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Faculty of Science

Role of transcription factors in early male gametophyte development of *Arabidopsis*

Ph.D. Thesis

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Annotation: In the presented work the relationship between transcription factors and male gametophyte development was studied. The Ph.D. Thesis covers selection of candidate genes, wide-scale screening of T-DNA mutant lines and detailed analysis of a selected transcription factor on pollen development.

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Declaration of originality

The coauthors listed below fully acknowledgement that David Reňák performed the major works in respective papers with the mention of his contribution.

(1) Honys D, **Reňák D**, Twell D. Male gametophyte development and function. Global Science Books, pp.76-87, 2007.

D. Reňák: Progamic phase of the male gametophyte development

(2) **Reňák D**, Dupľáková N, Honys D (2010) Wide-scale screening of T-DNA lines for transcription factor genes affecting male gametophyte development in Arabidopsis. Plant Sex Rep, submitted.

D. Reňák: Analysis of the early transcription factors, manuscript preparation

(3) Gibalová A, **Reňák D**, Matczuk K, Dupľáková N, Cháb D, Twell D, Honys D (2009) AtbZIP34 is required for Arabidopsis pollen wall patterning and the control of several metabolic pathways in developing pollen. Plant Mol Biol (2009) 70: 581-601.

D. Reňák: *in vivo* and *in vitro* pollen tube analysis, microscopy, manuscript preparation of respective part

(4) Honys D, Oh SA, **Reňák D**, Donders M, Šolcová B, Johnson JA, Boudová R, Twell D (2006) Identification of microspore-active promoters that allow targeted manipulation of gene expression at early stages of microgametogenesis in Arabidopsis. BMC Plant Biol 6: 31-39.

D. Reňák: microscopy analysis, image and manuscript preparation of respective part

(5) Dupľáková N, **Reňák D**, Hovanec P, Honysová B, Twell D, Honys D (2007) Arabidopsis Gene Family Profile (aGFP) – user-oriented transcriptomic database with easy-to-se graphic interface. BMC Plant Biol 7: 39-46.

D. Reňák: gene family arrangement, processing of transcriptomic data

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D. Reňák: analysis of four databases, manuscript preparation of respective part

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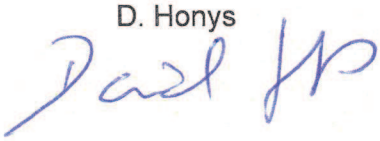

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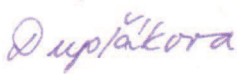

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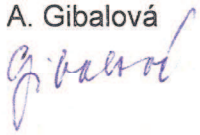

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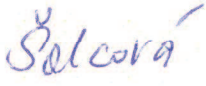

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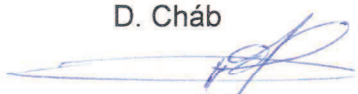

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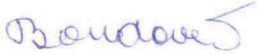

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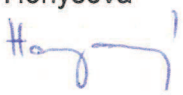

Contents

1. Cell biology of the male gametophyte

1.1. Stamen development

The developmental events leading to a stamen formation follows the pattern of floral meristem identification. Stamen primordia are initiated on the meristem surface within the third whorl as a result of specific floral homeotic MADS-box genes co-expression, including *APETALA3 (AP3), PISLILLATA (PI), AGAMOUS (AG),* and *SEPALLATA1/2/3/4 (SEP1/2/3/4)* in *Arabidopsis.* Misexpression of participating transcriptional regulators is well known to produce messy floral architecture, for instance additional stamens replacing carpels in the neighbouring fourth whorl, if male part is considered in *superman* mutation (Bowman et al. 1992). Stamen primordia appear after the outer sepal and petal primordia have initiated but prior to the initiation of inner carpel primordia. Later, the stamen primordium prolongs and differentiates into two compartments, filament and anther. The filament stands as a holder carrying conducting tissue while the anther contains both nonreproductive and reproductive tissues that are responsible for the production and release of mature pollen grains (Scott et al. 2004).

The anther forms two bilaterally symmetrical pollen sacks with two loculi in each. Developmentally, the anther surrounding pollen sacks consists of several cellular layers originating from three layers called L1, L2, and L3 of the floral meristem that can be recognized histogically and functionally and that play a role in the anther differentiation process. The outermost L1 layer establishes the epidermis and stomium which is responsible for splitting of anther wall during dehiscence. Thus the programmed cell death (PCD) of the stomial cells is necessary for pollen release and general plant fertility. The middle L2 layer produces archesporal cells that divide periclinally into outer parietal cells and inner sporogenous cells. Parietal cells by further divisions form the outer part of the tapetum, middle wall layer(s) and endothecium, which enforces the power for mechanical opening of the anther by the unique mechanism of the cell wall thickening (Scott et al. 2004). The mutation in *MYB26* gene causes the male sterility by defective cell wall fortification in this endothecial layer which unables the opening (Steiner-Lange et al. 2003). The sporogenous cells give rise by further divisions to microsporocytes or meiocytes and such differentiation establishes the male germ lineage (Yang and Sundaresan 2000). The last L3 layer contributes

to the formation of a connective, vascular bundle, and the inner part of tapetum (Goldberg et al. 1993). The tapetum is a nutritive tissue forming the inner wall of the pollen sack and supporting the development of microspore mother cells due to numerous lateral interconnections by plasmodesmata (Heslop-Harrison 1971a, 1971b). However, their lack plasmodesmata on their face towards middle layer. Sugars destinated to pollen in the loculus have then to cross the middle layer and the tapetal layers by the apoplasmic pathway; it is suggested that these two envelopes could be involved in the control of sugar transport from the outer anther wall layers to the locular fluid (Clément and Audran 1995).

Several genes have been identified genetically that are critical for early anther development and microsporogenesis (reviewed by Wilson and Yang 2004). One of the earliest acting gene is the *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*) playing a role at early anther differentiation with strong expression in microsporocytes and tapetum (Fig. 1). *spl/nzz* mutant plants are male-sterile producing no sporogenous and tapetal cells (Schiefhaler et al. 1999, Yang et al. 1999, Ito et al. 2004). This gene encodes a transcription factor that directly regulates a gene of *EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS* (*EMS/EXS*). Its mutation causes the formation of additional male sporocytes along with lack of tapetal cells but resulting in non-viable pollen, which implicates the irreplaceable role of tapetum (Canales et al. 2002, Zhao et al. 2002). *EMS/EXS* gene encodes a putative leucine-rich repeat receptor protein kinase. Another mutation in the *TAPETUM DETERMINANT1* (*TPD1*) gene showed the same phenotype as that of *ems/exc* (Yang at al. 2003) and it is known to be a putative signal peptide for secretion. Taken together, EMS/EXC and TPD1 may interact as a receptor and a ligand determining the microsporocytes and tapetal cell formation (Ma 2005, Wilson and Zhang 2009). Next, DYSFUNCTIONAL TAPETUM (DYT1), a putative bHLH transcription factor, is likely to be involved in regulation of *AMS* and *MS1* (Zhang at al. 2006). *ABORTED MICROSPORES* (*AMS*) is a member of MYC class bHLH transcription factor family. Although *ams* plants have both tapetum and male sporocytes that undergo meiosis, both tapetum and microspores degenerate soon afterwards (Sorensen et al. 2003). MALE STERILITY (MS1), a nuclear protein with a PHD-finger domain accompanying RING finger and putative leucine zipper motifs, is also required for early microspore development and proper exine formation (Wilson et al. 2001, Ito and Shinozaki 2002, Ariizumi et al. 2004, Vizcay-Barrena and Wilson 2006, Ito et al. 2007). MYB33/65 are GYMYB-like TFs regulated by SPL/NZZ either directly or via DYT1 (Fig. 1). Their mutations caused premeiotic abortion of

microsporocytes probably due to aberrant tapetum development (Millar and Gubler 2005). MYB103 is regulated by EMS/EXS and it is necessary for proper tapetum and microspore development (Zhang et al. 2007). In addition, *MYB32* is expressed in tapetum and its mutation leads to aberrant pollen development and partial male sterility (Preston et al. 2004) (Fig. 1).

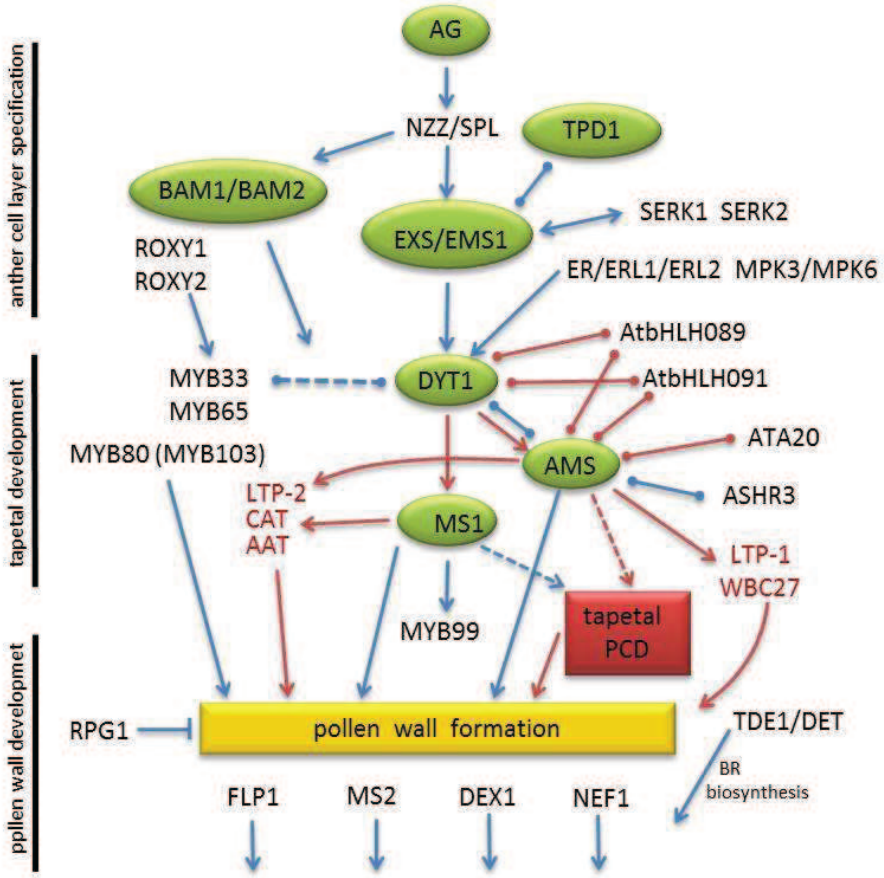

Fig 1. Regulatory network determining the anther cell wall specification, tapetum and pollen wall development indicating downstream regulation (→), and a protein-protein interaction (–) compiled from two sources: Wilson and Zhang, 2009 (blue lines) and Xu et al. 2010 (red lines).

After microsporogenesis, tapetal cells play an important role in the production of callase that helps to release individual microspores from the tetrads. In addition, tapetal cells secrete and deposit various compounds, including flavonoids and structural polymers such as sporopolenin, the most resistant biopolymer in the nature making the exine of the pollen wall. The tapetal cells eventually disintegrate and the material is deposited as a part of the pollen coat. Some of their cytoplasmic contents, including lipids and proteins, may cause allergic reaction to a human.

1.2. Pollen development

Microsporogenesis is the initial process of the male gametophyte development producing haploid microspores from the diploid microsporocytes. It is initiated by meiotic divisions I and II giving rise to tetrads of microspores. The tetrad is enclosed in a callose (β-1,3-gluan) cell wall that physiologically isolates the nascent microspores from the surrounding sporophytic tissue for the establishment, as believed, of the new developmental programme. Thick callose capsule is subsequently digested by callase secreted by tapetal cells. Such events demonstrate the tight co-operation between sporophytic and gametophytic cells for a successful process of pollen formation. At this point, *quartet3* mutant plants produce mature pollen grains remaining attached in tetrad position for the mutation in polygalacturonase which is normally secreted by tapetum.

As microspore grows, the initial three-lobed cell surface gradually evens and smoothes. The nucleus of an early microspore is located in the centre but later it is pushed towards the cell wall due to rapid vacuole biogenesis. The large central vacuole seems to be important for further haploid pollen mitosis I, an extremely asymmetric cell division producing larger vegetative and smaller generative cell. The vegetative cell makes the pollen grain itself and includes most of the former cytoplasm enriched by organelles and contains a large disperse and transcriptionally active nucleus while the generative cell encloses only very limited cytoplasm and rather condense and less active nucleus. Disruption of cell polarization dramatically changes the developmental programme and may even lead to a haploid embryogenesis as often used in plant biotechnologies (Hause et al. 1993, Eady et al. 1995). After pollen mitosis I, the generative cell migrates inward the pollen grain that then represents the unique cell-within-a-cell structure. In number of plants including *Arabidopsis*, the generative cell undergoes pollen mitosis II to form two sperm cells within a pollen grain (three-celled pollen). However, in most species (producing two-celled pollen) the second mitosis occurs during pollen tube growth when penetrating style tissues. Considering the male germline, the generative cell and later derived sperm cells involve processes that trigger different development and cell specification including chromatin rearrangement and histone modification plus germline specific histone H3.3 incorporation and transcriptional and post-transcriptional regulation by small RNAs pathways (Jones-Rhoades et al. 2006, Berger and Twell 2011, Borges et al. 2011, Twell 2011). On the cellular level, generative cell and sperm cells are characterized by condensed chromatin and very limited cytoplasm.

During the maturation of Arabidopsis pollen, the vegetative cell accumulates carbohydrate and lipid reserves for later demand during pollen tube growth (Pacini 1996) and similarly synthesizes mRNAs and proteins which are functionally required for rapid pollen tube growth after pollination as described earlier in other species (Tupý 1982, Mascarenhas et al. 1984, Mašek et al. 2000) (Fig. 2).

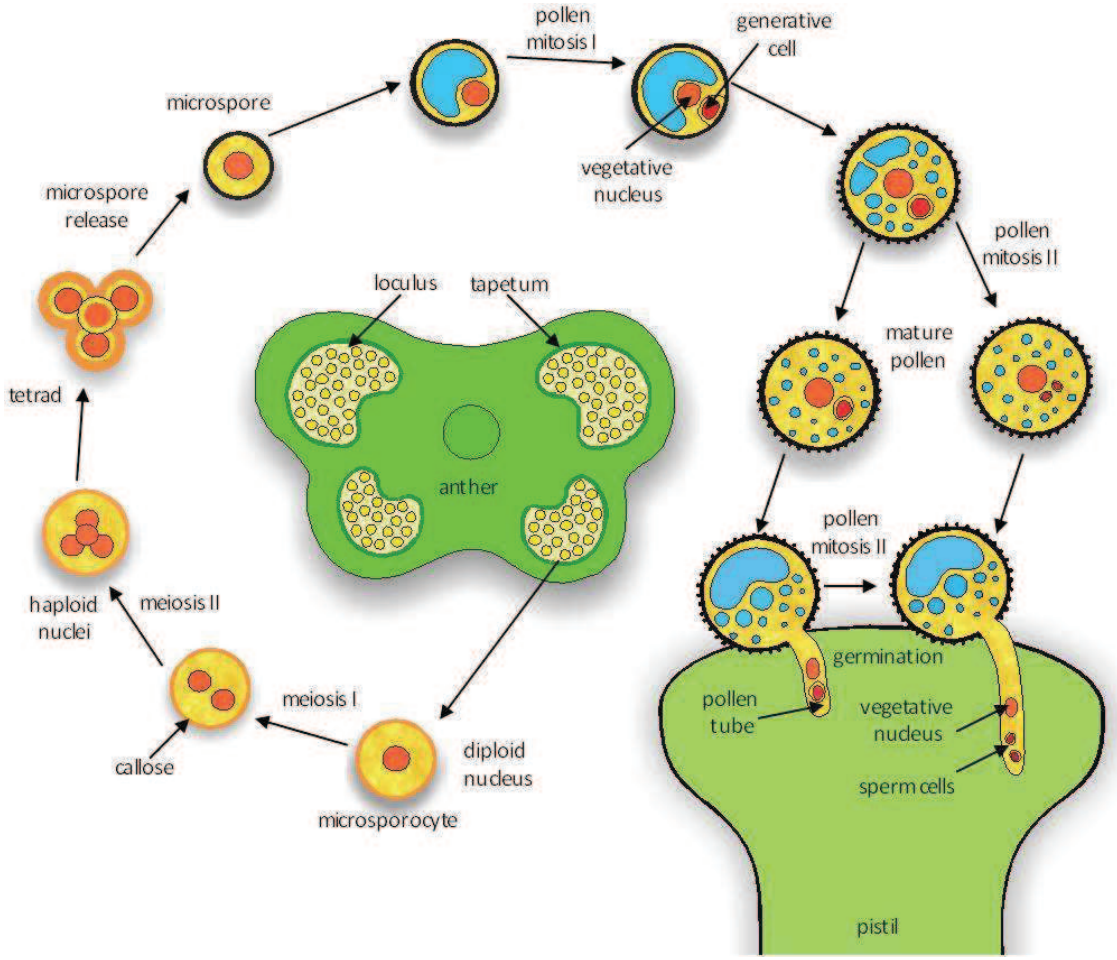

Fig. 2. Male gametophyte development. Diploid microsporocyte undergoes meiosis to produce a tetrad of haploid microspores embedded in callose wall. After enzymatic digestion by tapetal callase, individual microspores are released. After two haploid mitosis, the mature pollen grain is ready to land on stigma (*Arabidopsis*, tricellular pollen) and growth into the pollen tube delivering two sperm cells into female gametophyte. In case of bicellular pollen (on the left), the second mitosis of generative cell occurs later within the pollen tube.

Pollen cell wall displays a complex structure of different materials arranged in several distinguished layers. The internally laid intine is enriched with cellulose and pectines while outer exine, having additional sublayers of nexine and sexine, is made predominantly of sporopollenin, the complex of phenylpropanoids, phenolics, fatty acids, and carotenoids. The

exine often exhibits a highly decorated pattern characteristic for each plant species. The origin and genetic control of all pollen wall layers is different. Synthesis of exine is under the sporophytic control; however, the pattern is initiated by primexine at the tetrad stage. On the contrary, intine is completely formed and maintained from gametophytic side of the pollen wall. Exine does not form a completely enclosed layer; at specific spots (apertures) it is weakened to enable the outgrowth of a pollen tube. The final stage of pollen development is significant for pollen dehydration and production of osmoprotectants such as sugars, proline, and glycine-betain (Schwacke et al. 1999).

1.3. Pollen tube growth

Rehydration of a pollen grain after the landing on a stigma is a crucial initiation step for following vegetative cell activation. Rapid water uptake is accompanied by massive efflux of ions, sugars, amino acids, enzymes and other compounds. It boosts metabolic activity, respiration and various subcellular events including formation of vesicles, lipid droplets, vacuoles, and endomembrane biogenesis. Dynamic cytoskeletal rearrangements, mainly actin microfilaments, support the aperture, which was already determined during pollen maturation, for the outgrowth of intine when pollen germinates. Other significant features include higher dictyosome activity, massive RNA and protein synthesis (Raghavan 1997), and polysome formation to provide a translational site for stored mRNA/mRNP molecules, which are essential for pollen germination and early tube growth (Čapková et al. 1994, 1997, Honys et al. 2000 and 2009). There is the great evidence that the application of translational inhibitors (cycloheximide) arrests pollen tube growth (Čapková et al. 1980) unlike the transcriptional inhibitors (actinomycin D) that have no or very mild effect on pollen tube growth (Lafleur and Mascarenhas 1978, Honys and Twell 2003).

After germination, the vegetative cell becomes highly polarised and self-arranged into four subcellular zones for the whole period of tube growth: apical, subapical, nuclear and vacuolar zones from the tip backwards (Raghavan 1997). The apical zone is typical of the presence of vesicles derived from dictyosomes carrying carbohydrates and fusing with plasma membranes in the process of exocytosis (Pierson et al. 1990) and endocytic vesicles bringing various materials into the cell. The very tip is free of cytoskeleton and keeps sharp but oscillating gradient of Ca^{2+} ions (Malhó et al. 1994, Pierson et al. 1994) which correlates with oscillating vesicle-trafficking system and depositing methyl-esterified, and deesterified

and Ca^{2+}-bound pectins into the cell wall and oscillating tip growth (Pierson et al. 1995, Geitmann et al. 1996). The subapical zone is enriched by most organelles especially dictyosomes and mitochondria, while vegetative nucleus and sperm cells stay in nuclear zone, and the last vacuolar zone is occupied by large vacuoles regularly separated by callose plugs (Cresti et al. 1977) to keep the cytoplasm towards in constant volume when pollen tubes grows. Pollen tube zonation and intracellular movements are directly generated by molecular motors (myosin, kinesin) with association to cytoskeletal elements (Pierson et al. 1985, Lancelle et al. 1987, Raghavan 1997). The composition of the cell wall depends on the location along with pollen tube; at the very tip it is composed of pectins providing very elastic property for the active tip growth, subapically the inner cellulose wall is added for mechanical support and further backwards callose is deposited from the inner site to add even more resistance during the growth through transmitting tissue making the cell wall finally three-layered.

2. Molecular biology and genetics of the male gametophyte

2.1. Transcriptional regulation

Initiation:

Gene expression is the fundamental process to support the life of the cell and it involves multiple steps of regulation occuring in various subcellular compartments. The process has been traditionally divided into following events: signalling, chromatin remodelling, transcription, mRNA processing, post-transcriptional regulation and distribution of mRNA, translation, post-translational regulation, and degradation. It is obvious that such cascade of information flow represents the complex machinery and needs to be very well orchestrated. Each step also allows the cell to regulate the temporary need according to the internal or external signals at multi-level regulatory manner and therefore each step plays its own important role during the gene expression. However, the transcriptional control is considered as the primary level of regulation of the fate of a single cell/multicellular organism. The key point of the transcriptional regulation is the initiation of transcription that is tightly associated with the chromatin remodelling. The whole process is controlled by a diversity of proteins involved in specific DNA-protein and protein-protein interactions that finally help to place the RNA polymerase on promoters of regulated genes. In eukaryotes, three different enzymes (*RNA polymerase I, II,* and *III*) catalyse the transcription of different RNA molecules. *RNA polymerases I*, located in the nucleolus, generate precursors of ribosomal RNA (45S pre-rRNA) later processed into 25/28S (plant/animal), 5.8S and 18S rRNAs. *RNA polymerase II* transcripts all protein-coding genes producing pre-mRNA. The regulation of transcription by *RNA polymerase II* is the considered subject of this chapter. Finally, *RNA polymerase III* catalyses the origin of tRNAs, 5S rRNA, and a variety of small RNAs including spliceosomal U6 and SRP-specific RNA.

The expression of protein-coding genes, as mentioned, is regulated by multiple protein-binding DNA sequences. These include promoter elements, promoter-proximal elements and distant enhancers. The promoter is a regulatory part overlapping tens of nucleotides upstream of translational initiation site and includes TATA box, CpG islands, and other elements that control the position of RNA polymerase II for the transcription. The sequence which is recognized as a TATA box usually lies 25-35 bp upstream of the start site and is very conserved. Even a single mutation significantly decreases the level of

transcription. Instead of TATA-box, some eukaryotic genes contain an alternative but less conserved promoter element called the initiator. Despite those well-defined elements, many protein-coding genes, typically low-rate expressing house keeping genes require GC-rich stretch, the islands of 20-50 nucleotides situated within about 100 base pairs upstream of the start region. Besides those elementary promoter elements, there are other sequences known as promoter-proximal elements lying within 100-200 nucleotides upstream of the start site important for cell-type specific gene expression in terms of tissue and developmental stage delimitations. Additionally, the transcription of many genes can be remarkably stimulated by enhancers located thousands/tens-of-thousands base pairs from the start site but also they are frequently found downstream in 5'UTRs, intron or exon sequences or even behind the stop codon of the controlled gene. Like promoter-proximal elements, many enhancers are cell-type-specific. Taking together, it is notable that (not only) plant promoters harbour surprisingly high amount of variation, mostly due to specific *cis*-elements, that significantly affect expression levels and support the stability yet plasticity of plant development under various biotic and abiotic conditions.

Proteins controlling the pre-mRNA biogenesis can be classified into four different functional groups: (1) chromatin-associated proteins, (2) the basic transcription apparatus and intrinsic associated factors, (3) large multi-subunit coactivators and other cofactors, and (4) gene-specific DNA-binding transcriptional factors.

(1) ***Chromatin-associated proteins***. Chromatin structure can operate in two distinct states: euchromatin which is open and accessible for transcriptional machinery and heterochromatin which is closed and inactive for gene expression. The ability of the flexible change between these two states is the presumption for active transcriptional regulation. Proteins responsible for such dynamic rearrangement include factors that covalently modify histones and remodelling complexes necessary for chromatin reorganization. Covalent modification – histone acetylation – promotes chromatin unfolding and is characteristic for actively transcribed regions. It is catalysed by acetyltransferase and associated with coactivators. On the other hand, histone deacetylation triggers chromatin condensation and deacetylase activity is often associated with corepresors (Li et al. 2002). Chromatin remodelling complexes (such as SWI/SNF and ISWI complexes) actively hydrolyze ATP for local reorganization of nucleosomes in two pathways; by sliding nucleosomes in *cis* or displacing nucleosome octamers in *trans* manner. SWI/SNF are placed with the help of

enhancer-binding factor operating upstream of the initiation site. Increasing evidence suggests that the putative plant SWI/SNF complexes control transcription of genes involved in many developmental processes such as floral organ identification and embryogenesis (Hurtado et al. 2006, Archacki et al. 2009).

(2) The ***basic transcription apparatus and intrinsic associated factors*** represent the general transcriptional factors that play an important role in correct placing the RNA polymerase II on promoter sequences. These general transcription factors comprise TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. Most of them are multimeric proteins. The largest general TF is TFIID, which consists of TATA box-binding protein (TBP) and thirteen TBP-associated factors (TAFs). TBP is extremely conserved in all eukaryotes and binds the TATA box in a saddle-shape position. Once TBP has bound, it recruits TFIIB and subsequently a preformed complex of TFIIE, TFIIF, and TFIIH. The last has a kinase activity necessary for phosphorylation of the C-terminal domain (CTD) of RNA polymerase II, its separation from the associated factors and transcription start. Moreover, TFIIH harbours two key activities. First, it has a helicase activity important for DNA accessibility and second, it exhibits a histone acetylase activity and so it can function as a transcription coactivator by histone acetylation in the TATA box vicinity. The TAF subunits of TFIID appear to play a role in initiating of the transcription from promoters lacking the TATA box.

(3) ***Large multi-subunit coactivators and other cofactors*** represent heterogenous class of regulatory proteins including Mediator complex of about 20 subunits that essentially act as a molecular bridge between activation domains and RNA polymerase II by binding to those components directly and thus contributing to the integration of signals from several activators at a single promoter.

(4) ***Gene-specific DNA-binding transcriptional factors*** can act as activators or repressors and can interact with chromatin-remodelling complexes. In principle, these specific transcription factors bound to gene specific *cis*-elements and themselves are expressed in a tissue/cell-type-, temporal- or stimulus-dependent-specific manner and are therefore responsible for the specificity of gene expression. Transcription factors can interact directly with different components of the general machinery and with coactivators, affecting complex formation and provide, by combinational manner, another level of transcription regulation. Specific transcription factors are modular proteins with distinct and functionally separable domains, such as DNA-binding and activation domains. According

their DNA-binding domain and other properties they can be grouped into distinct families that are described later in an extra chapter.

Elongation and termination:

Once the polymerase (RNPII) has transcribed about 25 bases and pre-mRNA emerged from the protein complex, the 5' end is modified by capping enzyme complex, the C-terminus of RNPII is phosphorylated and further elongation is highly processive until it passes the polyadenylation signal that directs pre-mRNA 3' end cleavage and enables its further polyadenylation (Phatnami and Greenleaf 2006). RNA polymerase then can terminate at multiple sites located over a distance of 0.5-2 kb of this poly(A) additional tail. The protein complex responsible for such action is associated with phosphorylated carboxyl-terminal domain of the RNA polymerase II which serve as a flexible functional platform not only for polyadenylation but also other post-transcriptional modification including capping of nascent pre-mRNA, splicing of introns and cleavage behind polyadenylation signal.

2.2. Regulatory sequences

Up to date, a bulk of pollen-specific genes has been identified, making it possible to analyse their promoters including the potential regulatory sequences driving the specificity of their expression. Several pollen-specific *cis*-elements such as AGAAA, TCCACCATA and GTGA, have been already identified in many plant species (Twell et al. 1989, Twell et al. 1991, Eyal et al. 1995, Bate and Twell 1998, Hamilton et al. 1998, Rogers et al. 2001). Indeed, precise analysis of function of such motifs is a fruitful approach to reveal and understand the mechanisms of cell-specific gene regulation during pollen development in plants.

AGAAA and TCCACCATA motifs were well described within tomato *LAT52* promoter by Bate and Twell (1998). The promoter is active from pollen mitosis I but the accumulation of respective transcripts substantially increases during pollen maturation. Sequence analysis of the *LAT52* promoter revealed the presence of all major *cis*-regulatory elements required for pollen-specific transcription within the upstream region of -492 to -52. This region was shown to comprise three independent activator domains A, B, and C, each sufficient for transcriptional regulation. The PBII motif (TGTGGTT) within the domain B functions as pollen-specific enhancer element. Domain C contains an activator unit -72 to -52 (sub-domain C2) embodying two novel co-dependent regulatory elements AGAAA and

TCCACCATA. It was demonstrated that the transcriptional activity of *LAT52* promoter is controlled by a complex of pollen-specific *cis*-regulatory elements in a highly cooperative manner.

Similarly, AGAAA and GTGA motifs were described to regulate anther- and pollen stage-specific expression of two distinct genes, *OsIPA* and *OsIPK*. *OsIPA* encodes a protein similar to expansins, pollen allergens, and is activated during the late stage of pollen development, whereas, *OsIPK* promoter is active in developing anther till the pollen maturation and encodes a CDPK, a calcium-dependent protein kinases (Gupta et al. 2007).

In addition to known regulatory sequences, recently described *cis*-elements in *SBgLR* gene of *Solanum tuberosum* were demonstrated to drive the late pollen specific expression. A detailed promoter dissection analysis revealed that the region from -345 to -9 (counted upstream from ATG as +1 site) was sufficient in restricting gene expression specifically in pollen (Zhou et al. 2010). Moreover, the region between -345 and -269 was found as an enhancer while the newly described motif lies in the region of -253 to -227 and suppresses the function of nearby palindrome TTTCTATTATAATAGAAA in the region of -227 to -209. Interestingly, two pollen-specific palindrome motifs here TTTCT and AGAAA surround a putative TATA box and from this site downstream to ATG they are presented in additional nine copies plus one of GTGA. Besides the two known motifs (AGAAA/TTTCT and GTGA), the authors showed their spatial and combinational distribution within the promoter to affect the specific regulation of *SBgLR* gene expression. Its protein shares an 81% homology to SB401, encoded by a pollen-specific gene, that bounds to microtubules and actin filaments. Hence the SBgLR may be involved in cytoskeletal organization during pollen development (Zhou et al. 2010).

Detailed analysis of another well-described tobacco pollen-specific gene *NTP303* revealed an AAATGA regulatory element of which TGA triplet was shown to comprise an active part of the motif (Weterings et al. 1995, Verelst et al. 2007). This motif is completely conserved within the similarly regulated promoter of the *Bp10* gene from *Brassica napus* encoding the *NTP303* homologue. In addition, distal region of *NTP303* promoter shows an extensive overlap (CTTGTGTGGTTAAT) with a BPIII region of tomato *LAT52* promoter.

In *Arabidopsis*, three nuclear-encoded genes for mitochondrial Complex I (nCI) subunits (PSST, TYKY, and NADH binding protein) were found to share promoter regulatory sequences responsible for enhancement of their expression in pollen and anther wall. In

those genes, the common motif TGTGGTT involved in such specific regulation has a similarity to a “pollen box” (52/56 box) in inverted orientation. It is present in two tomato pollen-specific genes *LAT52* and *LAT56* (Twell et al. 1990, 1991) and in promoters of two pollen-specific pectin esterase genes from *Brassica campestris* (Kim et al. 1997). Obviously, regulatory sequences in those three mitochondrial *Complex I* genes play an essential role for correct spatial and temporal activity since anther/pollen development is highly energy-demanding process in plants.

2.3. Specific transcription factors

The genome-wide comparison of transcription factors among eukaryotic organisms revealed the evolutionary consequence of diversity in the regulation of transcription reflecting the biology, life style, strategy, and adaptation of various species. Each of individual eukaryotic kingdoms (*plants, animals, fungi*) has its own set of particular transcription factor families and genes.

Among 19 families that are shared between animals and plants, more than 14 are larger in plants than in animals. Such dramatic expansion in plants is not only as a result of gene/genome duplication but also as a frequent adaptive response to selection pressure in plants. Interestingly, bioinformatic analysis indicated that TF families have higher duplicatibility than genes involved in most functions in *Arabidopsis* (Shiu et al. 2005, Qu and Zhu 2006). In most cases, the TF family counterparts in *Arabidopsis* and rice genomes are of similar sizes. There are, however, a few exceptions in which the numbers of members within a family differ substantially between *Arabidopsis* and rice including WRKY, NAC, bZIP, MADS, ALFIN-like, GRAS and C2C2-dof families (Qu and Zhu 2006). There are about 20 families represented specifically in plants and several of them are at the same time the most abundant families in *Arabidopsis* at all, namely AP2/EREBP, NAC, and WRKY. That clearly reflects the uniqueness of the plant kingdom and the evolutionary adaptation for plant-specific functions (considering settled lifestyle, balancing environmental changes etc.). Members of AP2/EREBP families are involved in development, cell proliferation, secondary metabolism, abiotic and biotic stress response and ABA/ethylen response. Genes of NAC specifically regulate developmental processes including pattern formation and organ separation, whereas WRKY transcription factors are mostly involved in defence response.

The other highly represented and plant-specific gene families represent Dof, CO-like, YABBY (all from C2H2 superfamily), GRAS, Trihelix, TCP, ARF, C3H-type, SBP, Win-like, AB13/VP1, Alfin-like, EIL, LFY, and ARF-Aux/IAA, HB, MADS TFs. On the other hand, there are several gene families comprising transcription factors playing roles specifically in animals and therefore not represented in the Arabidopsis genome like NHR (C8-Zn2), Adf-1, T-Box, ETS, and Fork head. In addition to other abundant plant families, the MYB superfamily (consisted of MYB (R1)R2R3 and MYB-related) which is otherwise present in all model species, is dominant just in plants where it represents the largest superfamily of all counting about 180 gene members.

The classification of transcription factor families follows the functional organization of the factors with the most significance atributed to the DNA-binding domain playing the cognitive role in targeting specific *cis*-elements. The other prominent feature is the protein-protein interaction domain enabling the interaction with the RNA polymerase II or with other co-factors, either positively or negatively. Regarding the protein secondary structure, several distinct motifs were identified to mediate DNA binding properties. Such motifs have been used to classify transcription factors into families.

(1) **Helix-turn-helix** was the first described motif in a large number of various transcription factor genes of different classes suggesting its importance in regulatory activities including homeobox proteins, MYB, HSF factors (Riechmann et al. 2000, Stracke et al. 2001, von Koskull-Döring et al 2007). It contains two α-helixes separated by three amino acid spacer in rigid 120° angle. The complete factor is set together as homo- or heterodimer. This structure is embedded in a homeodomain of 60 amino acids that consists of three α-helices and a flexible N-terminal arm. Helix III, the recognition helix, packs against the DNA major groove and is responsible for specific contacts with DNA (Gehring et al. 1994, Wolberger 1996). Homeodomain-encoding genes share not only sequence similarity, but in general they play related roles *in planta* as well, predominantly during development.

(2) **Zinc-finger motif** is a proposed structure in which a loop of two β-sheets and one α-helix contains twelve amino acids including, in the typical case, two invariant cysteine and two invariant histidine residues that coordinate one zinc atom, so called class I C2H2 finger (Ciftci-Yilmaz and Mittler 2008). However, this structure can be modified into class IV C3H by replacing one histidine residue with another cystein; this group is typical for plants (Wang et

al. 2008). Alternatively, there is a pair of fingers binding two zinc atoms in class II C8 or class III C6 found in yeast. Zinc-finger factors operate as homodimers.

(3) **Leucine zipper/bZIP TFs** contain characteristic leucine-rich region in which successive leucine residues occur at every seventh position supporting an α-helix structure. Moreover, this long side chain tends to dimerize into parallel coiled-coil structure, the leucine zipper (Oas et al.1990, Zhou et al. 1992). Hence, the leucine zipper is not responsible only for DNA binding but also for such protein-protein interaction. However, the zipper is not itself the DNA binding domain, unlike zinc finger or helix-turn-helix, but it helps DNA binding by facilitating of adjacent region of basic amino acids which interact with DNA directly. In general, bZIP TFs bind DNA as homo- or heterodimers (Ellenberger et al. 1992). Heterodimers were shown to convey synergistic activation properties to target genes, suggesting that heterodimerization serves as an efficient mechanism of signal integration (Sibéril et al. 2001, Jakoby et al. 2002, Weltmeier et al. 2006, Alonso et al. 2009).

(4) **Helix-loop-helix/bHLH TFs** contains two α-helices spaced by a loop of several amino acids. This motif is distinct from the helix-turn-helix in that it can form two amphipathic helixes, containing all charged amino acids on one side of the helix. Helix-loop-helix motif plays a role similar to that of leucine zipper allowing dimerization of the transcription factors and thereby facilitating binding to DNA by the basic motif (Murre et al. 1989). Again, these factors pair as a homodimers or restrict their heterodimerization activity to closely related members of the family (Toledo-Ortiz et al. 2003). However, the others can dimerize with bZIP factors or members of R2R3 MYB proteins providing extensive combinational and various functional activities (Kuras et al. 1997, Heim et al. 2003).

2.4. Male gametophytic gene functions

After releasing microspores from tetrads they need to undergo certain structural and functional events during the development till the pollen maturation. Those includes cytoskeletal rearrangement, migration of nucleus, two haploid mitoses, synthesis of reserve metabolites, cell wall development, etc. Up to date, there are tens of genes playing role during the male gametophyte development (Table 2), whose mutations, functions and coding genes were mostly described. Probably the most striking event in pollen development is the cell cycle regulation which will be described more. It controls the essential asymmetric division of microspore nucleus during pollen mitosis I producing a

vegetative and generative nucleus. The later is further divided into two sperm cells during pollen mitosis II. Microspore enlarges and produces a large vacuole that pushes the nucleus towards the cell wall with the cooperation of microtubule rearrangement that essentially establishes the asymmetrical division. This event seems to be critical for the formation of germline since induced equal division results in two daughter cells that both exhibit vegetative cell fate (Eady et al. 1995). Several mutants have been isolated that demonstrate importance of asymmetric division including *sidecar pollen* (*scp*), *gemini pollen1* (*gem1*), *gemini pollen2* (*gem2*), and *two-in-one* (*tio*).

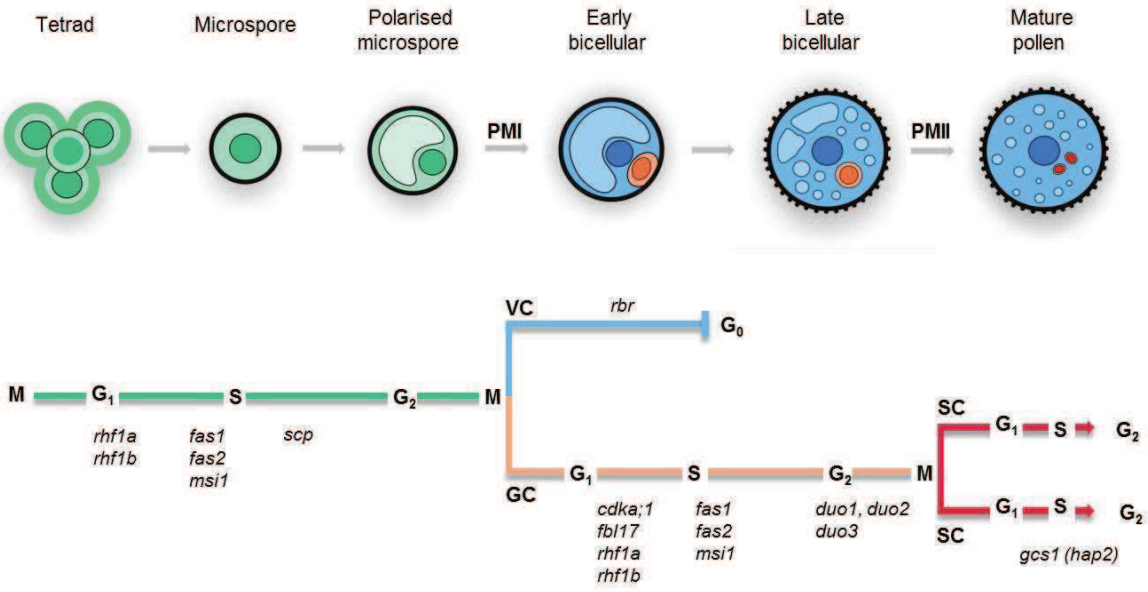

Fig. 3. Male gametophyte development and mutants affecting cell division, patterning and cellular fate in *Arabidopsis*. During microgametogenesis, the haploid microspores undergo a highly asymmetrical division, called pollen mitosis I (PMI), to produce a bicellular pollen within one vegetative nucleus (blue) and one generative cell (orange). Later, the generative cell enters further mitotic division, pollen mitosis II (PMII) to make two sperm cells (red). Compiled from two sources: Borg et al. 2009 and Twell 2011.

sidecar pollen is a male-specific mutant characterized by symmetrical division, followed by asymmetric division of only one of the daughter cells to produce mature pollen with an additional vegetative cell. *gemini pollen1* displays a range of microspore division phenotypes including equal, unequal, and partial division (Park et al 1998). GEM1 is identical to MOR1 (Whittington et al. 2001) which belongs to MAP215 family of microtubule-associated proteins and plays a vital role in microspore polarity and cytokinesis by stimulating growth of

the interface spindle and phragmoplast microtubule arrays (Twell at al. 2002). *gem2* displays the similar defects in establishing germ cell fate (Park et al. 2004). In the *tio* mutant, microspores complete nuclear division but fail to complete cytokinesis resulting in binucleate pollen grains. TIO is a plant homologue of a Ser/Thr protein kinase FUSED (Oh et al. 2005), which is localised to the phragmoplast where it plays an essential role in centrifugal cell plate expansion. Similar role to TIO could be linked with Kinesin-12A/12-B, functional redundant microtubule motor kinesins, localised also to the phragmoplast midline (Oh et al. 2010). TUBG1 and TUBG2, functionally redundant γ-tubulins, are required for spindle and phragmoplast organization in Arabidopsis microspores (Pastuglia et al. 2006). Microtubules form a basket-like nuclear cap connected to nuclear envelope and cell cortex and could be therefore involved in generating asymmetrical position of nucleus prior to division.

After pollen mitosis I, the fate of vegetative nucleus is co-determined by RBP (RETINOBLASTOMA-RELATED PROTEIN), the conserved repressor of cell proliferation. *rbp* mutation results in hyper-proliferation of the vegetative cell and to a lesser extent the germline, it perturbs cell differentiation to various degrees leading to pollen with four sperm cells (Chen et al. 2009). Hyperproliferation in the absence of RBP is dependent on CDKA;1 activity since introduction of a *cdka;1* mutant allele prevents cell proliferation in *rbp* pollen. This places RBP repression of the E2F-DP pathway downstream of KRP-dependent CDKA;1 inhibition, so that both mechanisms may cooperate to enforce cell cycle exit that is associated with commitment to vegetative cell fate (Fig 3, Fig. 4).

Considering the generative cell, the cell cycle is co-regulated by A-type cyclin-dependent kinase (CDKA;1) mentioned above, which mutation results in retarded S-phase, when germ cell enters mitosis during pollen germination (Iwakawa et al. 2006, Nowack et al. 2006) and pollen tube growth (Aw et al. 2010). Disruption of the *F-box-Like17* (*FBL17*) gene, which is only transiently expressed in the male germ cell was shown to phenocopy the cdk;1 germ cell defect (Kim et al. 2008, Gusti et al. 2009). FBL17 normally targets the CDK inhibitors KRP6 and KRP7 for proteasome-dependent degradation (Fig. 3, Fig. 4), enabling the germ cell to progress through S-phase (Kim et al. 2008). Conversely, the persistence of KRP6/7 in the vegetative cell is proposed to maintain the inhibition of CDKA activity and vegetative cell cycle progression. Germline specific expression of FBL17 thus enables differential control of the cell cycle in the germ and vegetative cells, effectively licensing

germ cell for progression through S-phase (Twell 2011). Similarly, the proteasome-mediated degradation of KRP6 by two RING-finger E3 ligases, RHF1a and RHF2a is required for regular progression through both microspore and the subsequent germ cell mitotic cycle. RHF1a and RHF2a are proposed to reduce the level of KRP6 accumulated during meiosis, thereby preventing the inhibition of CDK activity in microspores and germ cells (Liu et al. 2008).

Recent analysis of Chromatin assembly Factor-1 (CAF-1) pathway mutants (*fas1, fas2, msi1*) indicates that chromatine integrity is also important for germ cell division. The mutants display a range of phenotypes with some failing to divide at pollen mitosis I, some failing to divide at pollen mitosis II, and some successfully dividing to produce tricellular pollen. Surprisingly, pollen grains with single germ cell are able to fertilize either the egg or central cell (Chen et al. 2008).

A single germ cell phenotype is also present in *duo pollen* (*duo*) mutants. In these mutants, asymmetric microspore division at pollen mitosis I is completed, however, the resulting germ cell fails to undergo cell division at pollen mitosis II (Durbarry et al. 2005, Borg et al. 2009), *duo1* and *duo2* pollen then contains only single germ cell and vegetative nucleus. DUO1 encodes a novel R2R3 MYB transcription factor specifically expressed in germline cell (Rotman et al. 2005, Singh et al. 2008). Unlike *fbl17*, *cdk;1* and CAF-1 pathway-deficient mutant pollen, *duo1* pollen cannot fertilize. DUO3 is required for G2/M transition and for the expression of a subset of DUO1 target genes. However, unlike DUO1, the requirement for DUO3 in the germline during G2/M transition acts independently of CYCB1;1 (Brownfield et al. 2009). The mechanism by which DUO3 influences the expression of a subset of DUO1 targets remains unknown, but it has been proposed that DUO1 and DUO3 may cooperate in a transcriptional complex (Fig. 4).

Considering the sperm cells, their surface proteins are likely to play important roles in the guidance, recognition, and/or fusion of gametes during double fertilization. This is the case of Hap2 that has been identified in *Arabidopsis* (von Besser et al. 2006). It is a homolog of GCS1 (Generative Cell-Specific1), encoding a gamete surface protein required for pollen tube guidance and fertilization (Mori et al. 2006, von Besser et al 2006). In addition to the gene expression, germline and sperm cell specific histon H3 variant H3.3 (MGH3) is expressed under the control of DUO1 transcription factor. Histon H3.3 is incorporated into chromatin and corregulates its rearrangement to support the global reprogramming events (Twell 2011).

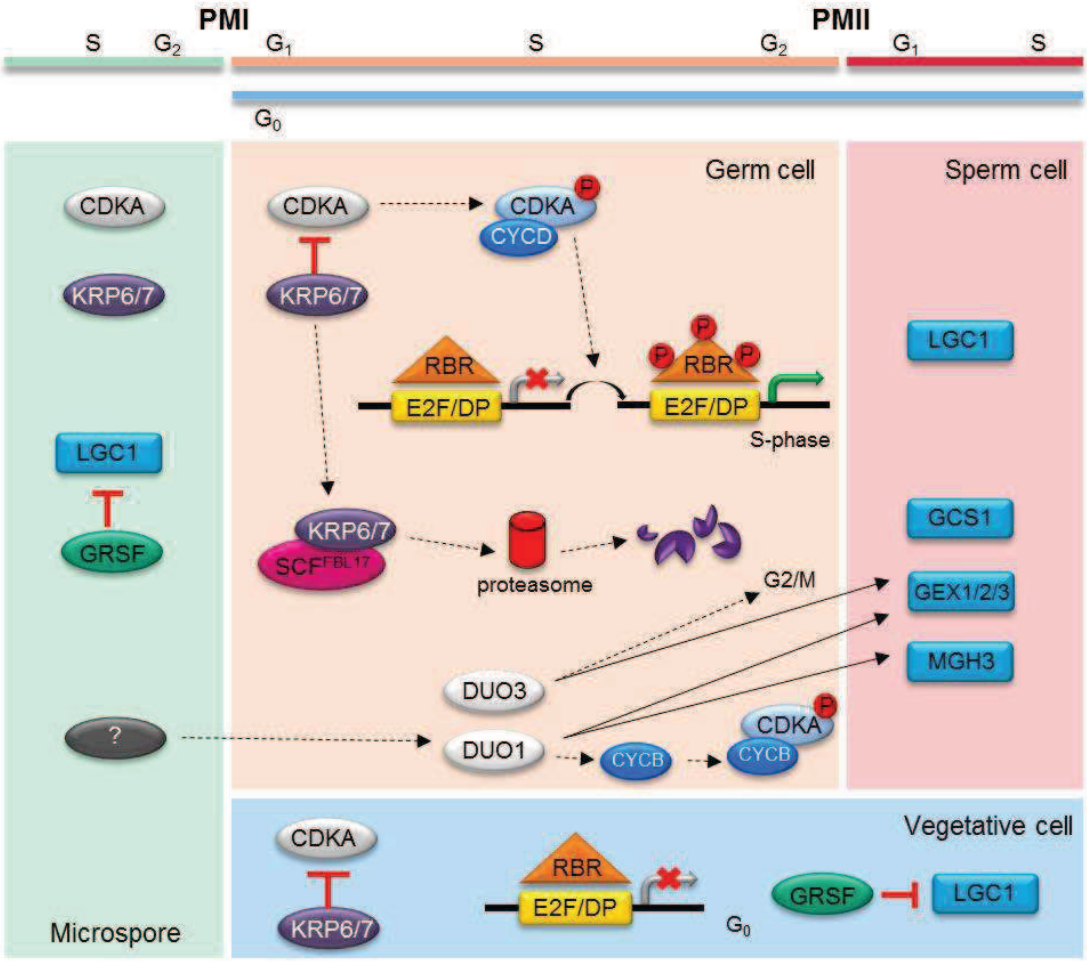

Fig. 4. A model of male germline specification and maintenance. After PMI, the cell cycle inhibitors KRP6 abd KRP7 are present in both the germ and vegetative cells. Transient expression of FBL17 in the germ cell, unlike in vegetative cell, leads to the degradation of these inhibitors, enabling CDKA activation and entry into S-phase. Once S-phase has been completed, the DUO1-dependent activation of G_2/M phase regulators coupled to CDKA activation, promotes the germ cell to progress through the G_2/M checkpoint and enter mitosis. Ultimately, the co-ordinated association of these parallel pathways results in a pair of fully differentiated sperm cells. Compiled from Borg et al. 2009, Berger and Twell 2011, Twell 2011.

During the male gametophyte development, the structural and functional changes are obviously connected to regulation of transcriptome dynamics. Pollen-specific MIKC class of MADS-domain transcription factors play a significant role in such regulation. The Arabidopsis MIKC genes can be subdivided into two groups whose members make functional heterodimers; S-class involving AGL66, AGL67 and AGL104 etc., and P-class involving AGL30, AGL 65 and AGL94 etc. AGL30, AGL65, AGL66, AGL94 and AGL104 are expressed specifically in pollen while AGL18, AGL29, AGL49 and AGL84 appear to be enriched in pollen. Five

heterodimers have been postulated to form in pollen: AGL65- AGL66, AGL65- AGL104, AGL30- AGL66, AGL30- AGL104 and they may be considered as the major regulators of pollen maturation programs (Verelst et al. 2007, Adamczyk and Fernandez 2009). It was intriguing that even though more than 1,300 transcripts were misregulated in a triple mutant pollen (*agl65/66/104*), the basic cellular organization and characteristic properties of mature pollen and the ability to germinate and fertilize ovules appeared to be largely unaffected (Verelst et al. 2007). It proves the great plasticity and potential dynamics in pollen transcriptome.

3. Male gametophyte transcriptomics and microarray databases

3.1. Male gametophyte developmental transcriptomics

The male gametophyte represents a unique plant structure and plays an unsubstitutable role during plant life cycle. This fact is reflected in various cellular events including specific gene expression pattern. Despite the long-term knowledge of overlapping gene expression during pollen development based on isozyme (Tanksley et al. 1981, Sari-Gorla et al. 1986, Pedersen et al. 1987) and hybridization studies (Stinson et al. 1987, Mascarenhas 1990), it was advanced microarray technology that helped to reveal the transcriptional profile of male gametophyte in great detail (Becker et al. 2003, Honys and Twell 2003, Pina 2005, Borges et al. 2008). For pollen developmental transcriptomics, it was necessary to carry out gene expression profiling over the whole developmental process, including microspores (UNM), bicellular pollen (BCP), tricellular pollen (TCP), and mature pollen grain (MPG). Additionally, the potential to effectively isolate RNA from pollen developmental stages, pollen tubes and sperm cells in required quantity and purity enabled further extensive transcriptomic studies (Honys and Twell 2004, Borges et al. 2008, Wang et al. 2008, Qin et al. 2009).

With the use of ATH1 Genome Array harbouring 22,591 genes that represent 80.7% of estimated 28,000 protein coding genes in *Arabidopsis*, it was revealed that 13,977 genes were actively transcribed providing significant and consistently positive expression signal in at least one stage of male gametophyte development. It represented 61.9% genes on the chip and suggested about 17,000 genes playing a role during pollen development. This huge number was in accord with previous estimations. However, it evoked a surprising finding that such specialized cell lineage with unique gametophytic function shared almost two thirds of its genes involved in sporophytic regulation as well (Honys and Twell 2004). The uniqueness of the pollen transcriptome was dissected by analysing the frequencies of representation of gene ontology categories. The most significant categories included general and cell wall metabolism, cytoskeleton, signalling, transport and vesicle trafficking. It clearly demonstrated the functional specialization of pollen and its predetermination to the pollen tube growth (Honys and Twell 2003, Pina et al. 2005).

Among all 13,977 genes expressed in pollen, 1,355 genes were considered pollen-specific based on their expression at least one stage of pollen development and no expression signal in any sporophytic tissue. The second key finding was the quantification of the gradual decrease of number of expressed genes from early to late phase. In numbers, UNM – BCP – TCP – MPG – PT4 – SPC stages expressed 11,565 – 11,909 – 8,788 – 7,235 – 6,148 – 5,801 genes, respectively (Fig. 5). The bulk of genes transcribed during early pollen development might reflect the intense metabolism and cell growth of microspores and young pollen. On the contrary, during pollen maturation, the steady decrease could be connected to the cessation of metabolic activity towards mature pollen grain until pollen germination and tube growth. However, the proportion of pollen-specific genes expressed over male gametophyte development steadily increased as calculated 6.9% - 7.2% - 8.0% - 8.6% - 10.9% - 11.5% for each respective stage (Fig. 5). The rising proportion of pollen-specific genes expressed in later pollen phase may be associated with desiccation and other specific pollen functions during maturation (Honys and Twell 2004).

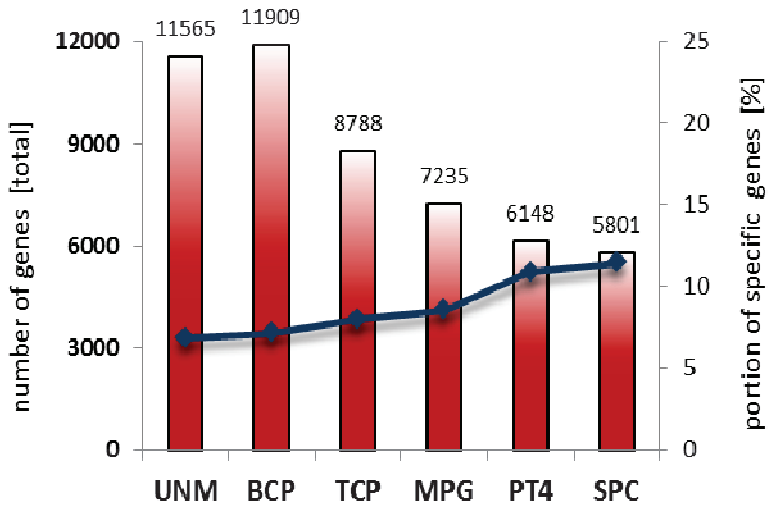

Fig. 5. Transcriptome profile of total expressed genes during male gametophyte development with the portion of specific genes active in each developmental stage. Columns from UNM to PT4 (4hr pollen tubes) represent the transcriptome of vegetative nucleus while SPC represents the transcriptome of sperm cells. Data from: Honys and Twell 2004, Borges et al. 2008, Wang et al 2008, Qin et al 2009.

Another striking phenomenon of pollen transcriptomic was a dramatic change of two subsequent global developmental programmes between early and late stages. There is a significant group of genes expressed specifically/predominantly in UNM and BCP with sharp decrease in expression at later stages and vice versa. Basically, such reprogramming reflects

the transition between BCP and TCP stages at the cellular level. When comparing UNM and BCP by scatter plot analysis, relatively high correlation coefficient of R=0.96 indicated close relation between these stages (Fig. 6). Similarly, the coefficient of R=0.86 between TCP and MPG also suggested great similarity in terms of expression profile of respective genes. On the contrary, BCP and TCP showed very poor correlation and even less similarity between UNM and MPG (Fig 6). It simply reflected the change in "cell status" from relatively undifferentiated and proliferating population of microspores into highly differentiated mature pollen (Honys and Twell 2004).

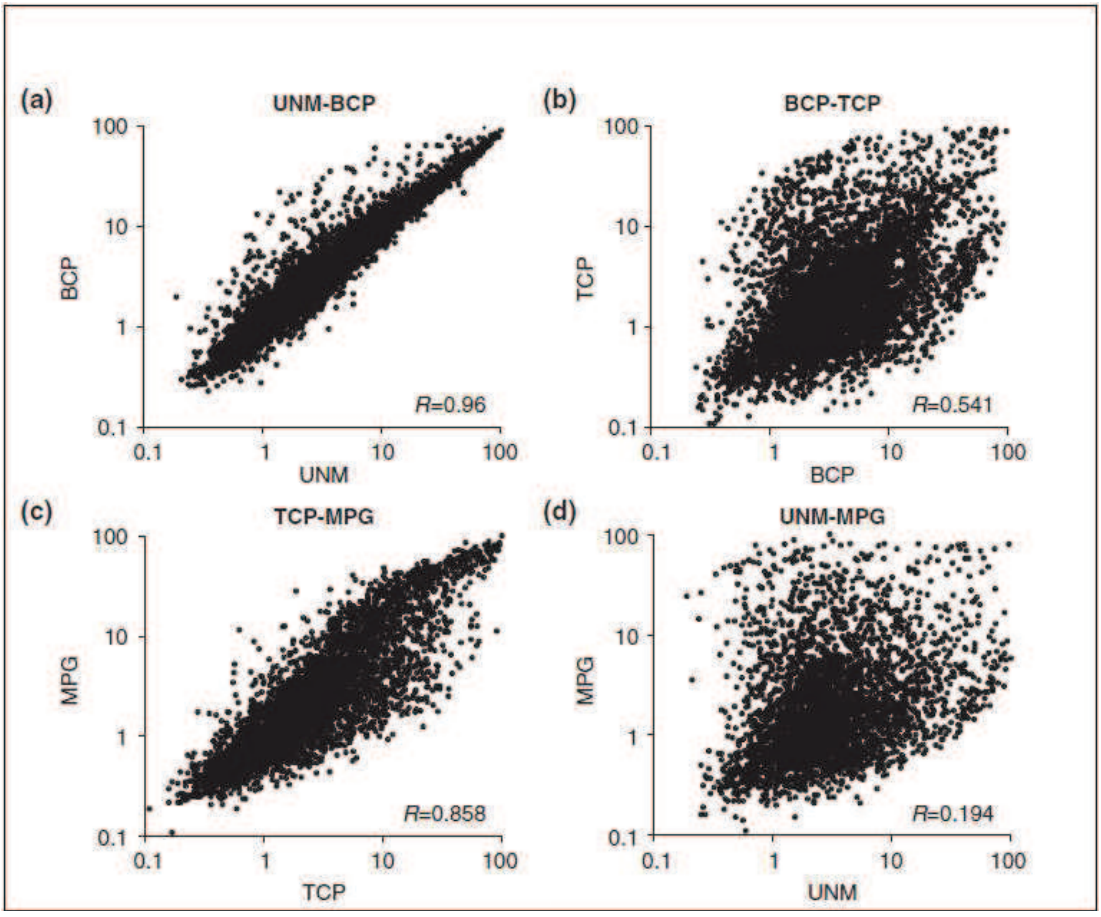

Fig. 6. Scatter-plots comparing gene expression between two respective developmental stages. (a) UNM versus BCP, (b) BCP versus TCP, (c) TCP versus MPG, (d) UNM versus MPG. Honys and Twell, 2004.

Considering transcription factors (TF), ATH1 harbours 1,350 genes that represent about 85% of total 1,594 annotated TF genes (Honys and Twell 2003). Of them, 608 (45%) TF genes were expressed at least one stage of male gametophyte development and 54 genes (15.7%) were pollen-specific. The family size also mattered rather than proportionally equal representation of genes in each TF family. There was a sharp difference between over-

represented families including p-coumarate 3-hydroxylase (C3H) family (67%), CAAT family (64%), C2H2 zinc finger proteins (57%), WRKY family (53%), bZIP family (51%), TCP family (50%), GRAS family (50%) and, in contrast, families under-represented such as AUX/IAA (20%), HSF (33%), bHLH (34%), NAC (34%), AP2-EREBP (35%), HB (36%), R2R3-MYB (37%), MADS (37%), and C2C2 zinc finger gene family (37%).

Besides pollen (vegetative cell) transcriptome, the data from isolated Arabidopsis sperm cells are also available (Borges et al. 2008). With the use of ATH1 Genome arrays (22,392), the amount of 5,829 (27%) gene transcripts was detected to be expressed in sperm cells. The functional classification of genes revealed that most represented genes were associated with DNA repair, cell cycle progression and ubiquitin-meditated proteolysis. Moreover, significant presence of components involved in small RNA metabolism and DNA methylation suggested their convincing role in epigenetic regulation. It may not be surprising that the vast majority of genes (3,813) expressed in sperm cells were also detected in pollen vegetative cell. However, the overlap between sperm cells and seedlings comprised 4,757 genes, representing almost the whole sperm-cell transcriptome with the half showing predominant expression in sperm cells. In relation to transcriptional regulation, the TF families were rather weakly represented with the exception for MYB-type TF family with relatively high expression of their members in sperm cells. Families of homeobox and bHLH genes, which were otherwise very abundantly expressed in sporophyte, were represented only by one or few genes in sperm cells, respectively. Focusing on transcription factors managing specific sperm cell functions, one candidate (At4g35700) could be traced into zinc finger C2H2 family, which was specifically expressed just in sperm cells and at the highest expression level of all TF genes on top of that, and possibly one member (at2g42300) of bHLH family being expressed specifically in male gametophyte and was not detected in pollen vegetative cell.

3.2. Microarray databases

The gene expression in its specific combinatory pattern and dynamics during whole development represents the essential background of the life with direct connection to structural and physiological behaviour of the organism. Therefore the study of transcriptome as a spatial and temporal result of inner and outer stimuli is of the great interest. In past decades, number of techniques have been used to discover the secret of transcriptional

activity using Nothern blot analysis, quantitative reverse transcription-PCR, cDNA library screening, serial analysis of gene expression, manual/mechanical spotting of probes on nylon/glass macro/microarrays, etc. (Schena et al. 1995, Velculescu et al.1995, Lockhart et al. 1996, Lipshutz et al. 1999). Those approaches were able to detect obviously very limited number of genes. It was the introduction of sophisticated high-throughput microarray technologies that significantly supported the parallel large-scale transcriptome analyses. Nowadays, microarray ATH1 GeneChip developed by Affymetrix covering 76% of Arabidopsis thaliana genes is considered as a standard tool in genome wide transcriptomic studies in plants.

Not surprisingly, the technological reliability, reproducibility, price availability and last but not least the experimental requirement have contributed to the massive production of transcriptomic data. Along with continuous data accumulation and the customers' need for the access to the gene expression information, numerous databases have been established. Currently, there are dozens of different databases covering different species, experimental conditions, and developmental stages which may result in navigation difficulties. Over last years, many articles have been published to deal with microarray databases, providing relevant introduction and help researchers direct to resources and tools they require. Basically, there are two types of microarray databases; one serves as a publicly available data repository and the others provide some additional data analysis tools. In both cases, experimental data comes directly from Affymetrix Service via Nottingham Arabidopsis Stock Centre (NASC) or from extensive bioinformatic projects run by large institutions like European Bioinformatics Institute (EMBL-EBI) or National Centre for Biotechnology Information (NCBI) as the largest data repositories. Alternatively data originates from numerous laboratories' projects dealing with transcriptomic analyses. Bioinformatic tools associated with most databases represent the main instrument to effectively select and analyse data of interest. Essentially, such facilities make the difference among each database and may attract different scientific questions. To mention several key data mining tools, it would include normalization and data filtering tools, global statistical data analysis, analysis of co-regulated genes, and hierarchical or K-means clustering tools. Some specialized databases offer visualizing tools, promoter element detection or potential upstream elements. Other helpful possibilities represent the selection from various experimental setups including physiological and stress conditions, different developmental stages and

tissue/cell lines. Moreover, most databases permit data download, either for free or charged, for further data processing if needed. Because of the diverse data sources and experimental conditions, the large number of databases is strictly MIAME-compliant, asking for the Minimum Information About Microarray Experiment (MIAME) to enable comparability across numerous datasets. Further criteria also comprise the assessment of expression data quality (QC, Quality Control) to evaluate the relevance of providing data. Ongoing microarray experiments and continuous generation of transcriptomic data have led to establish various microarray databases. Many applications are being supplemented with more specialized products offering various visualization and data mining tools to fulfil growing demands of researchers. It was one aim of this Ph.D. thesis to overview the most frequent databases (Honys et al. 2008) and setup a new portal using own expressional data including pollen transcriptome as described in Results of the thesis.

4. Aims of the study

The study of male gametophyte represents a very interesting field in the plant science for the uniqueness of the object of interest including structural features (cell-in-cell organization, cell wall composition) and functional properties (unique transcriptional pattern, posttranscriptional regulation, tip growth) not speaking about amazing cellular and life-span reduction during the gametophyte evolution in plants. Due to massive use of microarray technologies, 608 TF genes were identified to be expressed during pollen development and some of them have been functionally characterized. However, the complete understanding of the whole regulatory network is still rather limited.

Hence, the main goal of this Ph.D. thesis was to employ large-scale screening of T-DNA mutant lines in specific transcription factors to bona fide determine their importance in pollen development. Therefore this work intended to provide knowledge on TF mutant phenotype in terms of their structural and functional defects as a basement for further detailed studies of their regulatory roles. In addition, this thesis also reviewed the male gametophyte development on the whole and reflected gene expression data and transcriptomic databases. The particular aims were:

(1) To summarise the male gametophyte development with the respect to the structure and function of pollen grain and growing pollen tube, mutants affecting male gametophyte development and pollen transcriptomics.

(2) To select TF genes with either specific or enriched expression pattern during pollen development based on microarray data with the special focus on early stages of male gametophyte development and to perform large-scale screening of T-DNA mutant lines in selected TF genes and their evaluation regarding their effect on male gametophyte development.

(3) To participate on the characterization of a candidate gene *AtbZIP34* selected from previous large-scale screening as a case study including structural and functional analysis of the mutant.

(4) To identify promoter sequences driving gene expression in early stages of pollen development and their use as a molecular tool for the manipulation of gene expression.

(5) To build a gene expression database based on microarray data and to make it public to a broad scientific community in user-friendly and graphic environment.
Moreover, to compile and compare available transcriptomic databases world-wide to help potential users to target their questions on particular gene expression.

5. The list of publications

In accord to the aims of this Ph.D. thesis, I participated in preparation of several articles and book chapters.

(1) Honys D, **Reňák D**, Twell D. Male gametophyte development and function. Global Science Books, pp.76-87, 2007.

(2) **Reňák D**, Dupľáková N, Honys D (2011) Wide-scale screening of T-DNA lines for transcription factor genes affecting male gametophyte development in *Arabidopsis*. Plant Sex Rep, *resubmitted*.

(3) Gibalová A, **Reňák D**, Matczuk K, Dupľáková N, Cháb D, Twell D, Honys D (2009) *AtbZIP34* is required for Arabidopsis pollen wall patterning and the control of several metabolic pathways in developing pollen. Plant Mol Biol (2009) 70: 581-601.

(4) Honys D, Oh SA, **Reňák D**, Donders M, Šolcová B, Johnson JA, Boudová R, Twell D (2006) Identification of microspore-active promoters that allow targeted manipulation of gene expression at early stages of microgametogenesis in *Arabidopsis*. BMC Plant Biol 6: 31-39.

(5) Dupľáková N, **Reňák D**, Patrik Hovanec, Honysová B, Twell D, Honys D (2007) Arabidopsis Gene Family Profiler (aGFP) – user-oriented transcriptomic database with easy-to-se graphic interface. BMC Plant Biol 7: 39-46.

(6) Honys D, Dupľáková N, **Reňák D**. Online tools for presentation and analysis of plant microarray data. Nova Science Publishers, Inc. In: Oligonucleotide array sequence analysis, eds: M.K. Moretti and L.J. Rizzo, pp. 265-295, 2008.

6. Results

This chapter considers four author's research papers on wide-scale screening of T-DNA lines, AtbZIP34 TF functional analysis, identification of microspore-active promoters, and building the aGFP database, only (excluding two book chapters). Since all results are fully presented in each of respective papers, this part is only to summarise them in a united manner.

Aiming to identify TFs affecting male gametophyte development, we screened 74 T-DNA insertion lines for pollen phenotypic defects. We focused on structural and functional defects occurring during pollen development using bright field and epifluorescence microscopy. In comparison to the wild-type phenotype, five complex disorder classes were observed. Those included (A) pollen abortion, (B) presence of cytoplasmic inclusions, (C) pollen grain size and cell wall structure, (D) cell cycle defects, and (E) MGU (male germ unit) organization. However, the precise phenotype analyses have led to refined classification of above dysfunctions into tens categories: A: (1) abortion; B: (2) inclusions; C: (3) small grain, (4) oval grain, (5) deformed cell wall; D: (6) one-celled pollen, (7) two-celled pollen; E: (8) eccentric MGU, (9) separated MGU, and (10) linear MGU. We mostly observed abnormalities connected to cytoskeletal organization and function leading to the defective arrangement of MGUs with their eccentric, separated or linear position in the pollen grain unlike their characteristic central arrangement in wild-type pollen. Because of the sole positional variation we described those phenotypes simply as misarranged MGU. Other classes of mutations were likely to affect cell cycle and cell division. Namely, PMI defects resulted in one-celled pollen and/or PMII defects resulted in two-celled pollen with one vegetative nucleus and one generative cell. Cytoplasmic inclusions represented more specific class of phenotype defects. The most serious and complex phenotypic defect(s) led to a complete cell abortion with squashed pollen wall. Considering the pollen shape, we observed unusual pollen grain size and shape such as small and oval pollen grains. However, these phenotypic defects were rare. Only one insertion line produced pollen with cell wall deformation with rough cell surface. Among 61 confirmed lines, about a half (29 lines) showed strong phenotypic changes (i.e. ≥25% aberrant pollen). Yet only few lines producing heavily disturbed pollen grains were observed reaching very high penetrance. The most striking phenotypes were observed in lines of SALK_140819 with 100% abnormal pollen (At2g20400;

G2-like), 80-85% in SALK_018864 (At2g42380; bZIP), 70-75% in SALK_084050 (At3g15740; C3H), 70-75% in SALK_010704 (At5g54470; C2C2-CO-like), and 50-55% in SALK_090677 (At4g05330; C2C2) (Table 1).

AGI	T-DNA line	TF family	mutant phenotype
At2g20400	SALK_140819	G2-like	100%
At2g42380	SALK_018864	bZIP	80-85%
At3g15740	SALK_084050	C3H	70-75%
At5g54470	SALK_010704	C2C2-CO-like	70-75%
At4g05330	SALK_090677	C2C2	50-55%

Table 1. The list of the most affected lines by T-DNA insert in pollen development

But generally, in the population of homozygous plants we mostly identified remarkable proportion of pollen with wild-type or mild phenotype defects counting the average penetrance in between 10-30%. Several T-DNA lines with strong phenotype defects were further genetically tested using backcross analysis. Out of eight selected lines, six ones segregated close to the Mendelian 1:1 ratio. Reminding two lines performed heavy segregation distortion of 1:0.04 (SALK_002235) and 1:0.39 (SALK_043690) indicating the significant role of the TFs in male gametophyte development.

Our wide-screening led to an evaluation of selected TF mutant lines in terms of impact on pollen development and hence served as a good information basement for further studies. Based on this fact, we have selected a few lines with considerable impact on male gametophyte development which have been analysed in our laboratory. One of them was the transcription factor AtbZIP34, published by our laboratory (Gibalová et al. 2009). AtbZIP34 belongs to a large family (75 annotated genes) of bZIP transcription factors whose members have been shown to be involved in male gametophyte development (Iven et al. 2010). AtbZIP34 was confirmed to be specifically and increasingly expressed during pollen maturation both by RT-PCR and promoter:GUS analyses, that were in agreement with previously published microarray data (Honys and Twell 2004). Pollen grains of mutant *atbzip34* plants showed several phenotypic features including 15% aborted pollen and 27% malformed/misarranged MGU. In addition, the typical sign was the presence of lipid/oil

bodies. Electron microscopy confirmed the abundance of such inclusions in mutant pollen accompanied by under-developed ER and vacuoles increased in number and size. Farther observation revealed abnormal exine patterning with collapsed baculae and tectae and with areas of extra material deposition plus characteristic wrinkled intine detached from the outer nexine layer. Furthermore, its dysfunction was significant in progamic phase as well, as demonstrated by *in vitro/in vivo* pollen tube growth and segregation ratio distortion. Pollen bearing mutant allele showed weaker germination (85% reduction compared to wild-type) and slower pollen tube growth rate. According to microarray experiment, AtbZIP34 affected expression of genes involved in various metabolic pathways besides other functions. Over 1,300 genes were upregulated and, in contrast, over 800 genes were downregulated; the later subset included most significant changes in functional categories representing transcriptome, transport, metabolism and protein fate. Among the strongest downregulated genes belonged those encoding proteins involved in lipid catabolism and two lipid transfer proteins, and ABC transporter. All those findings indicated to the missing role of AtbZIP34 TF during male gametophyte development and therefore its unsubstitutable role in the regulatory network. Moreover, it is the first up to date described factor being involved in both sporophytic and gametophytic regulation.

Manipulation of gene expression during pollen development using differently active promotors may serve as a convenient tool for functional analysis. For such need, Lat52 as well characterised promoter active in late pollen development and in progamic phase has been a favourite tool for decades. In addition to Lat52 isolated from tomato, LGC1 promoter was characterized in lily generative cell (Singh et al. 2003, Singh and Bhalla 2008). However, promoters active in early pollen development were mostly missing. Therefore we focused on the identification of promoters driving expression in early stages and then isolated three microspore-active promoters enabling the spatially and temporally controlled expression pattern. Based on previous transcriptomic data from four pollen developmental stages (UNM, BCP, TCP, MPG) and several sporophytic tissues, a group of seven genes was selected exhibiting strict expression patterns only at early stages of male gametophyte development. Those candidate genes were verified by RT-PCR analysis but only three of them (At5g59040, At5g46795, and At4g26440) passed strict selective criteria on specific early expression and were named MSP1, MSP2, and MSP3, respectively. The activity of those promoters was tested using GUS:GFP markers. MSP1 showed earlier signal in microspores followed by a

sharp decrease in mature flowers while MSP2 and MSP3 initiated expression in microspores but accumulated in mature pollen as well. To evaluate MSP promoters as a suitable tool for early pollen expression *in planta*, two constructs for functional complementation using MSP1 and MSP2 promoters and *TIO* coding sequence were prepared. *tio* heterozygous plants produced 50% aberrant pollen and MSP1 construct were able to significantly (more than MSP2 construct) restore the phenotype confirming the usability of MSP1 promoter for targeted manipulation of gene expression at early pollen development.

With the increasing demand for transcriptomic studies, loads of expression data have been generated by number of research groups round the world including our and colaborative laboratory as published previously (Honys and Twell, 2003, 2004). These datasets included unique expression profiles of four pollen successive stages (UNM, BCP, TCP, MPG) of male gametophyte development plus numerous sporophytic tissues. To access those data to wide scientific community and provide it with an alternative web-based toolbox, we established a database named Arabidopsis Gene Family Profiler (aGFP). The database works with transcriptomic datasets from NASC Array and AtGenExpress and presents them in user friendly graphic environment introducing a unique virtual plant concept with coloured organs and tissues in a scale reflecting the level of gene expression. Furthermore, the data can be searched according to AGI codes, BAC loci, gene names, gene families or as custom genesets with the further possibility of keyword search. The user can also choose from MAS 4.0 or MAS 5.0 normalised datasets and from NASC Array/AtGenExpressed source to name some options. aGFP database covers only data from Affymetrix ATH1 arrays using wild-type *Arabidopsis* of different ecotypes grown under normal physiological (AtGenExpressed) or various (NASCArrays) conditions.

7. Discussion

The wide-screen analysis of T-DNA insertional lines brought in a sense surprising result. The first was, despite of numerous 21 TF families represented by 61 confirmed insertion lines, relatively small variance among individual pollen mutant phenotypes. Such limited portfolio of pollen defects may have seemed unexpected at this wide-scale screening but one should regard the role of transcription factors in complex networks when different regulators can trigger various biochemical processes that finally share and often overlap similar cellular events and functions like cytoskeleton organization, cell cycle, transport, cell wall metabolism etc. Such pleiotropic activity, but overlapping when comparing different mutants, has repeatedly been reported (Twell et al. 2006, Verelst et al. 2007, Gibalová et al. 2009). Second, we could not detect any significant bias towards any particular phenotypic category when comparing early and late TFs. Moreover, there were no preferences among individual TF families in disrupting cellular events. The common cellular disruption over all TF families affected MGU position resulted into three phenotypic defects: eccentric, separated or linear MGU. Third, among 61 verified lines we did not find many lines with considerably high penetrance with the exception of four lines reaching over 70% of aberrant pollen. On the contrary, the vast majority of lines showed wild-type-like appearance or mild phenotype defects in a range of 10-30%. Many authors suggested gametophytic mutations as pleiotropic, incompletely penetrant and displaying variable expressivity due to functional redundancy (Feldmann et al. 1997, Bonhomme et al. 1998, Grini et al. 1999, Drews and Yadegari 2002, Verelst et al. 2007) or due to a conditional-based phenotype. This phenomenon was especially apparent among transcription factors (Blanc et al. 2000, Verelst et al. 2007). Indeed, the vast majority of several hundred knockouts in *Arabidopsis* did not give rise to visible, directly informative phenotypes (Bouché and Bouchez 2001). However, out of six selected T-DNA lines for further genetic analysis using backcross, two lines performed heavy segregation distortion counting 1:0.04 (SALK_002235) and 1:0.39 (SALK_043690) in comparison to the Mendelian 1:1 segregation. It is likely that the corresponding genes (At3g20910, CCAAT-HAP and At5g54680, bHLH, respectively) play significant regulatory role in the male gametophyte development.

Further study on one selected transcription factor, AtbZIP34, revealed characteristic phenotypic and genetic transmission defects providing several lines of evidence that support

both sporophytic and gametophytic roles of this factor in male gametophyte development and function (Gibalová et al. 2009). Various phenotypic features including cell wall organization, changes in endomembrane system, vacuole and vesicle content in addition to handicapped pollen tube growth, were enlightened by microarray analyses. As a result, we identified over 1,300 genes up regulated and over 800 genes down regulated which disclosed the key position of the AtbZIP34 factor in male gametophyte regulatory network. For example, the abnormal presence of lipid/oil bodies in mutant pollen nicely correlated with significant down regulation of genes encoding proteins involved in lipid catabolism, lipid transfer and ABC transporter. Similar links could be associated to the cell wall biogenesis as the mutant pollen sustained in the structure of both intone (gametophytic management) and exile wall patterning (sporophytic management). The further analysis of downstream regulated genes including the regulator MYB97 may be promising to reveal new connections in the cellular network that control male gametophyte development.

For the functional analysis of transcription factors, manipulated gene expression using a set of promoters with various spatial and temporary activities is a very useful approach. Yet only two Arabidopsis promoters (*BCP1 and AtSTP2)* were known to be active in microspores (Xu et al. 1995, Truernit et al. 1999). However, *BCP1* also showed the expression in tapetum and *AtSTP2* was active from the tetrad stage. Similarly for other species (rapeseed, potato), microspore-active promoters have been identified but functionally overlapping to other stages (Fourgoux-Nicol et al. 1999, Maddison et al. 1999) with the exception for tobacco *MTM19,* whose promoter is highly microspore-specific (Clusters et al. 1997). Because of the absence of such tool for an early expression in Arabidopsis pollen development, three early active promoters were selected, isolated and verified by RT-PCR and GUS expression. Moreover, two of them, MSP1 and MSP2, were functionally confirmed by complementation analysis using MSP promoter and TIO coding sequence. Interestingly, the expression in early stages of male gametophyte development was steadily accompanied with the expression in tapetum, which tightly surrounds developing microspores in anther microsporangia. In this regard, the co-regulation of gene expression in both microspores and tapetum at early stages of anther development is not surprising since they have the same origin in archesporic tissue, tapetal inicials develop side by side with pollen mother cells the microsporogenesis itself is vitally dependent on secretory activity of tapetal cells.

Since the massive use of microarray experiments, the wealth of transcriptomic datasets have been widely expanding and it has been naturally followed by the introduction of transcriptomic databases collecting such data and presenting them in various manners either as a repository or with a web-based tools (Honys et al. 2008). aGFP database launched by our lab provides data from AtGenExpress and NASCArrays in easy intuitive graphic environment, which makes it unique among the others. The selection from those data source provides researchers transcriptomic data from Columbia-0 of numerous developmental stages grown under comparable conditions (AtGenExpress) and data coming from different ecotypes grown under various conditions (NASCArrays). Furthermore, none of the databases had till then offered instruments such as profiling according to gene families. In addition, interactive "virtual plant" represents another innovation helping users simply check transcriptomic data. The option between MAS 4.0 or MAS 5.0 normalized data are also rare among other databases round the world, which enables direct comparison of the influence of the detection algorithm. Empirical MAS 4.0, unlike the statistical MAS 5.0, is thought to yield more false-positive calls, our analysis of four pollen developmental stages showed that MAS 5.0 detection algorithm tended to eliminate a number of genes identified by MAS 4.0 as expressed genes and which were experimentally verified to be so, highlighting the extra value of MAS 4.0 detection algorithm. The uses can also switch between virtual plant or simple bar chart (standard or log-scaled) or tabulated display. The database is freely available on the internet and hopefully providing quick and easy steps to mine transcriptomic data as needed.

8. References

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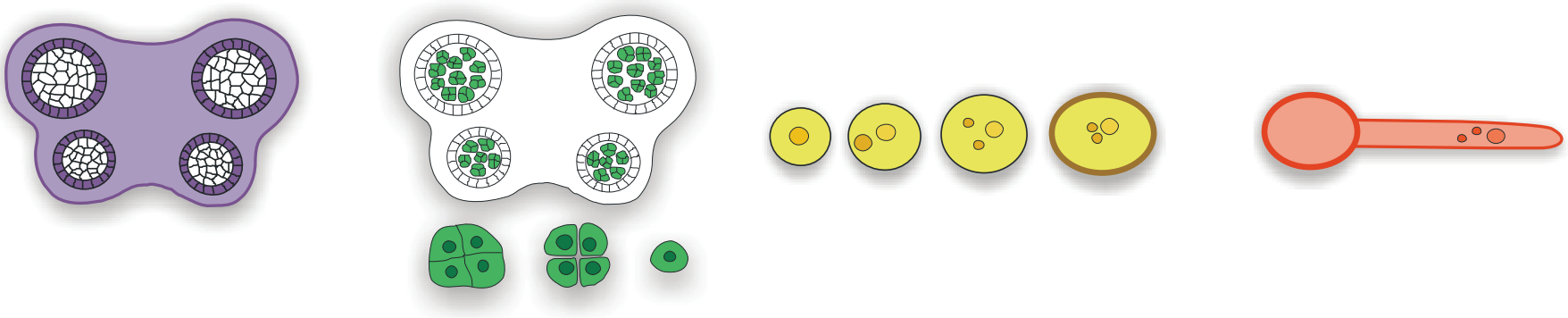

Fig. 7. Development of male sporofytic tissue (purple) and gametophyte structures (green, yelow, pink), colours correspond with Table 2.

Table 2. The list of genes affecting development of stamen, microsporogenesis, pollen development and pollen tube growth. TF genes are in red.

	AGI	Gene	Mutant	Mutant phenotype	Protein Identity	Protein Function	Reference
stamen development	At2g06050	*DDE1 /OPR3*	*DELAYED DEHISCENCE 1*	anther do not dehiscence	12-oxophytodienoate reductase that is required for jasmonate biosynthesis	jasmonic acid biosynthetic process	Sanders et al. 2000
	At2g39940	*COI1*	*coronatine insensitive1*	short filament, anther no dehiscence, conspicuous pollen	protein containing Leu-rich repeats and a degenerate F-box motif	SCF-dependent proteasomal ubiquitin-dependent protein catabolic process	Feys et al. 1994
	At2g44810	*DAD1*	*defective anther dehiscence1*	defects in anther dehiscence, pollen maturation	chloroplastic phospholipase A1	catalyzes the initial step of jasmonic acid biosynthesis	Ishiguro et al. 2001
	At3g13890	*AtMYB26*	*AtMYB26 , male sterile 35*	male sterile because of anther no dehiscence, fertile pollen	MYB transcription factor	transcriptional activation	Steiner-Lange et al. 2003
	At3g23130	*SUP*	*superman*	extra stamen at the expense of carpel	similar to zinc finger transcription factors	negative regulator, suppresion of AP3 and PI in fourh flower whorl	Gaiser et al. 1995
	At3g27810	*AtMYB21*	*AtMYB21*	male-sterile, short filaments, delayed dehiscence	MYB TF	regulatio of jasmonate pathway, anther maturation	Mandaokar et al. 2006
	At4g24972	*TPD1*	*tapetum determinant1*	excess of sporocytes, luck of tapetal cells	small putative signal peptide	hypoth.ligand for EMS1/EXC, meiotic regulation	Yang et al. 2003
	At4g25640	*FFT*	*flower flavonoid transporter*	anther dehiscence perturbed, male sterility	multidrug and toxin efflux family transporter	involved in flavonoid transport network	Thompson et al. 2010
	At4g27330	*SPZ/NZZ*	*sporocytless/nozzle*	defect in anther wall senecsence and sporocytes development	nuclear protein, similr to MADS box TFs	regulation of sporocytes development	Yang et al. 1999
	At5g07280	*EMS1/EXC*	*excess male sporocytes1/ extra sporogenous cells*	additional sporocytes, luck of tapetal cells	leucine-rich repeat receptor protein-kinase	required for meiotic cytokinesis, normal tapetum development	Canales et al. 2002, Zhao et al. 2002
	At5g40350	*AtMYB24*	*AtMYB24*	double mutant with MYB21: complete male-sterile	MYB TF	regulatio of jasmonate pathway	Mandaokar et al. 2006
	At5g56110	*AtMYB103*	*AtMYB103*	tapetum degeneration, male sterility	putative MYB TF	required for normal tapetum and pollen development	Higginson et al. 2003, Zhang et al. 2007
	unknown	*FAT TAPETUM*	*fat tapetum*	enlarged tapetum and middle layer at the time of meiosis	unknown	may be critical for proper differentiation af middle layer	Sanders et al. 1999, Sorensen et al. 2002
	unknown	*GNE1*	*gus-negative1*	similar to *fat tapetum*	unknown	unknown	Sorensen et al. 2002
	unknown	*GNE4*	*gus-negative4*	similar to *fat tapetum*	unknown	unknown	Sorensen et al. 2002

microsporogenesis	At1g06520	*AtGPAT1*	*glycerol-3-phosphate acyl transferase1*	abnormal tapetum, defects in pollen wall formation	membrane bond glycerol-3-phosphate acyl transferase	unknown	Zheng et al. 2003
	At1g07340	*AtSTP2*	*atstp2*	not observed ?	monosacharide carrier, sugar transporter	uptake of glucose from degradating callose	Truernit et al. 1999
	At1g13330	*AHP2*	*arabidopsis homologue pairing 2*	reduced, fertility, defects in chrom. pairing and bivalent formation	similar to yeast protein involved in homolog pairing, HP2 homolog	involved in chromosome synapsis and bivalent formation	Schommer et al. 2003
	At1g14750	*SDS*	*solo dancer*	reduced fertility, defective in synapsis and bivalent formation	meiotic cyclin-like protein	involved in chromosome synapsis and recombination	Azumi et al. 2002
	At1g63990	*SPO11-2*	*sporulation11-2*	male and female meiosis defects	homolog of topoisomerase subunit	homoloque synapsis, recombination and bivalent formation	Keeney et al. 1997
	At1g66170	*DUET**	*duet*	defects in meiosis telophase II, pollen abortion	PHD-domain containing protein	regulation of meiotic cell cycle progression	Reddy et al. 2003, Yang et al. 2003
	At1g66170	*MMD1**	*male meiocyte death1*	defects in meiosis telophase II, pollen abortion	PHD-domain containing protein	regulation of meiotic cell cycle progression	Reddy et al. 2003, Yang et al. 2003
	At1g67370	*ASY1*	*asynaptic1*	reduced, fertility, meiotic defects, reduced fertility	homolog to HOP1 important in chrom. synapsis in yeast	involved in chromosome synapsis and recombination	Ross et al. 1997, Armstrong et al. 2002
	At1g75950	*ASK1*	*arabidopsis skp1-like1*	polyads production of variable size and content	homologous to yeast SKP1 subunit of SCF complex	chromosome separation and segregation	Yang et al. 1999
	At1g77320	*MEI1*	*meiosis defective1*	abnormal tetrade, variable size and content	BRCT-domain-containing protein, simimar to acrosin-trypsin inhibitor	involved meiotic cell cycle	He and Mascarenhas 1998
	At1g77390	*TAM1*	*tardy asynchronous meiosis1*	defects in timing of meiotic division	core cell cycle protein involved in meiosis II during microsporogenesis	may control entry into metaphase II	Magnard et al. 2001, Wang et al 2004
	At2g31970	*AtRAD50*	*atrad50*	chromosome fragmentation, hypersensitivity to DNA damage	homolog of RAD50, component of meiotic recombination complex	involved in chromosome synapsis and recombination	Gallego et al. 2001
	At3g09090	*DEX1*	*defective in exine formation1*	defective exine, microspore degeneration	hypoth.membrane and calcium bonding protein	involved in sporopolenin deposition	Paxson-Souders 1997
	At3g11440	*MYB65*	*myb65*	myb64,myb33	nucleus, pollen sperm cell differentiation	regulation of gene expression	Millar and Gubler 2005
	At3g11980	*MS2*	*male sterile2*	thin pollen wall, reduce exine layer	similar to yoyoba protein, fatty acid reductase	may promote synthesis of long chain aliphatic molecules	Aarts et al. 1997
	At3g13170	*AtSPO11-1*	*atspo11-1*	production of polyads, defective chromosome pairing	homolog of the archaeal DNA topoisomerase VIA subunit (topo VIA)	involved in chromosome synapsis and recombination	Grelon et al. 2001
	At3g22880	*AtDMC1*	*atdmc1*	low male fertility, defects in bivalent formation	similar to meiosis-specific yeast DMC gene	bivalent formation	Couteau et al. 1999
	At3g25100	*AtCDC45*	*atdcd45*	polyads production, reduced male fertility	meiotic regulator	involved in DNA synthesis	Stevens et al. 2004
	At3g43210	*STD**	*stud*	blocked meiotic cytokinesis, failure in callose wall formation after PMII	kinesin with n-terminal motor domain	regulation of cytokinesis after meiosis	Hulskamp et al. 1997, Spielman et al. 1997
	At3g43210	*TES**	*tetraspore*	giant microspore with 4 nuclei, blocked meiotic cytokinesis	kinesin with n-terminal motor domain	regulation of cytokinesis after meiosis	Hulskamp et al. 1997, Spielman et al. 1997
	At4g14220	*RHF1*	*ring-h2 group f1*	male/female gamet formation	RING-type E3 ubiquitin ligase implicated in gametogenesis	cell cycle regulation	Liu et al. 2008
	At4g20900	*MS5/TDM1*	*male sterile5/three-division mutant*	microspores in polyads, degeneration, third division after meiosis II without DNA replication	similarity with SC protein and regulatory subunit of CDK	meiosis/cytokinesis control	Glover et al. 1998, Sanders et al. 1999
	At4g21270	*KATA/ATK1*	*arab. thaliana kinesin1*	abnormal meiosis	kinesin-like protein	possibly involved in meiotic spindle formation	Chen et al. 2002
	At4g34990	*MYB32*	*Myb32*	defective in meiosis, abnormal microspores,partial male sterility	R2R3 MYB transcription factor	highly expressed in tapetum,	Preston et al. 2004
	At5g05490	*SYN1/DIF1*	*determinate infertile1*	multiple meiotic defects, abnormal chromosome pairing	homolog to yeast meiotic-specific adhesion subunit	role in sister chromosome cohesion	Bai et al. 1999, Bhatt et al. 1999
	At5g06100	*MYB33*	*myb33*	double mutant:premeiotic abortion of meiocytes		during anther development	Millar and Gubler 2005
	At5g13390	*NEF1*	*no exine formation1*	lack of sporopolenine deposition, reduce lipid contant in tapetal plastids	membrane/transporter protein	accumulation/transport of lipids in tapetum	Ariizumi et al. 2004
	At5g20850	*AtRAD51*	*atrad51*	low male fertility, defects in chromosome pairing	encodes a homolog of yeast RAD51	chromosome repairing, synapsis	Li et al. 2004
	At5g22000	*RHF2*	*ring-h2 group f2*	male/female gamet formation	RING-type E3 ubiquitin ligase implicated in gametogenesis	cell cycle regulation, targeting of ICK4/KRD6, a CDK inhibitor	Liu et al. 2008

pollen development	At1g14830	*ADL1C*	*Arabidopsis dynamin-like1C*	pollen abortion during maturation	dynamin-like protein	pollen plasma membrane maintenance, cell wall	Kang et al. 2003
	At1g19890	*MGH3*	*male-gamete-specific histone H3*	no phenotype due to redundancy	male-germline-specific histone H3.3	germ cell and sperm cell specific gene expression	Okada et al. 2005, Ingouff et al. 2007
	At1g24520	*AGP1*	*arabinogalactan protein1 (AtBCP1)*	pollen aborts and collapsed at bicellular stage	arabinogalactan protein	tapetum, microspore and bicellular pollen viability	Xu et al. 1995
	At1g30450	*HAP5*	*hapless5*	aborted mature pollen	cation-chloride cotransporter	ion homeostasis during development	Johnson et al. 2004
	At1g50230	*TIO/SOLO POLLEN*	*two-in-one/solo pollen*	failure of cytokinesis at PMI	homology to Ser/Thr protein kinase FUSED	essential in centrifugal cell plate expansion	Oh et al. 2005
	At1g64570	*DUO3*	*duo pollen3*	germ cell fails to devide or delay	nuclear regulátory protein	required for G2/M transition	Brownfield et al. 2009
	At1g65470	*FAS1*	*fasciata1*	microspore and germ cycle arrested at G2/M	chromatin assembly factor 1 (CAF-1)	nucleosome/chromatine assemby during S phase	Chen et al. 2008
	At1g78900	*VHA-A*	*vacuolar ATPase V_1 subunit A*	pollen aborted from bicellular stage, swollen ER	vacuolar-ATPase V1 subunit A	Golgi organization, Ph homeostasis, secondary active transport	Dettmer et al. 2005
	At2g21870	*MGP1*	*gametophyte defective1*	destruction od mitochondria, pollen death	subunit of F1F0 ATP-synthase	co-regulation of ATP-synthase activity	Li et al. 2010
	At2g29980	*FAD3*	*fatty acid desaturase3*	fad3 fad7 fad8 triple mutant:	ER enzyme responsible for the synthesis of 18:3 fatty acids	omega-3 fatty acid desaturase activity	McConn and Browse 1996
	At2g34550	*UNG4*	*ungud4*	pollen aborts at bicellular stage	GA-2-oxidase	regulation of giberelins	Lalanne et al. 2004b
	At2g35630	*GEM1*	*gemini pollen1*	binucleate pollen, defective cytokinesis at PMI	MOR1/GEM1 microtubule associated protein	microspore polarity/cytokinesis	Park 1998, 2001, Twell et al 2002, Lee et al. 2007
	At2g42380	*AtbZIP34*	*atbzip34*	defects in cell wall patterning and lipid metabol.	bZIP transcription factor	Regulation of gene expression	Gibalová et al. 2009
	At3g11170	*FAD7*	*fatty acid desaturase7*	pollen abortion	chloroplastic enzyme responsible for the synthesis of 16:3 and 18:3 fatty acids	omega-3 fatty acid desaturase activity	McConn and Browse 1996
	At3g12280	*RBR*	*retinoblastoma-related*	microspore proliferation, cell fate defects	cell-cycle repressor protein	G1/S transition of mitotic cell cycle, cell fate specification	Chen et al. 2009
	At3g23670	*Kinesin-12Bki*	*kinesin-12b*	microspores fail to complete PMI with spindle defects	kinesin-12 family	phragmoplast microtubule formation	Lee et al. 2007
	At3g45150	*TCP16*	*tcp16*	microspore nuclear DNA loss and abortion	bHLH protein, TCP PCF-subfamily	regulator of microspore gene expression	Takeda et al. 2006
	At3g48750	*CDKA;1*	*cyclin-dependent kinase A;1*	bicellular pollen, germ cell division fails, S-phase delayed	cyclin-dependent kinase;1	cell cycle regulation, germ cell S-phase progression	Iwakawa et al. 2006, Nowak et al. 2006
	At3g54650	*FBL17*	*F-box-like17*	biccelular pollen, S-phase inhibited	E3 ubiquitin protein ligase, F-box protein	targeted proteolysis of CDKA inhibitor KRP6	Kim et al. 2008
	At3g57390	*AGL18*	*agl18*	not given	MADS-box containing protein	potential role in microspore gene exprssion	Alvarez-Buylla et al. 2000, Varelst et al. 2007
	At3g60460	*DUO1*	*duo pollen1*	mature pollen at bicellular stage, germ cell fails to enter PMII	novel R2R3 MYB protein in GC (MYB125)	regulation of gene expression for PMII entry, GC division	Twell and Howden 1998ab, Durbarry et al. 2005, Rotman et al. 2005
	At4g14450	*Kinesin-12A*	*kinesin-12a*	microspores fail to complete PMI with phragmoplast defects,	kinesin-12 family, microtubular motor kinesin	phragmoplast microtubule formation	Lee et al. 2007
	At4g26440	*WRKY34*	*wrky34*	less sensitive to cold stress	WRKY transcription factor	mediates cold sensitivity	Honys et al. 2006, Zou et al. 2010
	At4g36900	*HAP12*	*hapless12*	aborted mature pollen	contains AP2 domain	refulator of pollen gene expression	Johnson et al. 2004
	At5g05580	*FAD8*	*fatty acid desaturase8*	reduced germination and PT growth	temperature sensitive plastidic fatty acid desaturase	omega-3 fatty acid desaturase activity	McConn and Browse 1996
	At5g22260	*MS1*	*male sterile1*	microspore degeneration after releasing from tetrade	PHD finger domain	required for early microspore development	Yang et al. 2007
	At5g39400	*PTEN*	*arabidopsis phosphatase, tensin homologue*	pollen death after pollen mitosi II	phosphatase and tensin homologue (tyrosine/PIP3 phosphatase)	pollen maturation	Gupta et al. 2002
	At5g44860	*AGM*	*abnormal gametophytes*	pollen degenerates at late microspore stage	putative transmembrane protein	microspore development and/or division	Sorensen et al. 2004
	At5g49150	*GEX2*	*gamete expressed2*	affected pollen tube growth and fertility	plasma membrane associated, 6 transmembrane domains predicted	potentially involved in pollen tube guidance and fertilization	Engel et al. 2005

pollen development	At5g51330	*SWITCH1/DYAD*	*switch1/dyad*	uneven segregation of chromatides	member of sister chromatide complex	role in sister chromosome cohesion	Siddiqi et al, 2000, Mercier et al. 2001
	At5g54260	*AtMRE11*	*atmre11*	chromosome fragmentation, hypersensitivity to DNA damage	DNA repair and meiotic recombination protein	double strand break repair	Puizina et al. 2004
	At5g54800	*GPT1*	*glucose-6-phosphate translocator1*	aborted pollen, reduced lipid bodies, vesicles and vacuoles	glucose-6-phosphate translocator	Glc6P import for plastidic starch and fatty acids biosynthesis	Niewiadomski et al. 2005
	At5g55490	*GEX1*	*gamete expressed1*	affected pollen tube growth and fertility	transmembrane domain containing protein expressed in sperm cells	potentially involved in gamete guidance/interaction	Engel et al. 2005
	At5g57800	*FLP1*	*faceless pollen1*	defective sporopolenin and abnormal tryphine	transmembrane protein with similarity to the sterol desaturase family	role in synthesis of tryphine and sporopolenin	Ariizumi et al. 2003
	At5g58230	*MSI1*	*multicopy supessor of IRA1*	microspore and germ cycle arrested at G2/M	chromatin assembly factor 1 (CAF-1) p48 sbunit/pRbAp48	role in DNA replication	Chen et al. 2008
	At5g5t350	*AHA3*	*arabidopsis H+-ATPase3*	collapsed and aborted mature pollen	plasma membrane K+-ATPase	microspore/pollen maturation	Robertson et al. 2004
	At5g64630	*FAS2*	*fasciata2*	microspore and germ cycle arrested at G2/M	chromatin assembly factor 1 (CAF-1)	nucleosome/chrom. assemby during S phase	Chen et al. 2008
	unknown	*APG*	*apg*	defective pollen wall	unknown	involved in intine wall formation	Roberts et al. 1991
	unknown	*DSY1*	*dsynaptic1*	reduced fertility	unknown	involved in chromosome synapsis and recombination	Peirson et al. 1996, Ross et al. 1997
	unknown	*BCP1*	*bcp1*	aborted pollen development, cell death	unknown	structural component of pollen wall	Xu et al. 1995
	unknown	*DSY10*	*dsynaptic10*	male sterile, meiosis defects	unknown	involved in chromosome synapsis and recomb.	Ross et al. 1997
	unknown	*DUO2*	*duo pollen2*	bicellular pollen grain, germ cell arrested at prometaphase	unknown	mitotic progression at PMII, prometaphase	Durbarry et al. 2005
	unknown	*GEM2*	*gemini pollen2*	defective cytokinesis at PMI, range of pollen phenotype	unknown	asymmetric division and cytokinesis at PM I	Park et al. 2004
	unknown	*GUM*	*germ unit malformed*	dislocation of the MGU	unknown	MGU organization and integrity	Lalanne and Twell 2002
	unknown	*LIP*	*limpet pollen*	defective migration of GC into the centre after PMI	unknown	may regulate delivery of β-1,3-glucans/factor involved in cell migration	Howden et al. 1998
	unknown	*MAD1*	*male gametophytic defective1*	variable fenotype of generative cell	unknown	essential for PMII, GC division	Grini et al. 1999
	unknown	*MAD2*	*male gametophytic defective2*	generative cell defects	unknown	essential for PMII, GC division	Grini et al. 1999
	unknown	*MAD3*	*male gametophytic defective3*	formation of intine wall, PMI defect, double DNA content	unknown	essential for PMI, wall paterning	Grini et al. 1999
	unknown	*MS33*	*male sterile33*	defect in intine formation and tryphine deposition, abnormal pollen desication	unknown	unknown	Fei and Sawhney 2001
	unknown	*MUD*	*mgu displaced*	dislocation of the MGU	unknown	MGU organization and integrity	
	unknown	*SCP*	*sidecar pollen*	extra celled-pollen, abnormal divisions at PMI	unknown	involved in pollen asymmetric division	Chen and Mcormick 1996
pollen tube growth	At1g02140	*HAP1*	*hapless1*	disrupted guidance of pollentube growth, failure to leave the septum	high homology to Mago nashi	mRNA metabolism/localization	Johnson et al. 2004
	At1g04950	*AtTAF6*	*attaf6*	short pollen tube growth	TBP-associated factor 6	basal transcription factor	Lago et al. 2005
	At1g20200	*HAP15*	*hapless15*	short pollen tube growth, failure to exit style	26S proteasome regul. subunit protein S3	protein degradation	Johnson et al. 2004
	At1g60780	*HAP13*	*hapless13*	short pollen tube growth, failure to exit style	clathrin adaptor complexes medium subunit family protein	membrane trafficking	Johnson et al. 2004
	At1g66570/	*HAP3*	*hapless3*	short pollen tube growth,	sucrose transporter SUC1/	saccharide metabolism/	Johnson et al. 2004
	At1g71270	*POK*	*poky pollen tube*	short pollen tubes	Vps52/Sac2 family protein	polar tube growth, component of vesicle transport machinery	Lobstein et al. 2004
	At1g71880	*AtSUC1*	*sucrose-proton symporter*	pollen tube growth arrest	plasma membrane-H^+-sucrose symporter	sucrose import/cell specific modulation of water potential	Stadler et al. 1999, Feuerstein at al. 2010
	At2g03070	*SETH10*	*seth10*	reduced competitive ability, slower pt growth	putative protein	unknown	Lalanne et al. 2004b

pollen tube growth	At2g17800	*Rop3At*	*rop3at*	inhibition of pollen tube growth	member of ROP GTPase family	regulation of pollen tube growth	Li et al. 1998, 1999
	At2g25600	*ATK6*	*spik*	impaired pollen tube growth	shacker family inward K^+ channel	ion transport	Mouline et al. 2002
	At2g34680	*UNG9*	*ungud9*	reduced male transission	auxin-induced protein (AIR9), similar to leucine-rich repeat family protein	cell wall metaolism/signalling	Lalanne et al. 2004b
	At2g34980	*SETH1*	*seth1*	strongly reduced germination, abnormal callose deposition	phosphatidylinositol-glycan synthase subunit C	Component of GPI-anchor biosynthetic pathway	Lalanne et al. 2004b
	At2g35650	*AtCSLA7*	*cellulose synthase-like7*	slower pollen tube growth, defects in embryo development	glycosyltransferase	polysacharides	Goubet et al. 2003
	At2g41930	*SETH7*	*seth7*	strongly reduced pollen germination and pollen tube growth	Ser/Thr protein kinase	initiation of pollen germination	Lalanne et al. 2004b
	At2g43040	*NPG1*	*no pollen germination*	nongerminating pollen, no pollen tubes	calmodulin-binding protein	binding to calmodulin in calcium-dependent manner	Golovkin and Reddy 2003
	At2g47040	*VGD1*	*vanguard1*	retarded pollen tube growth	pectine methylesterase-homologous protein	cell wall metaolism	Jiang et al. 2005
	At2g47860	*SETH6*	*seth6*	strongly reduced pol. germination and ptgrowth	RPT2/NPH3-like protein	scaffold protein within signalling pathway	Lalanne et al. 2004b
	At3g04080+	*AtAPY1*	*apyrase1+2*	nongerminating pollen,	apyrase	mediation of gignalling +	Steiebrunner et al. 2003
	At3g08970	*TMS1*	*thermosensitive male sterile1*	lowered thermotolerance of pollen tube	DnaJ domain protein localized in ER lumen, homology to Hsp40	required for thermotolerance in progamic phase	Yang et al. 2009
	At3g10380	*SEC8*	*exocyst complex*	nongerminating pollen, no pollen tubes	subunits of exocyst complex	polarised exocytosis ofsecretory vesicles	Cole et al. 2005
	At3g45100	*SETH2*	*seth2*	strongly reduced germination, abnormal callose deposition	GPI-GnT cytalytic subunit PIG-A	Component of GPI-anchor biosynthetic pathway	Lalanne et al. 2004ab
	At3g51300	*Rop1At*	*rop1at*	inhibition of pollen tube growth	pollen-specific Rop GTPase	intracellulat Ca^{2+}-gradient, regulation of pollen tube growth	Li et al. 1998, 1999
	At3g52590	*HAP4*	*hapless4*	chaotic pollen tube growth in the ovary	ubiquitin extension protein1 (UBQ1)/60S ribosomal protein L40	preteosynthesis/ubiquitination	Johnson et al. 2004
	At3g54090	*UNG10*	*ungud10*	reducedmale transmitssion	fructokinase-like protein	sugar metabolism	Lalanne et al. 2004b
	At3g54690	*SETH3*	*seth3*	reduced pollen germination and pt growth	arabinose-5-phosphate isomerase	synthesis of rare rhamnogalactouronans	Lalanne et al. 2004b
	At4g00800	*SETH5*	*seth5*	strongly reduced pol. germination and growth	unknown transmembrane protein	unknown	Lalanne et al. 2004b
	At4g04710	*UNG6*	*ungud6*	reduced male transission	calcium-dependent protein kinase	calcium-depending signalling	Lalanne et al. 2004b
	At4g11720	*HAP2/GCS1*	*hapless2*	disrupter pollen tube guidance, blocked gamete fusion and fertilization	GCS1-like hypothetical protein, likely transmembrane protein	unknown	Johnson et al. 2004, von Besser et al. 2006
	At4g21150	*HAP6*	*hapless6*	short pollen tube growth, failure to exit style	ribophorin II (RPN2) family protein	membrane trafficking	Johnson et al. 2004
	At4g34940	*SETH4*	*seth4*	strongly reduced pollen germination	ARM repeat containing protein	unknown	Lalanne et al. 2004b
	At4g35950	*Rop5At*	*rop5at*	inhibition of pollen tube growth	member of ROP GTPases gene family-like	regulation of pollen tube growth	Li et al. 1998, 1999
	At5g12250	*SETH9*	*seth9*	reduced competitive ability, slower pollen tube growth	alcohol dehydrogenase-like protein	alcohol metabolism	Lalanne et al. 2004b
	At5g13650	*SETH8*	*seth8*	reduced competitive ability, slower pt growth	GTP-binding typA-related protein	unknown	Lalanne et al. 2004b
	At5g18280	*AtAPY2*	*double mutant*	no pollen tube	apyrase	nucleotide metabolism	Steiebrunner et al. 2003
	At5g47020/	*HAP11*	*hapless11*	normal pollen tube growth,	unknown protein/	unknown/	Johnson et al. 2004
	At5g47030			failure to enter the micropyle	mitochondrial ATP synthase δ chain	energy metabolism	Johnson et al. 2004
	At5g49680	*KIP*	*kinky pollen*	kinky-shaped pollen tubes	SABRE-like protein	component of the secretory pathway	Procissi et al. 2003
	At5g56250	*HAP8*	*hapless8*	short pollen tube growth, failure to exit style	expressed protein	unknown	Johnson et al. 2004
	unknown	*MAD4*	*mad4*	defective pollen tube elongation	unknown	unknown	
	unknown	*RTG*	*raring-to-go*	pollen germinated precociously within the anther	unknown	unknown	Johnson and McCormic 2001