliana*. Genome Biology **10:** R62 doi: 10.1186/gb-2009-10-6-r62

Zhang X, Clarenz O, Cokus S, Bernatavichute YV, PellegriniM, Goodrich J, Jacobsen SE (2007) Whole-genome analysis of histone H3 lysine 27 trimethylation in *Arabidopsis*. PLoS Biol **5:** 129

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

Zheng MY, Liu W, Weng Y, Polle E, Konzak CF (2001) Cultures of freshly isolated wheat (*Triticum aestivum* L.) microspore treated with inducer chemicals. Plant Cell Reports doi: 10.1007/s00299-001-0393-0

Zhou C, Labbe H, Sridha S, Wang L, Tian L, Latoszek-Green M, Yang Z, Brown D, Miki B, Wu K (2004) Expression and function of HD2-type histone deacetylases in *Arabidopsis* development. Plant Journal **38:** 715–724

Zhou J, Wang X, He K, Charron JB, Elling AA, Deng XW (2010) Genome-wide profiling of histone H3 lysine 9 acetylation and dimethylation in *Arabidopsis* reveals correlation between multiple histone marks and gene expression. Plant Molecular Biology **72:** 585–595

Zilberman D (2008) The evolving functions of DNA methylation. Current Opinion in Plant Biololgy **11:** 554–59

Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S (2007) Genome-wide analysis of *Arabidopsis thaliana* DNA methylation uncovers an interdependence between methylation and transcription. Nature Genetics **39:** 61–69

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell **5:** 1411–1423

7 PUBLICATIONS AND PROCEEDINGS RELATED TO THIS THESIS

Publications

P. Pandey, A. Houben, J. Kumlehn, M. Melzer, T. Rutten; Pollen embryogenesis is associated with reshuffling of post-translationally modified histones (in prep.).

P. Pandey, A. Houben, J. Kumlehn, M. Melzer, T. Rutten; Chromatin alterations during pollen development in *Hordeumvulgare*; Cytogenet Genome Res DOI: 10.1159/000351211 (2013). http://www.karger.com/Article/Citation/351211

Oral Presentations

P. Pandey, A. Houben, J. Kumlehn, M. Melzer and T. Rutten. ***Oral***: Epigenetic modifications associated with the change of cellular identity during pollen embryogenesis in *Hordeum vulgare*- Plant Biology Europe FESPB/EPSO Congress, Dublin, Ireland, 22-26 June 2014.

P. Pandey, A. Houben, J. Kumlehn, M. Melzer and T. Rutten. ***Oral***: Stress-induced chromatin modifications: a motor behind pollen embryogenesis?- 10th Plant Science Student Conference, IPK, Gatersleben, 2-5 June 2014.

P. Pandey, A. Houben, J. Kumlehn, M. Melzer and T. Rutten. Oral: Chromatin alterations: an approach to understand the initial mechanism of pollen embryogenesis in Hordeum vulgare- PhD Talk: PhD student Seminars, IPK, Gatersleben, Germany, 6 February 2014.

P. Pandey, A. Houben, J. Kumlehn, M. Melzer and T. Rutten. Oral: Chromatin alterations during pollen development and pollen embryogenesis in Hordeum vulgare- SEB (Society of Experimental Biology) annual main meeting, Valencia, Spain, 3-6 July 2013.

P. Pandey, A. Houben, J. Kumlehn, M. Melzer and T. Rutten. ***Oral***: Chromatin alterations during pollen development in *Hordeum vulagre*- 9th Plant Science Student Conference, IPB Halle, Germany, 28-31 May 2013.

P. Pandey, D. Daghma, T. Rutten, A. Houben, J. Kumlehn and M Melzer. ***Oral***: Chromatin modification during pollen embryogenesis (POEM) in *Hordeum vulgare* cv. Igri- Meeting of Siemens/DAAD scholars, Munich, Germany, 8-10 December 2011.

Poster Presentations

P. Pandey, J. Kumlehn, M. Benecke, A. Houben, T. Rutten & M. Melzer. ***Poster:*** Reshuffling of modified histones during pollen embryogenesis. – Institute Day, IPK, Gatersleben, 08-10 October 2014.

P. Pandey, A. Houben, J. Kumlehn, M. Melzer and T. Rutten. ***Poster***: Chromatin alterations: an approach to understand the initial mechanism of pollen embryogenesis in *Hordeum vulgare*-18th International Microscopy Congress, Prague, Czech Republic, 7-12 September 2014.

P. Pandey, T. Rutten, A. Houben, J. Kumlehn, M. Benecke and M. Melzer. ***Poster***: Pollen development and pollen embryogenesis: new insights by epigenetic modifications- Institute Day, IPK, Gatersleben, Germany, 25 July 2013.

P. Pandey, A. Houben, J. Kumlehn, M. Melzer and T. Rutten. ***Poster***: Chromatin alterations during pollen development in *Hordeum vulgare*- PANOS spring meeting, 'Current Methods in Subcellular Localization', EMBL, Heidelberg, Germany, 18-19 April 2013.

P. Pandey, D. Daghma, T. Rutten, J. Kumlehn, A. Houben and M. Melzer. ***Poster***: Natural Chromatin modification during pollen gametogenesis in *Hordeum vulgare*- Summer Institute Day, IPK, Gatersleben, 25-26 September 2012.

P. Pandey, D. Daghma, T. Rutten, A. Houben, J. Kumlehn and M Melzer. ***Poster***: Nuclear behavior and chromatin modification during pollen embryogenesis in barley- The 15th European Microscopy Congress, Manchester, UK, 16-21 September 2012.

P. Pandey, D. Daghma, M. Lietz, T. Rutten, J. Kumlehn, A. Houben and M. Melzer. ***Poster***: Chromatin behavior and modification during pollen gametogenesis in barley- 8th Plant Science Student Conference, IPK, Gatersleben, 4-7 June 2012.

P. Pandey, D. Daghma, M. Lietz, T. Rutten, J. Kumlehn, A. Houben and M. Melzer. ***Poster***: Chromatin modifications during pollen embryogenesis in barley- 11th Gatersleben Research Conference 2012 'Chromosome Biology, Genome Evolution and Speciation', IPK, Gatersleben, 23-25 April 2012.

8 CURRICULUM VITAE

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Experience

04/2015 to till date: Post-Doctoral Researcher, Imperial College London

Project: 'Effectors as molecular probes to uncover the mechanisms of microbial accommodation inside the plant cells'

Education

10/2011 to till date: Ph.D. (Biological Sciences), Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany

Thesis: 'A study on epigenetic modifications to dissect the initial mechanism of pollen embryogenesis in *Hordeum vulgare*'

8/2007 to 7/2009: M.Sc. Biotechnology, Integral University, Lucknow, India

Master's thesis: 'Genetic variation of *Aloe vera* cultivars through RAPD'

7/2004 to 6/2007: B.Sc. Biology Chhatrapati Shahu Ji Maharaj University (CSJMU), Kanpur, India

Publications

P. Pandey, A. Houben, J. Kumlehn, M. Melzer, T. Rutten; Pollen embryogenesis is associated with reshuffling of post-translationally modified histones (in prep.).

Y. F. Dagdas, K. Belhaj, A. Maqbool, A. Chaparro-Garcia, **P. Pandey**, B. Petre, N. Tabassum, R. K. Hughes, J. Sklenar, J Win, F. Menke, K. Findlay, M. J. Banfield, S. Kamoun, T. O. Bozkurt; An effector of the Irish potato famine pathogen antagonizes a host autophagy cargo receptor. Science (2015) (submitted)

P. Pandey, A. Houben, J. Kumlehn, M. Melzer, T. Rutten; Chromatin alterations during pollen development in *Hordeumvulgare*; Cytogenet Genome Res DOI: 10.1159/000351211 (2013). http://www.karger.com/Article/Citation/351211

Awards and grant

Siemens/DAAD Postgraduate scholarship award.
IFSM Young Scientist award.
IMC European Scholarship award.
FESPB travel grant for FESPB/EPSO 2014 Congress, Dublin, Ireland.
Talk award in Plant Science Student Conference, IPB, Halle, Germany.

Scientific activities

Workshop on Gene Technology, Biosafety and Biosecurity, Gatersleben, Germany, 17-18.02.2015.
The IFSM School for Young Scientists, Prague, Czech Republic, 06.09 2014.
13thZeiss Microscopy Workshop,Jena, Germany, 14.032013.
EMBL Introductory course: Confocal Microscopy, EMBL Heidelberg, Germany, 13-15.03.2012.
PANOS Spring meeting 'Super-Resolution Imaging: Light meets Electron Microscopy' Max Plank Institute of Experimental Medicine, Goettingen, Germany, 23.03.2012.

9 AFFIRMATION

I hereby declare that the submitted work has been completed by me, the undersigned, and that I have not used any other than permitted reference sources or materials or engaged any plagiarism. All the references and the other sources used in the presented work have been appropriately acknowledged in the work. I further declare that the work has not been previously submitted for the purpose of academic examination, either in its original or similar form, anywhere else.

Hiermit erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die den benutzten Hilfsmitteln wörtlich oder inhaltlich entnommenen Stellen habe ich unter Quellenangaben kenntlich gemacht. Die vorliegende Arbeit wurde in gleicher oder ähnlicher Form noch keiner anderen Institution oder Prüfungsbehörde vorgelegt.

Pooja Pandey
Gatersleben, 01.07.2015

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DEDICATION

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