Charles University in Prague

Faculty of Science

Department of Experimental Plant Biology

The role of bZIP transcription factors in male gametophyte of *Arabidopsis thaliana*

Ph.D. thesis

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David Honys

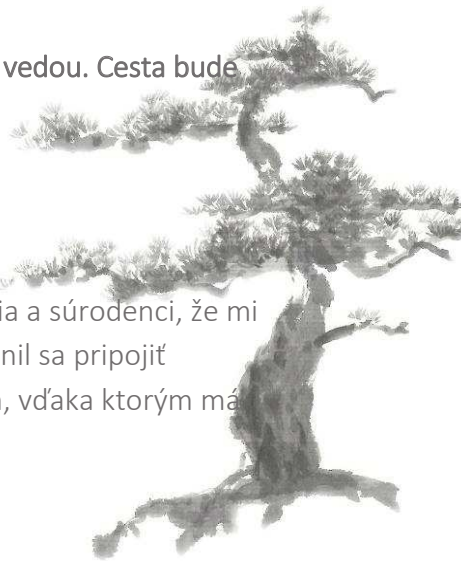
Prague, 16.10.2015

Poděkování

“Je v tom kus odvahy, pustit se do diskuze s přírodou a nechat fakta ať nás vedou. Cesta bude nejistá a zavede nás na netušená místa…“

~ M. O. Vácha ~

Ďakujem všetkým, ktorí na mojej ceste stoja pri mne. Ďakujem vám rodičia a súrodenci, že mi ukazujete smer a ste mi oporou. Ďakujem Davidovi Honysovi, že mi umožnil sa pripojiť k nadšeným objavovateľom mikrosveta peľu a všetkým drahým priateľom, vďaka ktorým má táto cesta zmysel.



CONTENTS

1. INTRODUCTION

1.1 Male gametogenesis - from gametes to seeds

1.1.1 Microsporogenesis, microgametogenesis and male germline specification

Germline cells in animals are determined in early embryogenesis and remain as distinct population of stem cells throughout life (see Hayashi and Surani 2009; see Twell, 2011). In contrast, flowering plants do not harbor a distinct germline in the sporophyte, but maintain populations of undifferentiated stem cells, until they switch the developmental program and start to produce reproductive organs containing diploid sporogenous cells. These mother cells serve as a gametophyte initials, undergo meiosis and give rise to haploid microspores and megaspores (Twell, 2011). Male gametogenesis begins with the division of a diploid sporophytic cell, giving rise to the tapetal initial and the sporogenous initial (pollen mother cell). The sporogenous cells undergo meiosis, giving rise to a tetrad of haploid microspores enclosed within a unique callosic (β-1,3-glucan) cell wall (Fig.1).

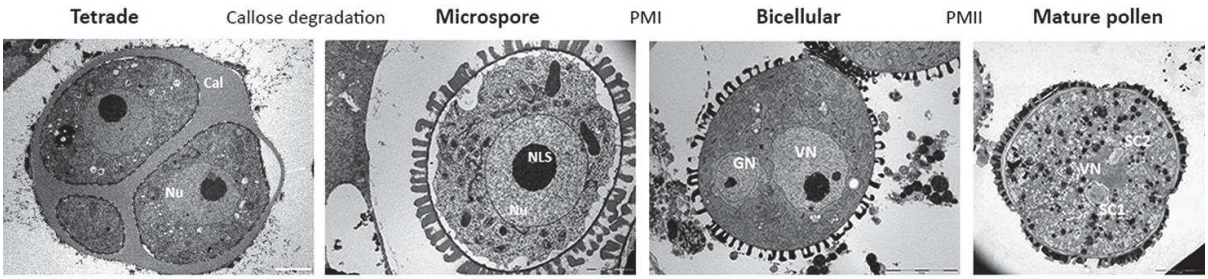


Figure 1. Arabidopsis pollen developmental stages. Scales are as follows: tetrade 2µm; microspores 2µm; bicellular 5 µm; mature pollen 5 µm. Cal – callose wall; Nu- nucleus; NLS – nucleolus; GN – generative nucleus; VN – vegetative nucleus; SC1,2 – sperm cell 1,2 (© Gibalová, 2011).

Callose is degraded by the activity of an enzyme complex – callase, secreted by the tapetum leading to the separation of tetrads into individual microspores (see Honys et al. 2006). Microspore growths and develops through a progressive cycle of vacuole biogenesis, fusion and fission events (Owen and Makaroff 1995, Yamamoto et al. 2003; Fig.2). Moreover, there is a compelling evidence that microtubules contribute to polar migration of the microspore nucleus in different species (Eady et al. 1995; Terasaka and Niitsu 1990). Two functionally redundant y-tubulin genes, TUBG1 and TUBG2, are required for spindle and phragmoplast organization in Arabidopsis microspores, further highlighting the importance of microtubule dynamics in

establishing polar division of the microspore (Pastuglia et al. 2006). Highly asymmetric division of the haploid microspore (pollen mitosis I; PMI) is a key event that is giving rise to the vegetative cell (VC) and the smaller generative (germ; GC) cell that further divides to produce the twin sperm cells (SC) (pollen mitosis II; PMII). Vegetative and generative daughter cells possess completely different structures and cell fates (Twell et al.1998). The large vegetative cell has dispersed nuclear chromatin and constitutes the bulk of the pollen cytoplasm. In contrast, the smaller generative cell has condensed nuclear chromatin and contains relatively few organelles and stored metabolites. Whereas the vegetative cell exits the cell cycle at G1 phase, the generative cell remains division-competent and completes pollen mitosis II (PMII) to form the two sperm cells required for double fertilization (see Honys et al. 2006). After this asymmetric division, the smaller generative cell is completely enclosed within the cytoplasm of the larger vegetative cell (McCue et al. 2011). PMII occurs within a membrane-bound compartment of the vegetative cell cytoplasm and in some angiosperms

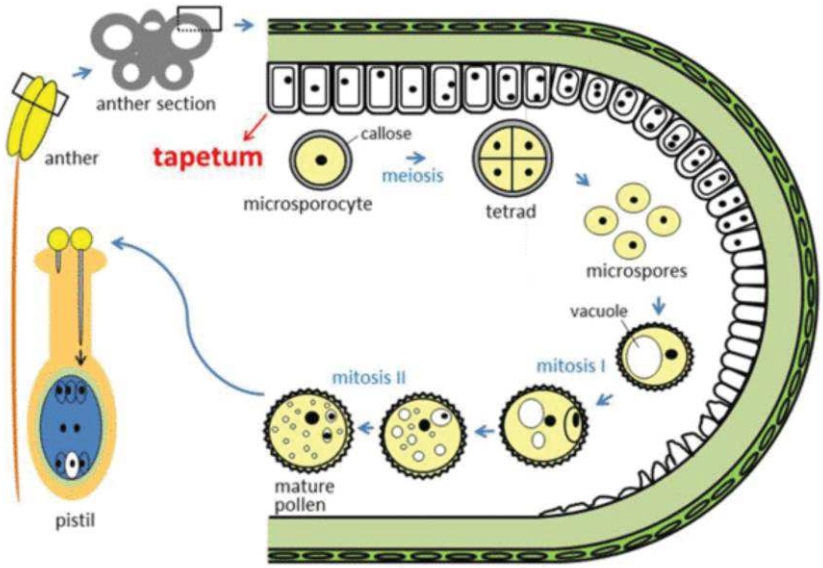


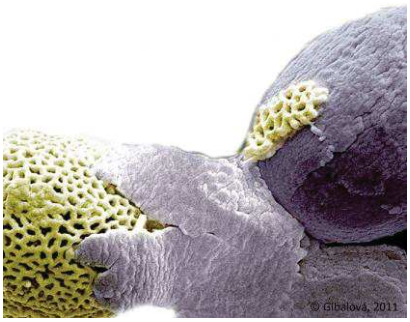
Figure 2. Schematic presentation of pollen developmental stages inside the anther locule. (http://abrc.sinica.edu.tw/)

a physical association is established between the gametes. The existence of this structural connection between the germinal SCs and the somatic vegetative nucleus (VN) has led to the proposal of a functional unit of fertilization in angiosperms (Russell and Cass, 1981) and was coined the 'male germ unit' (MGU) by Dumas et al. (1985). The connection between the SCs and VN ensures that the VN is associated with the sperm cells as all three nuclei travel as a unit through the pollen tube. Another role proposed for the cytoplasmic projection is that it may play a role in dictating the ordered arrival of each SC to the embryo sac, thereby determining which SC fertilizes the egg, as it was detected in Plumbago (Russel 1983, 1985). Mogensen (1992) envisioned that MGU can facilitate the transfer of material or information between these two entities. Although no transfer of RNA or protein

through the cytoplasmic projection has been observed directly, the cell non-autonomous impact of transcriptional products from the VN has been recently documented. The production of an artificial microRNA transcript from the VN is able to reduce the fluorescence of its GFP reporter target transcript in Arabidopsis SCs (Slotkin et al. 2009). However, there is more evidence needed to support the theory that the cytoplasmic projection aids the communication between the two pollen cell types. During pollen maturation the vegetative cell accumulates carbohydrate and/or lipid reserves required for the demands of plasma membrane and pollen tube wall synthesis (Pacini 1996). Pollen grains are usually dehydrated when finally released from the anthers. The accumulation of sugars and amino acids as osmoprotectants, including disaccharides and proline or glycine-betaine, is believed to protect vital membranes and proteins from damage during dehydration (reviewed in Twell et al. 2006).

Conclusively, specification and differentiation of the male germline requires two major steps, cytological events leading to polarized cell division and extensive gene expression reprogramming.

1.1.2. The unique pollen wall



The primary role of the outer layer of pollen wall - exine is to provide structural and physical support for the microspore cytoplasm containing the SCs and protection from harsh conditions, such as prolonged desiccation, high temperatures, ultraviolet (UV) light, and mechanical damage due to microbial attack; the exine prevents water loss from pollen grains and maintains their viability (Scott 1994; Scott et al. 2004). Except of the protective role, it also facilitates pollination by attracting vectors. Even before microspores are released from the tetrade, individual spores initiate to establish their wall. Pollen wall is in the end multilayered, whereas the first of several layers deposited at the microspore surface is an ephemeral callose wall. At the tetrad stage, the microspores are entirely covered by the callose wall (Fig.3A), the plasma membrane gradually assumes an undulating surface structure. Undulated plasma membrane may support the build-up and assembly of the elements of primexine (a precursor of the sexine). The primexine apparently acts as a template that guides the accumulation of sporopollenin, the main structural component of the pollen wall (Scott et

al. 2004). However, primexine is not deposited in areas where germinal apertures develop. It supports the build-up of solid proexine layers in a precise pattern (Tsou and Fu, 2002). After dissolution of the callose wall, the nexine, and finally the intine are formed by the microspore (Fig.3B; Fig.4) (Blackmore and Barnes, 1990). Until microspore release, sporopollenin is polymerized from precursors synthesized and secreted by the microspore; however, the bulk of sporopollenin precursors are secreted by the tapetum and incorporated into the wall after the dissolution of the tetrad (reviewed by Scott et al. 1991).

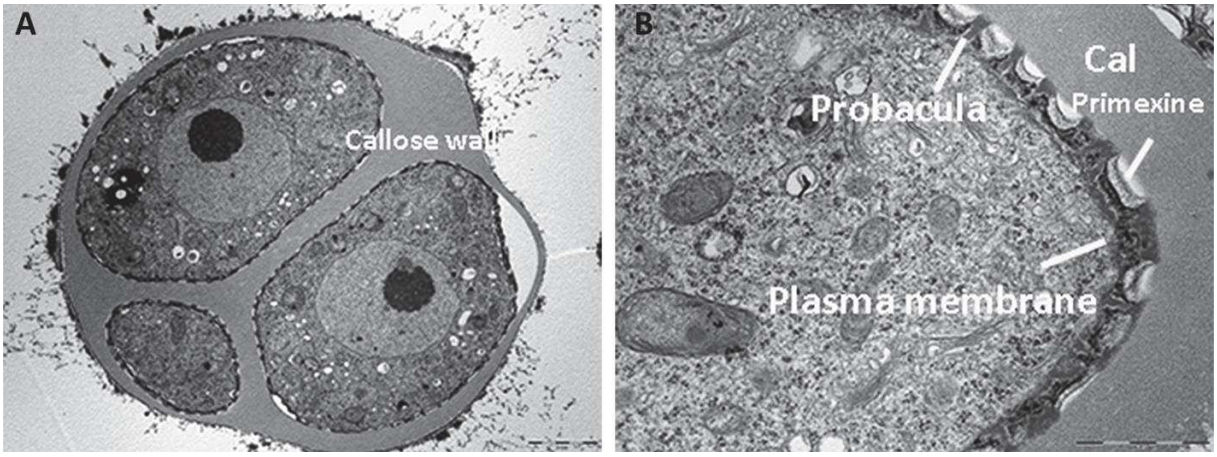


Figure 3. Transmission electron micrographs of tetrade developmental stage. The overall view on the whole tetrade; scale=2µm (a) detail of spore wall architecture and cytoplasm (b) scale=1 µm (© Gibalová, 2011).

Sporopolenin is synthetized by number of enzymes in tapetum, ACOS5 (ACYL-CoA SYNTHETASE 5; de Azevedo Souza et al. 2009) CYP703A, CYP704B (Cytochrome P450s; Morant et al. 2007; Dobritsa et al. 2009), MS2 (Male Sterility 2; Aarts et al. 1997), DRL1 (Arabidopsis Dehydroflavonol 4-Reductase-like1; Tang et al. 2009), LAP5, and LAP6 (Arabidopsis Less Adherent Pollen; Dobritsa et al. 2010). Progressively, the mechanism of the secretion and incorporation of the exine precursors into the microspore wall is being showed. Already for two decades transcripts of specific LTPs (Lipid Transfer Proteins) have been found to be present exclusively in anthers and/or tapetum, however their function related to exine formation is being deciphered nowadays. Previously, it was assumed that these proteins are secreted and bind to lipidic molecules based on amino-acid composition (N-terminal signal peptide for secretion). Huang et al. 2013 revealed localization and transfer pathway of type III LTPs specific for tapetum, where LTPs are bound or non-bound to exine precursors synthesized by ER-associated or cytosolic enzymes in the tapetum and they are secreted from the tapetum to the anther locule via ER-TGN system and become consequently constituents of the microspore

exine. Other tapetum-specific but non-type III LTPs likely move via similar paths but, per se, will not be a component of mature exine (Huang et al. 2013). Other exine precursors are synthesized in tapetum cytosol and are transported to the locule via the ABC transporter on the plasma membrane (Quilichini et al. 2010). Simultaneously, cellulosic precursors are produced in the microspore interior and transported to the microspore surface for the assembly of the cellulosic intine (Jiang et al. 2013). The molecules that compose the tryphine are also produced in the tapetum and deposited on the pollen wall when the tapetum degenerates through the process of programmed cell death (PCD) (Fig. 4b) (Edlund et al. 2004; Piffanelli et al. 1998). The tryphine contains a mixture of proteins, lipids, flavonoids and a mixture of carotenoids, both of which function in pollen pigmentation, serving as protectors from pathogen attacks, photo-oxidative damage, and UV radiation damage and as an attractant for pollination vectors (Hernandez-Pinzon et al. 1999).

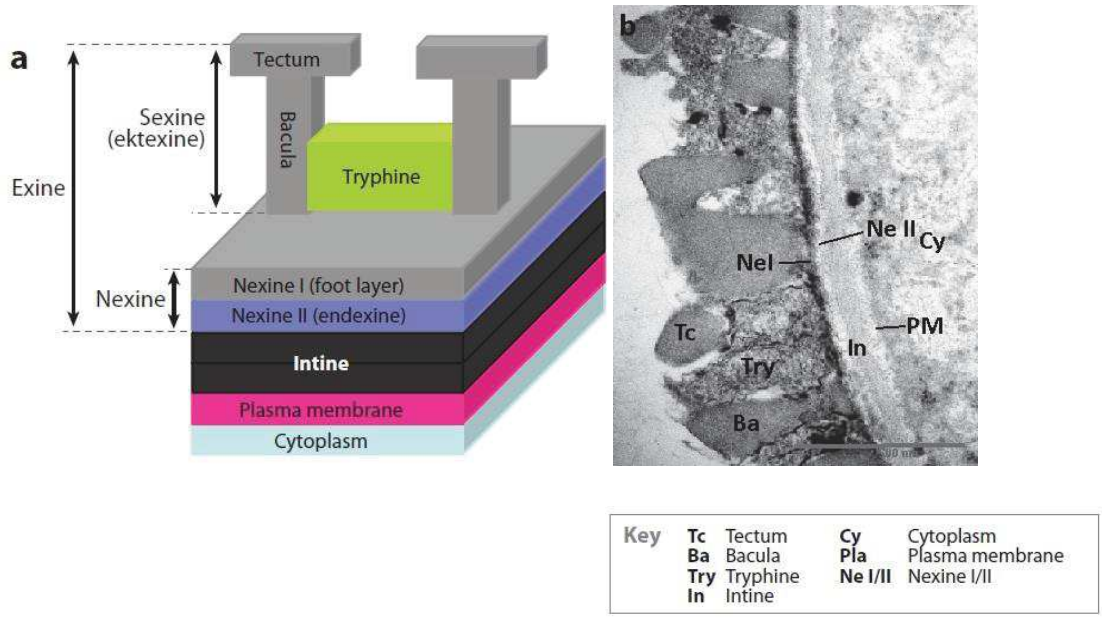


Figure 4. Schematic cross section through layers of a typical angiosperm mature pollen grain (a) (Ariizumi and, Toriyama, 2011); and transmission electron micrograph of a cross-section of Arabidopsis pollen grain, scale=500nm (b) (©Gibalová, 2011)

Except of supplying the wall material to the developing spores, tapetum layer also acts as a nurse tissue providing metabolites. Many key genes involved in exine formation have been cloned in Arabidopsis, but it would still be useful to saturate the exine-defective mutants to understand the whole molecular mechanism of exine pattern formation. To explore this, numerous mutants have been isolated and characterized, involved in several stages throughout pollen development (Fig. 5).

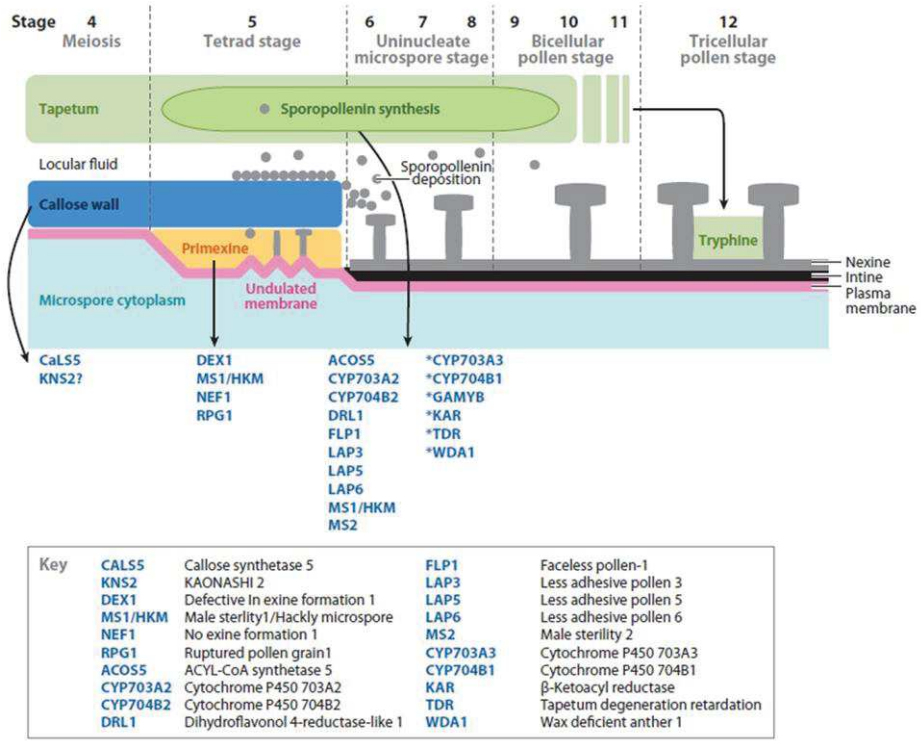


Figure 5. Schematic survey of mutants affecting pollen development through abberant exine or callose formation, sporopolenin synthesis (Ariizumi and Toriyama, 2011)

From the origin of the pollen components it is obvious that exine is predominantly of sporophytic origin, whereas intine formed by microspore is gametophytic (Owen and Makaroff 1995). It is apparent that the cross-talk between sporophytic and gametophytic molecules and tissues is crucial for the successful development of viable pollen grains within anthers.

1.1.3. Progamic phase of pollen development

Pollen tube germination represents unique cellular phenomenon with many critical changes in cellular metabolism. Just after pollen grains land on the stigma papillar cells, the pollen coat, composed of lipids and proteins (Piffanelli et al. 1998; Dickinson and Elleman, 2000), softens into a gelatinous mixture and flows onto the papillar surface. This bridge between the pollen grain and papillar cell, sometimes termed a foot, establishes the route of water flow into the desiccated pollen grain (Elleman et al. 1992). While pollen grain hydrates, Ca^{2+} influx triggers the activation, characterized by cytoplasmic reorganization within the pollen grain (Heslop-Harrison and Heslop-Harrison, 1992a, 1992b). This reorganization results in the formation of a cytoplasmic gradient of Ca^{2+} beneath the site of germination; this gradient is

critical for polar tip growth (Heslop-Harrison and Heslop-Harrison, 1992a; Franklin-Tong, 1999). Associated with Ca^{2+} influx, the pollen grain deposits callose, a β -1,3-glucan, at one of the three pores, where the pollen tube will emerge (reviewed in Johnson and McCormick, 2001).

The pollen tube grows through the female tissues in a polarized fashion similar to root-hair outgrowth, trichome specification, hyphal growth in fungi and extension of neuronal dendrites in the animal nervous system (Larkin et al. 1997; Gibson et al. 2011, reviewed in Hafidh et al. 2012). Nowadays, it is well established that cell polarity in pollen tube goes in hand with and relies on internal ion gradients (Konrad et al. 2011; Michard et al. 2009). This polarity serves the purpose of elongation exclusively at the tip of the cell by exocytosis, allowing some of the fastest cellular growth rates in nature (Konrad et al. 2011). Tip growing cells represent an extreme example of cell polarity, based on the vectorial sorting of organelles along the longitudinal axis of the cell, and the accumulation of secretory vesicles in their apex (Konrad et al. 2011). During pollen tube growth the tip needs to modulate the surrounding cell wall of stylar cells enabling its penetration through the extra-cellular space, most likely by interaction with extensin-like and arabinogalactan proteins as well as the secretion of cell wall softening enzymes and inhibitors such as polygalacturonases and pectinmethylesterase inhibitors (Nguema-Ona et al. 2012). The extra-cellular matrix (ECM) of the pistil transmitting tract provides essential nutrients as well as components for an accelerated, extended and guided pollen tube growth (Palanivelu and Preuss, 2006). Two important transcription factors are involved in ECM regulation. NO TRANSMITTING TRACT (NTT) encodes a C2H2/C2HC zinc finger transcription factor involved in ECM production and is essential for programmed cell death in the transmitting tract upon pollination (Crawford et al. 2007). HALF FILLED (HAF), encodes a bHLH transcription factor and is involved in NTT-dependent transmitting tract regulation (Crawford and Yanofsky, 2011).

The transport of cargo and cellular organelles over long distances in the pollen tube is ensured by a cytoskeletal array that consists of microtubules and actin filaments oriented parallel to the longitudinal axis of the cylindrical cell (Geitmann et al. 2000). Organelle motion along the actin bundles is mediated by myosin (Tang et al. 1989), whereas myosin IX belongs to the most abundant myosin subfamily in pollen. On the other hand, the largest feature, the male germ unit, moves relatively slowly, with approximately the same speed as that of the cellular growth rate and its movement relies largely on microtubules (Åström et al. 1995;

Miyake et al. 1995). The movement of both organelles and cytosol leads to an overall motion pattern called cytoplasmic streaming (Chebli et al. 2013). These forward and rearward movements result in a reverse fountain-like streaming pattern in the tip region enabling active and passive transport of molecules and organelles between cellular compartments (Lovy-Wheeler et al. 2005; Bove et al. 2008). The protoplast is present only in the distal part of the tube and becomes separated from the proximal region by the periodic deposition of callose plugs, keeping the protoplast volume nearly constant. Pollen tube growth is oscillatory and is correlated with an oscillatory influx of the cations Ca^{2+}, H^+, and K^+ and the anion Cl^- (Fig.6).

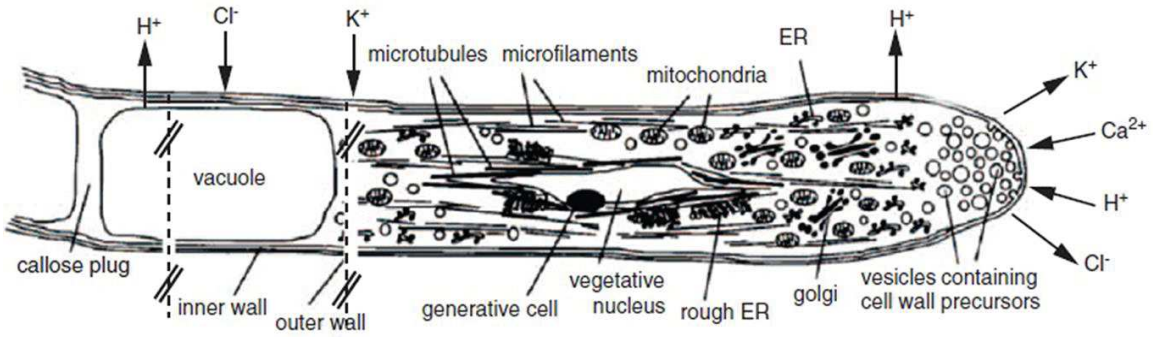
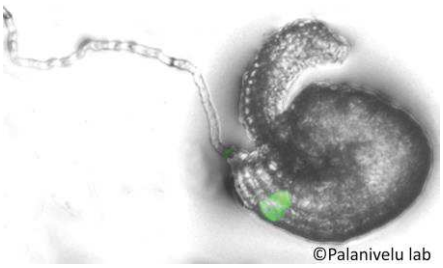


Figure 6. Diagrammatic representation of a pollen tube with the typical zonation, including the clear zone, subapical, nuclear and vacuolar domains, respectively (from right to left, not drawn to proportion), up to the first callose plug. Arrows represent ion fluxes known to be important for the establishment of polarity (adopted from Boavida et al. 2005, reviewed and modified in Konrad et al. 2011)

Periodic elongation of short actin bundles into the apical dome occurs between the exocytosis of synthetic materials delivered by the highly active vesicle-trafficking system (see Honys et al. 2006). In the pollen tube ultrastructure, four different zones are recognized - (a) the apical zone, enriched with vesicles; (b) the subapical zone, populated by organelles and especially active dictyosomes; (c) the nuclear zone, containing the vegetative nucleus and sperm cells, and (d) a vacuolar zone with large vacuoles separated by callose plugs (Cresti et al. 1977). There are three parts related to the pollen tube wall: the primary wall secreted in the growing tip is composed almost entirely of pectins, the callosic wall deposited behind the tip (secondary wall) and local callose depositions known as callose plugs at more or less regular distances behind the tip (Nasrallah et al. 1994). Just behind the pectic tip, cellulose synthases operate to form a very thin, pecto-cellulosic primary wall. Then, still in the subapical region, callose is synthesized beneath the thin primary wall to form a thick layer (Meikle et al. 1991). The pollen tube wall is an extension of the intine and is composed largely of callose [(1,3) β-glucan] forming 81% weight in Nicotiana (Schlupmann et al. 1994). Callose provides resistance to tensile and compression stress (Parre et al. 2005) and severely reduces wall permeability since angiosperm

pollen tube form septae ("callose plugs”) (Mogami et al. 2006; reviewed in Abercrombie et al. 2011).

1.1.4. Male-female crosstalk



Angiosperm pollen tubes (PT) enclosing non-motile male germ unit are growing through the maternal tissues towards the egg apparatus via polar tip growth. Upon pollen tube arrival, further signal transduction cascades are initiated leading to synergid cell death and pollen tube rupture (Kessler and Grossniklaus, 2011). Ultimately, fertilization occurs after successful gamete interaction and regulatory processes are activated to avoid polyspermy or to recover fertilization failures (see Dresselhaus and Sprunck, 2012). This complex process is being yet to be understood, leaning on transcriptomic as well as proteomic studies. These approaches enabled to reveal several molecular regulators crucial for the crosstalk between female (transmitting tract of the pistil and ovary) and male (pollen tube). Many of the recently discovered molecular players encode small cysteine-rich proteins (CRPs) comprising various subgroups such as defensin-like proteins (DEFLs), lipid-transfer proteins (LTPs), proteinase inhibitors, thionins, snakins, and others (Silverstein et al. 2007). From this point of view, it is possible to divide individual stages of te male-female crosstalk into several stages: PT guidance through and towards transmitting tract, ovular and micropylar PT guidance which is terminated by PT burst and sperm cells discharge (Bleckmann et al. 2014).

In the first stage, the growth direction of the pollen tube is regulated by the formation of different gradients including water, γ-amino butyric acid (GABA), calcium and other small molecules such as D-serine (Bleckmann et al. 2014). Growing pollen tubes possess and maintain spatially separated fluxes of a multitude of ions across the plasma membrane (PM) (Konrad et al. 2011). Especially, the investigation of Ca^{2+} channels and receptors is being quite extensive, bringing the first results a decade ago. For instance, Ca^{2+} ions enter the tube in the apex, frequently in an oscillatory manner (reviewed in Michard et al. 2009). Patch-clamp experiments showed clear Ca^{2+} channel activities in pollen (Shang et al. 2005; Wu et al. 2010), and transcriptomic data from Arabidopsis pollen revealed the presence of at least 20 putative Ca^{2+} transport systems (Konrad et al.2011). ACA9 (Autoinhibited Ca^{2+} -ATPase 9) encodes a pollen

specific calmodulin-binding Ca^{2+} pump localized to the pollen tube plasma membrane (Schiott et al. 2004). *aca9* pollen tubes have growth defects; however, they can reach ovules in the upper portion of the pistil and ~50% of these enter the ovule micropyle and arrest, but fail to burst (Schiott et al. 2004).

Little is known about signalling events that control pollen tube exit from the transmitting tract and guidance toward the ovule. Up to date, four important regulators have been discovered. In Arabidopsis, a gradient of GABA was reported in front of the ovule. The transaminase POLLEN ON PISTIL2 (POP2) forms this gradient through GABA degradation (Bleckmann et al. 2014). At moderate concentrations, GABA stimulates pollen tube growth and thus likely supports growth toward the ovule (Palanivelu et al. 2003). Another candidate involved in micropylar guidance is D-serine, which stimulates import of Ca^{2+} into the PT. MPK3 and MPK6, were identified in *Arabidopsis*, which are part of the ovular guidance network. MPK3/6 are two cytoplasmic protein kinases, which seem to be part of the signalling cascade mediating extracellular stimuli to changes in pollen tube growth direction (Guan et al. 2014). After arrival at the surface of the ovule, the pollen tube reaches the last phase of its journey, which is known as micropylar pollen tube guidance (Fig.7). It enters the micropyle, an opening between the two integuments, and directly grows toward the egg apparatus in species such as Arabidopsis. It was believed for a long time that the pollen tube grows through the filiform apparatus to enter one synergid cell, leading to pollen tube burst followed by a cell death of the receptive synergid cell. Recently, it was shown that the pollen tube is repelled by the filiform apparatus and instead grows along the cell wall of the synergid cells until it reaches a certain point after the filiform apparatus (FA) where its growth is arrested and burst occurs explosively (Leshem et al. 2013; Bleckmann et al. 2014). Filiform apparatus represents highly thickened structure at the micropylar end, consisting of numerous finger-like projections into the synergid

cytoplasm.

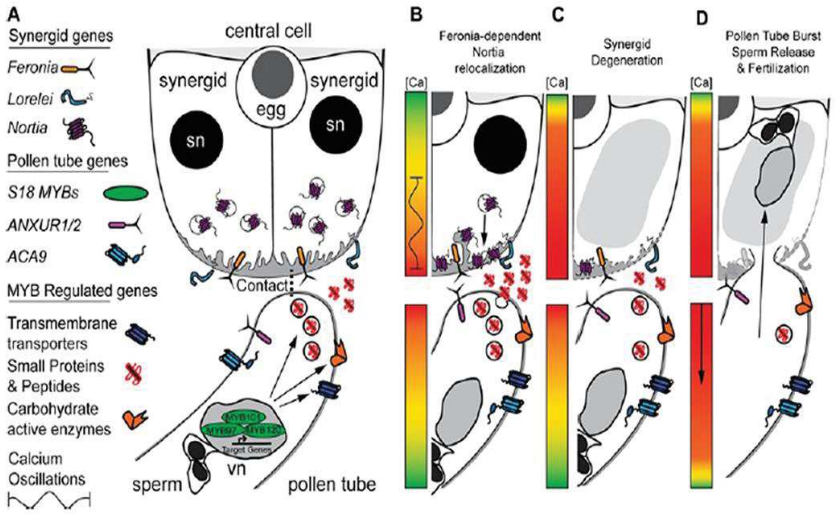


Figure 7. (**A**) Schematic diagram of the pollen tube and female gametophyte at the beginning of pollen tube reception. Pollen tube contact with one of the synergid cells is indicated (broken line): sn, synergid nucleus; vn, vegetative nucleus (**B** and **C**) Reception between a pollen tube and the synergid that will degenerate. Cytosolic Ca^{2+} concentrations are symbolized with a colour spectrum (red, high Ca^{2+}; green, low Ca^{2+}). (**B**) FER-dependent NTA relocalization occurs after pollen tube arrival. (**C**) Synergid degeneration is accompanied by loss of synergid nuclear integrity. (**D**) Pollen tube burst, sperm release and double fertilization (Leydon et al. 2014).

This structure greatly increases the surface area of the plasma membrane in this region, which is also associated with an elaborated endoplasmic reticulum. It is thought that the FA mediates the transport of molecules into and out of the synergid cells (Willemse and van Went, 1984; Huang and Russell, 1992; reviewed in Punwani et al. 2007). Many known components required for pollen tube growth and guidance at this stage are membrane-associated and accumulate at the FA. In Arabidopsis the formation of the FA as well as the expression of different attractants such as cysteine-rich proteins (CRPs) and group of defensin-like (DEFL) polypeptides, in the synergid cells depend on the activity of the R2R3-type MYB transcription factor MYB98 (Kasahara et al. 2005; Punwani et al. 2007). Important member of CRPs called LUREs secreted from the synergid cells and accumulating at the FA, attract pollen tubes in a species-preferential manner from a distance of about 100–150µm and were recently shown to bind to the tip region of pollen tubes (Okuda et al.2009; 2013). Pollen tube reception requires a number of synergid expressed genes including FER (FERONIA) (Escobar-Restrepo et al. 2007), NTA (NORTIA) (Kessler et al. 2010) and LRE (LORELEI) Capron et al. 2008). Relatively little is known about the pollen tube-expressed genes involved in pollen tube reception. Interestingly, a pair of pollen tube-expressed members of the FER family members CrRLK receptor-like kinases, ANX1 (ANXUR1) and ANX2 (ANXUR2) may be negative regulators of pollen tube burst (Leydon et al. 2014). Recently, three MYB transcription factors - MYB97, MYB101 and MYB120 have been identified controlling pollen tube gene expression in response to the pistil and

function as male factors that control pollen tube-synergid Interaction during fertilization (Liang et al. 2013). MYB97, MYB101 and MYB120 are critical for the pollen tube to exchange signals with the female gametophyte required for successful fertilization (Leydon et al. 2013; 2014). Not only synergids but also central cell plays an important role in micropylar guidance. For example, *magatama* (*maa*) mutants show defects in central cell maturation; both haploid nuclei are smaller and often fail to fuse (Shimizu and Okada, 2000). Another example of central cell-dependent defects in micropylar pollen tube guidance is the transcriptional regulator CENTRAL CELL GUIDANCE (CCG), which is expressed exclusively in the central cell (Chen et al. 2007). Finally, also egg cell seems to play a role in micropylar PT guidance. GAMETE EXPRESSED 3 (GEX3) is a plasma membrane-localized protein, which is expressed in the unfertilized egg cell (Bleckmann et al. 2014). Until recently, male factors and signaling pathways reacting to attractants secreted from the egg apparatus were unknown. The receptor-like kinases (RLKs) LOST IN POLLEN TUBE GUIDANCE1 (LIP1) and 2 (LIP2) have been identified, which are preferentially expressed in the pollen tube and are responsible for the AtLURE1-dependent guidance mechanism (Liu et al. 2013, reviewed in Bleckamnn et al. 2014).

1.2 Brief survey of historical and previous advances in pollen biology

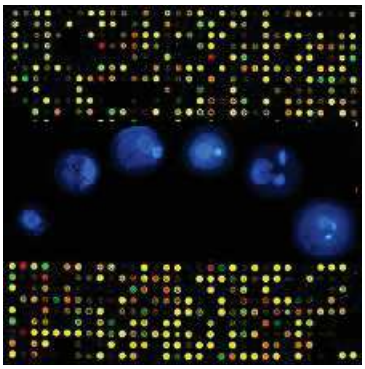
Focusing on pre-genomic era of the last century, one of the first evidences of experimental work within the frame of male gametophyte biology represents study of Parnel (1921), who observed that half of rice pollen heterozygous for glutinous or waxy endosperm phenotype, stained reddish rather than dark blue after iodine staining. Consequently, other group of MacGillivray (1924) observed reduced pollen transmission of “waxy” alleles and hypothesized that this may be caused by reduced pollen tube growth, ‘by the action of certain factors active in the tube nucleus’ (reviewed in Rutley and Twell 2015). The biochemistry of angiosperm pollen development was first reviewed by Mascarenhas (1975), and several subsequent reviews have appeared (McCormick, 1993) e.g.,Mascarenhas, 1989, 1990, 1993, McCormick, 1991; Bedinger, 1992. However, even earlier, in the 30´s attempts to study sporopollenin, the main component of the pollen wall, started. The term, a compound of “sporonin” and “pollenin” was first used by Zetzsche in 1932 as a collective appellation for the resistant wall material found in spores of angiosperms and gymnosperms (book chapter by J.Heslop-Herrison, 1970). Sporopollenin confers on the exine an unparalleled combination of physical strength, chemical inertness, and resistance to biological attack; these features have

greatly hampered progress in understanding both its chemical composition and details of its biosynthesis (Scott et al. 2004). Early literature frequently cites carotenoids as the main constituents of sporopollenin (Shaw, 1971; reviewed by Scott, 1994; 2004). However, the demonstration that a potent inhibitor of carotenoid biosynthesis, norflurazon, failed to prevent sporopollenin biosynthesis in *Cucurbita pepo* (Prahl et al. 1985) led to the re-evaluation of sporopollenin composition. Subsequently, a large body of experimental evidence established that sporopollenin consists mainly of long-chain fatty acids and a minor component of phenolic compounds (Scott et al. 1994).

First attempts to select genes important for the male gametophyte based on segregation ratio distortion and revealed few examples where mutant alleles affected pollen development, germination, or pollen tube growth (Ottaviano and Mulcahy, 1989, Scott and Stead, 1994). At the same time, a great deal of progress has been made in determining the different cell fates of the generative and vegetative cells of pollen grain dependent on asymmetric cell division e.g. Tanaka and Ito 1980; 1981; Zaki and Dickinson 1991 – studies based on applying of microtubule inhibitors to microspores; Park et al. 1989 and 2004 – revealed failure of establishment of the germ cell fate in *gemini pollen* - *gem1* and *gem2*; Oh et al. 2005 – showed failure of cytokinesis, which causes confinement of cell fate determinants; Kim et al. 2008; Brownfield et al. 2009 a, b – identified crucial regulators of male germ cell cycle progression and differentiation. The latter studies already took an advantage from the sequenced Arabidopsis genome (Arabidopsis Genome Initiative 2000) and enabled direct selection and functional characterization of pollen-specific and/or enriched genes, by means of forward or reverse genetic screens. Up to date, numerous such genes have been characterized and shown to be important for the pollen development. However, considering the size of pollen transcriptome, these data are covering although crucial but still minor part of pollen active genes.

1.3 Pollen transcriptomic studies – a decade of investigation

1.3.1 Transcriptome of pollen developmental stages



Since the genomic era started, it is possible to study anther and/or pollen specific genes to establish regulatory schemes crucial for functional male gametophyte. Consequently microarrays of individual stages of pollen development allowed to identify and study gene expression dynamics in this highly reduced and specialized cell lineage (Fig.8). First genomic assays were based on Affymetrix 8K ATH1 and contributed to reveal gametophytic transcripts in mature pollen (Honys and Twell, 2003; Becker et al. 2003). These analyses were provided on approximately one-third of the Arabidopsis genome. Further refinement was enabled by the availability of Affymetrix 23K Arabidopsis ATH1 arrays. There are three publicly available independent data sets for the Arabidopsis male gametophyte. The first contains microarray data covering four stages of the male gametophyte development (uninucleate microspores, bicellular pollen, tricellular pollen and mature pollen) and was performed from ecotype *Landsberg erecta* (Honys and Twell, 2004). The two remaining datasets were obtained from mature pollen grains from the ecotype Columbia (Zimmermann et al. 2004, Pina et al. 2005) (reviewed in Honys et al. 2006). Male gametophytic transcriptome revealed that there are two major phases of gene expression, early, covering uninucleate microspores (UNM) and bicellular pollen (BCP) and late - tricellular (TCP) and mature pollen (MPG).

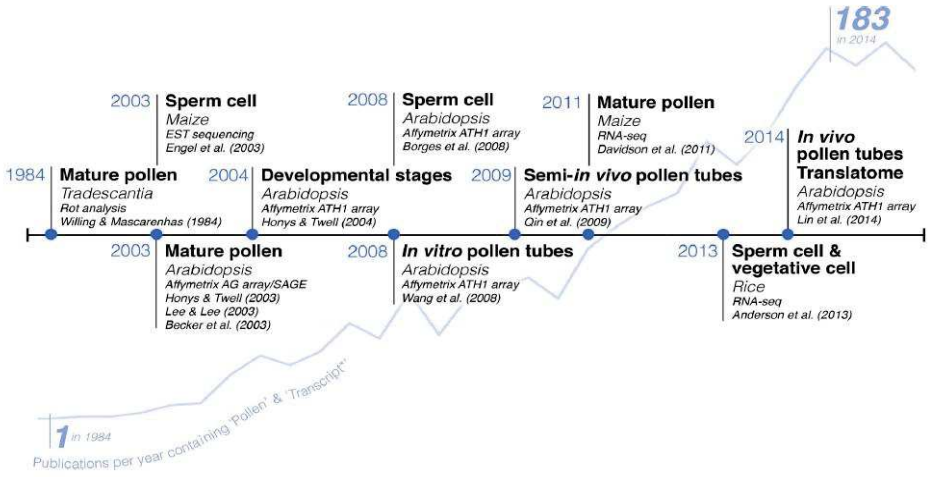
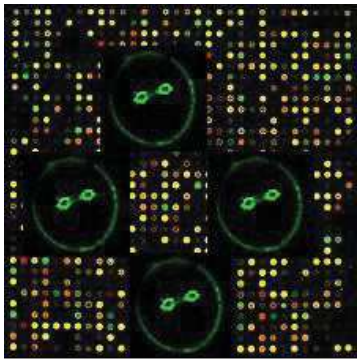


Figure 8. Advances in male gametophyte transcriptomic studies are indicated over time (Rutley and Twell, 2015).

The switch between both developmental programs occurs prior to PMII (Twell et al. 2006) Number of transcripts decreases towards pollen maturity, however the specificity has an increasing tendency and microarray data are showing also higher expression signals of pollen specific or enriched genes when compared to constitutive transcripts. Core cell cycle genes and transcription factors were enriched in UNM-BCP, while genes involved in signalling and cell wall metabolism were overrepresented in TCP-MPG, consistent with the early proliferative and late differentiation phases of pollen development (Rutley and Twell 2015). Among genes specific for the male gametophyte, the most dominant and mostly expressed category represented genes involved in cell wall associated proteins (e.g. glycoside hydrolases, polygalacturonases and cellulases), transport and cytoskeletal components (e.g. actin and profilin), both in microspores and mature pollen. The major difference between early and late stages of pollen development is represented by characteristic gene expression profiles, which possess high abundance of genes involved in protein synthesis in microspores, highlighting the importance of proteosynthesis initiated early during male gametophyte development. On the contrary, in mature pollen down-regulation of most microspore-expressed genes is apparent, especially those involved in protein synthesis and the most over-represented gene categories fall into cell wall metabolism, signalling and cytoskeleton, which are most likely to play important roles in post-pollination events.

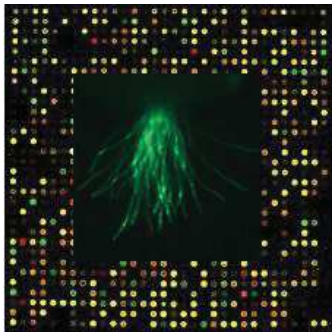
1.3.2 Sperm cell transcriptome



As mature pollen represents so called “cell within cell” structure, it was presumptive to separately reveal the sperm cells transcriptome. The first genomic study by Borges et al. 2008 of what may constitute a canonical sperm transcriptome in *Arabidopsis thaliana* revealed 5829 transcribed genes using an Affymetrix 24K microarray (reviewed in Russel et al. 2012). Authors reported set of over-represented genes associated with DNA repair, ubiquitin-mediated proteolysis, epigenetic labelling and cell cycle progression, which were also reported in prior studies of sperm cells expressed sequenced tag (EST) (Gou et al. 2001, 2009; Engel et al. 2003; Okada et al. 2006). Even more understanding of the unique contribution of sperm cells to sexual reproduction and their role in fertility and crop productivity brought other studies of sperm cell transcriptome in *Oryza sativa* (Russel et al.

2012) and *Lilium longiflorum* (Okada et al. 2006). These authors came to interesting findings, particularly that despite small size and diminishing volume of sperm cells, their transcriptome is substantially autonomous, some sperm transcripts appear not to be translated into protein in sperm cells, but may display delayed expression (Bayer et al. 2009), others are clearly transcribed and translated inside sperm cells (Ge et al. 2011). Other sperm cell transcripts present at fusion may be transmitted through plasmogamy during double fertilization to effect immediate post-fertilization expression of early embryo and (or) endosperm development (Russel et al. 2012). Numerous sperm cell-expressed genes were proven to be crucial for fertilization and normal embryo establishment, including, for example, HAPLESS2 - surface-linked protein required for fertilization and implication in directing pollen tubes to their female targets (HAP2, von Besser et al. 2006), SHORT SUSPENSOR – is transmitted as a sperm transcript into the egg cell during gamete fusion and encodes the protein SSP, which activates the developmentally critical asymmetrical division of the zygote, producing a polarized proembryo, (SSP, Bayer et al. 2009), DUO POLLEN 1 - plays a key role in activating Arabidopsis male germline genes (DUO1, Borg et al. 2011), GAMETE EXPRESSED 2 – is required for gamete attachment prior fertilization and GAMETE EXPRESSED 3 – necessary for micropylar guidance of pollen tube and embryogenesis (GEX2, GEX3, Mori et al. 2014; Alandete-Saez et al. 2008) and Arabidopsis male-germline histone H3 – is a sperm-specific histone H3 variant (MGH3, Okada et al. 2005). Small non-coding RNA pathways and DNA methylation pathways were also upregulated in sperm compared with the vegetative cell (Borges et al. 2008). For example, the DNA methyltransferase (MET1) is enriched in sperm, consistent with the active role of MET1 in the maintenance and epigenetic inheritance of CG-context methylation (Saze et al. 2003; 2008; Calarco et al. 2012) (reviewed in Rutley and Twell, 2015).

1.3.3 Pollen tube transcriptome



It is well established that transcriptional processes play important roles in global and specific gene expression patterns during pollen maturation. On the contrary, pollen germination in many species has been shown previously to be largely independent of transcription but vitally dependent on translation (Twell 1994; 2002). Arabidopsis was shown to follow this general trend (Honys and Twell 2004) and there is compelling evidence that many mRNAs and mRNPs are stored in

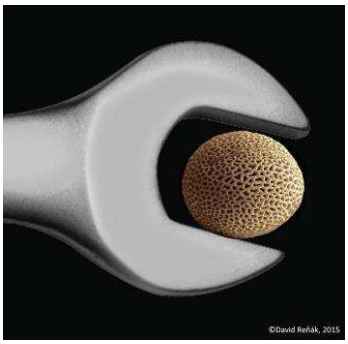
preparation for translation during tube growth (Honys et al. 2000, Twell 2002; reviewed in Honys et al. 2006). Transcriptome of pollen tubes mostly in Arabidopsis and tobacco was extensively studied in several time points post-germination. The first genomic analysis of tobacco mature pollen and 4 hours pollen tubes (Hafidh et al. 2012 a) followed by extended study for 24 hours pollen tubes (Hafidh et al. 2012 b) showed, that there is moderate but significant increase in transcription accompanied with stronger expression signals during pollen tube growth. These results confirmed the ongoing transcription activity and specific transcript accumulation in tobacco pollen tubes after PMII (Hafidh et al. 2012 b). Another approach to reveal transcriptional dynamics of pollen tubes in Arabidopsis brought study by Qin et al. 2009. Authors developed semi-*in vivo* (SIV) pollen tube growth assay and showed that they indeed can grow in synthetic medium, but their trajectory is random and growth rates are slower when compared to *in vivo* conditions. Therefore they used semi-*in vivo* conditions, allowing the growth of pollen tubes through the pistil tissues. Indeed the gene expression profiles of *in vitro* and SIV grown pollen tubes were distinct. Semi-*in vivo* grown pollen tubes express a substantially larger fraction of the Arabidopsis genome than pollen grains or pollen tubes grown *in vitro*. Genes involved in signal transduction, transcription, and pollen tube growth are overrepresented in the subset of the Arabidopsis genome that is enriched in pistil-interacted pollen tubes, suggesting the possibility of a regulatory network that orchestrates gene expression as pollen tubes migrate through the pistil (Qin et al. 2009). Authors also identified a set of genes that are specifically expressed in pollen tubes in response to their growth in the pistil and are not expressed during other stages of pollen or plant development. Set of 383 genes, uniquely expressed in SIV-PT, was enriched for genes involved in signalling (e.g. transmembrane receptors and protein kinases), defence response (e.g. TIR-NBS-LRR receptors), and cell extension (transporters and antiporters) (reviewed in Rutley and Twell et al. 2015).

The novel approach that investigates gene expression profiles represents high throughput RNA sequencing (RNA-seq) (Loraine et al. 2013). Most Arabidopsis pollen transcriptome studies have used the ATH1 microarray, which does not assay splice variants and lacks specific probe sets for many genes (Loraine et al. 2013). Study by Loraine et al. 2013 led to the identification of 1,908 high-confidence new splicing events and several unannotated (59) and untranslated (39) regions for pollen-expressed genes. The overlap between pollen ATH1

and RNA-seq data was almost complete, however 11 % of the genes detected by RNA-seq had no corresponding probe sets on the ATH1 array, revealing a previously unknown group of pollen-expressed genes, including two well-known male germline-specific genes, DUO1 and GCS1/HAP2.

Taken together, pollen transcriptomic studies imply differential gene expression dynamics for individual stages of the male germline reflecting its rapid progress in development and following stages leading to successful fertilization. Based on pollen transcritomic studies, the unique composition of the pollen transcriptome and its striking reduction in complexity compared with sporophytic tissues and purified sporophytic cell types, such as root hair cells (11,696 genes; Becker et al. 2014) and stomatal guard cells is apparent (13,222 genes; Bates et al. 2012) (reviewed in Rutley and Twell, 2015).

1.4 Genetic tools to study pollen expressed genes



A common way to dissect a developmental pathway is to isolate mutants that disrupt it (McCormick, 2004). The gain-off function approach supported with another analysis contributed to answer the fundamental questions related to the cell fate of vegetative and generative cells of pollen grain, cell polarity, cell signalling and many others. From such point of view, pollen serves as a microcosm for all the interesting questions facing plant biologists today (McCormick, 2004). Extensive studies of the transcriptomes of e.g. Arabidopsis pollen developmental stages, including germ cells, tobacco pollen and pollen tubes both *in vitro* a and semi-*in vivo* together with other plant species brought a valuable data sets still using for uncovering of new important genes and regulatory pathways during pollen development. Except of Arabidopsis and tobacco, other species for which mature pollen transcriptome data have been published include rice (Suwabe et al. 2008; Hobo et al. 2008; Wei et al. 2010) maize (Ma et al. 2008; Davidson et al. 2011; Chettoor et al. 2014), soybean (Haerizadeh et al. 2009), grapevine (Fasoli et al. 2012), potato (Sanetomo and Hosaka 2013), woodland strawberry (Hollender et al. 2014), and most recently lily (Lang et al. 2015) (reviewed in Rutley and Twell, 2015).

For elucidating the role of numerous pollen specific/enriched genes, pollen biologists has to face the fact, that the use of CaMVΩ35S promoter is not suitable for pollen studies. Since

early 90`s scientists attempt to uncover genes that are pollen-specific and moreover specific for early and late stages of pollen development. The first identified genes fell into the late class of messages: the Lat genes of tomato, Lat51 (McCormick, 1991), Lat52, Lat56 and Lat59 (Twell et al. 1991), the Bp10 gene of *Brassica napus* (Albani et al. 1992) and the NTP303 gene of tobacco (Weterings et al. 1992). It was shown that these genes reach the maximal expression in mature pollen. Bp10, Lat51 and NTP303 genes encode proteins that show sequence similarity to cucumber ascorbate oxidase (Ohkawa et al. 1989). Lat56 and Lat59 encode proteins whose putative sequences are highly similar (54% amino acid identity) and that show significant sequence similarity to the pectate -lyase genes of the plant pathogen Erwinia (Wing et al. 1990), implying that pectin degradation is important for pollen germination and/or PT growth. Twell et al. 1991 showed that the 5`- flanking regions of the Lat52 and Lat59 promoters, when fused to GUS (β-glucurinidase) reporter gene were sufficient to direct expression in an essentially pollen specific manner in transgenic tomato, tobacco and Arabidospsis plants. These promoters were analyzed into detail, and respective cis-elements necessary for pollen gene expression, as well as upstream regulatory elements functioning as enhancers were identified. Especially, Lat52 promoter is broadly used for targeted manipulation of gene expression that is restricted to the vegetative cell during pollen maturation after pollen mitosis I (Twell et al. 1990). Following step in uncovering the molecular components that control spatial and temporal patterns of gene expression was the identification of an “early” class of pollen expressed genes. Honys et al. (2006) described three Arabidopsis promoters MSP1, MSP2 and MSP3 that are active in microspores and are otherwise specific to the male gametophyte and tapetum. These promoters therefore provide important tools for the functional analysis of genes and proteins expressed during microspore development. Interestingly, in histochemical GUS assay, MSP1 promoters showed earlier expression and a decline in mature pollen, whereas expression of MSP2 and MSP3 increased towards pollen maturation. These differences in GUS expression profiles were not predicted by the MSP microarray expression profiles that were very similar. As the previously mentioned Lat52 promoter is restricted to the vegetative cell of pollen grain, it was necessary to identify also promoters specifically active in sperm cells. For example, the promoter of the LILY GENERATIVE CELL-SPECIFIC 1 (LGC1) gene of lily (*Lilium longiflorum*) directs reporter gene expression in the generative cells and sperm cells of transgenic *Nicotiana tabacum* (Singh et al. 2003; reviewed in Engel et al. 2005). An Arabidopsis MYB transcription factor gene, DUO POLLEN 1 (DUO1), is specifically expressed in the generative cells and sperm

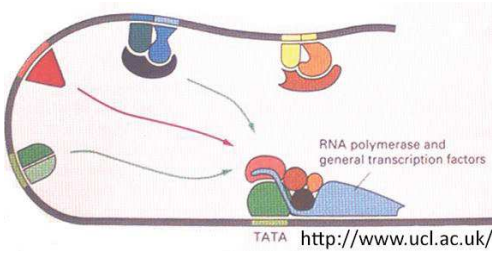
cells (Rotman et al. 2005). However, it was equally important to identify promoters useful for driving the expression of reporter genes in the sperm cytoplasm. Engel et al. 2005 identified two Arabidopsis promoters – Gamete Expressed 1 (AtGEX1 active in the sperm cells and not in the progenitor generative cell or in the vegetative cell) and – Gamete Expressed 2 AtGEX2 active only in the sperm cells and in the progenitor generative cell, but not in the vegetative cell or in other tissues. The AtGEX1 and AtGEX2 promoters therefore represent useful tools for manipulating gene expression in sperm cells, for localization and functional analyses of sperm proteins, and for imaging of sperm dynamics as they are transported in the pollen tube to the embryo sac (Engel et al. 2005). Finally, AtGEX3 gene coding for plasma membrane (PM) protein, is important for micropylar pollen tube guidance. This protein was shown to be expressed in PM of sperm cells, vegetative cell and egg cell of the female gametophyte (Alandete-Saez et al. 2008). The above mentioned transcription factor DUO1 regulates three genes crucial for successful fertilization – GEX1, MGH3 coding for a male germline-specific histone H3.3 variant (Okada et al. 2005; Ingouff et al. 2007) and GCS1/HAP2, encoding an ancestral membrane-associated protein required for gamete fusion (Mori et al. 2006; von Besser et al. 2006). These promoters are useful tools in marker lines, protein localization studies, and cell specific over-expression or targeted down-regulation/silencing of the transcripts. For instance, vegetative cell expressed Lat52 or germline specific MGH3 promoters driving the expression of artificial amiRNA are being used to silence target transcripts in cell specific manner.

Except of localization studies, it is important to study pollen expressed genes in genetic manner. Especially, two mutations have greatly facilitated the analysis of male gametophytic genes. In *quartet 1/-* plants, all products of a single meiosis are held together in a tetrad through pollen development, whereas each individual pollen grain is normal and can germinate (Preuss et al. 1994; reviewed in McCormick 2004). Two *Arabidopsis thaliana* genes, QRT1 and QRT2, are required for pollen separation during normal development. In *qrt* mutants, the outer walls of the four meiotic products of the pollen mother cell are fused, and pollen grains are released in tetrads. Tetrad analysis is often used to test whether pollen phenotypes result from a gametophytic mutation or from dominant sporophytic mutation. *qrt1* pollen is viable and fertile and the cytoplasmic pollen contents are discrete. Pollination with a single tetrad usually yields four seeds, and genetic analysis confirmed that marker loci segregates in a 2:2 ratio within these tetrads, however it is not in and itself proof of gametophytic action (Preuss et al. 1994;

McCormick 2004). Many gametophytic mutants exhibit variable expressivity and penetrance, therefore quartet mutation represents a useful tool to judge the nature of the mutation. Because pollen is haploid, it is not straightforward to determine if a mutation is dominant or recessive (McCormick 2004). To combine a mutant allele with a wild-type allele in one cell for a dominance/recessiveness test, one option is to use *tetraspore* mutation (sporophytic recessive mutant), where pollen grains fail to undergo cytokinesis after meiosis and large multinucleate pollen grains are formed (Hulskamp et al. 1997; Yang et al. 2003). In a tetraspore (*tes/tes*) homozygote that also is heterozygous for the gametophytic gene being tested, pollen grains carrying both a mutant allele and a wild-type allele will exist (McCormick 2004). For instance, by this approach *polka dot pollen* (*pdp*) mutation was analyzed (Johnson and McCormick, 2001). For gametophytic mutations crosses with *tes* is important to know whether to introduce a wild-type copy of the gene into the mutant background or to introduce the mutant version of the gene into the wild-type background (McCormick 2004).

Another important tools for pollen biology represent male gametophyte transcriptomes. Transcriptional dynamics significantly change through the development, thus these data represent valuable source of information about regulatory mechanism in this cellular lineage. However, these assays will be impossible to provide without the suitable protocols to isolate needed developmental pollen stages. It was important to provide pure fractions containing appropriate developmental spore stage with minimum contaminants. Such protocol based on collecting immature flowers to 0.3M Manitol and performing Percoll gradient for isolating pollen developmental stages was published by Honys and Twell 2004 (protocol modified according to Kyo and Harada, 1985, 1986). Further optimization and refinement of the protocol was provided by Dupľáková et al. submitted).

1.5 Transcriptional regulation of the male germline



DNA binding proteins, so called transcription factors (TFs) are key nodes of regulatory networks in eukaryotic organisms. Most eukaryotic genes are regulated by multiple transcription control elements, however TFs possessing executive activity in regulation are responsible whether or not a specific gene in multicellular organism is expressed in a

particular cell, at a particular time. Recent progress has been made in cloning and characterization of Arabidopsis TFs on the genome scale predicating numerous crucial functions in plant development, biosynthesis, cell growth, stress responses, hormone signalling, differentiation and many others (Qu and Zhu, 2006). Given this critical roles of TFs during plant development, it is necessary to further study transcription regulation. Pollen development offers great opportunity to study genes regulating cell fate, cell patterning, cell polarity and cell signalling (McCormick, 1993), moreover it also represents easily accessible cell type and its haploid nature eases functional and genetic analyses of the transformants.

To identify novel TFs, new high-throughput technologies, mostly microarrays or next generation sequencing are being used, which enable analysis of the haploid pollen transcriptome on the global scale (Twell et al. 2006). The microarray studies led to the identification of 992 (Honys and Twell, 2003) and 1587 (Becker et al. 2003) genes expressed in mature pollen of which 39% / 10% were considered to be pollen specific (Twell et al. 2006). Out of approximately 1350 predicted Arabidopsis TFs (Davuluri et al. 2003; Riechman et al. 2000; Parenicova et al. 2003; Toledo-Ortiz et al. 2003), 612 were expressed in developing male gametophyte. Of these, 49 were pollen enhanced and only 27 were pollen specific. These genes represent strong candidates for transcriptional regulators of the male gametophyte development (reviewed in Twell et al. 2006). Several large TF families were overrepresented among male gametophyte, including C3H and C2H2 zinc finger proteins, WRKY, bZIP and TCP proteins. On the contrary, basic loop helix (bHLH) and APETALA2/ethylene response element binding protein like (AP2/EREBP), MADS and R2R3-MYB gene families were underrepresented (Honys and Twell 2004). Several genes belonging to the above mentioned TF families have been identified and their roles in male gametophyte have been characterized.

Great progress has been done in revealing of several TFs and their target genes. DUO1 TF belongs to R2R3 MYB family and it was shown to be a key regulator of germ cell division and sperm cell differentiation (Brownfield et al. 2009 a,b; Durbarry et al. 2005; Rotman et al. 2005). As an integrator of these processes, DUO1 operates through DAZ1 and DAZ2, which encode EAR motif-containing C_2H_2-type zinc finger proteins required for germ cell division and for the proper accumulation of mitotic cyclins (Borg et al. 2011; 2014). Function of another MYB TFs was demonstrated further in pollen development and during progamic phase. Three MYB TFs (MYB101, MYB97 and MYB120) were shown to control *de novo* transcription of genes

required for pollen tube differentiation, pollen tube-female interactions and subsequent release of sperm cells prior to fertilization (Leydon et al. 2013).

Another well characterized TF network reported by Verelst et al. 2007 belongs to MADS - box family. Their subgroup AtMICK* proteins are directing pollen maturation through an active repression of early male gametophytic program. The AtMIKC* complexes repress immature pollen-specific TF genes such as WRKY34, and activate mature pollen-specific TFs such as AGL18 and AGL29. Conclusively, pollen TFs were shown to regulate cellular processes leading to cell division and cell identity (DUO1), or they act as master regulators of gene expression (AtMICK* complexes). Except of these functions it was shown that pollen TFs also regulate metabolic pathways. For instance Aborted Microspores (AMS) TF network affects pollen wall formation, anther and pollen development through tapetal PCD and lipid deposition (Xu et al. 2010, 2014). AMS gene encodes a postmeiotic, tapetally expressed bHLH TF. *ams* mutant displays an extended tapetal layer and aborted microspores (Sorensen et al. 2003). Previously we have shown, that also bZIP family TFs seem to be important players during the pollen development. *bzip34* mutant shows multiple defects in the development of gametophytic and sporophytic tissues. Characteristic phenotype together with genetic transmission defects demonstrated a requirement for AtbZIP34 for correct formation of pollen wall, lipid metabolism and cellular transport (Gibalová et al. 2009). Another bZIP TF BZI-1 studied in tobacco, most likely regulates carbohydrate supply of the developing pollen. Authors have revealed reciprocal action between BZI-1 co-regulators performing opposing functions as positive and negative regulators of pollen development (Iven et al. 2010).

1.6 bZIP family of transcription factors

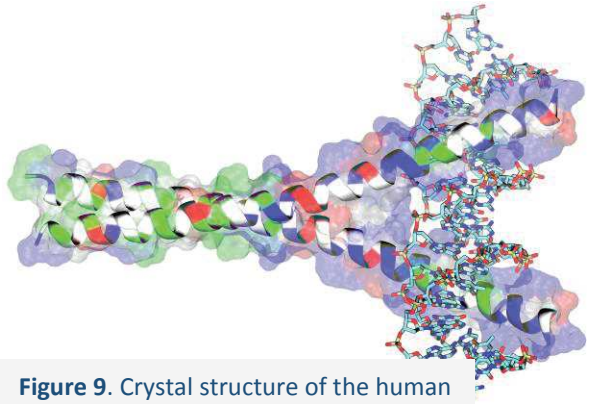


Figure 9. Crystal structure of the human Jun/CRE complex 1JN (https://rcbs.org)

Dimeric basic leucine zipper (bZIP) factors constitute an important class of predominantly enhancer-type TFs consisting of 75 members in Arabidopsis clustered into 10 subgroups (A-I; S group) based on sequence similarity of their basic region and the presence of additional motifs (Jakoby et al. 2002). bZIP proteins are involved in many crucial processes across

eukaryotic organisms (Deppmann et al. 2006). Some proteins with bZIP domain such as Jun/Fos or CREB have been studied extensively in animals and serve as models for understanding TF-DNA interactions, ternary complex formation and TF post-translational modifications (Jakoby et al. 2002) (Fig.9). In plants, bZIP TFs were shown to be employed in seed maturation (Alonso et al., 2009), flowering (Abe et al. 2005), pollen development (Iven et al. 2010; Gibalová et al. 2009), senescence (Smykowski et al. 2010), unfolded protein response (Liu et al. 2007; Iwata et al. 2008), abiotic stress signaling (Fujita et al. 2005) and energy metabolism (Baena-González et al. 2007). bZIP domain consists of two structural features located on contiguous α helices (Fig.9). These contain basic region with nuclear localization signal and a heptad repeat of leucines or similar bulky hydrophobic amino acids positioned exactly nine amino acids towards the C-terminus creating amphiphatic helix (Jakoby et al. 2002). Deppmann et al. (2006) pointed out that bZIP domains are indeed stereotyped, however at the same time they influence a broad range of functions. The explanation has to do with bZIP TFs dimerization and DNA binding preferences as well as their transactivation and/or repression properties. One of the main characteristics of bZIP TFs is that they exist as dimers, however, they are not dimerizing promiscuously, and specific interactions are preferred (Newman et al. 2003). Dimerization represents one major way of creating a large repertoire of regulatory responses without multiplication of TF genes, as organisms increase in complexity (Amoutzias et al. 2007). Having this functional feature, bZIP proteins belong to the second largest family of dimerizing TFs and therefore became an excellent model in understanding certain aspects in transcriptional regulation pathways. Different bZIP heterodimers show intermediate effects, depending on the monomers combined. Such system was described in the regulation of late embryogenesis by A group bZIPs ABA-insensitive 5 (ABI5) and Enhanced Em Level (EEL). These two bZIPs compete for the same binding site, conferring antagonistic transactivation functions: ABI5 homodimers activate gene expression, whereas EEL homodimers and ABI5/EEL heterodimers function as repressors (Bensmihen et al. 2002). Another regulatory model was identified in tobacco, where BZI-4 homodimers and BZI-1/BZI-2 heterodimers perform opposing functions and act as negative and positive transcriptional regulators during pollen development (Iven et al. 2010). Similarly, the expression of RBCS1a is modulated by Elongated Hypocotyl 5 (HY5) and homologue HY5 (HYH) and GBF1, where GBF1 acts as a repressor whereas HYH and HY5 are activators of RBCS1a expression in in Arabidopsis seedling development (Singh et al. 2012). Taken together, protein-protein interactions represent important post-translational

mechanism modulating bZIP TFs function. Because of their sessile nature, plants require an effective system of gene expression regulation for rapid response to the variation of actual environmental and developmental conditions. In recent years, several studies have been describing dimerization preferences for bZIP TFs from different species, including *Homo sapiens*, *Drosophila melanogaster*, *Arabidopsis thaliana* and *Saccharomyces cerivisiae* (Deppmann et al. 2006). These authors have developed a network mapping approach to summarize the dimerization potentials of all bZIP TFs among above mentioned genomes. When compared to human (56 bZIPs) and yeast (14 bZIP genes) genomes, Arabidopsis dimerization network with 67 members represents the largest network theoretically creating 175 possible dimer combinations (Deppmann et al. 2006). The specificity of dimerization is however achieved by three principal regulators: affinity and specificity of interaction, and local protein concentration. From the evolutionary point of view, bZIPs represent very old proteins that evolved some of their DNA-binding specificities before the divergence of the metazoa and fungi (Amoutzias et al. 2007). Later, at the origin of multicellular animals around 950 MYA, they probably underwent extended gene duplications that allowed them to evolve new DNA-binding specificities as well as complex dimerization networks (Amoutzias et al. 2007). The recognition of new DNA surfaces, when coupled with hetero-dimerization, must have had a tremendous impact at the organismal level because it increased the complexity and inventory of possible regulatory responses manifold. The same group revealed possibility that three to five gene duplication events, possibly at the origin of bilaterian animals, created new families. These retained their DNA-binding specificity but evolved new interactions, thus further increasing the complexity of the network (Amoutzias et al. 2007).

2. OBJECTIVES

Submitted thesis "The role of bZIP transcription factors in the male gametophyte of *Arabidopsis thaliana*" has two main objectives further described in the following chapters.

The first objective is to demonstrate functional involvement of selected bZIP transcription factors in the transcriptional regulation during male gametophyte development. We have functionally characterized two pollen expressed bZIP TFs - AtbZIP18 and AtbZIP34. For that reason it was necessary to individually describe their roles using several methodological

approaches, including reverse genetic approach, transmission of mutant alleles, pollen germination tests, promoter activities, protein localization etc.

The second objective is focused on the identification of putative Arabidopsis bZIP TFs network involved in transcriptional regulation during male gametophyte development. To achieve this goal, it is necessary to select candidate genes within bZIP family according to pollen microarray data and subsequently predict and experimentally verify putative interactions between these candidates.

3. RESULTS

3.1 Selection of bZIP candidate genes putatively involved in the regulation of the male gametophyte development

Given this critical roles of TFs during plant development, we aimed to shed more light on several aspects of transcriptional regulation on model plant *Arabidopsis thaliana* during male germline development. To select the TF candidates, we have provided wide-scale screening of T-DNA lines to search for putative TFs affecting male gametophyte development (Reňák et al. 2012), based on our previous pollen microarray data (Honys and Twell 2003, 2004). Out of approximately 1595 predicted Arabidopsis TFs (Honys and Twell, 2004), 1358 were reliably expressed in at least one stage of male gametophyte. Of these, using more stringent criteria, 27 “early” and 22 “late” expressed TFs were selected (Reňák et al. 2012). These genes represent strong candidates for transcriptional regulators of the pollen development and were screened for putative changes in pollen morphology of respective T-DNA lines. One of these T-DNA lines affected the “late” gene At2g42380 coding for AtbZIP34 TF and showed promising and reproducible pollen phenotype. It is very well established, that bZIP TFs are functional as dimers and they possess important functions during plant development. For that reason, we have selected another pollen expressed bZIP candidate – At2g40620 coding for AtbZIP18 TF.

On top of the detailed characterization of the two bZIP genes, the next objective was to uncover the putative bZIP regulatory network operating in the male gametophyte. To select candidate genes, we used AtbZIP family expression data and the composition of bZIP dimerization domains, according to analysis of Deppmann et al. 2004. We compared the

expression profiles of 75 AtbZIP genes in the publicly available databases: Genevestigator, (www.genevestigator.ethz.ch); Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgibin/efpWeb.cgi); Arabidopsis Gene Family Profiler (http://agfp.ueb.cas.cz/, Dupľáková et al. 2007) and selected only those candidates having the mean expression signal in pollen over 400 (for each bZIP gene, Affymetrix Arabidpsis ATH1GeneChip). In the end, 17 genes were selected and highlighted in green boxes (Fig.10).

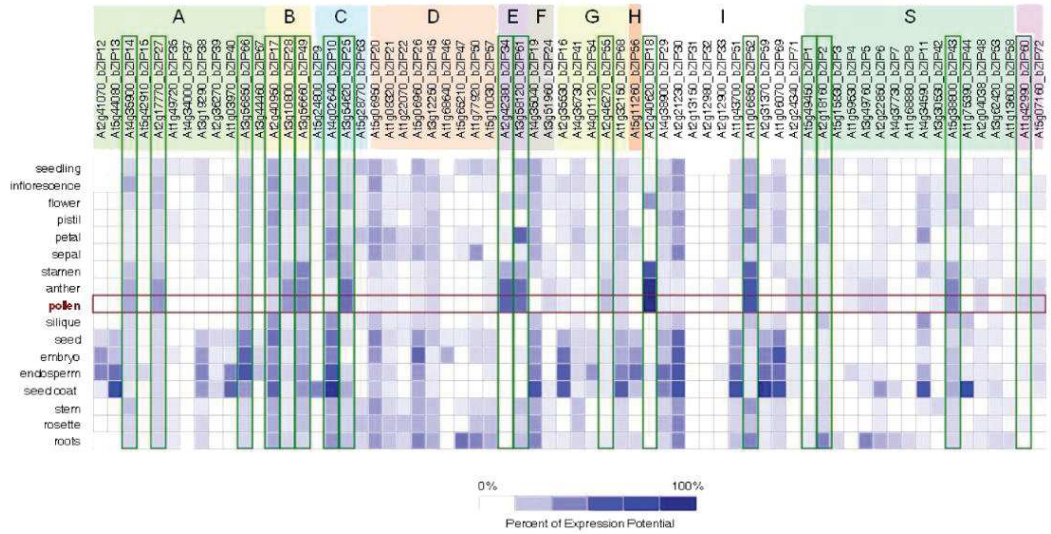


Figure 10. Expression profiles of the AtbZIP family transcription factors (www.genevestigator.ethz.ch). 17 candidate genes possessing mean expression signal over 400 (Affymetrix ATH1 GeneChip) in pollen are highlighted in green boxes. Relative level of gene expression is illustrated at the bottom scale.

To narrow down the number of candidate genes and to increase the specificity of the putative bZIP network, we have analysed the amino acids composition of leucine dimerization domains. For this purpose, we adopted the *in silico* analysis of Deppmann et al. (2004) who predicted the dimerization potential of 67 AtbZIP proteins out of 75 members of the AtbZIP family. The authors excluded eight TFs because these proteins did not meet their selection criteria. However, two out of eight excluded bZIP factors are present in our study, AtbZIP34 (At2g42380) and AtbZIP61 (At3g58120), both belonging to the subgroup E.

The bZIP domain consists of two structural features located on contiguous α helices. These contain basic region with nuclear localization signal and a heptad repeat of leucines or similar bulky hydrophobic amino acids positioned exactly nine amino acids towards the C-terminus creating amphiphatic helix (Jakoby et al. 2002). Based on multiple amino-acid alignment of 17 pollen expressed AtbZIP proteins, we have identified the basic region and adjacent leucine

domains (LD; Fig.11). Positions in every leucine heptade were marked as **"g a b c d e f"**, whereas positions **"a e g"** represented amino acid

Figure 11. Amino acid sequences of 17 bZIP dimerization domains. Individual leucine domains were identified based on the alignment and divided into heptades. Positions in individual leucine heptades are marked "gabcdef" to visualize the putative dimerization, whereas g-e pairs are critical. Amino acid residues predicted to regulate the dimerization specificity are color-coded (positions "g a e"). Positively charged amino acid residues (K, R) in positions "g" and "e" are marked in green. Negatively charged amino acids (D, E) are marked in red. Purple colour is used in case of electrostatic attraction of amino acid residues (g-e positions). N residue at position "a" is marked in blue and in case that the same residue is present in other bZIP at the position "a", dimerization is favourable.

residues determining the attraction or repulsion of the two proteins (Fig.11). Charged amino acids in position "**a**" inhibited homodimer formation, while lysine at the same position was favourable for heterodimer formation. Charged amino-acids in positions **"g e"** allowed the electrostatic attraction of α helices (adopted from Deppmann et al. 2004). Therefore, formation of electrostatic interaction between R and E localized at positions **"g e",** and/or the presence of N at position **"a a"** enhance the probability of dimerization. Based on this analysis we hypothesize that interaction partners of AtbZIP34 in pollen are AtbZIP18 and AtbZIP52 belonging to the group I. We can also assume formation of heterodimer between AtbZIP18 with AtbZIP61 and AtbZIP18 forming homodimers. Moreover, proline residue in the LD of AtbZIP34 and AtbZIP61 interferes with the formation of homodimer, what was corroborated by previously published results by Shen at al. 2007. Finally, we have selected eight candidate proteins for the dimerization study: AtbZIP1 (At5g49450; group S), AtbZIP18 (At2g40620; group

I), AtbZIP25 (At3g54620; group C), AtbZIP28 (At3g10800; group B), AtbZIP34 (At2g42380; group E), AtbZIP52 (At1g06850; group I), AtbZIP60 (At1g42990; group S) and AtbZIP61 (At3g58120; group E). As the putative interactions among six of these proteins have been reported by Deppmann et al. (2004) *in silico*, we have focused on the interactions of one of the excluded gene – AtbZIP34.

3.2 Functional characterization of bZIP34 and bZIP18 TFs

3.2.1 AtbZIP34 and AtbZIP18 represent "late" pollen enriched TFs

The presence of both AtbZIP34 and AtbZIP18 mRNAs was studied in complex using 1) publicly available microarray data; 2) RT-PCR and 3) promoter fused to GUS transgenic plants. Microarray data showed significant pollen-enriched expression pattern of AtbZIP34 (absolute expression signal 203), suggesting its role in late male gametophyte development compared to weak and therefore not reliable expression signal among leaves, stems and flowers (~25; http://bar.utoronto.ca). Further analyses of transcriptomic data including reproductive organs revealed that At2g42380 was active in the second and third whorls of flowers (stage 15; Smyth et al. 1990; Zimmermann et al. 2005). RT-PCR using RNA isolated from four stages of the male gametophyte development, unicellular, bicellular, tricellular and mature pollen, and four sporophytic tissues revealed its cumulative expression in the male gametophyte and weak expression in whole flowers (Fig 12).

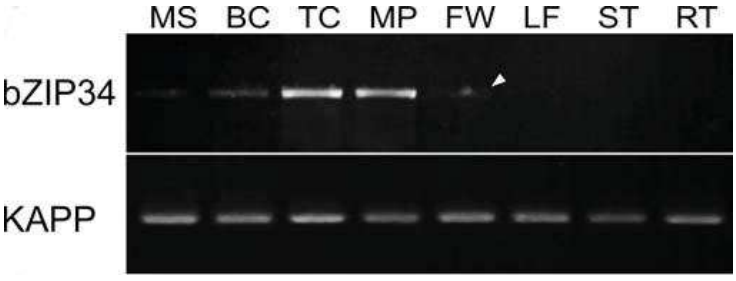


Figure 12. Verification of At2g42380 expression profile and control KAPP gene expression by RT-PCR in microspores (MS), bicellular (BC), tricellular (TC) and mature pollen (MP), whole flowers (FW), leaves (LF), stems (ST) and roots (RT). White arrowhead shows expression of At2g42380 in flowers.

Finally, the activity of pAtbZIP34 was observed in GUS-transgenic plants with following pattern (Fig. 13).

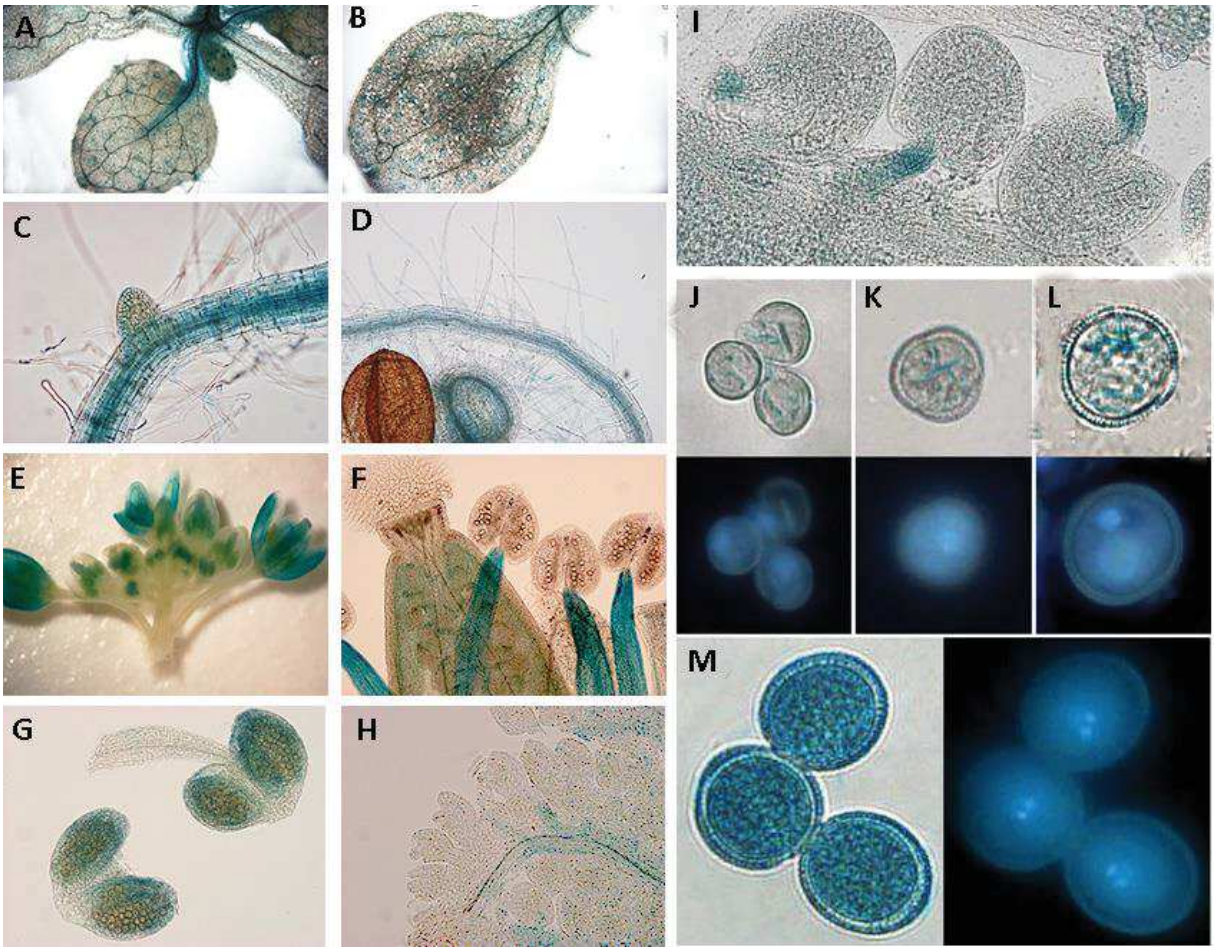


Figure 13. Activity of the AtbZIP34 promoter. Bright field microscopy of the shoot - first true leaf (A) and cotyledon with patches of GUS staining (B). Root with primordia (C), primary root with root hairs (D) inflorescence (E), later developmental flower stage with detailed view on anthers and pistil (stage 15, F) young developmental stage of anthers (stage 8) (G), young ovary (H) three ovules (I) bright field and epifluorescent micrographs of pollen developmental stages - uninucleate microspores (J), early bicellular (K), late bicellular (L) and mature pollen (M).

In sporophytic tissues, patches of GUS stain were visible on the cotlyledons and first true leaves (Fig. 13A, B). Roots and root hairs also exhibited bZIP34 promoter activity (Fig. 14C, D). Moreover, AtbZIP34 promoter activity was always associated with vascular tissues in the distal regions of stems and leaves. However, microarray data together with RT-PCR possessed absence of the AtbZIP34 transcript in shoots and roots, when compared to its promoter activity. This discrepancy was probably caused by very low expression of AtbZIP34 in sporophytic tissues. Whole seedlings were incubated in GUS buffer for 36 hrs in comparison with inflorescences and mature flowers incubated for 24 hrs when GUS staining first appeared. Moreover, according to ATH1 microarray data (Dupľáková et al. 2007), AtbZIP34 possesses although very low expression values in several sporophytic tissues including leaf, stem and root, however these values cannot be considered as reliable, because of low detection call ≤0.3. In

whole inflorescences (Fig. 13E), the GUS signal was detectable in petals and sepals and throughout young anthers in the tapetum (Fig. 13G). In developed flowers (stage 14), GUS staining extended to anthers filaments (Fig. 13F). In carpels, GUS staining was first detected in pistil vascular tissues and young female gametophytes before complete development of the integuments (Fig. 13H). After the developmental shift, the highest GUS activity was localized in funiculi connecting mature ovules with the placenta (Fig. 13I). On the contrary, in the male gametophyte, GUS signal gradually accumulated from microspores to mature tricellular pollen grains (Fig. 13J–M).

The microarray data showed significantly overlapping expression patterns of AtbZIP18 and AtbZIP34, whereas AtbZIP18 possessed substantially higher absolute expression signal ~ 1918 (http://bar.utoronto.ca), or 3904 (data Dupľáková et al. 2007) in mature pollen compared to AtbZIP34 and other tissues within AtbZIP18. Microarray data were verified by RT-PCR analysis in four stages of pollen development and four sporophytic tissues revealing its expression in all templates (Fig. 14A).

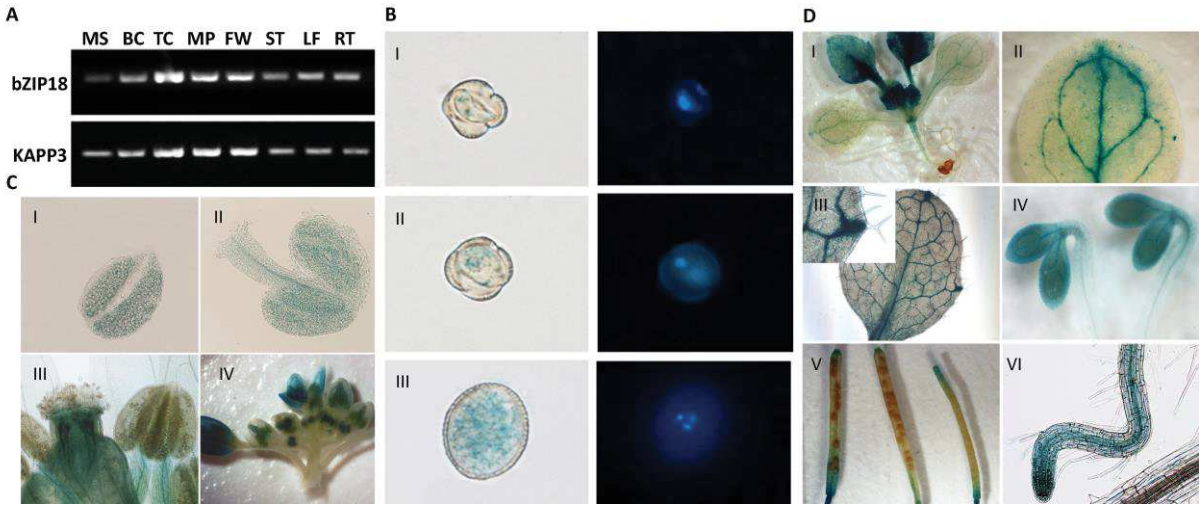


Figure 14. Verification of AtbZIP18 expression in four stages of pollen development: microspores (MS), bicellular pollen (BC), tricellular pollen (TC) and mature pollen (MP) and among four sporophytic tissues: mature flowers (FW), stem (ST), leaf (LF) and root (RT), KAPP3 – control of expression (A). Activity of the AtbZIP18 promoter in the male gametophyte (B) and among sporophytic tissues (C).

In pAtbZIP18_GUS transgenic plants, a weak GUS signal was observed at the microspore stage graduating towards pollen maturity (Fig.14B, I-III). In inflorescences, the GUS signal was present in young flower buds with the localized activities of the AtbZIP18 promoter in tapetum and sepals (Fig. 14C, I, II, IV). In mature flowers, AtbZIP18 promoter expressed GUS in carpels, petals and anther filaments (Fig. 14C, IV). In the sporophyte, promoter activity was detected in

cotelydon vascular tissues of five days old seedlings (Fig. 14D, I, II). In true leaves, the AtbZIP18 promoter activity was observed in vasculature and in areas surrounding hydatodes (Fig. 14D, III). Stems and roots of normally grown and etiolated seedlings also showed GUS signal associated with vascular tissues (Fig. 14D, IV, VI). In siliques, we observed specific GUS staining only in proximal and distal parts (Fig. 14D, V). Taken together, analysis of AtbZIP18 expression pattern by three independent approaches demonstrate a wider expression profile of AtbZIP18 with its indeed significant enrichment in mature pollen, lacking apparent tissue specificity.

3.2.2 AtbZIP34 and AtbZIP18 transcription factors are differently localized

All Arabidopsis bZIP genes are annotated as transcription factors containing DNA binding domain and their localization in the nucleus is therefore prerequisite. Localization of both AtbZIP proteins was tested as C- and N-terminal bZIP_GFP fusions. In transiently transformed tobacco plants, AtbZIP18 protein fully co-localized with the endoplasmic reticulum (ER) marker in the nucleus and the perinuclear region (Fig.15A-C).

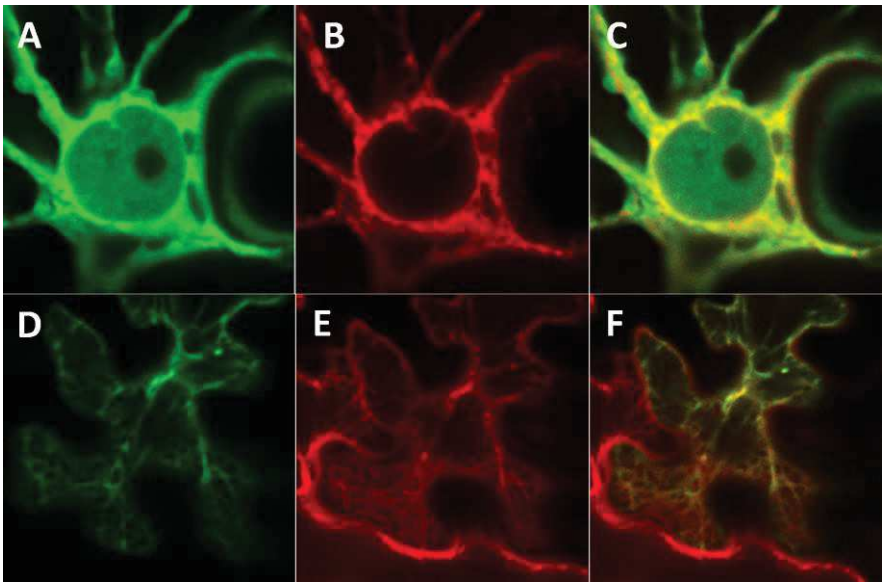


Figure 15. Localization of AtbZIP18_GFP fusion protein in tobacco epidermal cells. Fusion protein was localized in the nucleoplasm and perinuclear region (A) confirmed by co-localization of ER marker (B). Merged image (C). AtbZIP18 is partially or fully associated with the ER in the cytoplasm (D) ER marker (E) merged image (F).

Portion of the fusion protein was observed in the cytoplasm, partially or fully associated with the ER (Fig. 15D-F). As a control, localization of free GFP co-infiltrated with the ER-marker was not showing such co-localization as AtbZIP18 in all observed cells (data not shown). For independent verification of this localization pattern, we have prepared stable transformation lines, expressing the whole genomic sequence of AtbZIP18 C-terminally fused to GFP (Fig. 16; 17).

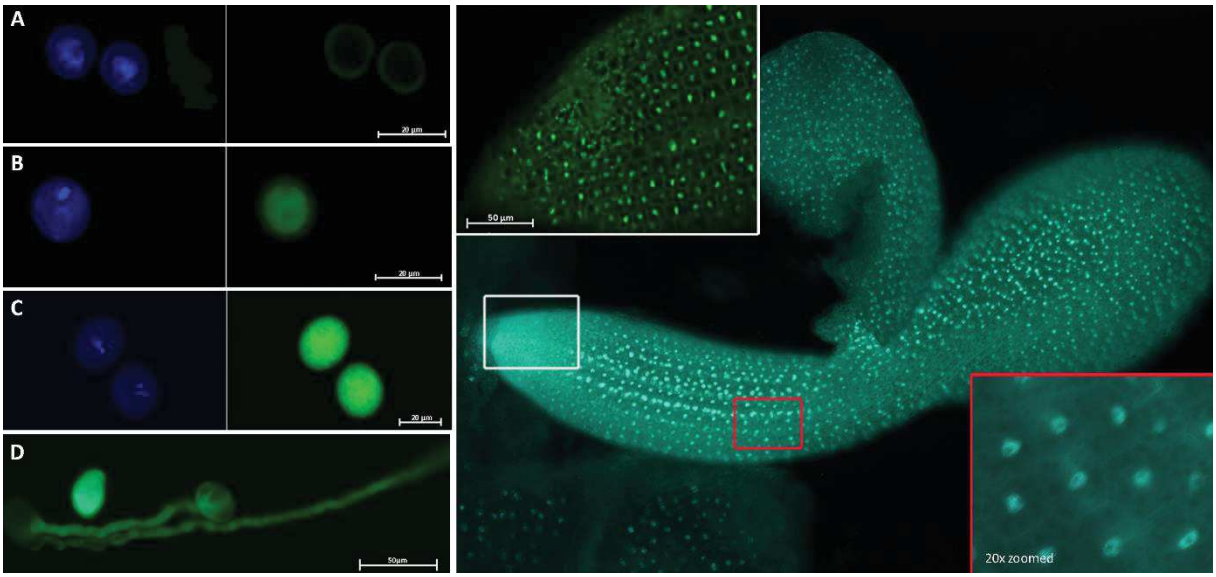


Figure 16. Localization of the whole AtbZIP18 genomic sequence fused to the GFP observed during male gametophyte development. GFP signal was absent in microspores (A) and started to be accumulated from bicellular pollen (B), and increases in mature pollen grains (C). During pollen germination, the AtbZIP18_GFP signal was also detected in the cytosol of pollen tubes (D). Localization of AtbZIP18-GFP fusion protein in mature embryo (right panel). The signal was restricted to the nuclei and ER and/or cytoplasm of individual cells – zoomed windows.

In the male gametophyte, GFP signal was not detected in microspores (Fig. 16A), but a weak GFP signal emerged in bicellular pollen (Fig. 16B) that significantly increased in mature pollen grains (Fig. 16C) and pollen tubes (Fig. 16D). After fertilization, GFP signal was observed in whole mature embryo restricted to the nuclei (Fig. 16-right panel). In shoots of 6-days old seedlings, the GFP signal was localized in cotyledons and guard cells nuclei (Fig. 17A), later also in the true leaves (Fig. 17B), stems (Fig. 17C) and nuclei of the trichomes (Fig. 17D). Further analysis of roots showed the GFP signal in primary roots (Fig. 17E) and secondary root primordia (Fig. 17F). GFP expression was observed further at the elongation zone (Fig. 17G) and in the nuclei of the root tip (Fig. 17H). Apart of the predominant nuclear localization, we occasionally detected the AtbZIP18-GFP protein in the cytoplasm (Fig. 16 and 17- zoomed windows).

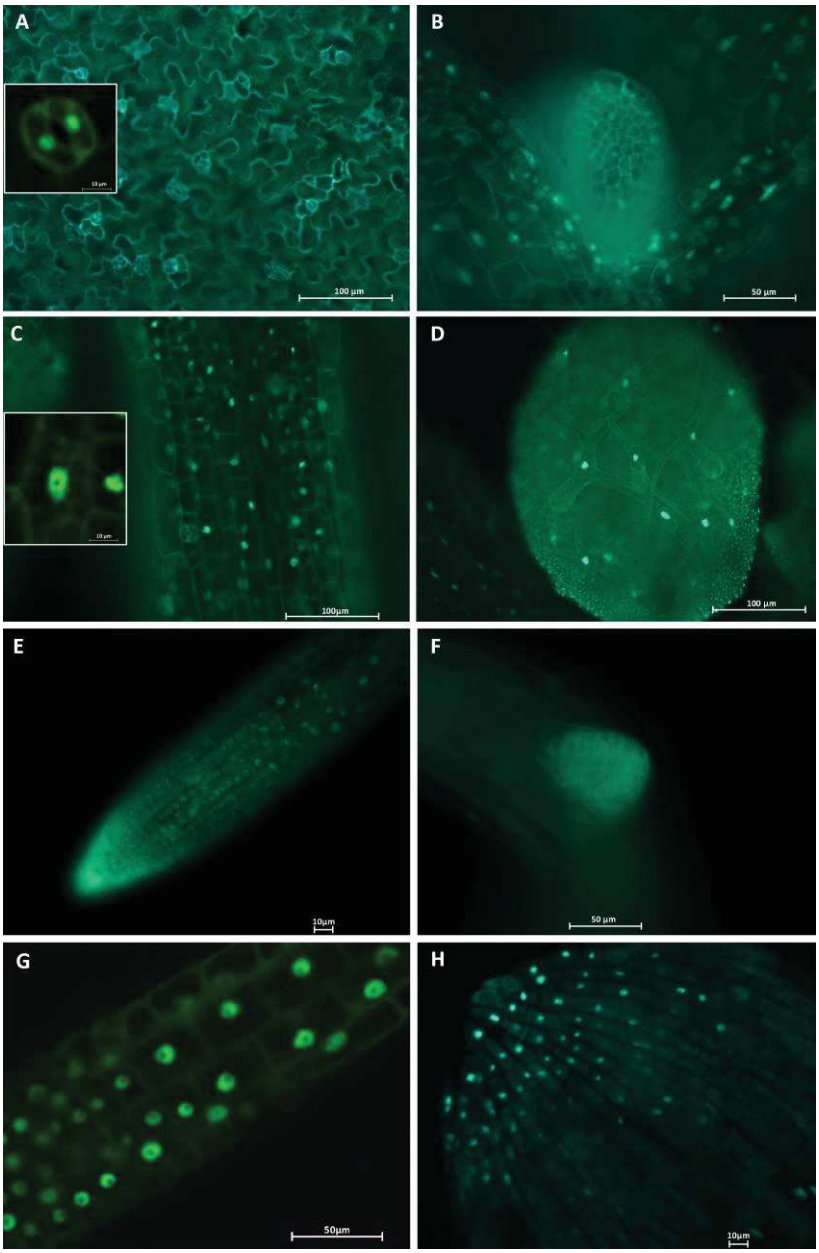


Figure 17. AtbZIP18-GFP fusion protein is localized in the cotyledons of six days-old seedlings. Bottom epidermis with guard cells-zoomed window (A). True leaf between cotyledons (B) stem, zoomed window showing details of AtbZIP18 localization (C) and nuclei of trichomes (D). AtbZIP18 localizes in primary root (E), root primordial cells of secondary roots (F) at the elongation zone of the primary root showing nucleoplasm localization (G) and at nuclei of the root tip (H).

Transient expression of AtbZIP34 fused to GFP showed specific localization of the fusion protein in the nuclei of transiently transformed tobacco leaf epidermal cells (Fig.18).

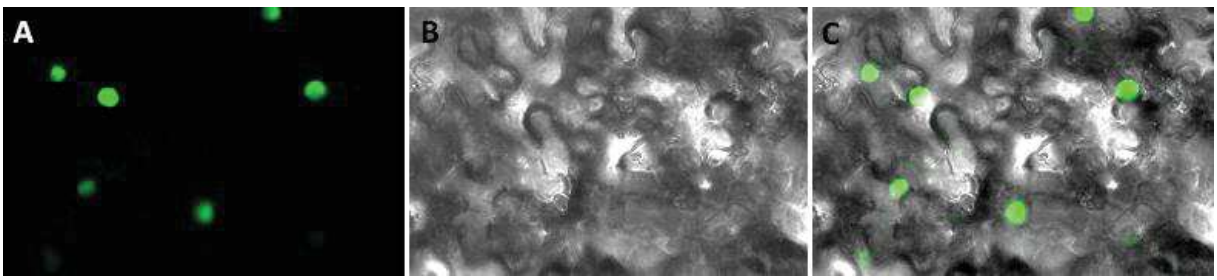


Figure 18. Localization of AtbZIP34_GFP fusion protein in epidermal cells of transiently transformed tobacco leaves is restricted specifically to the nuclei (A), bright field (B), merged image (C).

3.2.3 Revealing biological function of AtbZIP34 TF in the male gametophyte

3.2.3.1 Cellular and pollen wall defects in *atbzip34* mutant pollen

As a strategy to address the involvement of AtbZIP34 and AtbZIP18 during the pollen development, we have employed reverse genetic approach using respective T-DNA lines. In case of AtbZIP34, SALK_18864 line was used, harbouring T-DNA insertion at the beginning of exon 4. We still detected the 3` truncated transcripts upstream of the insertion site by RT-PCR (Fig. 19) indicating that SALK_18864 represents a partial loss of function allele.

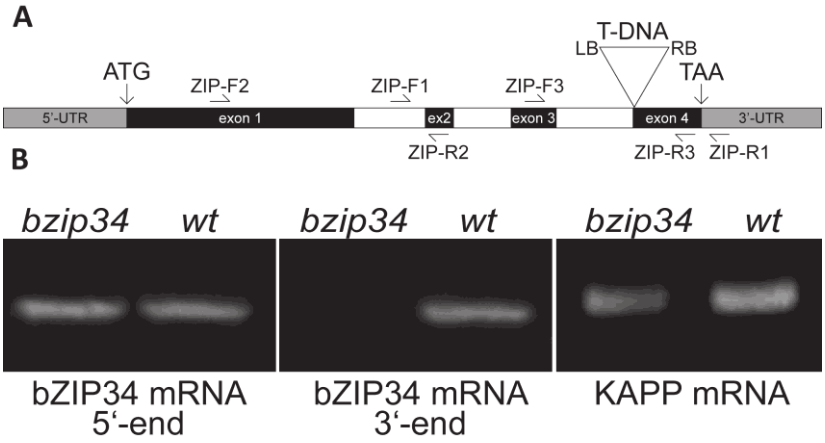


Figure 19. Diagram showing At2g42380 gene model (A) including T-DNA insertion site (triangle) and positions of respective primers - arrows, introns - white boxes, exons - black boxes, untranslated regions - light grey boxes, proximal promoter region - dark grey box, LB and RB - left and right borders of T-DNA. Expression analysis of both end regions of AtbZIP34 transcript in wild type and atbZIP34 pollen (B) RT PCR of AtbZIP34 mRNA 5'-end (upstream of T-DNA insertion, primers ZIP-F2/ZIP-R2) and 3'-end regions (downstream of T-DNA insertion, primers ZIP-F3/ZIP-R3) as well as KAPP control transcript (primers KAPP-F/KAPP-R) (Gibalová et al. 2009).

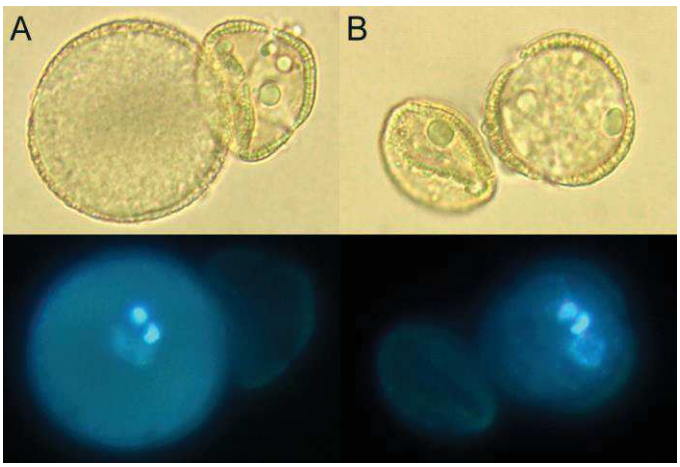


Figure 20. Phenotypic defects in *atbzip34* pollen. Bright field and corresponding fluorescence images after DAPI-staining on the bottom, wild type and *atbzip34* pollen (A), *atbzip34* collapsed pollen (B) (Gibalová et al. 2009).

The transcript abundance in mature pollen isolated from *Atbzip34* mutant plants was verified using both semi RT-PCR and qPCR confirming a significant down-regulation of the AtbZIP34 mRNA (Fig. 25). Because expression of this gene is increasing towards pollen maturity, we have provided phenotypic screen of mature pollen grains using bright field (BF), fluorescence and electron microscopy. In the BF observations, we detected unusual *atbzip34* pollen

grains smaller in diameter, containing cytoplasmic inclusions evoking lipid or oil bodies (Fig. 20-upper panel). Fluorescence microscopy revealed fraction of tricellular pollen (26.7 ± 5.5%) contained malformed or displaced male germ units, often with unusual vegetative nuclei and the occurrence of collapsed pollen was 15.5 ± 3.9% (Fig.20-bottom panel). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were employed to observe cell wall patterning, membrane structure and ultrastructure of developing *atbzip34* pollen. The most obvious differences from wild type pollen observed by SEM were irregular pollen shape and abnormal exine patterning (Fig. 21). Aberrant exine patterning appeared as regions of collapsed baculae and tecta together with areas with extra material deposited onto them. This phenotype was observed in all *atbzip34* pollen grains. There were no significant differences in the frequency of exine patterning defects in wild type pollen and pollen from heterozygous *Atbzip34* plants (data not shown), consistent with the sporophytic control of exine patterning defects. More thorough ultrastructural analysis was performed by TEM. Because of presumed sporophytic nature of cell wall patterning defects, the ultrastructure of both tapetum and spores was examined at several developmental stages (tetrads, uninucleate microspores, bicellular pollen). When observing tapetum development,apart from the general ultrastructure of tapetal cells (Ariizumi et al. 2004; Vizcay-Barrena and Wilson 2006; Yang et al. 2007), special attention was paid to the number and organization of secretory vesicles, vacuolization, plastid development (number and size of plastoglobules, lipid bodies, elaioplasts) and cell wall degeneration. Nevertheless, tapetum development seemed less affected by *atbzip34* mutation, as tapetal cells of wild type and mutant were similar throughout development

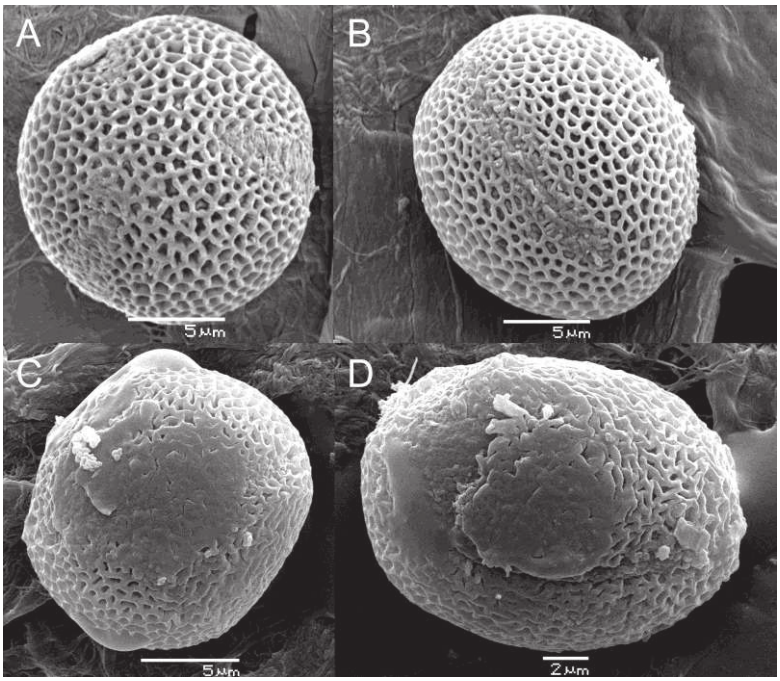


Figure 21. Scanning electron micrographs of wild type pollen (A), *atbzip34* pollen complemented with At2g42380 genomic fragment (B) and *atbzip34* pollen grains defective in exine pattern formation with often irregular shape (C, D) (Gibalová et al. 2009).

(Supplementary Fig. 1). In mature spores, TEM observations confirmed differences in pollen wall structure between wt and *atbzip34* pollen (Fig. 22). Mature *atbzip34* pollen possessed a characteristic wrinkled intine, sparse and deformed baculae and tecta, and under-developed ER cisternae (Fig. 22 B, D, F, H), when compared to the wt (Fig.22 A, C, E, G). There were no apparent differences in cell wall structure of microspores in tetrads; the first differences were found in bicellular stage. Unlike the exine-patterning defect, the unusual intine shape was observed in approximately one half of pollen grains isolated from *Atbzip34* heterozygous plants, indicating gametophytic control of intine development.

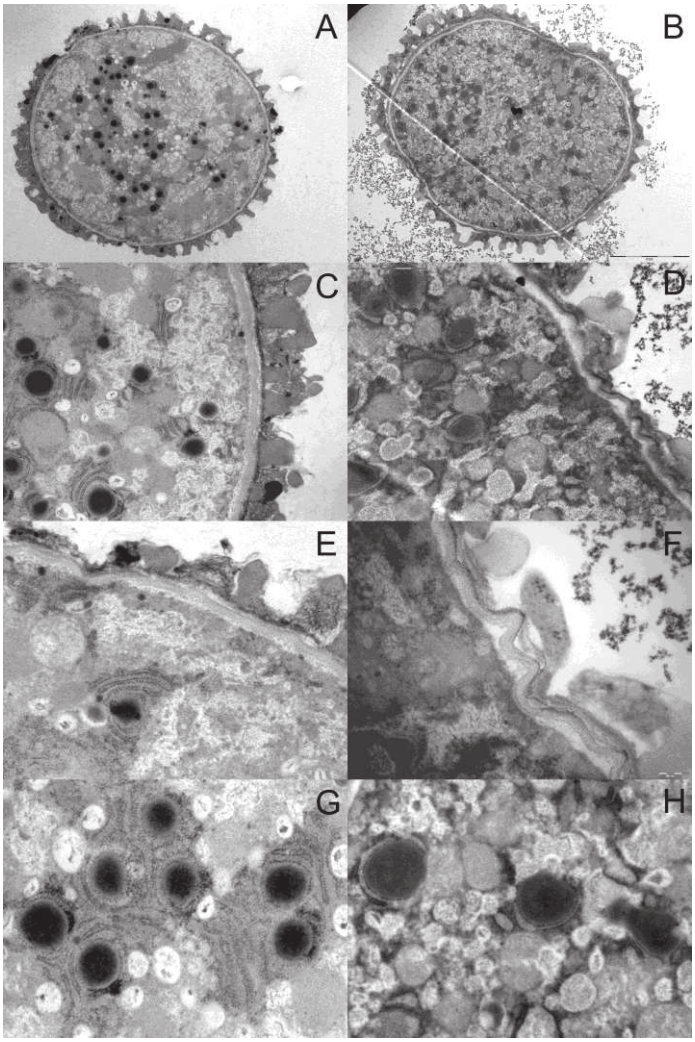


Figure 22. Transmission electron micrographs of mature wild type (A, C, E, G) and *atbzip34* (B, D, F, H) pollen grains. *atbzip34* pollen has an irregular, wrinkled intine and exine with misplaced tecta and baculi (D, F). Mutant pollen has less developed endomembrane system and higher number of clustered lipid bodies that are surrounded by one or very rarely more layers of ER (D). In wild type, these lipid bodies are enclosed by several layers of ER (E) (Gibalová et al. 2009).

3.2.3.2 *atbzip34* pollen shows reduced viability and progamic phase defects

Since AtbZIP34 affects late stages of pollen development, defects in the progamic phase were expected. The *in vitro* germination rate of mutant *atbzip34* pollen was reduced by 85% compared to that of wild type pollen (n = 300). Moreover, mutant pollen tube growth rate was slower compared to wild type and after 10 h, mutant pollen tubes were 53% shorter than wild type tubes (n = 100). *In vivo* pollen tubes growth tests confirmed slower growth rate of *atbzip34* mutant pollen tubes to the embryo sac when compared to wild type. However, resulting differences in length were less dramatic than observed *in vitro*. After 7 h post-pollination, the

longest *atbzip34* pollen tubes only reached the ninth ovule from the base of the ovary (l = 1,438 ± 53 µm; n = 5 pistils), whereas wild type pollen tubes reached the third ovule from the base (l = 1,818 ± 65 µm; n = 5 pistils).

To verify phenotypic defects caused by AtbZIP34 mRNA down-regulation, complementation analysis was performed in which homozygous *Atbzip34* plants were transformed with a vector containing a 3,232 bp AtbZIP34 genomic fragment (pKGW:AtbZIP34). Pollen from 12 independent transformed lines was analyzed by bright field and fluorescence microscopy after DAPI staining. Ten out of twelve pKGW:AtbZIP34 lines showed a reduced frequency of aberrant pollen. The percentage of normal pollen in *Atbzip34* plants complemented with pKGW:AtbZIP34 ranged between 95 and 99%, with only 1–5% of pollen exhibiting phenotypic defects characteristic of *atbzip34* pollen (Fig. 23).

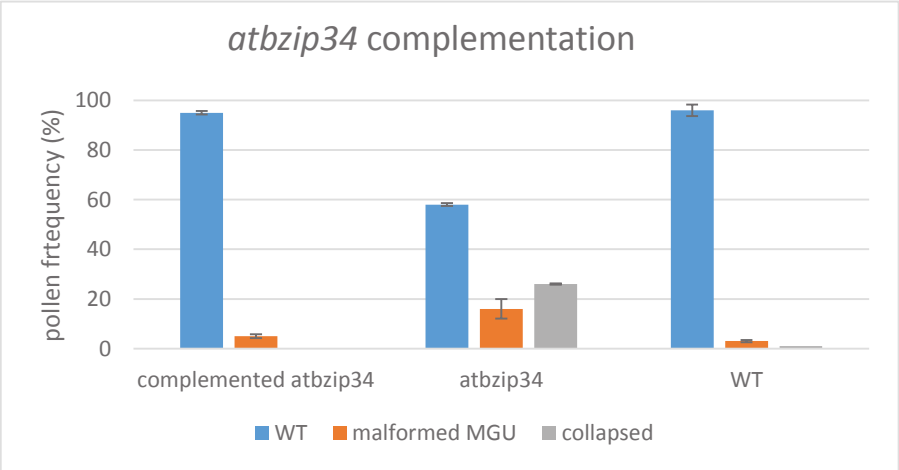


Figure 23. Quantitative demonstration of ATbZIP34 complementation analysis compared with *atbzip34/-* pollen isolated from homozygous plants and wt pollen population.

3.2.3.3 AtbZIP34 directly or indirectly affects several metabolic pathways

Some characteristics of *atbzip34* pollen analysed suggested impairment of certain metabolic pathways such as lipid metabolism and cellular transport during pollen maturation. To test this hypothesis, Affymetrix Arabidopsis ATH1 Genome Arrays were used to explore gene expression in *atbzip34* pollen in comparison with wt (Fig. 24). The set of AtbZIP34-downstream genes shared several characteristic features. First, it was enriched with membrane-associated proteins as 49 out of 100 most highly down-regulated genes in *atbzip34* pollen fell into this category. A fraction of these genes encoded various transporters including the ATP-binding cassette (ABC) transporter, AtABCB9 (At4g18050, 14.7X downregulated), lipid transfer proteins (At4g08670, 6.6X; At1g18280, 4.3X), mitochondrial import inner membrane translocase

(At3g46560, 5.5X), lysine and histidine specific transporter (At1g67640, 5X), potassium transporter family protein (At4g19960, 4.57X), sugar transporter family protein (At4g16480, 4X), sucrose transporter (At1g71880, 3.8X), porin (At5g15090, 3.95X), cation/hydrogen exchanger (At3g17630, 3.7X), acyl carrier protein (At3g05020, 3.7X).

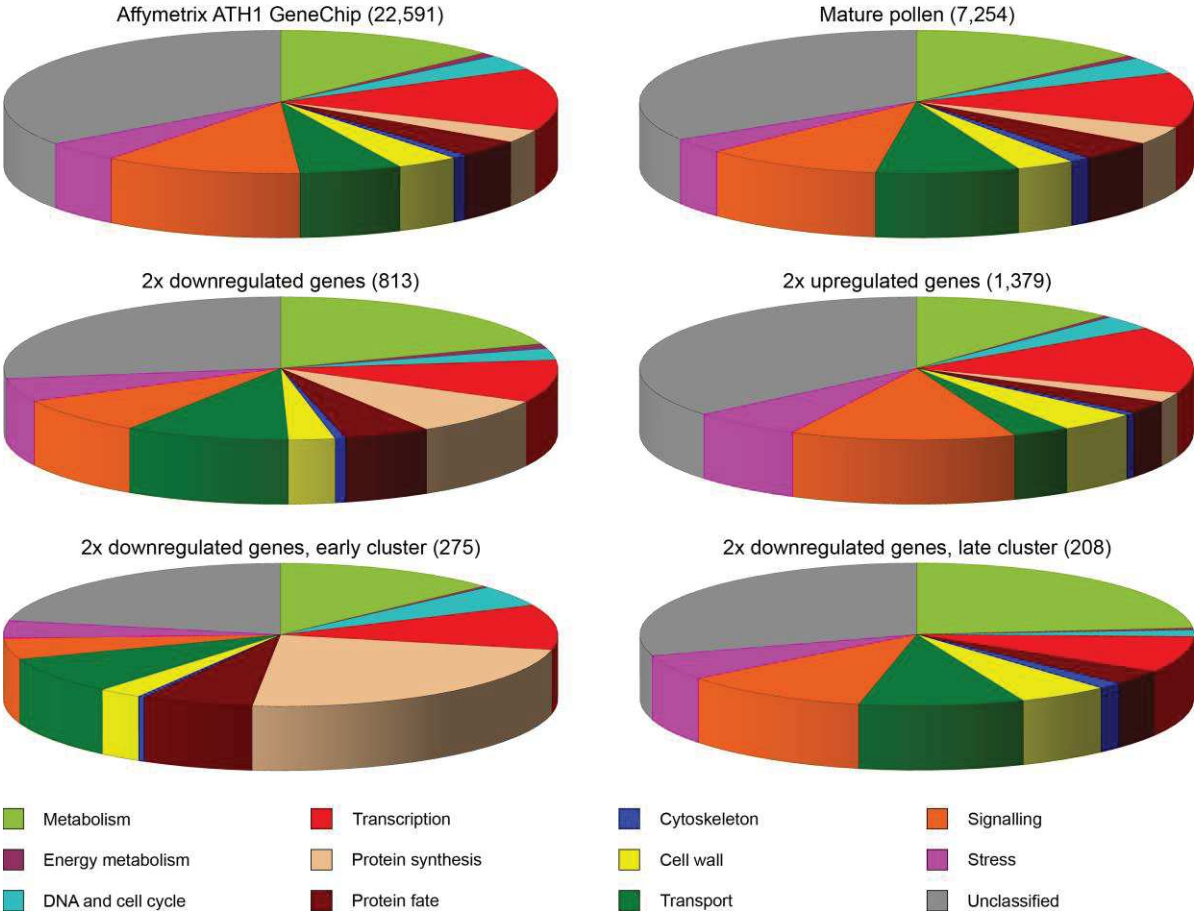


Figure 24. Proportional representation of expressed mRNA among gene function categories. Data is presented for up and down regulated genes in *atbzip34* pollen in comparison with wild type (Gibalová et al. 2009).

These proteins are involved in transport of ions and various metabolites. The importance of membrane-associated transporters for male gametophyte development was already demonstrated (Bock et al. 2006; Sze et al. 2004). Moreover, two lipid transfer proteins and ABC transporter AtABCB9 are also likely involved in lipid transport (Martinoia et al. 2002; Verrier et al. 2008) and all three genes were amongst those most down-regulated in *atbzip34* pollen. Another set of proteins overrepresented among *atbzip34* pollen down-regulated genes included those involved in several steps of lipid catabolism: aspartate aminotransferase (At2g30970, 5.09X), family II extracellular lipase (At5g42170, 4.77X), malate dehydrogenase (At3g15020, 4.17X) (Kindl 1993; Pracharoenwattana et al. 2007; Teller et al. 1990; Zhou et al. 1995). All these genes were abundantly expressed in wild type pollen and significantly down-

regulated in *atbzip34* pollen. To verify *atbzip34* microarray data, we have tested expression profiles of selected transporter genes (all subunits of Sec61 translocon, encoded for 10 genes) as well as genes involved in pathway leading to cell wall precursors(UDP-glucose epimerases). In both cases, qRT-PCR showed significant down-regulation when compared to the wt. More details about *atbzip34* microarray analysis are summarized in attached publication Gibalová et al. 2009.

3.2.4 Characterization of AtbZIP18 knock-out and AtbZIP18 overexpression lines

AtbZIP18 T-DNA SALK_111120 line harbours the insertion in 5' UTR, at the position -266 nt upstream of the ATG start codon, representing knock-out allele of AtbZIP18. The absence of the AtbZIP18 transcript was verified in mature pollen cDNA by quantitative RT-PCR (Fig. 25).

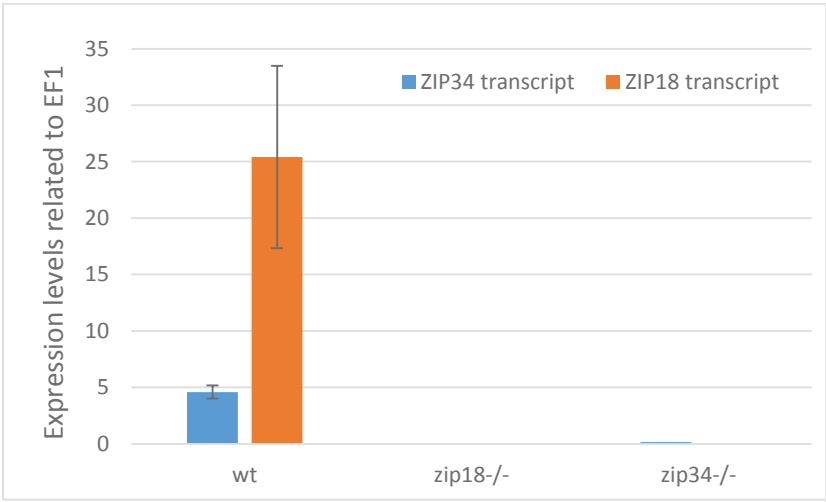


Figure 25. Schematic representation of T-DNA insertion of SALK_111120 localized at the 5´UTR (triangle), starting at- 266bp upstream of the ATG start codon. Positions of primers used for genotyping are marked by arrows. Quantification of the AtbZIP18 and AtbZIP34 transcript abundance in Col-0 and *atbzip18* and *atbzip34* pollen cDNA.

However, unlike in case of AtbZIP34, phenotypic screen of SALK_111120 line didn`t reveal any significant disturbances in mature pollen or earlier developmental stages. As a next step, we have focused on the events following the progamic phase of the male gametophyte development and assessed the frequency of seed gaps in siliques of *Atbzip18* homozygous plants that reached 4 ± 6% (mean ± SD; n=45) representing slight but significant increase (P-value 0.02) when compared to the frequency of abolished seed set in wild type plants (1 ± 2%; n=35). As the SALK_111120 knockout allele didn't show severe phenotypic aberrations in

mature pollen, we explored the AtbZIP18 function by means of overexpression to identify pathways that might remain undetected by traditional los-of-function analysis.

Overexpression (OEx) lines were designed to drive the expression of AtbZIP18 specifically in a vegetative cell of mature pollen by strong pollen-specific LAT52 promoter. Wild type Col-0 plants transformed with the LAT52::AtbZIP18 construct were subjected to microscopic analysis. Our observation showed an increased proportion of aborted pollen grains, the only phenotype category. Among T1 plants it was 20 ± 3% (mean ± SD) at average (n=3). To verify the stability and genetic identity of the individuals with the observed phenotype, we selected plants harbouring disturbed pollen grains and analysed the progeny of T2 generation after selfing. As a control, we also analysed wt, as well as T1 plants showing no phenotype. Seeds from six parent T1 plants in total were sown individually and 15 segregating plants from each parent were selected for microscopic observation. Our results showed the reoccurrence of the aborted pollen in two out of three parent T1 plants, counting 23 ± 4% at average (n=15) among heterozygous plants, 6 ± 10% at average (n=15) among homozygous plants and absence of the phenotype in the control set of plants (n=60). Similar results were observed in AtbZIP34 overexpression lines. We have scored the number of aborted pollen grains among T1 generation, reaching 22 ± 4%. In T2 generation, 26 ± 4% at average (n=16) aborted pollen grains of heterozygous plants and 1 ± 1% at average (n=13) of disturbed pollen of homozygous plants were observed.

All together, these results show a significant incidence of pollen abortion in heterozygous individuals and a decrease of phenotypic defects of pollen isolated from homozygous plants of AtbZIP34 and AtbZIP18 OEx lines.

3.2.5 Genetic analysis of AtbZIP34 and AtbZIP18 T-DNA lines

As Mendelian segregation ratio distortion is a good indicator of transmission defects and gametophytic gene function (Lalanne et al. 2004), transmission efficiency of the *atbzip34* and *atbzip18* mutant alleles through male and female gametophytes was assessed together with the analysis of selfing progeny.

A non-Mendelian segregation ratio 1.86:1 (R:S) was observed among self-progeny (n = 448), of AtbZIP34 plants indicating reduced gametophytic transmission. Analysis of reciprocal crosses progeny demonstrated that both gametophytes were affected. Through the male, *atbzip34* was

transmitted with moderately reduced efficiency resulting in a distorted segregation ratio of 0.66:1 (n = 186). Through the female, the transmission of *atbzip34* was reduced further to 0.55:1 (n = 219). Thus gametophytic transmission of *atbzip34* was reduced by 34% through the male and 45% through the female compared with the wild type AtbZIP34 allele (Tab.1).

SALK_18864

Female transmission			Male transmission			Self		
atbzip34/- ♀ x Col_0 ♂			Col_0 ♀ x *atbzip34/-* ♂			*atbzip34/-* ♂ x atbzip34/- ♀		
+/–	wt	TE^f	+/–	wt	TE^m	wt	+/-	-/-
78	141	55	74	112	66	156	243	49
						calculated ratio 1	1.55	0.31

Table 1. Genetic transmission analysis of *atbzip34* allele. Numbers of progeny arisen from reciprocal test crosses and selfing progeny are illustrated in the second row together with the calculated transmission efficiencies (TE), through the male (TE^m) and the female (TE^f) gametophytes. Calculated ratio of the self-progeny differs from the Mendelian ratio 1:2:1.

atbzip18 allele showed an apparent decrease transmission through the male gametophyte by 18%, while the transmission through the female gametophyte remained unaffected. After self-pollination, the progeny of heterozygous *Atbzip18* plants showed 1: 1.51: 1.27 ratio diverging from Mendelian ratio 1: 2: 1, further supporting the reduced gametophytic transmission (Tab. 2).

SALK_11120

Female transmission			Male transmission			Self		
atbzip18/- ♀ x Col_0 ♂			Col_0 ♀ x *atbzip18/-* ♂			*atbzip18/-* ♂ x atbzip18/- ♀		
+/–	wt	TE^f	+/–	wt	TE^m	wt	+/-	-/-
82	79	103	73	89	82	55	83	70
						calculated ratio 1	1.51	1.27

Table 2. Genetic transmission analysis of *atbzip18* allele. Transmission efficiencies (TE), through the male (TE^m; P-value 0.81) and the female gametophytes (TE^f; P-value 0.21). Calculated ratio of the self-progeny differs from the Mendelian ratio 1:2:1.

3.3 Identification of putative bZIP transcriptional regulation network

In many eukaryotic TF gene families, proteins require a physical interaction between identical or different protein molecules within the same family to form a functional dimer binding DNA (Amoutzias et al. 2008). Deppmann et al. (2006) pointed out that bZIP domains are indeed stereotyped, however, at the same time they influence a broad range of functions.

The explanation has to do with bZIP TFs dimerization and DNA binding preferences as well as their transactivation and/or repression properties.

Arabidopsis bZIP network consists of 67 members, which in theory can generate 175 possible dimeric combinations (Deppmann et al. 2004). Regulation of the dimer formation is achieved by protein affinity, specificity and local protein concentration (Deppmann et al. 2006).

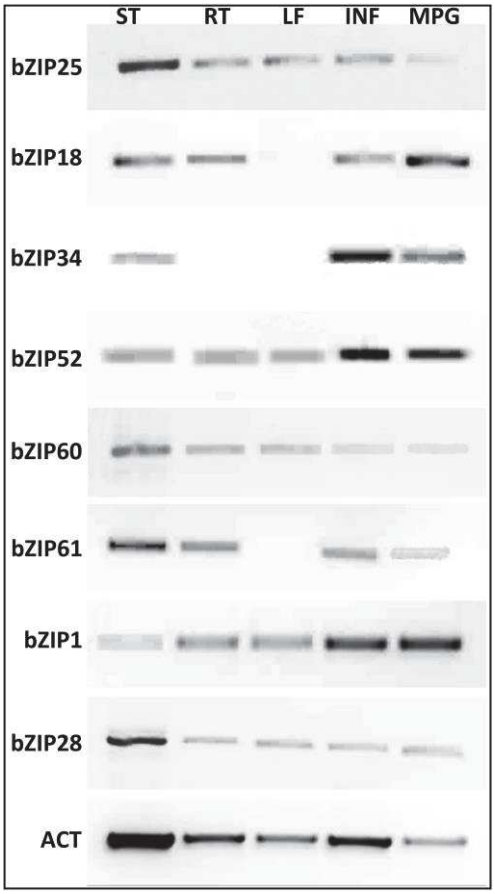


Figure 26 .Semi-quantitative RT-PCR of candidate genes among sporophytic tissues; ST (stem), RT (root), LF (leaf), INF (inflorescence) and mature pollen grains (MPG). Actin (ACT) was used as a control of expression.

Taken together, the evidence of the bZIP TFs importance in a wide range of cellular functions in plants is broad. So far, several interaction studies within bZIP family have been conducted (Shen et al. 2007; 2008; Strathmann et al. 2001; Alonso et al. 2009; Weltmeier et al. 2009; Dietrich et al. 2011; Ehlert et al. 2006), however, the information about bZIP networks in male gametophyte is very limited. The only published example demonstrated the functional cooperation of several bZIP TFs during pollen development in tobacco (Iven et al. 2010). Therefore we aimed to extend our knowledge about the bZIP network in pollen and to shed more light onto genomic plasticity and transcriptional control in the male gametophyte. We have selected eight bZIP candidates as described in section 3.1 and the gene microarray expression patterns were verified. Semi-quantitative RT-PCR showed pollen expression among all tested bZIP genes (Fig. 26). The expression patterns of selected bZIP TFs were indeed not tissue-specific, as it was shown also for other genes in the bZIP family. Specificity of their function lies rather in post-translational mechanisms, e.g. protein dimerization.

Based on our preliminary results, eight candidate genes were selected for yeast-two hybrid (Y2H) assay: AtbZIP1, AtbZIP18, AtbZIP25, AtbZIP28, AtbZIP34, AtbZIP52, AtbZIP6 and AtbZIP61, and cloned as C-terminal fragments to avoid self-activation. However, bZIP1 and bZIP25 were excluded due the impossibility to design their further truncated forms without influencing part

of the BRLZ domain important for dimerization. Testing of AtbZIP52 bait resulted in self-activation as well and further designing of the second truncated version drove the expression of reporter genes without the presence of the prey, even after increasing the selection stringency by titrating 3-Amino Triazole.

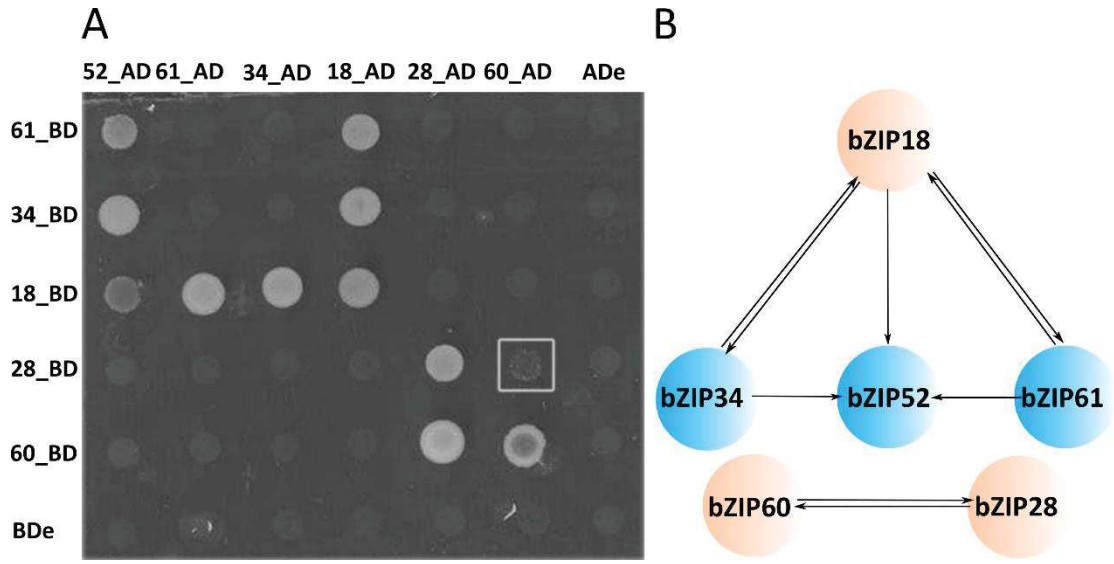


Figure 27. Pairwise interactions of six pollen bZIP TFs. Binding domain (BD-bait) fusions are illustrated in rows, activation domain (AD-prey) fusions are illustrated in columns. bZIP52 was used as a prey only. Tested colonies were resuspended in water to reach OD600 = 0.1 and dropped (10µL) on selection media lacking W, L, A, H. Weak interaction between bZIP28 and bZIP60 is framed (A). Graphical illustration of individual bZIP interactions observed in Y2H analysis (B). bZIP proteins in orange circles are also homo-dimerizing. Double lines represent reciprocal interactions, simple lines are showing interactions carried out in one direction.

Nevertheless, we kept this highly pollen expressed TF in our Y2H assay and considered only those interactions where bZIP52 was used as a prey. From the spotting of individual pairs of bait and prey colonies, we identified three homodimerization events: bZIP18/bZIP18; bZIP28/bZIP28 and bZIP60/bZIP60. Hetero-dimerization occurred between bZIP61/bZIP18; bZIP34/bZIP18; bZIP28/bZIP60 in reciprocal manner and proteins bZIP61, bZIP34 and bZIP18 interacted with bZIP52 (Fig. 27A,B). These results are in agreement with our *in silico* prediction for dimerization (according to Deppmann et al. 2004).

4. MATERIALS AND METHODS

Plant material and growth conditions

Plant material and growth conditions *Arabidopsis thaliana* ecotype Columbia-0 plants were grown in controlled environment cabinets (Phytotrons; Conviron, Winnipeg, Canada) at 21°C under illumination of 150 µmol m^{-2} s^{-1} with a 16h photoperiod. Seeds of SALK 111120 (*atbzip18*) and SALK_18864 (*atbzip34*) T-DNA insertion lines obtained from NASC (The European Arabidopsis Stock Centre) were sown on Jiffy 7 soil pellets (Jiffy International AS, Kristiansand, Norway) due to silenced Kanamycin resistance. Plants were subjected to genotyping using gene-specific and insert-specific primers (Table S1). Sequencing of *Atbzip18* lines revealed T-DNA insertion in the 5'UTR at the position -266 nt upstream of the ATG start codon, in *Atbzip34* it was localized at position 1222nt downstream start codon. Genomic DNA for genotyping was isolated using CTAB method modified from Weigel and Glazebrook (2002).

Transgenic plants (10 days-old and 6 days-old ethiolated seedlings, whole inflorescences and siliques) harbouring AtbZIP18 or AtbZIP34 promoter fused to GUS reporter gene were incubated in GUS buffer (0.1 M phosphate buffer, pH 7.0; 10 mM EDTA, pH 8.0; 0.1% triton X-100 supplemented with 1 mM X-glcA and 1 mM ferricyanide) at 37°C for 48h. Samples were analysed using bright field (BF) microscopy.

Genetic analysis of SALK_18864 and SALK_111120 T-DNA lines

Transmission efficiency of mutant *atbzip18* or *atbzip34* alleles through male and female gametophytes was determined by genotyping of self-fertilized progeny and progeny of reciprocal test crosses. Primers for genotyping were used as a combination of gene specific primers for wt allele and left border SALK T-DNA insertion primer.

DNA constructs

In order to reveal promoter activity of AtbZIP18 and AtbZIP34 during development in sporophytic and gametophytic tissues, 978 bp and 1060 bp promoter regions were PCR-amplified and cloned into Gateway-compatible pENTR-D/TOPO entry vector (Invitrogen, Carlsbad, CA) and further into pKGWFS7,0 (Karimi et al. 2002; http://www.psb.ugent.be/gateway) expression vector.

Localization of AtbZIP18 was studied using transgenic lines expressing the complete genomic sequence of AtbZIP18 fused to GFP. Coding region of AtbZIP18 gene was PCR-amplified from genomic DNA and cloned into pENTR-D/TOPO (Invitrogen) vector and consequently into pB7FWG,0 (Karimi et al. 2002; http://www.psb.ugent.be/gateway) expression vector. Protein localization was further studied as follows. Coding sequences of AtbZIP18 and AtbZIP34 was PCR-amplified from pollen cDNA and cloned into pDONR221 entry vector (Invitrogen) and consequently into pGWB5 (C-terminal GFP) and pGWB6 (N-terminal GFP fusion) expression vectors (Nakagawa et al. 2007). AtbZIP18 was co-localized with the ER marker (ER-rk CD3-959, http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2007.03212.x/full) fused to mCherry.

The overexpression study was performed on transgenic lines expressing AtbZIP18 specifically in vegetative cell. Coding sequence of AtbZIP18 including stop codon was cloned into pDONR221 entry clone and further into pHLat52-7GW7 expression vector harbouring pollen-specific promoter LAT52 (Grant-Downton et al. 2013).

Expression vectors for Yeast two Hybrid (Y2H) assay were prepared by PCR-amplification of At3g10800, At1g42990, At3g58120, At1g06850, At2g42380, At2g40620 CDS fragments into pDONR221 entry clone in full length, as well as versions lacking N-terminal domain (ZIPΔN). Verified entry clones were subsequently cloned into pDEST32 and pDEST22 expression vectors (Invitrogen). C-terminal versions were cloned as follows: bZIP25ΔN$_{227-403}$, bZIP28ΔN $_{161-298}$, bZIP60ΔN $_{138-208}$, bZIP61ΔN $_{200-329}$, bZIP52ΔN $_{149-337}$, bZIP34ΔN $_{175-321}$, bZIP18ΔN $_{146-367}$. All expression clones were transformed into yeast strain AH109 using PEG based transformation, according to Matchmaker Gal4 Two hybrid system3 manual (Clontech, Palo Alto, CA). All clones were verified by restriction analysis and sequencing.

For complementation analysis, 3,232 bp genomic fragment including the complete AtbZIP34 gene and 720 bp of 5` flanking DNA was PCR amplified and recombined into the pENTR2B vector (Invitrogen, Carlsbad, CA). This entry clone was further recombined into pKGW,0 GATEWAY-destination vector (VIB, Ghent, Belgium, Karimi et al. 2005). Constructs were verified by restriction analysis and sequenced.

Cell specific localization of AtbZIP34 promoter in pollen was performed by cloning AtbZIP34 promoter into pENTRP4-P1R^{TM} entry clone, H2B coding sequence into pDONR221^{TM} and GFP

into pDONRP2-RP3^{TM} using BP Clonase® II Enzyme Mix (Invitrogen, Carlsbad, CA). Consequently multisite GATEWAY LR reaction was carried out according to manufacturer`s instructions using LR Clonase® II Plus Enzyme Mix (Invitrogen, Carlsbad, CA) and all three entry clones were sub-cloned into pK7m34GW destination vector (http://gateway.psb.ugent.be/). LR reaction was transformed into One Shot® TOP10 Chemically Competent E. coli (Invitrogen, Carlsbad, CA). Construct was verified by restriction analysis and sequenced.

Plant transformation

Expression clones for promoter activity, AtbZIP18 protein localization (pB7FWG,0), AtbZIP34 promoter activity localization (pK7m34GW7) and overexpression were transformed into Agrobacterium tumefaciens, strain GV3101 and consecutively into *Arabidopsis thaliana* plants using floral dip method (Clough and Benth, 1998). Transformants were selected on ½ MS medium (0.66 g Murashige and Skoog basal medium, 3 g sucrose, 30 mg Myo-inositol, 150 mg MES (2-(N morpholino) ethanesulfonic acid), 0.8% agar, pH 5.7 with KOH) containing the appropriate antibiotic selection.

Transient assay of AtbZIP18 and AtbZIP34 (pGWB5,6) was performed as follows: expression clones were transformed into Agrobacterium strain GV3101 and incubated in YEB media containing the appropriate antibiotic selection at 28°C and 221 rpm. Bacterial culture was pelleted after overnight cultivation and rinsed twice with an infiltration media (10mM MES, 10mM MgCl2, 200µM Acetosyringon – 3,5-dimethoxy-4-hydroxy-acetophenone). Finally, bacterial pellet was resuspended in the infiltration media to an OD_{600} =0.1 and the mixture was incubated at room temperature in the dark for 3h. Bacterial suspension was then infiltrated into abaxial epidermis of tobacco leaves using a syringe. Plants were grown at normal conditions for 36h and infiltrated leaf discs were subjected to confocal laser scanning microscopy (Zeiss LSM 5 DUO confocal laser scanning microscope).

Microscopy

Pollen for phenotype analysis of individual transgenic lines was collected from freshly opened mature flowers into DAPI (4'-6'-diamino-phenylindole) solution according to Park et al. 1998 and observed by Nikon Eclipse TE 2000-E inverted microscope. Fluorescence microscopy was applied to reveal possible cell-division defects and male germ unit disorganization and BF microscopy was used to explore pollen morphology. For transient assay, *Nicotiana*

benthamiana transformed leaf discs were observed using Zeiss LSM 5 DUO confocal laser scanning microscope. Fluorescence and confocal laser scan microscopy were used for the observation of transgenic lines harbouring AtbZIP18 genomic sequence fused to GFP.

Electron microscopy was performed as follows: Freshly harvested material was fixed in a 2.5% (w/v) glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) for 24 h at room temperature, washed with 4% glucose in 0.1 M PBS (NaH2PO4 9 H2O, pH 7.0) for 15 min, post-fixed in 2% (w/v) osmium tetroxide in 0.1 M PBS buffer, washed with 4% glucose in 0.1 M PBS for 15 min, dehydrated through an ascending ethanol series (30–100% ethanol), and, via ethanol: acetone, to acetone. Samples were embedded in Poly/Bed 812/Araldite resins. Thin sections (70 nm) were cut on a Reichert–Jung Ultracut E ultra-microtome and stained using uranyl acetate and lead citrate. Sections were analyzed and photographed using the JEM-1011 electron microscopes with Megaview III camera and analySIS 3.2 software (Soft Imaging System). For scanning electron microscopy, freshly harvested material was fixed in a 2.5% (w/v) glutaraldehyde in 0.1 M PBS for 24 h at room temperature, washed with 4% glucose in 0.1 M PBS for 15 min, dehydrated through an ascending ethanol series (30–100% ethanol), and, via ethanol: acetone, to acetone. Pollen samples for scanning electron microscopy was then critical point dried in CO_2, mounted on a stub, sputter coated with gold, and observed and photographed with a JEOL 6300 scanning microscope.

Statistical evaluation

Statistical evaluation (the percentage of seed gaps in siliques of SALK_111120 and Col-0 plants was performed using Number Cruncher Statistical System (NCSS software, Kaysville, UT). Statistical significance was analyzed by non-parametric Kruskal-Wallis test. Statistical evaluation of the transmission efficiency of progeny arisen from reciprocal test crosses was performed by Chi-squared test using MS Excell 2010 (Microsoft Corp., Redmont, WA). A P value<0.05 was considered statistically significant.

qPCR and RT-PCR

Pollen, stem, leaf and inflorescence RNA was isolated from Col-0, *Atbzip34* and *Atbzip18* homozygous plants as described in Honys and Twell (2003). RNA in total amount of 1 500 ng was DNase-treated (Promega, Madison, WI) and subsequently reverse-transcribed using ImProm ImProm-II Reverse Transcription 214 System (Promega). For PCR amplifications, 1µL of

20x diluted cDNA was used. Quantitative real-time PCR was carried out on a Light-Cycler 480, (Roche Applied Science, Mannheim, Germany) using GoTaq® qPCR Master Mix (Promega). The reaction was performed in 96-well plastic plate (Roche). Real-time PCR data were collected on the light cycler with following cycling conditions: 5 min of initial denaturation at 95°C, then 45 cycles of 10s at 95°C, 10s at 58°C and 15s at 72°C. Cp values were normalized with the reference gene KAPP (kinase associated protein phosphatase, At5g19280). The ratio of the relative amount of the target and reference gene was calculated as follows: E_R^{CpR}/E_T^{CpT} (E_T, E_R: efficiency for reference or target gene qRT PCR assay; CpT, CpR: a crossing point for target or reference genes).

Yeast two Hybrid assay

Small-scale yeast transformation was performed according to Matchmaker Gal4 Two Hybrid System3 manual (Clontech) based on PEG/Lithium acetate using yeast strain AH109 [MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS}$ $GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ]. All bZIP genes were first transformed as full-length versions (bZIPs in pDEST32-baits) together with pDEST22 empty prey. In auto-activation test all full-length baits promoted self-activation of reporter genes. For that reason NΔ versions, negative for self-activation were used together with the respective preys to include all combinations. Yeast cells were grown on –LEU-TRP selection media and interaction were screened on –LEU-TRP-ADE; -LEU-TRP-HIS and –LEU -TRP-ADE-HIS selections. Single colonies were resuspended in 1mL of water and OD600 was adjusted to 0.5 for all interactions tested. Interactions were dropped out on 3 types of selection media and on –LEU-TRP as a growth control and incubated at 28°C. Transformations and interaction tests were repeated 4 times.

RNA extraction, probe preparation and DNA chip hybridization

Total RNA was extracted from 50 mg of isolated pollen using the RNeasy Plant Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). For microarray experiments, RNA integrity was checked using an Agilent 2100 Bioanalyser (Agilent Technologies, Boblingen, Germany) at NASC. Biotinylated target RNA was prepared from 20 lg of total RNA as described in the Affymetrix GeneChip Technical Analysis Manual (Affymetrix, Santa Clara, CA). Preparation of cRNA probes and hybridization to ATH1 Genome Arrays and scanning were carried out as

described (Honys and Twell 2003). Publicly available transcriptomic datasets were downloaded from Arabidopsis GFP database (http://aGFP.ueb.cas.cz; Dupl'áková et al. 2007). All transcriptomics datasets were normalized using freely available dChip 1.3 software (http://www.dchip.org). The reliability and reproducibility of analyses was ensured by the use of duplicates or triplicates in each experiment, the normalization of all arrays to the median probe intensity level and the use of normalized CEL intensities of all arrays for the calculation of model-based gene-expression values based on the Perfect Match-only model (Li and Wong 2001a, b). As a reference, all four available mature pollen transcriptomic datasets were used and labelled MP1 (Ler, 2 repeats; Honys and Twell 2004), MP2 (Col-0, 3 repeats; Zimmermann et al. 2005), MP3 (Col-0, 2 repeats; Pina et al. 2005) and MP4 (2 repeats, wild type in this study). For each gene, the most deviant expression value was eliminated and the mean from the remaining three values was calculated (MPG).

5. DISCUSSION

Main goal of the submitted thesis was the identification and characterization of novel regulators involved in *Arabidopsis thaliana* male germline development. The identification of candidate genes leaned on the male gametophyte transcriptomic data (Honys and Twell, 2004). Microarray analysis led to the selection of several transcription factors, based on pollen-specific or pollen-enriched expression patterns. Respective T-DNA lines of these genes were screened for pollen phenotype defects using bright field and fluorescence microscopy (Reňák et al. 2012). One of the selected candidates belonged to the bZIP family of transcription factors. Numerous studies conducted so far uncovered various roles of bZIP proteins during plant development, however their involvement in the male gametophyte was still missing. Therefore we performed complex functional characterization of the two highly pollen expressed bZIP TFs (AtbZIP34 and AtbZIP18) using several experimental approaches and decipher their interaction properties within the group of other pollen-expressed genes to understand their roles in broader biological context.

AtbZIP18 AND AtbZIP34 REPRESENT LATE POLLEN ENRICHED TRANSCRIPTION FACTORS WITH OVERLAPPING EXPRESSION PROFILES

Expression patterns of AtbZIP34 and AtbZIP18 TFs were verified in complex, at three levels using microarray data, RT-PCR and microscopic observation of bZIP promoter_GUS transgenic plants. These analyses showed overlapping expression patterns. Quantitatively, AtbZIP18 mRNA was significantly more abundant in mature pollen when compared to AtbZIP34 mRNA. Preferentially focusing on the male germline, their expression started at the microspore stage and increased towards pollen maturity. Several differences were observed in expression patterns of both genes in flowers, where AtbZIP34 promoter drove GUS expression in papillar cells of the pistil, in the integuments of young female gametophytes and in funiculi of mature ovules. Apart from the reproductive tissues, both promoters expressed GUS in majority of vegetative organs, suggesting their pleiotropic activity. AtbZIP18 and AtbZIP34 GUS signals were also associated with the vasculature of cotyledons, true leaves, stems and roots. This trend was observed also for bZIP proteins belonging to the group C/S1, where AtbZIP1 and AtbZIP11 promoters express GUS in phloem and xylem and AtbZIP9 in phloem (Weltmeier et al. 2009). Authors hypothesize, based on GUS results and previously published work showing that AtbZIP11 functions in source-sink control (Rook et al. 1998), that these members of C/S1 network regulate allocating nutrients in response to the energy status of the cell. Moreover, AtbZIP1, AtbZIP11 and AtbZIP9 are dimerizing in Plant two Hybrid analysis (P2H) (Ehlert et al. 2006), further supporting the above proposed hypothesis.

SEVERAL POLLEN EXPRESSED AtbZIP PROTEINS DIMERIZE IN Y2H ANALYSIS AND PROBABLY ACT AS ACTIVATORS AND/OR REPRESSORS OF TRANSCRIPTION DEPENDING ON MONOMERS COMBINED

To further support that bZIP proteins are involved in the male germline development, we investigated their possible interactions, as they function only as dimers. Dimerization ability brings significant enlargement of regulatory potential without the multiplication of TF genes, as organisms increase in complexity (Amoutzias et al. 2007). However, bZIP interactions are specific, dependent on binding preferences of the basic domain to DNA and leucine zipper domain constitution. Good example of the specific heterodimer formation is binding of bZIP53 to the albumin 2S2 promoter, which is significantly enhanced when combined with bZIP25 or bZIP10 (Alonso et al. 2009, reviewed in Llorca et al. 2014). Conversely, other bZIPs lose their

DNA-binding potential, when associated to particular partners, as bZIP1, whose DNA-binding activity is prevented in combination with bZIP63 or bZIP10 (Kang et al. 2010).

To identify bZIP dimers associated with pollen development, we have selected bZIP genes expressed during male gametophyte development. This selection counted for 17 out of 75 annotated bZIP genes (Jakoby et al. 2002). To increase the specificity of the pollen bZIP network and to narrow down the number of selected genes, we have employed *in silico* method based on amino-acid composition of the leucine zipper domains, which favours or disables formation of dimers according to Deppmann et al. (2004). Finally, we have identified eight candidate bZIP genes and performed Y2H assay between six of them in a reciprocal manner (AtbZIP1 and AtbZIP25 were excluded due to bait auto-activation). We identified three homodimers - bZIP18/bZIP18; bZIP28/bZIP28 bZIP60/bZIP60 and three heterodimer combinations - bZIP18/bZIP61; bZIP18/bZIP34; bZIP28/bZIP60. The Y2H assay also showed that proteins bZIP18, bZIP34 and bZIP61 are able to interact with bZIP52. Out of all proteins tested, bZIP18 interacted with most of the selected proteins including the formation of homodimers.

Focusing on the mode of regulatory action of the bZIP proteins we plan to focus on dimers composed of bZIP18 and/or bZIP52 possessing so called "EAR" motif (ethylene-responsive element binding factor–associated amphiphilic repression; Kagale et al. 2010). The EAR motif, defined by the consensus sequence patterns of either LxLxL or DLNxxP, is the most predominant form of transcriptional repression motif so far identified in plants (Kagale et al. 2011). Published *in vitro* and *in planta* experiments support that approximately 40% of proteins containing EAR motif function as negative regulators of gene expression and play key roles in diverse biological functions by negatively regulating genes involved in developmental, hormonal, and stress signalling pathways (Kagale et al. 2010). EAR motif containing proteins fall into the category of active repressors - generally defined as proteins containing a distinct, small, and portable repression domains, that inhibit the activation of transcription by different pathways. They either interact with components of basal transcription machinery or positive transcriptional regulators and/or recruit histone deacetylases (HDACs) modifying chromatin structure, precluding other transcriptional activators from binding to their target *cis*-elements (Hanna-Rose and Hansen, 1996; Pazin and Kadonaga, 1997). One of such examples represents TOPLESS (TPL), a member of the Groucho/Tup1 family of co-repressors, which works in conjunction with HISTONE DEACETYLASE19 (HDA19) during the transition stage of

embryogenesis, facilitating EAR motif-mediated gene regulation through chromatin modification (Long et al. 2006; Kagale et al. 2010). Recently published work on DUO1 regulatory network confirmed the involvement of EAR containing proteins also in regulation of the male germline specification, where two EAR motifs in DAZ1/DAZ2 are required for transcriptional repression and for physical interaction with the co-repressor TOPLESS mediating germ cell division and DUO1 dependent gamete differentiation (Borg et al. 2014). Among bZIP TF family, there are several examples of dimers mediating repression of transcription without EAR motif, falling into the category of passive repressors. During late embryogenesis interaction of A group bZIPs ABA-insensitive 5 (ABI5) and Enhanced Em Level (EEL) compete for the same binding site conferring antagonistic transactivation functions: ABI5 homo-dimers activate gene expression, whereas EEL homo-dimers and ABI5/EEL heterodimers function as repressors (Bensmihen et al. 2002). Another regulatory model was identified in tobacco, where BZI-4 homodimers and BZI-1/BZI-2 heterodimers perform opposing functions and act as negative and positive transcriptional regulators during pollen development (Iven et al. 2010). Similarly, the expression of RBCS1a is modulated by HY5, HYH and GBF1, where GBF1 acts as a repressor, whereas HYH and HY5 are activators of RBCS1a expression (Singh et al. 2012). It has been shown that the progression from proliferating microspores to terminally differentiated pollen is characterized by large-scale repression of early male gametophytic genes and the activation of a unique late gene-expression program in late Arabidopsis pollen development (Honys and Twell, 2004). As AtbZIP18 was detected to be expressed from the microspore stage, we cannot exclude the possibility of its involvement in such regulatory machinery.

LOCALIZATION OF AtbZIP34 AND AtbZIP18 TRANSCRIPTION FACTORS

Transcription factors usually localize in the nucleus, driving the expression of their target genes. This was the case of AtbZIP34 protein, which was specifically present in the nuclei of transiently transformed tobacco leaf epidermal cells. However, the situation of AtbZIP18 protein was different. We observed its partial or full co-localization with the ER marker in the perinuclear region and on the cytoplasmic ER network in tobacco transient assay. To exclude potential artefacts that could arise from the strong CaMV 35S promoter activities, we generated and analysed stably transformed Arabidopsis plants expressing whole genomic sequence of AtbZIP18 fused with GFP. In the male gametophyte, we detected the GFP signal in

the whole pollen grain. Additionally, in the sporophyte, AtbZIP18-GFP fusion protein was observed in the cytoplasm and nuclei of developing embryos and seedlings. Such redistribution of TFs between two cellular compartments, was already described in the bZIP family. One of such examples represents AtbZIP10, which shuttles between the nucleus and the cytoplasm, where is retained by a plant-specific zinc-finger protein - Lesions Simulating Disease resistance 1 protein (LSD1; Kaminaka et al. 2006). LSD1 interferes with the nuclear localization signal (NLS), mediating nuclear import of AtbZIP10. Another explanation of AtbZIP18 co-localization with the ER marker could be its indirect association to the membranes, as AtbZIP18 doesn`t possess membrane binding domain. Nevertheless, there are other bZIP proteins, associated with the ER. AtbZIP28 and AtbZIP17 localize on the ER, oriented by their N-terminal parts containing DNA-binding domains towards the cytoplasm (Gao et al. 2008). After stress treatment resulting in the aggregation of unfolded proteins in the ER (unfolded protein response; UPRE), N-terminal parts of both bZIP proteins are released by S1P and S2P proteases and imported to the nucleus, where they activate the expression of chaperons, such as BiP genes. AtbZIP60 and AtbZIP49 represent another example of ER-resident bZIP transcription factors executing UPRE (Iwata et al. 2005; Iwata et al. 2008).

CHARACTERIZATION OF *atbzip34* AND *atbzip18* MUTANTS DURING MALE GAMETOPHYTE DEVELOPMENT SUPPORTS THEIR INVOLVEMET IN THE MALE GERMLINE

Except of their pollen co-expression, nuclear localization and dimerization, AtbZIP18 and AtbZIP34 represented candidate TFs involved in male gametophyte development also by means of genetic and phenotypic analyses of the respective mutants.

atbzip34 mutant characterization showed that the most striking difference compared to wt was the presence of lipid-like inclusions in the cytoplasm of mature pollen grains observed by bright field microscopy, often containing malformed male germ units. Moreover, detailed observation of the outer pollen wall structure (exine) by scanning electron microscopy revealed collapsed baculae and tecta, which however did not influence *atbzip34* male fertility. On the contrary, a number of mutants have been characterized in Arabidopsis, that show defects in exine structure and sporopollenin deposition often leading to pollen abortion and male sterility: *dex1* (Paxson-Sowders et al. 2001), *ms2* (Aarts et al. 1997), *nef1* (Ariizumi et al. 2004), *tde1*

(Ariizumi et al. 2008), *rpg1* (Guan et al. 2008). Some characteristics of *atbzip34* pollen analysed suggest impairment of certain metabolic pathways leading to correct pollen wall formation, such as lipid metabolism and/or cellular transport during pollen maturation confirmed by the set of genes with changed expression patterns in the mutant microarray analysis. Moreover, altered cellular transport from the tapetum could also explain the defects in exine synthesis and cell wall patterning. Cross-sections of *atbzip34* pollen grains confirmed presence of numerous lipid bodies, localized in clusters close to cell surface and, most interestingly surrounded by a less dense ER network as compared to wt. The encirclement of pollen cytosolic lipid bodies by ER is proposed to prevent their coalescence (Piffanelli et al. 1998). This ER network persists throughout pollen release and has been proposed to facilitate the direct mobilization of the lipid body TAGs required to support the rapid pollen tube growth (Murphy 2001; Piffanelli et al. 1998). Later in the *atbzip34* male gametophyte development, defects in progamic phase both *in vitro* and *in vivo* pollen tubes growth tests showed slower growth rate than in the wt. Genetic analysis of *atbzip34* allele through both gametophytes showed reduced transmission through the male by 34% and by 45% through the female suggesting importance of AtbZIP34 for the haploid cell linage.

On the other hand, the complete knock-down of *atbzip18* mRNA did not influence morphological or functional properties of the mutant pollen grains, implicating possible redundancy in this large TF family. To decipher the functional involvement of AtbZIP18 in pollen, we overexpressed AtbZIP18 in cell specific manner. Microscopic observations showed that transgenic lines overexpressing AtbZIP18 under control of Lat52 promoter produced aborted pollen population, which represented the most abundant phenotypic category found repeatedly among generations. Surprisingly, aborted pollen was isolated most prominently from heterozygous plants and was reverted to a near wild type phenotype in homozygous plants. This phenomenon is still enigmatic particularly in pollen. In diploid situation, there are few examples of protein equilibrium state importance represented by overexpression of histone proteins. Overexpression of either histone H2A-H2B or histone H3-H4 gene pairs causes aberrant chromosome segregation (Meeks-Wagner and Hartwell, 1986) and gene defects (Clark-Adams and Winston, 1987), yet co-overexpression of all four-core histones reverts the phenotypic effects due to restoration of the normal histone stoichiometry (Prelich, 2012). Moreover, disruption of protein equilibrium has been reported to be relatively common to

cause ~ 23% of observed phenotypes in cell morphology in systematic overexpression studies (Sopko et al. 2006). We are realizing, that uncovering the function of AtbZIP18 might lie rather on focusing on individual dimers or downstream targets, rather than looking on one component of the regulatory system. For this purpose we are analysing microarray data of the *atbzip18* mutant and preparing double knock-out mutants accordingly the Y2H analysis.

This study originated from the necessity to understand the roles of the transcription factors, which are critical components of transcriptional regulatory schemes globally controlling gene expression in time- and tissue-specific manner. Together with the complex characterization of the single genes we are realizing requisiteness to elevate the study from the single entity level, to higher hierarchies, such as groups of co-expressed genes, functional models and networks of interactions.

6. CONCLUSIONS

Collectively, our work on two bZIP transcription factors - AtbZIP18 and AtbZIP34 together with the identification of the interaction network among several pollen expressed bZIP proteins represents the first study of the transcription regulation mediated by bZIP TFs in the Arabidopsis male germline. Accordingly, we achieved following results.

1. AtbZIP34 – was the first bZIP transcription factor shown to have multiple roles in the development of gametophytic and sporophytic reproductive tissues.

1.1. *atbzip34* partial loss of function mutant showed characteristic pollen phenotype and functional defects of both gametophytic and sporophytic origin – it possessed misshaped and misplaced nuclei, lipid-like inclusions in the vegetative cell cytoplasm and, most significantly, defects in the cell wall (exine) patterning, endomembrane systems and wrinkled shape of intine in ~50% of pollen grains isolated from heterozygous plants. The viability of *atbzip34* pollen was reduced by 20% to that of wild type pollen. Similarly, *atbzip34* pollen tube growth both *in vitro* and *in vivo* was slower than that of wild type. Complementation analysis confirmed the association of the observed morphological and functional defects of *atbzip34* pollen and pollen tubes with the respective T-DNA line.

1.2. *atbzip34* knock-down mutation resulted in significant changes in pollen gene expression as demonstrated by the hybridization of Affymetrix ATH1 Genome Arrays. The subset of down-regulated genes was more distinguishable then the up-regulated set. Herein, the most significant changes were observed for genes involved in protein synthesis, transport, metabolism and protein fate. Among *atbzip34*-downstream genes, especially those encoding membrane-associated proteins including various transporters and proteins involved in lipid catabolism were enriched in particular.

1.3. AtbZIP34 affects both gametophytes. We observed a moderately reduced transmission efficiency of the *atbzip34* allele – by 34 % through the male and by 45 % through the female compared with the wild type AtbZIP34 allele.

1.4. AtbZIP34 is widely expressed in sporophytic and gametophytic tissues, with highly enriched expression in the mature pollen, confirmed by RT-PCR together with observation of AtbZIP34 promoter activity in GUS transgenic plants. AtbZIP34 promoter activity was restricted to the vegetative cell in transgenic lines expressing H2B_GFP protein. AtbZIP34_GFP fusion protein localized specifically in the nuclei of tobacco epidermal cells.

2. Complex functional characterization of the second bZIP candidate - AtbZIP18 - did not bring unambiguous conclusions about its function in the male gametophyte for now.

2.1. *atbzip18* knock-out mutant pollen did not display significant morphological deviations from the wild type.

2.2. The transmission efficiency of the *atbzip18* allele through pollen was reduced by 18% and remained unaffected through the female gametophyte. Accordingly, the frequency of seed gaps in *atbzip18/-* siliques reached 4% representing slight but significant increase (P-value 0.02) when compared to the frequency of abolished seed set (1.5%) in wild type plants.

2.3. Overexpression of AtbZIP18 mRNA in pollen vegetative cell resulted in the occurrence of aborted pollen population counting ~23% among heterozygous plants and ~6% at average among homozygous plants. Similarly, the overexpression of AtbZIP34 mRNA affected ~26% pollen grains leading to their abortion among heterozygous plants, and only ~1% of aborted pollen grains were observed in homozygous plants.

2.4. AtbZIP18 expression pattern/promoter activity was highly overlapping with that of AtbZIP34 in the tissue manner. Significant difference was observed quantitatively, as AtbZIP18 mRNA was 5 fold more abundant in mature pollen cDNA than AtbZIP34 transcript, confirmed also by transcriptomic data. Throughout the plant development, AtbZIP18 was localized in most sporophytic tissues of the developing embryos and seedlings. In the male gametophyte, the localization was restricted to the whole pollen grains and pollen tubes. Localization of AtbZIP18_GFP protein was studied both in transient assay and under native conditions. In subcellular context, the AtbZIP18_GFP protein was restricted to the nucleus and to the ER and/or cytoplasm, confirmed by the ER-marker co-localization.

3. The bZIP family of transcription factors contains 75 members, of which 17 are expressed in pollen. Therefore we evaluated the dimerization potential of pollen-expressed bZIP TFs. Six examined pollen-expressed bZIP proteins dimerized in Y2H assay and formed homo- as well as hetero-dimers. AtbZIP18 protein formed dimers with the majority of examined proteins. The results of interaction studies as well as the presence of EAR domain in AtbZIP18 and AtbZIP52 proteins tempted us to speculate that both AtbZIP18 and AtbZIP52 modulate AtbZIP34 activity.

Wide roles of bZIP family of TFs have been demonstrated in plants so far, such as organ and tissue differentiation, photomorphogenesis, cell elongation, energy metabolism, hormone and sugar signalling, ER stress response, abiotic stress response, therefore it is promising they play also critical roles during male gametophyte development.

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8. SUPPLEMENTS

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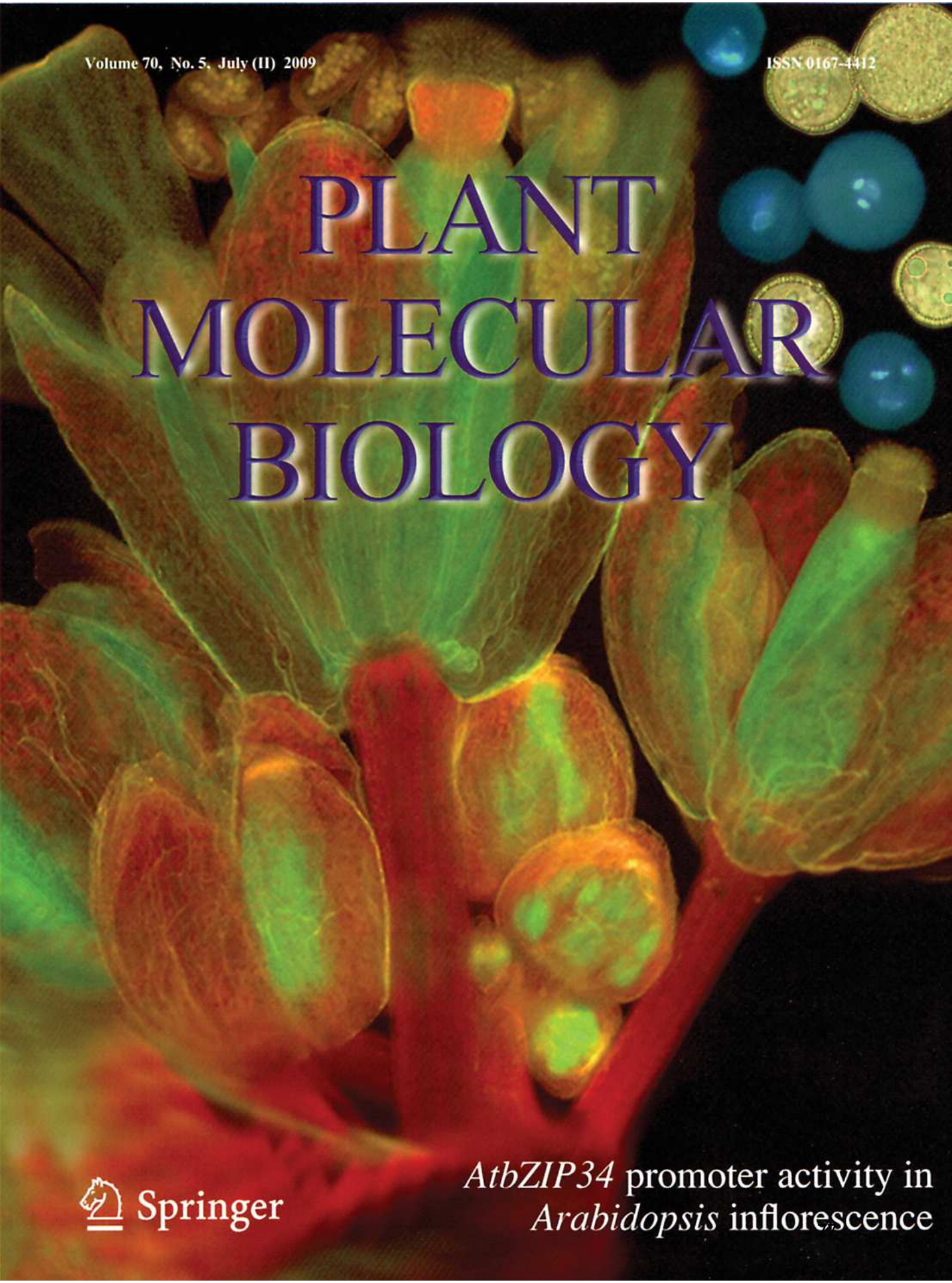

AtbZIP34 promoter activity in *Arabidopsis* inflorescence

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AtbZIP34 is required for Arabidopsis pollen wall patterning and the control of several metabolic pathways in developing pollen

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Abstract Sexual plant reproduction depends on the production and differentiation of functional gametes by the haploid gametophyte generation. Currently, we have a limited understanding of the regulatory mechanisms that have evolved to specify the gametophytic developmental programs. To unravel such mechanisms, it is necessary to identify transcription factors (TF) that are part of such haploid regulatory networks. Here we focus on bZIP TFs that have critical roles in plants, animals and other kingdoms. We report the functional characterization of *Arabidopsis thaliana AtbZIP34* that is expressed in both gametophytic and surrounding sporophytic tissues during flower development. T-DNA insertion mutants in *AtbZIP34* show pollen morphological defects that result in reduced pollen germination efficiency and slower pollen tube growth both in vitro and in vivo. Light and fluorescence microscopy revealed misshapen and misplaced nuclei with large lipid inclusions in the cytoplasm of *atbzip34* pollen. Scanning and transmission electron microscopy revealed defects in exine shape and micropatterning and a reduced endomembrane system. Several lines of evidence, including the *AtbZIP34* expression pattern and the phenotypic defects observed, suggest a complex role in male reproductive development that involves a sporophytic role in exine patterning, and a sporophytic and/or gametophytic mode of action of AtbZIP34 in several metabolic pathways, namely regulation of lipid metabolism and/or cellular transport.

Keywords bZIP transcription factor · AtbZIP34 · Male gametophyte development · Lipid metabolism · Cellular transport · Cell wall formation · Transcriptomics

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Introduction

Male gametophyte development is a complex process requiring the coordinated participation of various cell and tissue types in the flower. The developmental phase leading to the formation of mature pollen grains is followed by the functional or progamic phase beginning with the impact of the grains on the stigma surface and terminated by double fertilization. Although its accessibility and highly reduced structure makes the male gametophyte an ideal model for developmental studies, we still have a limited knowledge of the regulatory mechanisms that specify gametophytic

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development and function (McCormick 2004; Honys et al. 2006; Twell et al. 2006; Borg et al. 2009).

Previous genome-wide transcriptomic studies have led to the identification of 608 putative transcription factor (TF) genes active in at least one stage of male gametophyte development in *Arabidopsis* (Honys and Twell 2004). This represents ~45% of the 1,350 TF genes with corresponding probe sets on the Affymetrix ATH1 GeneChip. Of these 608 male gametophyte expressed TF transcripts, 54 (15.7%) were shown to be putatively pollen-specific. Several large protein families were overrepresented among male gametophyte expressed TFs, including C3H and C2H2 zinc finger proteins, WRKY, bZIP and TCP proteins. On the contrary, basic helix-loop-helix (bHLH) and APETALA2/ethylene response element binding protein-like (AP2/EREBP), MADS and R2R3-MYB gene families were underrepresented (Honys and Twell 2004). Interestingly, members of a few underrepresented gene families have been reported to function in male gametophyte and/or tapetum development (Ito et al. 2007; Takeda et al. 2006).

The most significant advances have originated from two studies of *Arabidopsis* MADS-box TFs (Verelst et al. 2007a, b). The authors identified pollen-specific MIKC* class of MADS-box proteins as major regulators of transcriptome dynamics during late stages of pollen development in *Arabidopsis*. MIKC* protein complexes were demonstrated to control a transcriptional switch directing pollen maturation that is essential for pollen competitive ability. The co-expression of five of the six AtMIKC* genes during late stages of pollen development suggests that they cooperate to establish a TF network active during the final stages of pollen development.

Several others TFs regulating male gametophyte development belong to the MYB family. An unusual R2R3 MYB gene, *DUO1* is specifically expressed in the male germline and has been shown to be a key regulator of germ cell division and sperm cell formation in *Arabidopsis* (Durbarry et al. 2005; Rotman et al. 2005; Brownfield et al. 2009). However, most of the known MYB-family factors act sporophytically. For example, knockout of two redundant genes, *MYB33* and *MYB65,* results in premeiotic abortion of pollen development (Millar and Gubler 2005). Moreover, expression of these genes is regulated at the post-transcriptional level by miRNAs miR159a and miR159b (Allen et al. 2007). Similarly, AtMYB103 is involved in the sporophytic control of microspore release and exine formation (Zhang et al. 2007) and forms part of regulatory network that acts downstream of another MYB gene, TDF1 (Zhu et al. 2008).

However, bZIP-family TFs have not yet been demonstrated to be directly involved in male gametophyte development. Compared to the largest TF gene families, the bZIP family is slightly smaller, consisting of 75 members in *Arabidopsis* (Jakoby et al. 2002), 89 or 92 in rice (Correa et al. 2008; Nijhawan et al. 2008) and 89 in *Populus trichocarpa* (Correa et al. 2008). The number of genes and distribution among subfamilies demonstrates the complexity and homogeneity of the bZIP gene family in angiosperms. Their chromosomal distribution and sequence similarities suggest that the bZIP TF family has diverged through multiple gene duplication events (Correa et al. 2008), contributing to their potential for regulating diverse gene networks. Putative AtbZIP proteins were clustered into 10 groups according to their domain structures and sequence similarities (Jakoby et al. 2002).

Basic leucine zipper (bZIP) proteins represent an exclusively eukaryotic class of enhancer-type TFs that are known to regulate many critical processes including histodifferentiation during embryogenesis (Darlington et al. 1998; Eferl et al. 1999; Wang et al. 1992). In adult animals, bZIP factors are involved in diverse processes such as metabolism, circadian rhythm, and learning and memory (Darlington et al. 1995, 1998; Sanyal et al. 2002; Yamaguchi et al. 2005). In yeast, bZIP proteins are necessary for sexual differentiation and entry into stationary phase (Takeda et al. 1995; Watanabe and Yamamoto 1996). In general, bZIP TFs appear to be mostly involved in regulatory processes of general metabolism and appear to act downstream in regulatory hierarchies.

In plants, bZIP factors have been shown to have important roles in organ and tissue differentiation, photomorphogenesis, cell elongation, nitrogen/carbon balance control, energy metabolism, hormone and sugar signalling, flower maturation, seed development and pathogen defence (Weltmeier et al. 2009, reviewed by Cluis et al. 2004; Correa et al. 2008; Jakoby et al. 2002). A group of bZIP TFs play important roles in the ABA signalling pathway in *Arabidopsis* and most ABA-responsive element-binding bZIPs belong to group A. Functional characterization of several group A bZIPs revealed that their expression is induced by ABA or abiotic stress (Choi et al. 2000; Finkelstein and Lynch 2000; Uno et al. 2000). Phylogenetic analysis showed that this group of bZIPs was evolutionarily conserved between *Arabidopsis* and rice. OsbZIP72, another member of group A, was recently shown to be a positive regulator of ABA response and drought tolerance in rice (Lu et al. 2008). Similarly another rice bZIP TF OsbZIP23 confers stress tolerance and ABA sensitivity (Xiang et al. 2008). Several bZIP TFs (*Arabidopsis thaliana* AtbZIP17, AtbZIP28, AtbZIP49 and AtbZIP60 with orthologues in *Nicotiana tabacum* NtbZIP60 and *N. benthamiana* NbbZIP60) were demonstrated to be membrane-bound in their cytoplasmic, inactive form (Iwata and Koizumi 2005; Liu et al. 2007a, b; Tajima et al. 2008; Tateda et al. 2008). These proteins are activated during the stress response by an intramembrane proteolysis

mechanism (RIP; reviewed by Seo et al. 2008) and have different sensitivities or responses to particular stimuli (reviewed in Chen et al. 2008). AtbZIP60 and AtbZIP28 are proteolysis-activated TFs directly involved in the endoplasmic reticulum stress response (Iwata et al. 2008; Liu et al. 2007a). The conserved presence of bZIP factors across all eukaryotic kingdoms, together with their roles in a myriad of cellular functions, underscores the importance of this class of enhancer-type TFs (Deppmann et al. 2006).

We carried out phenotypic screening of T-DNA insertion lines for candidate TFs potentially involved in regulation of male gametophyte development. A T-DNA insertion in *AtbZIP34*, encoded by At2g42380, resulted in obvious pollen morphological defects and was characterized further. Here we report the functional characterization of *AtbZIP34* and its expression in both gametophytic and surrounding sporophytic tissues during flower development. Our results demonstrate a role for AtbZIP34 in the sporophytic control of cell wall patterning and gametophytic control of pollen development. Transcriptomic analysis of *atbzip34* mutant pollen further identified altered patterns of gametophytic gene expression that highlight a role for AtbZIP34 in the control of pathways regulating cellular transport and lipid metabolism.

Materials and methods

Plant material and growth conditions

Arabidopsis T-DNA insertion line SALK 018864 (insertion in At2g42380 gene; kanamycin resistance) was used together with wild type ecotype Columbia-0 plants. Plants used for pollen isolation were grown in controlled-environment cabinets at 21°C under illumination of 150 μmol m^{-2} s^{-1} with a 16-h photoperiod. Pollen for microarray experiments was harvested from two independently grown populations according to Honys and Twell (2003). The purity of isolated pollen was determined by light microscopy and 4′,6-diamino-phenylindole-staining according to Park et al. (1998). Pollen viability was tested by fluorescence diacetate (FDA) staining according to Eady et al. (1995). In all tests, more than 1,000 grains were scored. Roots were grown from plants in liquid cultures as described previously (Honys and Twell 2003).

For genotyping of transgenic plants, gene-specific ZIP-F1, ZIP-R1 primers and insert-specific primer LB2 were used. Appropriate gene-specific primers were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and are listed in Supplementary Table 1. Genomic DNA was isolated by a CTAB DNA extraction method modified from Weigel and Glazebrook (2002) in which initial grinding of leaf tissue frozen in liquid nitrogen was carried out ground for 12 s with glass beads in a dental amalgam mixer ESME Cap mix (3 M, Maplewood, MN). For segregation analyses, heterozygous plants *AtbZIP34/atbzip34* were allowed to self-fertilize and seeds were aseptically sown on ½ Murashige–Skoog media containing 10 μg μl^{-1} kanamycin.

RNA extraction, probe preparation and DNA chip hybridization

Total RNA was extracted from 50 mg of isolated pollen using the RNeasy Plant Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). For microarray experiments, RNA integrity was checked using an Agilent 2100 Bioanalyser (Agilent Technologies, Boblingen, Germany) at NASC. Biotinylated target RNA was prepared from 20 μg of total RNA as described in the Affymetrix GeneChip Technical Analysis Manual (Affymetrix, Santa Clara, CA). Preparation of cRNA probes and hybridization to ATH1 Genome Arrays and scanning were carried out as described (Honys and Twell 2003). Publicly available transcriptomic datasets were downloaded from arabidopsis GFP database (http://aGFP.ueb.cas.cz; Dupl'áková et al. 2007). All transcriptomics datasets were normalized using freely available dChip 1.3 software (http://www.dchip.org). The reliability and reproducibility of analyses was ensured by the use of duplicates or triplicates in each experiment, the normalization of all arrays to the median probe intensity level and the use of normalized CEL intensities of all arrays for the calculation of model-based gene-expression values based on the Perfect Match-only model (Li and Wong 2001a, b). As a reference, all four available mature pollen transcriptomic datasets were used and labelled MP1 (Ler, 2 repeats; Honys and Twell 2004), MP2 (Col-0, 3 repeats; Zimmermann et al. 2005), MP3 (Col-0, 2 repeats; Pina et al. 2005) and MP4 (2 repeats, wild type in this study). For each gene, the most deviant expression value was eliminated and the mean from the remaining three values was calculated (MPG).

RT-PCR

Pollen, stem, leaf and inflorescence RNA was isolated from Col-0 and/or *atbzip34/atbzip34* plants grown as described (Honys and Twell 2003). Pollen RNA used for RT-PCR analyses was obtained from plants that were grown independently from those used for microarray analysis. Samples of 1 μg total RNA were reverse transcribed in a 20-μL reaction using the ImProm-II Reverse Transcription System (Promega, Madison, WI) following the manufacturer's instructions. For PCR amplification, 1 μl of 50× diluted RT mix was used. The PCR reaction was carried out in 25 μL with 0.5 unit of *Taq* DNA polymerase (MBI

Fermentas, Vilnius, Lithuania), 1.2 mM $MgCl_2$, and 20 pmol of genotyping primers SALK_018864_F1 and SALK_018864_R1. The PCR program was as follows: 2 min at 95°C, 35 cycles of 15 s at 94°C, 15 s at the optimal annealing temperature 55°C, and 30 s at 72°C, followed by 10 min at 72°C.

The presence or absence of *AtbZIP34* transcripts in *atbzip34* mutant pollen was verified by RT-PCR of 5′ and 3′ end gene fragments separately. Wild type-pollen cDNA and genomic DNA was used as a control. PCR was performed with exon-localised primers: ZIP-F2, ZIP-R2 (exons 1, 2; upstream of insertion site), ZIP-F3, ZIP-R3 (exon 3–4; downstream of insertion site; Supplementary Table 1). The PCR program was as follows: 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at the annealing temperature (51°C), and 1 min at 72°C, followed by 10 min at 72°C.

qRT-PCR

Quantitative real-time PCR was carried out on a LightCycler 480, (Roche Applied Science, Mannheim, Germany) using LightCycler 480 SYBR Green I Master (according manufacturer′s instructions). The primers used were specific for genes encoding UDP-glucose epimerases (Supplementary Table 1) cDNA was produced with 1.8 µg of total RNA and 2 µl of 35 µM oligo-$(dT)_{23}$ in a 20 µl reaction. First strand cDNA was 20× diluted in a final volume of 10 µl with 500 nM of each of the HPLC purified primers. Reaction was performed in 96-well plastic plate (Roche, Mannheim, Germany). Real-time PCR data were collected on the light cycler with cycling conditions: 5 min of initial denaturation at 95°C, then 45 cycles of 10 s at 95°C, 10 s at 58°C, and 15 s at 72°C. PCR efficiencies were estimated from calibration curves generated from serial dilution of cDNAs. Real time PCR expression measurements are frequently normalized with the expression of reference gene. We used KAPP (kinase associated protein phosphatase, At5g19280) as a reference gene. The ratio of the relative amount of the target and reference gene was calculated as follows: E_R^{CpR}/E_T^{CpT} (E_T, E_R: efficiency for target or reference gene qRT PCR assay; C_pT, C_pR: a crossing point for target or reference genes).

Promoter analysis

Developmental and tissue-specific expression profile of At2g42380 gene was evaluated using a promoter:eGFP:GUS construct. A 1,060 bp region upstream of *AtbZIP34* gene was PCR-amplified using pZIP-F and pZIP-R primers (Supplementary Table 1). An entry clone was prepared by cloning the promoter fragment into the pENTR2B vector (Invitrogen, Carlsbad, CA). From the entry clone, the *AtbZIP34* promoter fragment was further sub-cloned into the Gateway-destination vector pKGWFS7.0 (Karimi et al. 2005). Constructs were verified by restriction analysis and sequenced. *Arabidopsis* wt plants were transformed using the floral dip method (Clough and Bent 1998) and *Agrobacterium tumefaciens* strain GV3101. Transformants were selected on ½ MS medium-300 ml (0.66 g Murashige and Skoog basal medium, 3 g sucrose, 30 mg Myo-inositol, 150 mg MES (2-(*N*-morpholino)ethanesulfonic acid), 0.8% agar, pH 5.7 with KOH) containing 50 µg ml^{-1} kanamycin. Transformants were verified for T-DNA insertion by PCR. Flowers from T1 generation were collected to GUS buffer (0.1 M phosphate buffer, pH 7.0; 10 mM EDTA, pH 8.0; 0.1% triton X-100 supplemented with 1 mM X-glcA and 4 mM ferricyanide) After 48-h incubation at 37°C, samples were analyzed by bright field and fluorescence microscopy with Olympus DP50-CU microscope.

Complementation analysis

A 3,232 bp genomic fragment including the complete *AtbZIP34* gene and 720 bp of 5′ flanking DNA was PCR-amplified using ZIP-F and ZIP-R primers (Supplementary Table 1) and recombined into the pENTR2B vector (Invitrogen, Carlsbad, CA). This entry clone was further recombined into GATEWAY-compatible destination vector (VIB, Ghent, Belgium, Karimi et al. 2005). Constructs were verified by restriction analysis and sequenced. Homozygous *atbzip34* plants were transformed using the floral dip method (Clough and Bent 1998) and *Agrobacterium tumefaciens* strain GV3101. Transformants were selected on ½ MS medium-300 ml (0.66 g Murashige and Skoog basal medium, 3 g sucrose, 30 mg Myo-inositol, 150 mg MES, 0.8% agar, pH 5.7 with KOH) containing 50 µg ml^{-1} kanamycin. Transformants were verified for the presence of T-DNA by PCR with primers ZIP-F1 and ZIP-R1 (Supplementary Table 1). Phenotypic complementation was examined by bright field and fluorescence microscopy after DAPI staining as described (Park et al. 1998).

Electron microscopy

Freshly harvested material was fixed in a 2.5% (w/v) glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) for 24 h at room temperature, washed with 4% glucose in 0.1 M PBS ($NaH_2PO_4 \times H_2O$, pH 7.0) for 15 min, post-fixed in 2% (w/v) osmium tetroxide in 0.1 M PBS buffer, washed with 4% glucose in 0.1 M PBS for 15 min, dehydrated through an ascending ethanol series (30–100% ethanol), and, via ethanol: acetone, to acetone. Samples were embedded in Poly/Bed® 812/Araldite 502 resins. Thin

sections (70 nm) were cut on a Reichert–Jung Ultracut E ultra-microtome and stained using uranyl acetate and lead citrate. Sections were analyzed and photographed using the JEM-1011 electron microscopes with Megaview III camera and analySIS 3.2 software (Soft Imaging System®).

For scanning electron microscopy, freshly harvested material was fixed in a 2.5% (w/v) glutaraldehyde in 0.1 M PBS for 24 h at room temperature, washed with 4% glucose in 0.1 M PBS for 15 min, dehydrated through an ascending ethanol series (30–100% ethanol), and, via ethanol:acetone, to acetone. Pollen samples for scanning electron microscopy was then critical point dried in CO_2, mounted on a stub, sputter coated with gold, and observed and photographed with a JEOL 6300 scanning microscope.

Analysis of in vitro pollen tube growth

Pollen was collected from just-open flowers of wild type and *atbzip34/atbzip34* plants. Pollen grains were germinated on a germination medium on microscope slide according to Boavida and McCormick (2007) with several modifications. Pollen germination medium (final volume 25 ml) was always prepared fresh from 0.5 M stock solutions of the main components (5 mM KCl, 0.01% H_3BO_3, 5 mM $CaCl_2$, 1 mM $MgSO_4$) using autoclaved water. Sucrose (10%) was added and dissolved and pH was then adjusted to 7.5 with NaOH. About 1.5% of low-melting agarose (Amresco, Solon, Ohio) was added and briefly heated in a microwave oven, just long enough for the agarose to melt. Glass slide was then filled with 500 µl melted germination media. Pollen from individual flowers was spread on the surface of germination pad by inverting the flower with the help of tweezers and gently bringing it onto agarose surface after its solidification. The whole flower could be used as a "brush" to spread pollen uniformly on the surface of the germination medium. Glass slides were immediately placed inside a moisture incubation chamber to avoid media dehydration and incubated for 10 h in the dark at 24°C. Samples were examined by bright field and fluorescence microscopy with an Olympus DP50-CU microscope.

Analysis of in vivo pollen tube growth

Flower buds from wild-type and *atbzip34* plants were emasculated and hand-pollinated on the following day. Wild type plants were pollinated with *atbzip34* pollen, and *atbzip34* plants by wt pollen. After 7 h, the styles were collected separately and fixed in ethanol/acetic acid (3:1) for 1 h at room temperature. After overnight softening in 8 M NaOH, the flowers were washed several times with distilled water and incubated with aniline blue solution (0.1% aniline blue in 0.1 M K_2HPO_4-KOH buffer, pH 11.0) for 3 h in the dark. The stained flowers were placed in a drop of 50% glycerol on a microscope slide and observed by epifluorescence microscopy.

Results

bZIP family TFs are widely expressed in *Arabidopsis*

AtbZIP genes form a large family of TFs comprising 75 annotated genes in *Arabidopsis* (Jakoby et al. 2002). Of these, 66 genes are represented on the Affymetrix ATH1 GeneChip and 24 showed reliable signals in the developing male gametophyte (Supplementary Table 2). Although most AtbZIP genes do not show strict or preferential expression according to transcriptomic data (Dupl'áková et al. 2007), At2g42380 encoding AtbZIP34 showed a pollen-enriched expression pattern suggesting its role in late male gametophyte development (Honys and Twell 2004). Further analyses of transcriptomic data including reproductive organs revealed that At2g42380 was active in the second and third whorls of stage 15 flowers (Smyth et al. 1990; Zimmermann et al. 2005). RT-PCR using RNA isolated from four stages of male gametophyte development, unicellular, bicellular, tricellular and mature pollen, and four sporophytic tissues revealed its cumulative expression and weak expression in whole flowers (Fig. 1A). This expression pattern suggested that AtbZIP34 represents a late pollen-enriched TF.

The expression pattern of *AtbZIP34* was further investigated in transgenic plants harbouring the *AtbZIP34* promoter fused to the eGFP:GUS reporter (Fig. 2). GUS assay confirmed previously investigated expression pattern by RT-PCR together with transcriptomic data that AtbZIP34 represents a late pollen-enriched TF. In stamens the GUS signal was first detectable throughout young anthers and later became concentrated in the tapetum (Fig. 2B). In young flower buds (stage 7–9), GUS signal was localized in anthers and pistils (Fig. 2A). In developed flowers (stage 14), GUS staining extended to whole anthers and filaments (Fig. 2D, G). In carpels, GUS staining was first detected in pistil vascular tissues and young female gametophytes before complete development of the integuments (Fig. 2C). After the developmental shift, the highest GUS activity was localized in funiculi connecting mature ovules with the placenta (Fig. 2L) and in papillar cells and adjacent stigmatic tissue (stage 14) (Fig. 2E, F). In ovules, GUS activity was detected only in earlier developmental stages (Fig. 2C). On the contrary, in the male gametophyte, GUS signal gradually accumulated from microspores to mature tricellular pollen grains (Fig. 2H–K). *AtbZIP34* promoter activity was also observed in vegetative organs and was always associated

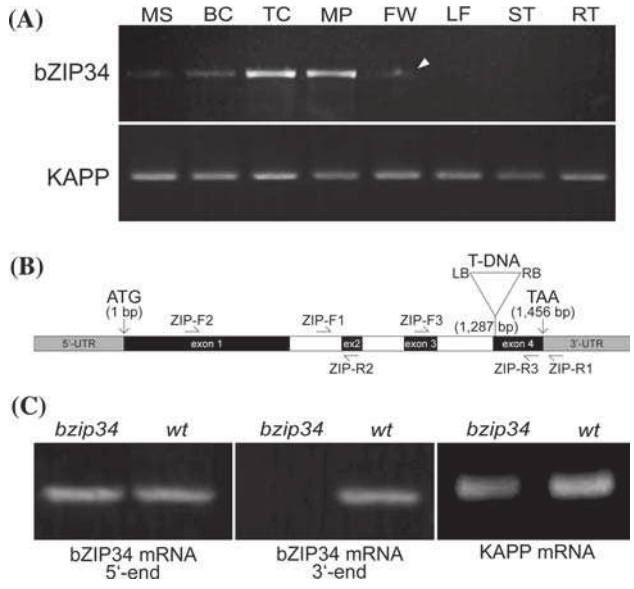

Fig. 1 Verification of At2g42380 expression profile and control KAPP gene expression by RT-PCR (**A**) in microspores (MS), bicellular (BC), tricellular (TC) and mature pollen (MP), whole flowers (FW), leaves (LF), stems (ST) and roots (RT). *White arrowhead* shows expression of At2g42380 in flowers. Diagram showing At2g42380 gene model (**B**) including T-DNA insertion site (*triangle*) and positions of respective primers—*arrows*, introns—*white boxes*, exons—*black boxes*, untranslated regions—*light grey boxes*, proximal promoter region—*dark grey box*, LB and RB—*left* and *right borders* of T-DNA. Expression analysis of both end regions of *AtbZIP34* transcript in wild type and *atbZIP34* pollen (**C**)—RT-PCR of *AtbZIP34* mRNA 5′-end (upstream of T-DNA insertion, primers ZIP-F2/ZIP-R2) and 3′-end regions (downstream of T-DNA insertion, primers ZIP-F3/ZIP-R3) as well as KAPP control transcript (primers KAPP-F/KAPP-R)

with vascular tissues in the distal regions of stems, leaves and siliques (data not shown).

Given its dynamic expression profile the regulatory function of AtbZIP34 TF is likely be complex. In this article, we focused our investigation on the role of AtbZIP34 in male gametophyte development and function.

Identification of an *AtbZIP34* T-DNA insertion mutant

We identified a T-DNA insertion in At2g42380 encoding AtbZIP34. In SALK_18864, the T-DNA insertion is located at the beginning of exon 4, after nucleotide 1,287 from the ATG initiation codon (Fig. 1B). The knock-down of *AtbZIP34* mRNA in pollen produced by homozygous SALK_18864 plants was verified by RT-PCR analysis of the transcripts upstream and downstream of the insertion site. The results confirm the absence of complete transcripts in *AtbZIP34* pollen using primer pair F1–R1, and partial transcripts downstream of the insertion site with primer pair F3–R3. However, 3′ truncated transcripts upstream of the insertion site were detected using primer pairs F2 and R2 (Fig. 1B), indicating that SALK_18864 represents a partial loss of function allele.

Cellular and pollen wall defects in *atbzip34* mutant pollen

The T-DNA insertion in the *AtbZIP34* gene is not lethal for gametophytic or sporophytic development as homozygous *atbzip34/atbzip34* plants were easily identified. However, pollen produced by homozygous plants showed characteristic phenotypic defects under bright field and fluorescence microscopy (Fig. 3). Five independent samples were observed ($n = 3{,}419$ pollen grains). In bright field observations, 56.2 ± 9.5% appeared similar to wild type pollen. After DAPI staining this percentage was lower (44.1 ± 5.5% of all pollen examined). The occurrence of collapsed pollen was 15.5 ± 3.9%. Despite the low percentage of unicellular microspores (2.8 ± 1.3%) and bicellular pollen (9.9 ± 2.3%), a fraction of tricellular pollen (26.7 ± 5.5%) contained malformed or displaced male germ units, often with unusual vegetative nuclei. These nuclei were larger and more diffuse than in wild type (Fig. 3B, D). Taken together, the majority of pollen grains exhibiting phenotypic abnormalities were tricellular, but these were smaller in diameter (*atbzip34*: $d = 12.05 \pm 1.54$ µm; $n = 30$; wt: $d = 15.87 \pm 0.66$ µm; $n = 30$) than wild type pollen. Moreover, *atbzip34* pollen contained characteristic cytoplasmic inclusions evoking lipid or oil bodies (Fig. 3) that were examined in more detail by electron microscopy.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were employed to observe cell wall patterning, membrane structure and ultrastructure of developing *atbzip34* pollen. The most obvious differences from wild type pollen observed by SEM were irregular pollen shape and abnormal exine patterning (Fig. 4). Aberrant exine patterning appeared as regions of collapsed baculae and tecta together with areas with extra material deposited onto them. This phenotype was observed in all *atbzip34* pollen grains. Some pollen grains were found still attached together (Fig. 4E). There were no significant differences in the frequency of exine patterning defects in wild type pollen and pollen from heterozygous *atbzip34* plants (data not shown), consistent with the sporophytic control of exine patterning defects.

More thorough ultrastructural analysis was performed by TEM. Because of presumed sporophytic nature of cell wall patterning defects, the ultrastructure of both tapetum and spores was examined at several developmental stages (tetrads, uninucleate microspores, bicellular pollen; Supplemental Fig. 1). When observing tapetum development, apart from the general ultrastructure of tapetal cells (Ariizumi et al. 2004; Vizcay-Barrena and Wilson 2006; Yang et al. 2007), special attention was paid to the number

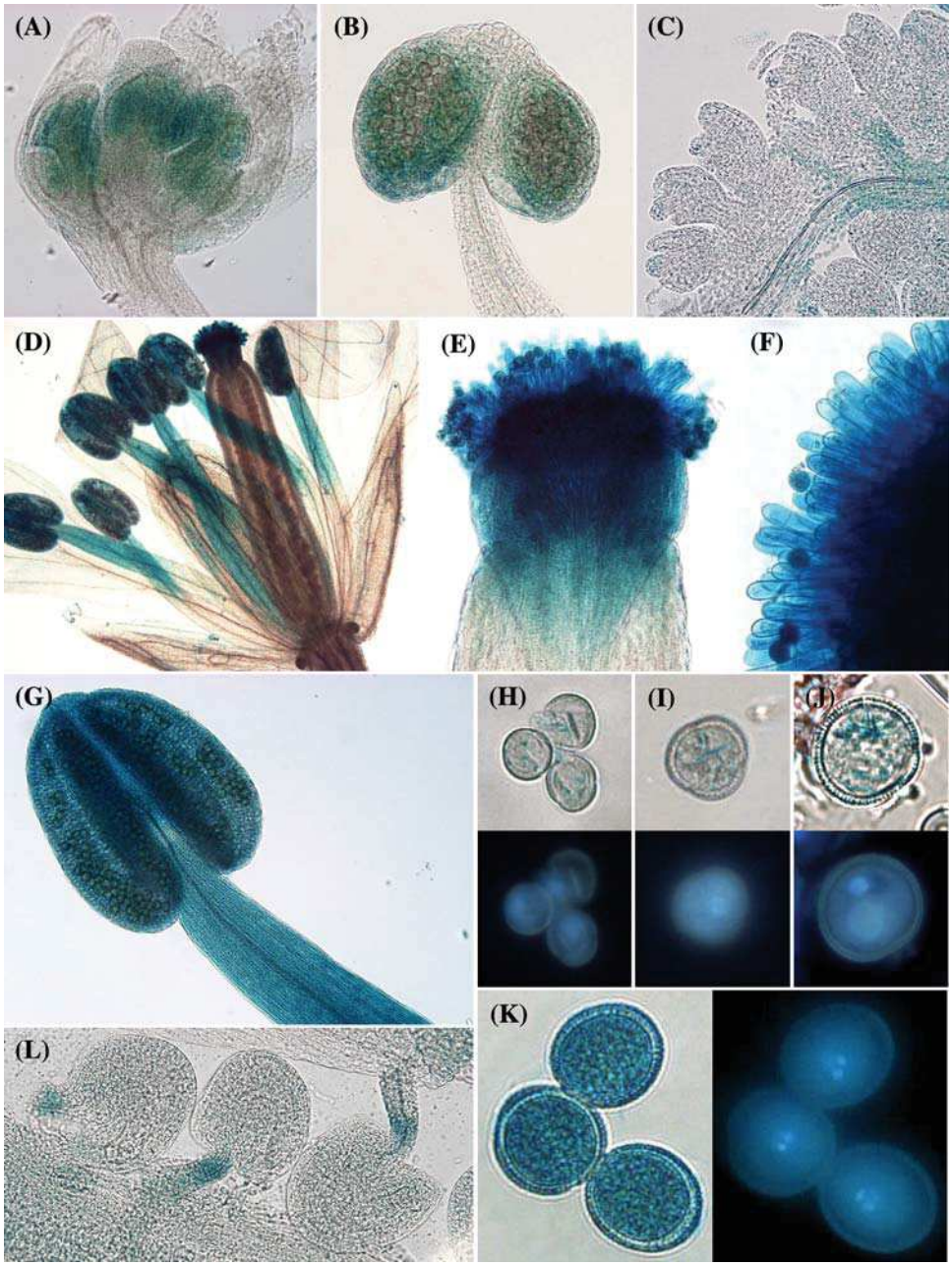

Fig. 2 Activity of the *AtbZIP34* promoter. Bright field microscopy of flower bud (stage 8, **A**) with detailed view of anther (**B**) and ovary (**C**). Later developmental stages are represented by an open flower (stage 15, **D**) with details of anther (**G**), pistil (**E**), papillar cells with attached pollen (**F**) and three ovules (**L**). Bright field and epifluorescent micrographs of several stages of pollen development including uninucleate microspores (**H**), early bicellular (**I**), late bicellular (**J**) and mature pollen (**K**)

and organization of secretory vesicles, vacuolization, plastid development (number and size of plastoglobules, lipid bodies, elaioplasts) and cell wall degeneration. In developing spores, cell wall structure and subcellular organization including endomembrane system and lipid bodies was analysed as phenotypic defects in these structures were suggested by bright field observations.

TEM observations confirmed differences in pollen wall structure between wt and *atbzip34* pollen (Fig. 5). Mature a*tbzip34* pollen possessed a characteristic wrinkled intine (Fig. 5D, F), which in wt is smooth and closely connected to the inner side of nexine (Fig. 5C, E). Mutant pollen also showed sparse and deformed baculae and tecta (Fig. 5D, F) that correspond with regions of unusual exine patterning observed by SEM. There were no apparent differences in cell wall structure of microspores in tetrads; the first differences were found in bicellular stage. Mutant pollen grains had wrinkled intine (and malformed exine characteristic of mature pollen grains) and vacuoles were increased in number and size. Unlike the exine-patterning defect, the unusual intine shape was observed also in approximately one half of pollen grains from *atbzip34* heterozygous plants. Moreover, there were differences in endomembrane systems together with the appearance of

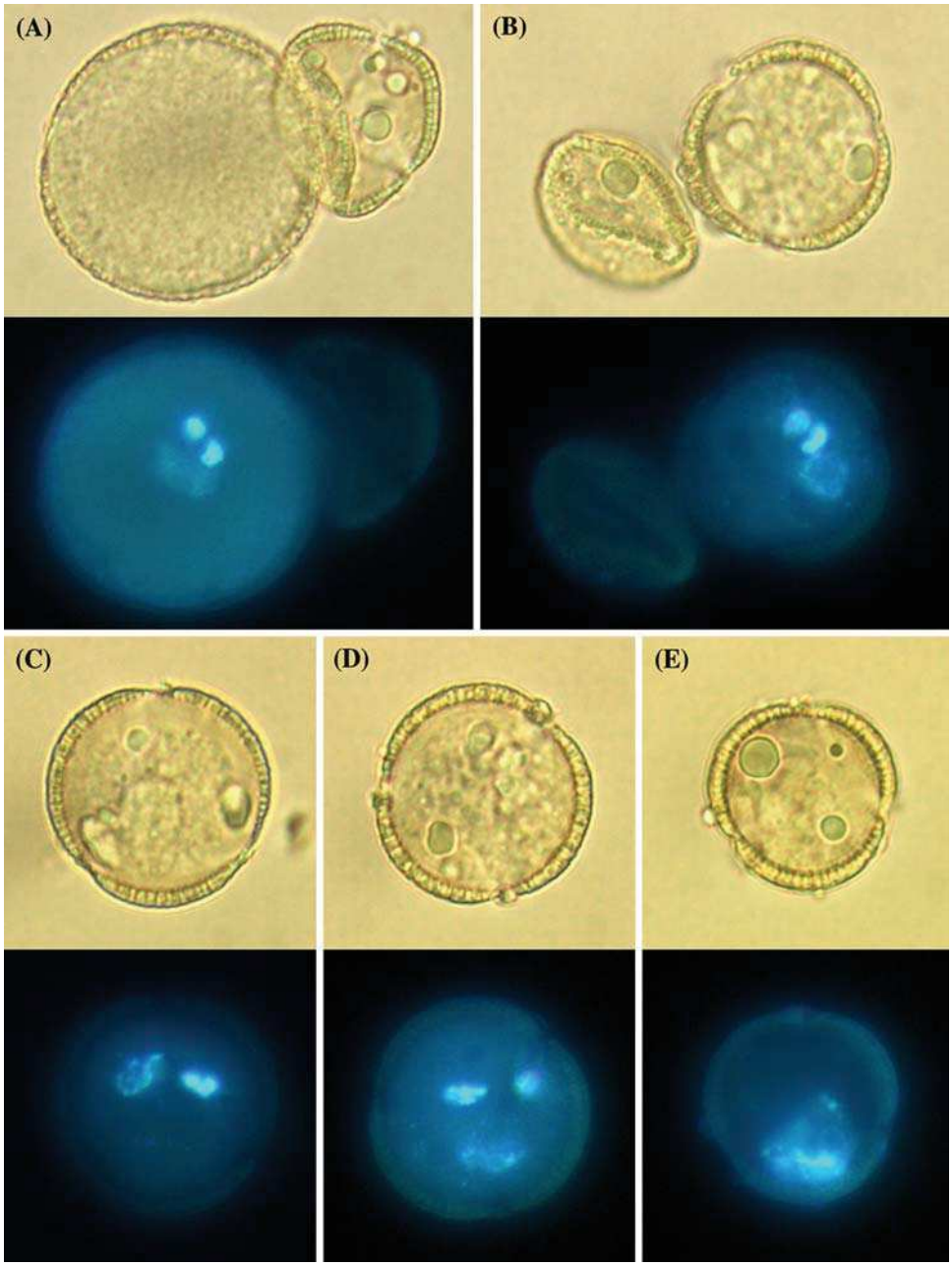

Fig. 3 Phenotypic defects in *atbzip34* pollen. Bright field and fluorescence images after DAPI-staining are shown, (**A**) wild type and *atbzip34* collapsed pollen, (**B**–**E**) *atbzip34* pollen

dense round inclusions (Fig. 5A, B). Generally, endoplasmic reticulum (ER) was underdeveloped in *atbzip34* pollen. Electron dense round inclusions were present both in wild type and *atbzip34* pollen. Their structure and co-localisation with rough ER correspond to lipid bodies that are formed during pollen maturation (Murphy 2001; Van Aelst et al. 1993). In wt pollen, lipid bodies were enclosed by one to mostly several layers of rough endoplasmic reticulum (Fig. 5E, G). However, more than one ER layer surrounding lipid bodies was rare in *atbzip34* pollen and often no surrounding ER was present (Fig. 5D, H). Lipid bodies were also more numerous in *atbzip34* pollen grains and localized in clusters in a cortical regions of the vegetative cell cytoplasm.

Tapetum development seemed less affected by *atbzip34* mutation. Tapetal cells of wild type and mutant were similar throughout development (Supplementary Fig. 1). The only apparent difference was the organization of round electron-dense inclusions at microspore stage. These structures were more numerous and clustered into larger groups (Supplementary Fig. 1h) clearly distinguishable from other structures found in tapetal cells, especially plastids. In heterozygotes, the ultrastructure of tapetal cells was unchanged (data not shown).

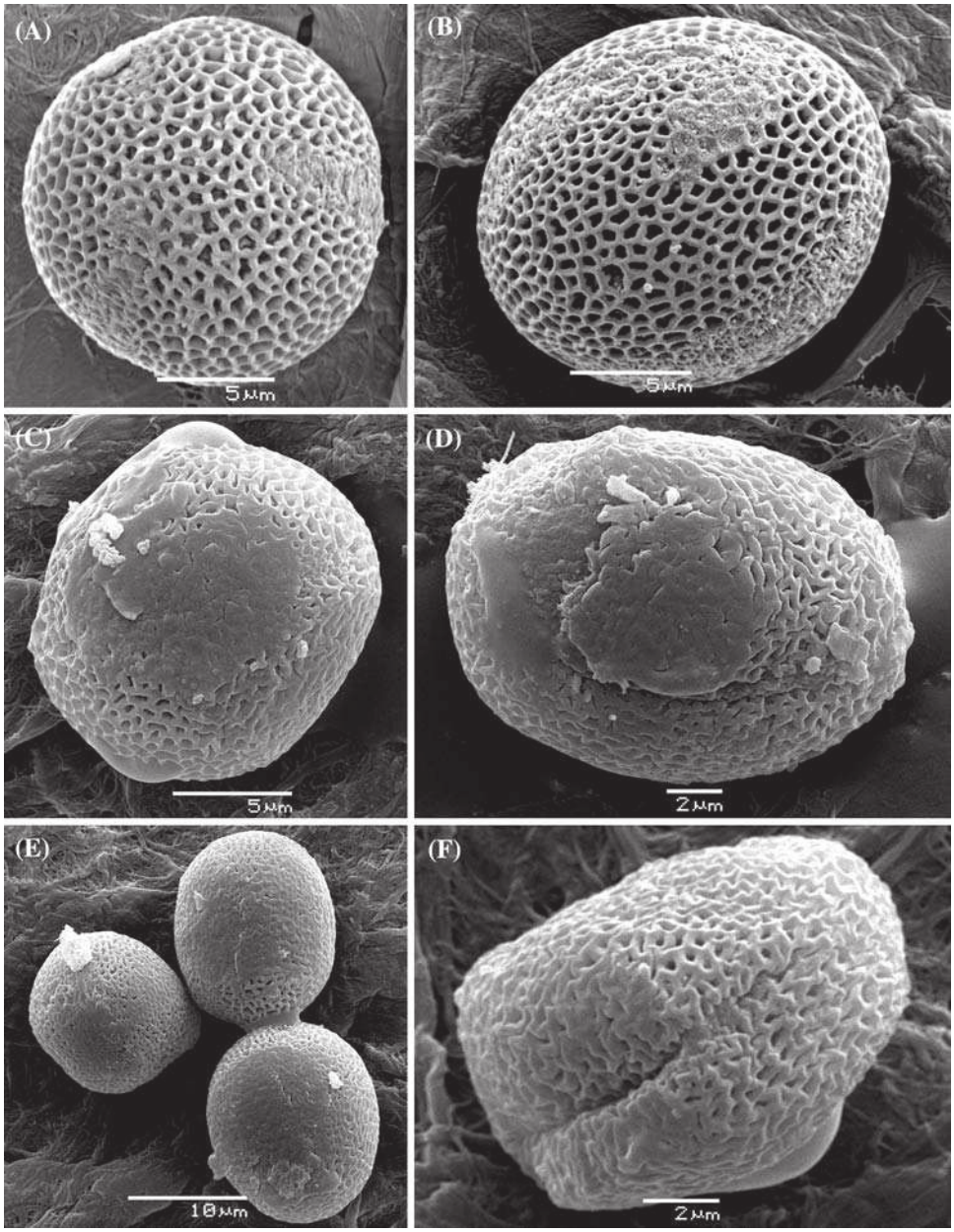

Fig. 4 Scanning electron micrographs of wild type pollen (**A**), *atbzip34* pollen complemented with At2g42380 genomic fragment (**B**) and *atbzip34* pollen grains (**C**–**F**). *atbzip34* pollen is defective in exine pattern formation (**C**–**F**) with often irregular shape (**F**). Pollen grains are frequently attached (**E**)

atbzip34 pollen shows reduced viability and progamic phase defects

Since AtbZIP34 affects early and late stages of pollen development, defects in the progamic phase were expected. First, pollen viability was calculated after FDA staining. In wild type plants over 90% of pollen was viable (91.8 ± 2.3%; $n = 412$). In *atbzip34* pollen population, this percentage was lower, 72.2 ± 4.3%; $n = 386$). To examine progamic phase defects we monitored pollen tube growth in vitro (Fig. 6A, B) and in vivo (Fig. 6C, D). Significant differences between *wt* and mutant pollen were observed in both assays. The in vitro germination rate of mutant *atbzip34* pollen was reduced by 85% compared to that of wild type pollen ($n = 300$). Moreover, mutant pollen tube growth rate was slower than that of wild type and after 10 h, mutant pollen tubes were ∼53% shorter than wild type tubes ($n = 100$). In vivo pollen tubes growth tests confirmed slower growth rate of *atbzip34* mutant pollen tubes to the embryo sac when compared to wild type (Fig. 6C, D). However, resulting differences in length were less dramatic than observed in vitro. After 7 h postpollination, the longest *atbzip34* pollen tubes only reached the ninth ovule from the base of the ovary ($l = 1{,}438 \pm 53$ µm; $n = 5$ pistils), whereas wild type pollen tubes had reached the third ovule from the base ($l = 1{,}818 \pm 65$ µm; $n = 5$ pistils).

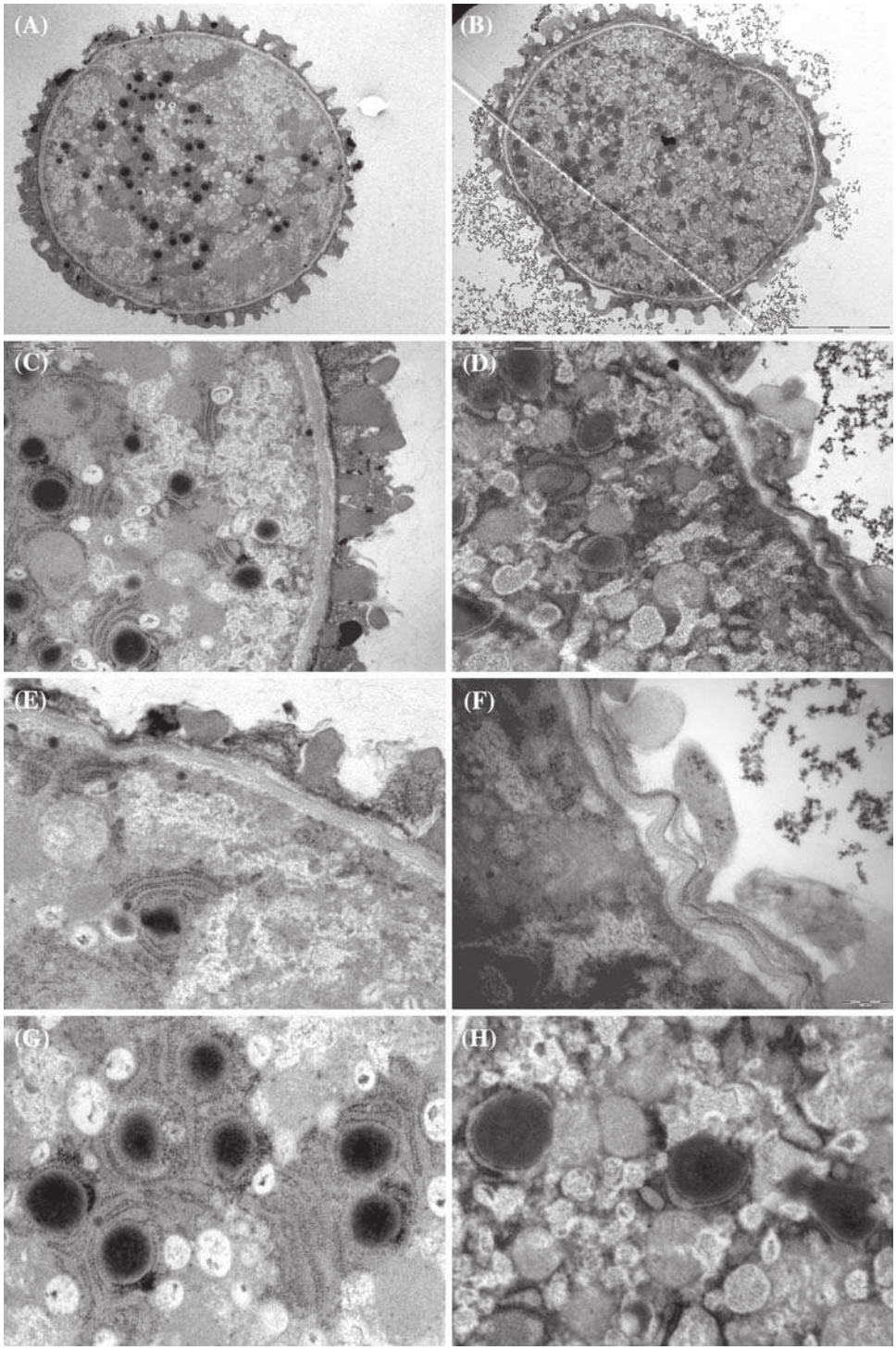

Fig. 5 Transmission electron micrographs of mature wild type (**A**, **C**, **E**, **G**) and *atbzip34* (**B**, **D**, **F**, **H**) pollen grains. *atbzip34* pollen has an irregular, wrinkled intine and exine with misplaced tecta and baculi (**D**, **F**). Mutant pollen has less developed endomembrane system and higher number of clustered lipid bodies that are surrounded by one or very rarely more layers of ER (**D**, **H**). In wild type, these lipid bodies are enclosed by several layers of ER (**E**, **G**)

atbzip34 shows gametophytic transmission defects

The transmission efficiency of mutant alleles and segregation ratio was examined. Heterozygous *atbzip34* plants were allowed to self-fertilize and seeds were sown onto kanamycin-containing plates. A non-Mendelian segregation ratio 1.87:1 (R:S) was observed among self progeny ($n = 448$) indicating reduced gametophytic transmission. Analysis of progeny from reciprocal crosses demonstrated that both gametophytes were affected. Through the male, *atbzip34* was transmitted with moderately reduced efficiency resulting in a distorted segregation ratio of 0.66:1 ($n = 186$). Through the female, the transmission of *atbzip34* was reduced further to 0.55:1 ($n = 219$). Thus

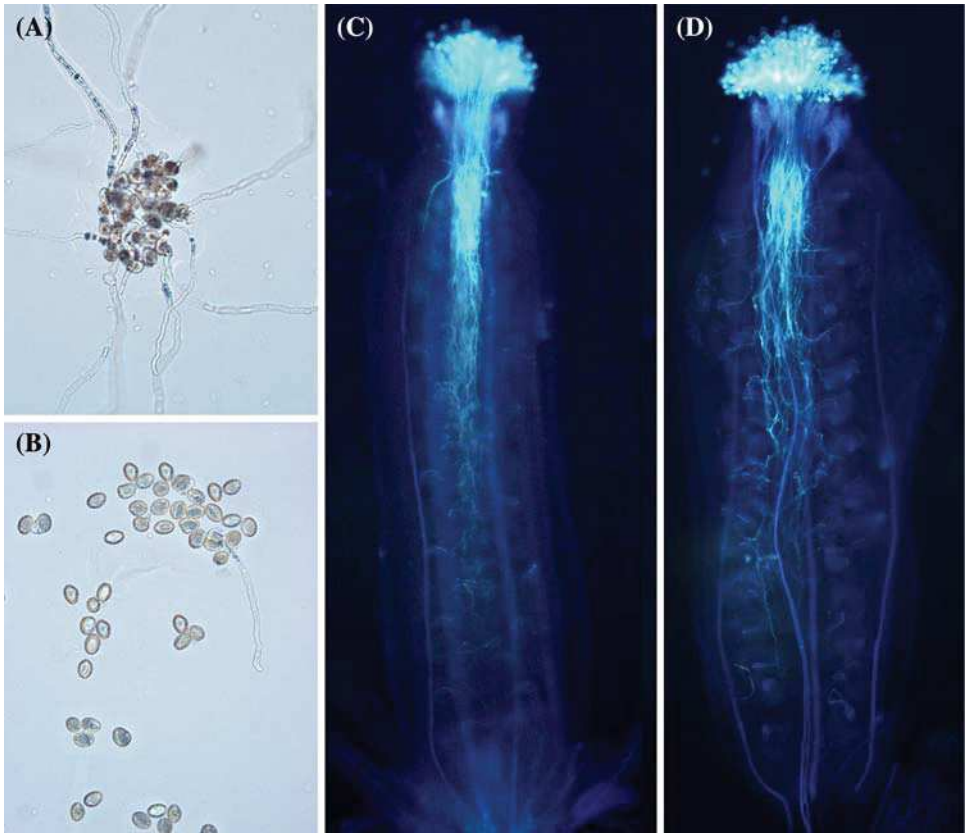

Fig. 6 Pollen tube growth tests. Wild type (**A**) and *atbzip34* (**B**) pollen tubes grown in vitro for 10 h. *atbzip34* pollen tubes were indistinguishable from wild-type pollen tubes, but there was markedly impaired germination. Wild type (**C**) and *atbzip34* (**D**) pollen tubes grown in wild type pistils. Tubes were observed 7 h after pollination

gametophytic transmission of *atbzip34* is reduced by 34% through the male and 45% through the female compared with the wild type *AtbZIP34* allele.

Complementation analysis was performed in which homozygous *atbzip34* plants were transformed with a vector containing a 3,232 bp *AtbZIP34* genomic fragment (pKGW:*AtbZIP34*). Pollen from 12 independent transformed lines was analysed by bright field and fluorescence microscopy after DAPI staining. Ten out of twelve pKGW:AtbZIP34 lines showed a reduced frequency of aberrant pollen. The percentage of normal pollen in *atbzip34* plants complemented with pKGW:*AtbZIP34* ranged between 95 and 99%, with only 1–5% of pollen exhibiting phenotypic defects characteristic of *atbzip34* pollen (Fig. 4B).

AtbZIP34 directly or indirectly affects the expression of genes involved in metabolic pathways

Some characteristics of *atbzip34* pollen analysed suggested impairment of certain metabolic pathways such as lipid metabolism and cellular transport during pollen maturation. To test this hypothesis, Affymetrix Arabidopsis ATH1 Genome Arrays were used to explore gene expression in *atbzip34* pollen in comparison with wt. Microarrays were hybridized with cRNA probes made from total RNA isolated from mature pollen of wild type and homozygous mutant *atbzip34* plants.

In *atbzip34* pollen, 813 genes were downregulated at least two-fold (Supplementary Table 3). Accordingly, another 1,379 genes were at least two-fold upregulated (Supplementary Table 4). Although nearly 70% more genes were upregulated in *atbzip34* pollen, the downregulated subset represented more distinguishable group for several reasons. First, downregulated transcripts were more highly expressed with average expression signal 863 compared to 225 in the upregulated set. Second, 760 of all downregulated genes (over 93%) had a relative expression signal over the threshold value of 100 compared to 937 genes (only 68%) in the upregulated subset. Third, the level of fold change was higher in the downregulated group reaching an average ratio of 2.71. On the contrary, the average upregulation was 2.42-fold. Finally, functional categorization of both subsets revealed specific composition of the group of downregulated genes (Fig. 7). Functional classes were defined as published previously (Honys and Twell 2004). Most significant changes were observed in these functional categories: protein synthesis (8.49% in downregulated subset to 2.94% in total pollen transcriptome; 2.89-fold change), transport (9.59 to 5.94; 1.61-fold), metabolism (19.43 to 13.05; 1.49-fold) and protein fate (5.04 to 3.92; 1.29-fold). However, the distribution of

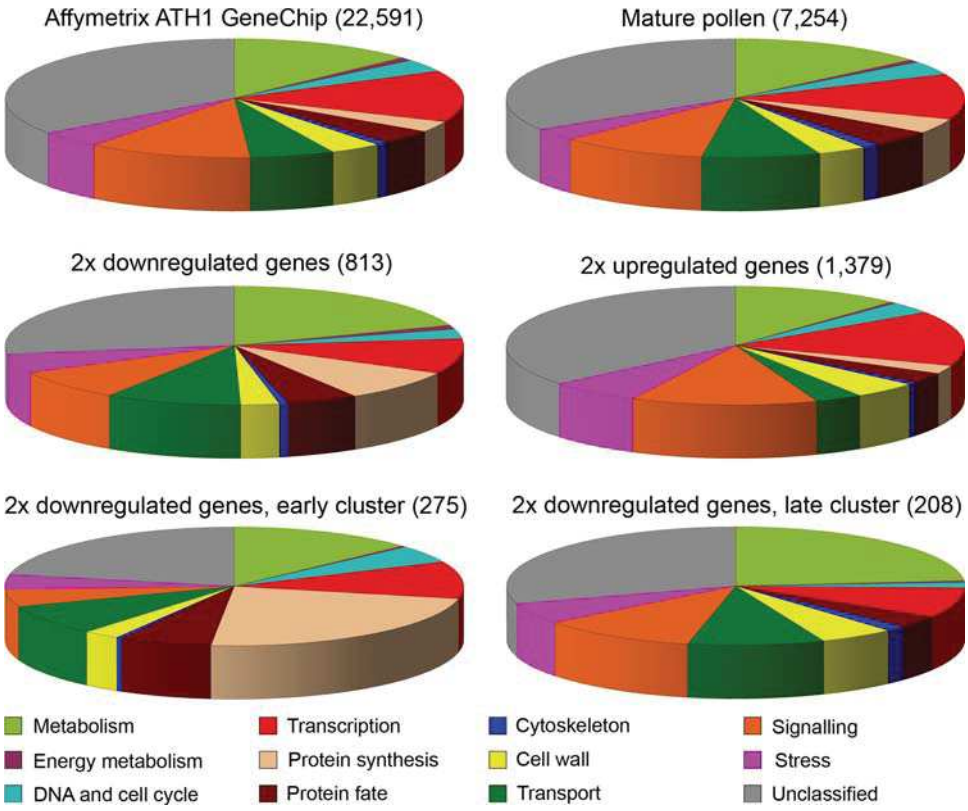

Fig. 7 Proportional representation of expressed mRNA among gene function categories. Data is presented for up and down regulated genes in *atbzip34* pollen in comparison with wt

genes among functional categories in the upregulated subset was very similar to that of the complete mature pollen transcriptome. The only exceptions were stress-related genes (6.53 to 4.59%) and those involved in cell wall metabolism (4.57 to 3.63%).

The weak activity of the *AtbZIP34* promoter was first detected in microspores and gradually increased until pollen maturity (Fig. 2H–K), so late pollen genes were more likely to be affected in its absence. This assumption was confirmed by cluster analysis of transcripts that were both two-fold down regulated, and upregulated according to their developmental expression profiles (Honys and Twell 2004). This led to the identification of three distinct groups within each geneset. These comprised genes with early, constitutive and late expression patterns. Among downregulated genes, 208 showed a late expression profile (25.6%), whereas within the upregulated set it was only 19 genes (1.4%). All affected late pollen genes are listed in Supplementary Tables 5 and 6. Gene ontology (GO) analysis of late downregulated genes revealed that several categories were affected more than others. Moreover, the GO profile of late genes is quite distinguishable from early genes (275 genes; Fig. 7E, F). All downregulated genes encoding ribosomal proteins (protein synthesis) were early. Similarly, most genes involved in cell cycle control comprised the early cluster. On the contrary, the most overrepresented categories in the late cluster were metabolism (23.6%), signalling (11.1%), transport (9.6%) and cell wall (5.3%).

The set of *AtbZIP34*-downstream genes shared several characteristic features. First, it was enriched with membrane-associated proteins as 49 out of 100 most highly downregulated genes in *atbzip34* pollen fell into this category. A fraction of these genes encoded various transporters including the ATP-binding cassette (ABC) transporter, AtABCB9 (At4g18050, 14.7X downregulated), lipid transfer proteins (At4g08670, 6.6X; At1g18280, 4.3X), mitochondrial import inner membrane translocase (At3g46560, 5.5X), lysine and histidine specific transporter (At1g67640, 5X), potassium transporter family protein (At4g19960, 4.57X), sugar transporter family protein (At4g16480, 4X), sucrose transporter (At1g71880, 3.8X), porin (At5g15090, 3.95X), cation/hydrogen exchanger (At3g17630, 3.7X), acyl carrier protein (At3g05020, 3.7X). These proteins were involved in transport of ions and various metabolites. The importance of membrane-associated transporters for male gametophyte development was already demonstrated (Bock et al. 2006; Sze et al. 2004). Moreover, there were two lipid transfer proteins and ABC transporter AtABCB9 is also likely involved in lipid transport (Martinoia et al. 2002; Verrier et al. 2008) and all

three genes were amongst those most downregulated in *atbzip34* pollen.

The complete Sec61 translocon complex was downregulated in *atbzip34* pollen. Translocons are sites of cotranslational protein translocation through ER membrane to its luminal compartment. They consist of core heterotrimeric Sec61 complexes (Sec61$\alpha\beta\gamma$) and associated proteins forming a cylindrical channel aligning with ribosomal large subunit during translocation (Beckmann et al. 1997). Selectivity of translocon function is facilitated by gating protein, luminal HSP70 chaperone BIP1, that seals its luminal side (Alder et al. 2005). In *Arabidopsis* each Sec61 subunit is encoded by three genes, whereas BIP1 by a single gene. All ten genes are expressed in male gametophyte and all but one are downregulated in *atbzip34* pollen (Fig. 8). For all subunits, the most abundant genes showed the highest level of downregulation.

Another set of proteins overrepresented among *atbzip34* pollen-downregulated genes included those involved in several steps of lipid catabolism: aspartate aminotransferase (At2g30970, 5.09X), family II extracellular lipase (At5g42170, 4.77X), malate dehydrogenase (At3g15020, 4.17X) (Kindl 1993; Pracharoenwattana et al. 2007; Teller et al. 1990; Zhou et al. 1995). All these genes were abundantly expressed in wild type pollen and significantly downregulated in *atbzip34* pollen.

We investigated potential metabolic pathways that may be controlled by AtbZIP34 factor using the MapMan visualization tool (http://gabi.rzpd.de/projects/MapMan//; Thimm et al. 2004). Most down- or up-regulated genes were scattered amongst various pathways. However, several metabolic pathways contained overrepresented down- or up-regulated genes with absolute value of ln(wt/*atbzip34*) over 1. These were transporters (Supplementary Fig. 2a) and genes involved in stress responses and development (Supplementary Fig. 2c). However, most genes with altered expression were associated with transport and cell wall-related pathways (Supplementary Fig. 2b). Although most visualized pathways contained both down- and up-regulated genes, genes involved in transport and metabolism of cell wall precursors were predominantly downregulated in *atbzip34* pollen.

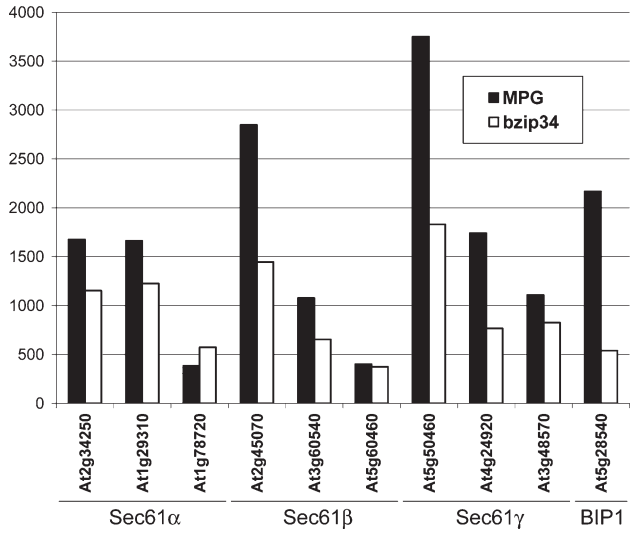

Fig. 8 Relative expression levels of nine genes encoding all Sec61 translocon subunits and gating protein AtBIP1 in wild type and *atbzip34* pollen

To verify microarray data visualised by Map Man tool, we selected several genes for quantitative RT-PCR tests. We selected the whole cluster formed by four genes from the metabolic pathway leading to the cell wall precursors, which were predominantly downregulated in *atbzip34* pollen (Supplementary Fig. 2b). This cluster was selected because it comprised two highly downregulated genes and two genes showing little or no change in expression. The selected genes encoded orthologues of UDP-glucose epimerases that are linked to the interconversion of sugar nucleotides UDP-glucose and UDP-galactose via UDP-4-hexo ketose intermediate. According to microarray data, two genes (At1g12780 and At1g63180) were downregulated and the remaining two (At4g23920 and At1g64440) showed no change (Supplementary Table 7). The expression of all four genes was verified by quantitative RT-PCR and related to the expression of the KAPP control gene (At5g19280). Expression profiles of genes putatively downregulated in bZIP microarray experiments were verified by qRT-PCR (Supplementary Table 7). Two genes, At1g12780 and At1g63180, downregulated on the microarrays were shown to be considerably downregulated also by RT-PCR. Expression of the third gene, At1g64440 was unchanged. The only exception was the At4g23920 gene that was expected to be unchanged as suggested by transcriptomic results. By quantitative RT-PCR, it was shown to be 4.4-fold downregulated in *atbzip34* pollen. This can be explained by the low expression signal (mean 135 in mature pollen, compared to 1,586 for At1g12780) and its low reliability (detection call P in only four out of nine mature pollen datasets). This low expression signal was confirmed by quantitative RT-PCR.

In addition to the above mentioned protein classes, we looked for TFs with changes in expression pattern in *atbzip34* mutant pollen. Putative TFs were defined according to MapMan. In general, TFs followed the above described scheme. Most genes were upregulated in *atbzip34* pollen (22 genes with ln(*atbzip34*/MPG) >1, Supplementary Fig. 3). However, both the relative expression signal and the ln-change were low, and only five upregulated genes had reliable detection calls in both biological replicates. On the contrary, twelve genes downregulated in *atbzip34* pollen with ln(MPG/*atbzip34*) >1 were identified. Again,

downregulated genes were more abundant; the mean relative expression signal of 22 upregulated TF genes was 166, whereas that of 12 downregulated ones was over 530.

Although downregulated TFs belonged among several gene families, there was no apparent cluster since in each family no more than one member was affected (Table 1). These families were as follows; AP2, ARR, bZIP, several zinc finger families (C2H2, C2C2-CO-like, C2C2-GATA), GeBP, MYB, NAC and general or unspecified factors. The expression of individual TF genes showed significant variation. MYB97 was the most abundant with a mean signal over 1,500. All the others had signals under 1,000 and only three reached values over 500. Moreover, MYB97 was the only downregulated TF with a late expression profile. According to microarray data, At4g26930 encoding MYB97, is also male gametophyte-specific, thus MYB97 may be controlled by AtbZIP34. The distribution of upregulated TFs was wider; they belonged to families AP2, APRR, bZIP, several zinc finger families (C2H2, C2C2-CO-like, C2C2-Dof, C3H), GeBP, MADS-box, MYB, NAC, WRKY and several general or unspecified factors. Most of these do not show reliable expression throughout male gametophyte development. Moreover, none of the reliably expressed genes are male gametophyte-specific nor have late expression profiles. The data presented seem to confirm previously published results (Jakoby et al. 2002) indicating that bZIP family TFs do not act as master regulators of TFs networks, but mainly act to regulate the expression of metabolic and structural genes.

Discussion

We have functionally characterized the role of TF AtbZIP34 in male gametophyte development. Characteristic phenotypic and genetic transmission defects provide several lines of evidence that support sporophytic and gametophytic roles for AtbZIP34 in male gametophyte development and function.

The T-DNA insertion line, SALK_18864 harbours an insertion in exon four of *AtbZIP34* gene, but *atbzip34* pollen express 3′ truncated transcripts (Fig. 1). Therefore, the corresponding truncated protein may be expressed in *atbzip34* pollen. The missing 3′-region encodes the bZIP dimerization domain and the truncated polypeptide would lack the dimerization potential of wild type protein. This T-DNA insertion was sufficient to cause transmission and phenotypic defects. However, the non-lethal nature of the mutation in *AtbZIP34* may also stem from redundancy among bZIP TFs co-expressed in pollen.

bZIP TFs possess a basic DNA binding domain adjacent to a leucine zipper region and act as homo- or heterodimers. In general, bZIP proteins do not heterodimerize promiscuously; specific interactions are preferred (Newman and Keating 2003). In *Arabidopsis*, GBF1-3 belonging to the G-group can form both homo- and heterodimers (Schindler et al. 1992), other G-group bZIP TFs AtbZIP16 and AtbZIP68, could form homodimers and heterodimers with other G-group members (Shen et al. 2008). S-group bZIP TFs can heterodimerize with C-group bZIPs (Ehlert et al. 2006; Weltmeier et al. 2009). AtbZIP43 (a member of S group bZIP TFs) can form heterodimers with members of E group (Shen et al. 2007). The basic region is relatively similar between members of groups E and I. E-group bZIPs (bZIP34 and bZIP61) and I-group bZIP51 were already shown to heterodimerize (Shen et al. 2007). Moreover, bZIP34 and bZIP61 could not form homodimers because they have a proline residue in the third heptad of their basic region distorting its α-helix structure (Shen et al. 2007). Comparative analyses of dimerization domains suggested that the most likely interactors of subfamily E AtbZIP proteins belong to subfamily I (Shen et al. 2007).

atbzip34 pollen showed characteristic phenotypic defects affecting cell wall as well as pollen ultrastructural organization (Figs. 3, 4, 5). There were misshaped and misplaced nuclei, inclusions in the cytoplasm and, most significantly, defects in cell wall patterning and endomembrane systems. Severe pollen surface defects were observed with scanning electron microscopy. These data were confirmed using transmission electron microscopy demonstrating that exine patterning is affected in *atbzip34* mutant pollen (Fig. 5F). A number of mutants have been characterized in *Arabidopsis* that show defects in exine structure and sporopollenin deposition that often lead to pollen abortion and male sterility (*dex1* (Paxson-Sowders et al. 2001), *ms2* (Aarts et al. 1997), *nef1* (Ariizumi et al. 2004), *tde1* (Ariizumi et al. 2008), *rpg1* (Guan et al. 2008)). Some of these pollen wall mutants affect callose accumulation (*calS5;* Dong et al. 2005; Nishikawa et al. 2005), or wax biosynthesis (*flp1/cer3–7;* Ariizumi et al. 2003). Mutant *atbzip34* pollen is distinguished by the characteristic wrinkled nexine and rare and deformed baculae and tecta, but does not lead to high levels of pollen abortion or male sterility. Interestingly, our transcriptomic analyses revealed that *RPG1* (ruptured pollen grain 1; At5g40620; Guan et al. 2008) is approximately four-fold downregulated in *atbzip34* pollen. Considering the expression profiles of both genes, RPG1 could represent a direct target of AtbZIP34 as the *RPG1* gene contains three copies of the core ACGT motif recognized by bZIP TFs within 1 kb of 5′ flanking sequence.

Exine pattern malformations were not the only phenotypic defects observed in *atbzip34 pollen*. Characteristic inclusions observed in the cytoplasm of *atbzip34* pollen (Fig. 3) suggested disturbance of metabolic pathways,

Table 1 Transcription factor genes with altered expression in *atbzip34* pollen

AGI	Family	Annotation	MS		BC		TC		MP1		MP2	
			Sig	DC	Sig	DC	Sig	DC	Sig	DC	Sig	DC
At5g15150	Homeobox	HAT7/HB-3	6	AA	6	AA	10	AA	4	AA	14	AAA
At3g04410	NAC	Hypothetical protein	135	PA	103	PA	157	PA	196	AA	309	AAA
At1g13370	Histone	Histone H3, putative	145	PA	125	AA	88	AA	99	AA	78	AAA
At1g52890	NAC	No apical meristem (NAM) family protein	198	AA	203	AA	125	AA	95	AA	123	AAA
At4g00940	C2C2-Dof	Dof-type zinc finger domain-containing protein	134	AA	117	AA	190	AA	276	AA	218	PAA
At3g61150	Homeobox	Homeobox-leucine zipper family protein HD-GL2-1	111	AA	100	PA	133	AA	154	AA	153	AAA
At2g41380	Unspecified	Embryo-abundant protein-related	24	AA	23	AA	58	AA	50	AA	131	AAA
At1g73870	C2C2-CO-like	Zinc finger family protein	28	AA	23	AA	36	AA	52	AA	112	PAA
At4g22950	MADS-box	MADS-box protein (AGL19)	66	PP	49	PA	64	PA	68	PA	88	AAA
At1g30650	WRKY	WRKY transcription factor	69	AA	54	AA	111	AA	102	AA	108	AAA
At1g07840	Unspecified	Leucine zipper factor-related	716	PP	607	PP	255	AA	264	AA	241	AAA
At5g06500	MADS-box	MADS-box family protein	260	AA	226	PA	121	PA	121	AA	180	PAA
At5g15310	MYB	MYB family transcription factor	65	AA	54	AA	77	AA	112	AA	131	PAA
At5g60890	MYB	MYB34	105	AA	83	AA	166	AA	209	AA	170	AAA
At5g06839	bZIP	bZIP family transcription factor	222	PA	183	PA	270	PA	355	PA	381	PAA
At1g27730	C2H2	ZAT10	129	AA	173	PP	190	AA	208	PA	244	PAA
At4g18020	APRR/GARP	APRR2/TOC2	253	AA	234	AA	276	AA	362	AA	401	AAA
At5g09240	General	Transcriptional coactivator p15 (PC4) family protein	490	PP	435	PP	383	PA	478	AA	532	AAA
At5g58890	MADS-box	MADS-box family protein	137	PP	128	PP	213	PP	232	PA	128	PAA
At3g55980	C3H	Zinc finger family protein	51	AA	72	PA	147	PP	86	AA	139	PAA
At3g17730	NAC	No apical meristem fam. protein	54	AA	51	AA	72	AA	107	AA	76	AAA
At2g25650	GeBP	DNA-binding storekeeper protein-related	15	AA	19	AA	22	AA	27	AA	59	AAA
At4g26930	MYB	MYB97	108	AA	122	PA	556	PP	1,466	PP	1,640	PPP
At1g61730	GeBP	DNA-binding storekeeper protein-related	1,924	PP	1,586	PP	330	AA	344	PA	243	PPA
At5g17580	Unspecified	Phototropic-responsive NPH3 family protein	1,303	PP	1,113	PP	170	AA	221	AA	230	AAA
At1g05290	C2C2-CO-like	Hypothetical protein	1,181	PP	1,543	PP	529	PP	204	PA	210	PPA
At1g26610	C2H2	Zinc finger family protein	1,771	PP	2,609	PP	1,737	PP	583	PP	624	PPP
At2g40670	ARR	ARR16	1,024	PP	1,605	PP	275	PP	265	PA	216	PPP
At1g48630	Unspecified	Guanine nucleotide-binding family protein	4,408	PP	3,951	PP	674	PP	822	PP	719	PPP
At5g25830	C2C2-GATA	Zinc finger family protein	895	PP	1,631	PP	1,353	PP	257	PA	375	PPA
At4g04830	General	Methionine sulfoxide reductase domain-containing protein	35	AA	41	AA	51	AA	67	AA	220	PPP
At5g05410	AP2	DREB2A	191	PP	338	PP	252	PP	240	PP	1,092	PPP
At5g44080	bZIP	bZIP family protein	445	PP	603	PP	545	PP	147	AA	337	PPA
At3g49530	NAC	No apical meristem fam. protein	228	PP	372	PP	250	AA	169	AA	847	PPP

Table 1 continued

AGI	Family	Annotation	MP3		MP4		MPG	bZIP		Fold change	
			Sig	DC	Sig	DC		Sig	DC	Down	Up
At5g15150	Homeobox	HAT7/HB-3	6	AA	42	AA	8	47	AA	0.17	6.03
At3g04410	NAC	Hypothetical protein	111	AA	188	AA	165	652	AA	0.25	3.95
At1g13370	Histone	Histone H3, putative	100	AA	129	AA	92	329	AA	0.28	3.57
At1g52890	NAC	No apical meristem (NAM) family protein	98	AA	1,279	PP	105	347	PP	0.3	3.29
At4g00940	C2C2-Dof	Dof-type zinc finger domain-containing protein	207	AA	241	AA	222	716	AA	0.31	3.23
At3g61150	Homeobox	Homeobox-leucine zipper family protein HD-GL2-1	170	AA	194	AA	159	510	AA	0.31	3.21
At2g41380	Unspecified	Embryo-abundant protein-related	138	AA	556	AA	106	340	AA	0.31	3.2
At1g73870	C2C2-CO-like	Zinc finger family protein	55	PA	208	PP	73	228	AA	0.32	3.12
At4g22950	MADS-box	MADS-box protein (AGL19)	98	PA	79	PA	88	274	AA	0.32	3.1
At1g30650	WRKY	WRKY transcription factor	166	AA	232	AA	126	388	PP	0.32	3.09
At1g07840	Unspecified	Leucine zipper factor-related	210	AA	362	AA	238	736	AA	0.32	3.09
At5g06500	MADS-box	MADS-box family protein	241	PA	149	AA	150	458	PA	0.33	3.05
At5g15310	MYB	MYB family transcription factor	149	AA	92	AA	130	398	AA	0.33	3.05
At5g60890	MYB	MYB34	172	AA	217	AA	184	551	AA	0.33	3
At5g06839	bZIP	bZIP family transcription factor	362	PP	419	PA	366	1,086	AA	0.34	2.97
At1g27730	C2H2	ZAT10	176	AA	2,331	PP	209	607	PP	0.34	2.9
At4g18020	APRR/GARP	APRR2/TOC2	354	AA	672	AA	372	1,079	AA	0.35	2.9
At5g09240	General	Transcriptional coactivator p15 (PC4) family protein	414	AA	922	PA	474	1,360	AA	0.35	2.87
At5g58890	MADS-box	MADS-box family protein	110	PP	120	AA	119	340	PP	0.35	2.85
At3g55980	C3H	Zinc finger family protein	119	AA	1,351	PP	114	320	PP	0.36	2.8
At3g17730	NAC	No apical meristem fam. protein	182	AA	145	AA	109	306	AA	0.36	2.79
At2g25650	GeBP	DNA-binding storekeeper protein-related	27	AA	71	AA	38	105	AA	0.36	2.79
At4g26930	MYB	MYB97	1,864	PP	886	PP	1,656	608	PP	2.72	0.37
At1g61730	GeBP	DNA-binding storekeeper protein-related	210	AA	356	PP	314	115	AA	2.73	0.37
At5g17580	Unspecified	Phototropic-responsive NPH3 family protein	222	AA	140	AA	224	80	AA	2.81	0.36
At1g05290	C2C2-CO-like	Hypothetical protein	70	PA	163	PP	192	65	AA	2.97	0.34
At1g26610	C2H2	Zinc finger family protein	314	PP	314	PP	404	129	PP	3.12	0.32
At2g40670	ARR	ARR16	177	AA	156	PP	183	58	AA	3.18	0.31
At1g48630	Unspecified	Guanine nucleotide-binding family protein	471	PP	974	PP	839	262	PA	3.2	0.31
At5g25830	C2C2-GATA	Zinc finger family protein	87	PA	274	PP	302	94	PA	3.21	0.31
At4g04830	General	Methionine sulfoxide reductase domain-containing protein	398	PP	280	PP	299	93	PA	3.23	0.31
At5g05410	AP2	DREB2A	1,243	PP	624	PP	986	273	PP	3.62	0.28
At5g44080	bZIP	bZIP family protein	304	AA	208	AA	283	77	AA	3.66	0.27
At3g49530	NAC	No apical meristem fam. protein	550	PP	637	PP	678	134	PA	5.06	0.2

The first three columns show AGI number, gene family and protein annotation. Following columns show mean expression value (Sig) and Detection call in all replicates (DC) of pollen developmental stages (MS, microspores; BC, bicellular pollen; TC, tricellular pollen), wild type mature pollen (MP1 (Honys and Twell 2004); MP2 (Zimmerman et al. 2005); MP3 (Pina et al. 2005); MP4 (this study); MPG (mean)) and *atbzip34* pollen (bZIP). Fold change columns show gene down- and up-regulation in *atbzip34* pollen

possibly related to cellular transport and/or lipid metabolism. This assumption was supported by transmission electron microscopy showing differences in structure of endomembrane systems and lipid bodies (Fig. 5). Lipid bodies were frequently found enclosed by an extensive network of ER especially at later developmental stages (see Murphy 2001 and references therein). The encirclement of pollen cytosolic lipid bodies by ER is proposed to prevent coalescence of lipid bodies (Piffanelli et al. 1998). This ER network persists throughout pollen release and has been proposed to facilitate the direct mobilization of the lipid-body TAGs required to support the rapid pollen tube growth (Murphy 2001; Piffanelli et al. 1998). In *atbzip34* pollen, lipid bodies were more numerous, localized in clusters close to cell surface and, most interestingly surrounded by a less dense ER network. In this respect the cytoplasm of *atbzip34* mature pollen also resembles that of immature pollen (Van Aelst et al. 1993; Yamamoto et al. 2003) that could indicate retarded pollen maturation. The observed higher frequency of unicellular and bicellular pollen as well as the smaller size of tricellular pollen supports this assumption.

It has been repeatedly demonstrated that pollen exine defects are generally of sporophytic origin, mainly caused by impaired tapetal cells development and/or function (Aarts et al. 1997; Ariizumi et al. 2003, 2004, 2008; Dong et al. 2005; Guan et al. 2008; Nishikawa et al. 2005; Paxson-Sowders et al. 2001). On the contrary, general pollen metabolism is likely to be under gametophytic control. To address these issues, developing male gametophyte and tapetum of both homozygous and heterozygous plants were observed for phenotypic defects. Light and electron microscopy observations (Figs. 3, 4, 5) confirmed the coordinated sporophytic and gametophytic modes of action already suggested by *AtbZIP34* expression pattern (Fig. 2). Exine patterning defects were shown to be under sporophytic control as indicated by the presence of defective exine in all pollen grains from *atbzip34* (-/-) plants and normal pollen shape from *atbzip34* heterozygous and wt plants. On the contrary, other metabolic defects as well as wrinkled intine were observed in around one half of pollen isolated from heterozygous plants and thus appear to be under gametophytic control. A similar mode of action can be expected for recently published AtbZIP60 that was also expressed in tapetum and male gametophyte besides number of other tissues. This membrane-bound TF was demonstrated to be involved in general ER stress response and its possible role in ER stress response function in normal development of secretory cells was suggested (Iwata et al. 2008).

To independently address the hypothesis of impaired cellular transport and metabolism, microarray analysis was performed to identify *AtbZIP34*-downstream genes. The reliability of microarray data was verified by quantitative RT-PCR of four orthologues of UDP-glucose epimerases. Treated microarray data were further analysed using Map Man to visualize metabolic pathways possibly affected by *atbzip34* mutation (Supplementary Fig. 2). Two diagrams showed relevant results, grouping transporters and genes involved in cell wall and lipid metabolism. There was apparent correlation between observed *atbzip34* pollen phenotype and microarray data supported by MapMan (Supplementary Fig. 2). First, there were no marked differential expression of other TFs (Fig. 7, Supplementary Fig. 3). This seems to confirm previously published findings of bZIP proteins mainly acting as "effector" TFs, controlling the expression of structural or metabolic genes, rather than other TFs. The only strong exception was the late pollen-expressed MYB97 gene. Although MYB97 is putatively pollen-specific, it is not a close orthologue of the MYB factor DUO1, that has an essential role in male germline development (Brownfield et al. 2009), moreover, its downregulation in *atbzip34* mutants does not support an important role in germ cell development.

On the contrary, six genes encoding proteins involved in lipid metabolism and/or transport were identified among the most highly downregulated genes. The most affected was the ABC transporter AtABCB9 that was downregulated 14.67-fold. AtABCB9 protein (synonyms AtMDR9 and AtPGP9) is a member of multidrug resistance subfamily of full ABC transporters (Sanchez-Fernandez et al. 2001a, b) and is also likely to be involved in lipid transport (Martinoia et al. 2002; Verrier et al. 2008). AtABCB9 expression was abundant and specific to the male gametophyte according to microarray data. Two seed storage/lipid transfer proteins downregulated in *atbzip34* pollen 6.6- and 4.4-fold are involved in lipid transfer from liposomes or microsomes to mitochondria and play a major role in membrane biogenesis by conveying phospholipids from their site of biogenesis to target membranes (Ohlrogge et al. 1991; Wirtz 1991). Aspartate aminotransferase (5.1-fold downregulated) is mainly involved in energy metabolism, in aspartate catabolic processes. However, besides its predominant role it was reported to be active also in fatty acid uptake in mitochondria in animal cells in a similar manner as its closely related plasma membrane fatty acid binding protein (FABPpm; Zhou et al. 1995). Moreover, aspartate aminotransferase was shown to interact with another downregulated enzyme, malate dehydrogenase (4.2-fold downregulated) in the inner mitochondrial membranes in various animal tissues (Teller et al. 1990). Apart from this, one study showed that malate dehydrogenase might be involved in plant peroxisomal fatty acid degradation (Kindl 1993) that in *Arabidopsis* seeds causes slow triacylglycerol mobilization and impaired growth (Pracharoenwattana et al. 2007). The last

affected enzyme involved in lipid catabolism was extracellular lipase that catalyses hydrolysis of triacylglycerols (Svendsen 2000).

Lipid storage, transport and catabolism were not the only affected metabolic pathways. The function of bZIP TFs RSG, RF2a and VSF-1 in vascular tissue development has been demonstrated in different species (Fukazawa et al. 2000; Ringli and Keller 1998; Yin et al. 1997). All these factors belong to group I bZIP proteins that heterodimerize with group E bZIP TFs (Shen et al. 2007). These facts together with the expression pattern of *AtbZIP34* (Fig. 2) provide indirect support for its involvement in regulation of transport tissue development and/or functions alongside its activity in gametophyte development.

Microarray data analyses further revealed that all subunits of Sec61 translocon were downregulated in *atbzip34* pollen (Fig. 8). Sec61 is an ER membrane protein translocator consisting of three subunits Sec61α, Sec61β and Sec61γ (Beckmann et al. 1997). Each subunit is encoded by three genes and all nine genes gave reliable signals in the male gametophyte. All genes but one (the least abundant) encoding all three Sec61 subunits were downregulated in *atbzip34* pollen. Moreover, gating protein AtBIP1 associated with the luminal side of Sec61 complex was downregulated to a greater extent that any Sec61 subunit. The orchestrated downregulation of almost all Sec61 subunits suggests common regulatory mechanism of Sec61 translocon synthesis in the male gametophyte. Many ribosomal proteins and proteins involved in protein posttranslational modifications were also downregulated in *atbzip34* pollen (Supplementary Table 3). Thus, the rate of protein synthesis together with protein translocation to ER and subsequent processing may be affected in *atbzip34* pollen, although its impact is not critical. Recently, a novel adenine nucleotide transporter (ER-ANT1, At5g17400) was identified in *Arabidopsis* ER (Leroch et al. 2008), but this gene is not significantly downregulated in *atbzip34* pollen. Among other phenotype defects, delayed flower bud development was observed in *er-ant1* knock-out lines. Moreover, several genes downregulated in *atbzip34* pollen were also downregulated in *er-ant1* plants (AtBIP1 (At5g28540), Sec61γ (At5g50460)). ER-ANT1 is involved in ATP/ADP antiport on ER membranes thus maintaining ATP concentration in the luminal space. Although unlikely because of coordinated downregulation of Sec61 subunits, the possibility of impaired protein translocation in *atbzip34* resulting from shortage of ATP cannot be completely ruled out.

Conclusions

Collectively our results indicate that AtbZIP34 has multiple roles in the development of gametophytic and sporophytic reproductive tissues. AtbZIP34 is the first bZIP-family TF with a demonstrated role in male gametophyte development. Characteristic phenotype and genetic transmission defects demonstrate a requirement for AtbZIP34 for correct formation of pollen cell walls. Although sporophytic control of exine patterning has been repeatedly shown in a number of mutants, analyses of *atbzip34* revealed sporophytic and gametophytic roles for AtbZIP34 in exine and intine formation. Wrinkled intine in $\sim$50% pollen of +/*atbzip34* plants, the presence of large inclusions in *atbzip34* pollen and the altered structure of ER in contact with lipid bodies indicate that AtbZIP34 is involved in gametophytic control of lipid metabolism, cellular transport and/or intine synthesis. This hypothesis is further supported by the downregulation of distinct subsets of genes. Moreover, altered cellular transport from the tapetum could also explain the defects observed in exine synthesis and cell wall patterning. The investigation of putative downstream genes including the regulator MYB97 will help to reveal new features in the cellular networks that control pollen wall development in relation to cellular transport.

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Electronic supplementary material
Below is the link to the electronic supplementary material.
Supplementary material 1 (PDF 51 kb)

Supplementary Table 2 Expression of AtbZIP transcription factors in various tissues and cell types according to aGFP database (Duplakova et al., 2007). (XLS 42 kb)

Supplementary Table 3 List of genes at least two-fold downregulated in atbzip34 pollen (XLS 322 kb) – available online

Supplementary Table 4 List of genes at least two-fold upregulated in atbzip34 pollen (XLS 500 kb) - available online

Supplementary table 5 List of genes showing late male gametophytic expression profile and at least two-fold downregulated in atbzip34 pollen (XLS 57 kb) - available online

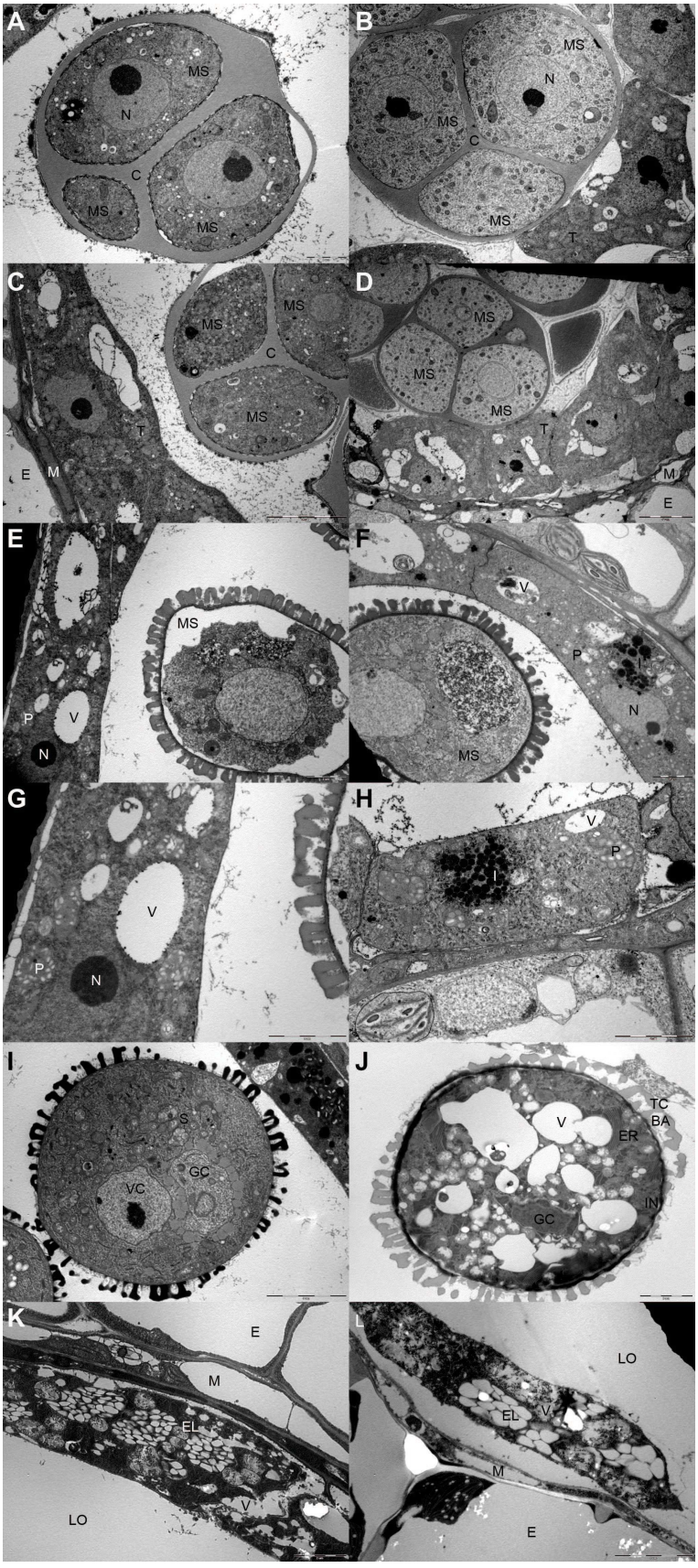
Supplementary Table 6 List of genes showing late male gametophytic expression profile and at least two-fold upregulated in atbzip34 pollen (XLS 23 kb) - available online

Supplementary material 7 (PDF 65 kb)

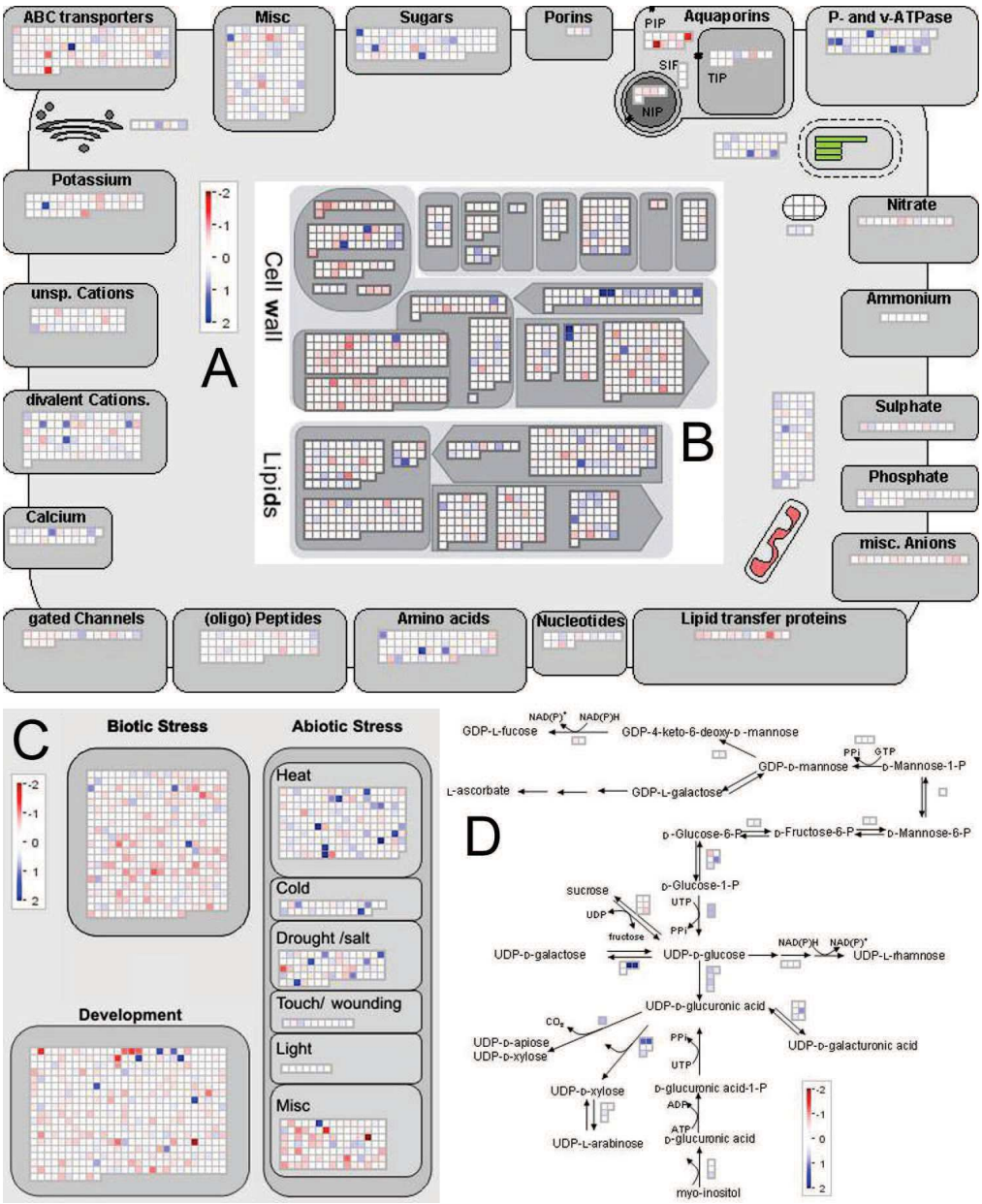
Supplementary Fig. 1 Transmission electron micrographs of cross-sections of developing male gametophyte from tetrad stage to bicellular pollen. wild type (A, C, E, G, I, K) and atbzip34 (B, D, F, H, J, L). Size bar corresponds to 2 μm (A, B, E-H, J-L) and 5 μm (C, D, I). Tetrad stage (A-D). Tetrads of haploid microspores are surrounded by callose wall and deposits of sporopollenin are visible on surface of outer callose wall in wt (A). Primexine forms characteristic undulations in wt, while the atbzip34 tetrads (B) seem to be younger with smooth plasma membrane and thinner callose wall with no sporopollenin deposits on the outher callosic wall. The structure of tapetum was similar in wt (C) and mutant (D). Uninucleate microspore stage (E-H). Microspores of wt (E) and mutant (F) looked similar with large nucleus and smooth cytoplasm. On the contrary, mutant tapetal cells (H) were less vacuolated than wt (G) and contained clusters of electron-dense granules along the locule wall. Late bicellular stage (I-L). Unlike wt (I), vegetative cell of atbzip34 bicellular pollen (J) was highly vacuolated and was enclosed in characteristic wrinkled intine On the contrary, there were no apparent ultrastructural differences in wt (K) and atbzip34 (L) tapetum; in both genotypes elaioplasts were fully developed. BA, baculae; C, callose wall; E, endothecium; EL, elaioplast; ER, endoplasmic reticulum; GC, generative cell; I, electron-dense inclusions; IN, intine; LO, anther locule; M, middle layer; MS, microspore; N, nucleus; P, plastid; S, starch; T, tapetum; TC, tectum; V, vacuole; VC vegetative cell (PDF 3107 kb)

Supplementary Fig. 2 MapMan visualization of genes with altered expression in atbzip34 pollen. General transporters (A), genes involved in development and cell wall and lipid metabolism (B), stress-response genes (C) and metabolic pathways leading to cell wall precursors (C) are shown. Logarithmic scale; downregulated genes in blue, upregulated genes in red (PDF 581 kb)

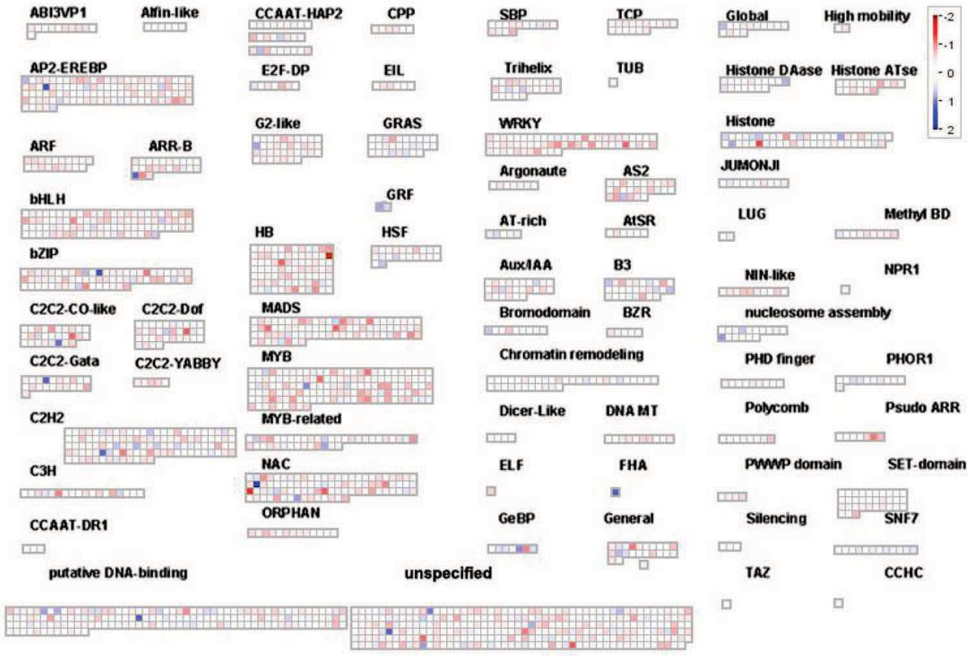
Supplementary Fig. 3 MapMan visualization of transcription factor genes with altered expression in atbzip34 pollen. Logarithmic scale; downregulated genes in blue, upregulated genes in red (PDF 245 kb)



Supplementary figure 1



Supplementary figure 2



Supplementary figure 3

Supplementary table 1. *Primers used for cloning, PCR, RT-PCR and qRT-PCR*

Primer	Primer sequence
Genotyping	
ZIP-F1	5′-CCAAGAAGCTAAAAGAGAGAAA-3′
ZIP-R1	5′-GAAGATTAGCGTCACATCACTA-3′
pROK-LB2	5′-CCCTATCTCGGGCTATTCTTTT-3′
5′ and 3′ end verification	
ZIP-F2	5′-TTCCTCGAAGCTCCAACAGT-3′
ZIP-R2	5′-CACCCTTGATCTCTGTGCTG-3′
ZIP-F3	5′-AAGGGTTAAGAGAATATTAGCAAA-3′
ZIP-R3	5′-TCTTGATGTGCGTCTTTGAAAA-3′
Complementation analysis	
ZIP-F	5′-TGTACAAAAAGCAGGCTTCCACTCGTCCACTACCCTACT-3′
ZIP-R	5′-GTACAAGAAAGCTGGGTTCCCAGAGTTTTGGTCTGCTAT-3′
Promoter analysis	
pZIP-F	5′-TGTATAGAAAAGTTGCTATGAGATTACTGTCCGACACACA-3′
pZIP-R	5′-TTTTGTACAAACTTGAACCAAAATGGCTATGTTTAACAAATGA-3′
qRT-PCR	
At4g23920-F	5′-TGGTACAGGGGTTAGAGATTACATT-5′
At4g23920-R	5′-ATACCTCACAACTGATTTTGAGATCATC-3′
At1g64440-F	5′-TGGCACTGGTGTACGAGACT3′
At1g64440-R	5′-GTGGGATTTTCATTCCAGAA-3′
At1g12780-F	5′-CTCCATATTTTGCCTTCCAGCCAAG-3′
At1g12780-R	5′-TTGCTGATCCAAAGATTGGTTGTA-3′
At1g63180-F	5′-TGGATGGTAGCGCGGTTAGAGACT-3′
At1g63180-R	5′-TAGCTTGATAGGTATTTTCTTGCCAGA-3′
KAPP-F	5′-TTGGCTAGTGACGGGTTATG-3′
KAPP-R	5′-GTTGTCCTTTGTACGCATCG-3′

Table 7. *qRT-PCR verification od microarray data.*

The first column shows AGI number. Four putative UDP-glucose-4-epimerases were tested against control KAPP gene. Following columns show mean expression value (Signal) and Detection call in all replicates (DC) of pollen developmental stages (MS, microspores; BC, bicellular pollen; TC, tricellular pollen), wild type mature pollen (MP1 (Honys and Twell 2004); MP2 (Zimmerman et al. 2004); MP3 (Pina et al. 2005) ; MP4 (this study); MPG (mean)) and *atbzip34* pollen (bZIP). Fold change columns show gene downregulation in *atbzip34* pollen according to microarray (Chip) and qRT-PCR experiment (qRT-PCR).

Gene	MS		BC		TC		MP1		MP2		MP3		MP4		MPG	bZIP		Fold change	
	Signal	DC	Signal	DC	Signal	DC	Signal	DC	Signal	DC	Signal	DC	Signal	DC		Signal	DC	Chip	qRT-PCR
At1g12780	687.47	PP	776.89	PP	1176.57	PP	3698.5	PP	1216.44	PPP	1654.65	PP	1887.8	PP	1586.3	235.49	AA	6.74	17.93 !"0.70
At1g63180	2466.06	PP	2995.89	PP	1197.6	PP	1192.41	PP	1078.42	PPP	780.16	PP	560.3	PP	1017	132.33	AA	7.69	4.21 !"0.14
At1g64440	252.57	PP	367.05	PP	532.04	PP	207.52	PP	269.76	PPP	223.5	PP	185.05	PP	205.36	212.87	PA	0.96	1.44 !"0.13
At4g23920	469.62	PP	426.68	PP	100.48	AA	140.64	PA	133.64	PPA	131.83	AA	99.25	PA	135.37	158.93	AA	0.85	4.38 ! 1.90
KAPP	367.64	PP	502.48	PP	1127.99	PP	1236.98	PP	1184.25	PPP	1309.9	PP	1722.71	PP	1243.71	1322.98	PP	0.94	0.82 !"0.005

NETWORKS OF bZIP TRANSCRIPTIONAL REGULATORS ACTING DURING MALE GAMETOPHYTE DEVELOPMENT IN ARABIDOPSIS THALIANA

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Highlight: We have identified the putative bZIP regulatory network in pollen of Arabidopsis thaliana and characterized to the greater impact confirmed member of this transcription factor network AtbZIP18.

ABSTRACT

Transcriptional control of gene expression represents an important mechanism guiding organisms through developmental processes and providing plasticity towards environmental stimuli. Because of their sessile nature, plants require an effective system of gene expression regulation for rapid response to the variation of actual environmental and developmental conditions. Transcription factors (TFs) provide such control ensuring correct gene expression in spatial and temporal manner. Our work reports the identification of putative bZIP TFs regulatory network potentially active in *Arabidopsis thaliana* pollen and highlights the important component of this pollen regulatory complex - AtbZIP18.

AtbZIP18 is highly expressed in pollen, but also possess lower promoter activities in several sporophytic tissues. This observation overlapped with the localization of AtbZIP18 protein throughout plant development and was restricted to the nucleus and cytoplasm/ER. To address the role of *AtbZIP18* in the male gametophyte, we performed phenotypic screen of the T-DNA knockout allele together with the genetic analysis and showed reduced transmission of the mutant *atbzip18* allele through male gametophyte. However, we did not observe phenotypic aberrations in the progeny of analysed T-DNA lines. We hypothesize the likely functional redundancy with other confirmed pollen-expressed bZIP genes. In contrast, overexpression lines showed the presence of aborted pollen grains occurring significantly more in heterozygous plants compared to homozygous individuals.

This study reports novel data required for the final assessment of the action of bZIP protein complexes in male germline.

Key words: bZIP, transcription factors, regulatory network, male gametophyte, Y2H, pollen development.

INTRODUCTION

The mature male gametophyte is uniquely specialized structure for the delivery of two sperm cells via pollen tube to the embryo sac within the ovary of the flower where double fertilization takes place (Bedinger, 1998). This event is essential for plant fertility and crop production. Despite this fact, molecular mechanisms underlying many aspects of this complex process, including the production of both male and female gametes, remain largely unknown (Brownfield *et al*., 2009). To uncover such mechanisms, one approach is to identify transcription factors (TFs) that are part of haploid regulatory networks. To our knowledge, reports of TF networks in pollen are very rare and limited to MADS TF regulatory network directing pollen maturation through active repression of early male gametophytic program (Verelst *et al*., 2007). Based on our previous characterization of the dynamics of Arabidopsis and tobacco male gametophyte transcriptomes (Honys and Twell, 2004; Hafidh *et al*., 2012*a, b*; Bokvaj *et al*., 2015), we have selected several candidate TFs belonging to the AtbZIP family for further studies.

Dimeric basic leucine zipper (bZIP) factors constitute an important class of predominantly enhancer-type transcription factors. They are involved in many crucial processes across eukaryotic organisms (Deppman *et al.*, 2006). In plants, they were shown to be employed in many important regulatory pathways involved in development including seed maturation (Alonso *et al*., 2009), flowering (Abe *et al.,* 2005), pollen development (Iven *et al.,* 2010; Gibalová *et al*., 2009), senescence (Smykowski *et al*., 2010), responses to various environmental cues such as unfolded protein response (Liu *et al*., 2007; Iwata *et al*., 2008), abiotic stress signaling (Fujita *et al*., 2005) and energy metabolism (Baena-González *et al*., 2007). In many eukaryotic TF gene families, proteins require a physical interaction between identical or different protein molecules within the same family to form a functional dimer binding DNA (Amoutzias *et al*., 2008). Dimerization represents one major way of creating a large repertoire of regulatory responses without multiplication of TF genes, as organisms increase in complexity (Amoutzias *et al*., 2007). Deppmann *et al.* (2006) pointed out that bZIP domains are indeed stereotyped, however, at the same time they influence a broad range of functions. The explanation has to do with bZIP TFs dimerization and DNA binding preferences as well as their transactivation and/or repression properties. The formation of bZIP homo- or heterodimers offers a tremendous

combinatorial flexibility to a regulatory system (Naar *et al*., 2001; Weltmeier *et al*., 2006). Arabidopsis bZIP network consists of 67 members, which in theory can generate 175 possible dimeric combinations (Deppmann *et al*., 2004). Regulation of the dimer formation is achieved by protein affinity, specificity and local protein concentration (Deppmann *et al.*, 2006). Taken together, the evidence of the importance of bZIP TFs in a wide range of cellular functions in plants is broad. So far, several interaction studies between bZIP family proteins have been conducted (Shen *et al*., 2007; Shen *et al*., 2008; Strathmann *et al*., 2001; Alonso et al., 2009; Weltmeier et al., 2008; Dietrich *et al*., 2011; Ehlert *et al*., 2006), however, the information about bZIP networks in male gametophyte is very limited. The only published example demonstrated the functional cooperation of several bZIP TFs during pollen development in tobacco (Iven *et al*., 2010). Therefore we aimed to extend our knowledge about the bZIP network in pollen and to shed more light onto genomic plasticity and transcriptional control in male gametophyte.

Previously, we have functionally characterized AtbZIP34 protein (Gibalová *et al*., 2009). Its expression patterns together with the phenotypic defects of *atbzip34* mutants suggested a complex sporophytic and gametophytic mode of action during late stages of male reproductive development. However, the pollen-enriched expression of other bZIP-family transcription factors together with the mild phenotype defects of *atbzip34* pollen suggested the involvement of other bZIP transcription factors acting redundantly during male gametophyte development. Here, we have aimed to uncover the bZIP regulatory network in Arabidopsis pollen using Y2H assay and to further investigate the importance of AtbZIP34-binding partner, AtbZIP18, for male gametophyte development.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia-0 plants were grown in controlled environment cabinets (Phytotrons; Conviron, Winnipeg, Canada) at 21°C under illumination of 150 µmol m^{-2} s^{-1} with a 16h photoperiod. Seeds of SALK 111120 T-DNA insertion line obtained from NASC (The European Arabidopsis Stock Centre) were sown on Jiffy 7 soil pellets (Jiffy International AS, Kristiansand, Norway) due to silenced Kanamycin resistance. Plants were subjected to genotyping using gene-specific and insert-specific

primers (Table S1). Sequencing revealed T-DNA insertion in the 5'UTR at the position -266 nt upstream of the ATG start codon. Genomic DNA was isolated using CTAB method modified from Weigel and Glazebrook (2002).

Transgenic plants (10 days-old and 6 days-old ethiolated seedlings, whole inflorescences and siliques) harboring *AtbZIP18* promoter fused to GUS reporter gene were incubated in GUS buffer (0.1 M phosphate buffer, pH 7.0; 10 mM EDTA, pH 8.0; 0.1% triton X-100 supplemented with 1 mM X-glcA and 1 mM ferricyanide) at 37°C for 48h. Samples were analyzed using bright field (BF) microscopy.

Genetic analysis of SALK 111120 T-DNA line

Transmission efficiency of mutant *atbzip18* allele through male and female gametophytes was determined by genotyping of self-fertilized progeny and progeny of reciprocal test crosses. Primers for genotyping were used as a combination of gene specific primers for wt allele and left border SALK T-DNA insertion primer (Table S1).

DNA constructs

In order to reveal promoter activity of *AtbZIP18* during development in sporophytic and gametophytic tissues, 978 bp promoter region was PCR-amplified and cloned into Gateway-compatible pENTR-D/TOPO entry vector (Invitrogen, Carlsbad, CA) and further into pKGWFS7,0 (Karimi *et al.*, 2002; http://www.psb.ugent.be/gateway) expression vector.

Localization of AtbZIP18 protein was studied using transgenic lines expressing the complete genomic sequence of *AtbZIP18* fused to GFP. Coding region of *AtbZIP18* gene was PCR-amplified from genomic DNA and cloned into pENTR-D/TOPO (Invitrogen) vector and consequently into pB7FWG,0 (Karimi *et al.*, 2002; http://www.psb.ugent.be/gateway) expression vector.

For protein localization, coding sequence of *AtbZIP18* was PCR-amplified from pollen cDNA and cloned into pDONR221 entry vector (Invitrogen) and pGWB5 (C-terminal GFP) and pGWB6 (N-terminal GFP fusion) expression vectors (Nakagawa *et al.*, 2007). AtbZIP18 was co-localized with the ER marker (ER-rk CD3-959, http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2007.03212.x/full) fused to mCherry.

The overexpression study was performed on transgenic lines expressing AtbZIP18 specifically in pollen. Coding sequence of *AtbZIP18* including stop codon was cloned into pHLat52-7GW7 vector harbouring pollen-specific promoter LAT52 (Grant-Downton *et al*., 2013).

Expression vectors for Yeast two Hybrid (Y2H) assay were prepared by PCR-amplification of At3g10800, At1g42990, At3g58120, At1g06850, At2g42380, At2g40620 CDS fragments into pDONR221 entry clone in full length, as well as versions lacking N-terminal domain (ZIPΔN). Verified entry clones were subsequently cloned into pDEST32 and pDEST22 expression vectors (Invitrogen). C-terminal versions were cloned as follows: bZIP25ΔN$_{227-403}$, bZIP28ΔN $_{161-298}$, bZIP60ΔN $_{138-208}$, bZIP61ΔN $_{200-329}$, bZIP52ΔN $_{149-337}$, bZIP34ΔN $_{175-321}$, bZIP18ΔN $_{146-367}$. All expression clones were transformed into yeast strain AH109 using PEG based transformation, according to Matchmaker Gal4 Two hybrid system3 manual (Clontech, Palo Alto, CA). All clones were verified by restriction analysis and sequencing.

Plant transformation

Expression clones for promoter activity, protein localization and overexpression were transformed into *Agrobacterium tumefaciens*, strain GV3101 and consecutively into *Arabidopsis thaliana* plants using floral dip method (Clough and Benth, 1998). Transformants were selected on ½ MS medium (0.66 g Murashige and Skoog basal medium, 3 g sucrose, 30 mg Myo-inositol, 150 mg MES (2-(N morpholino) ethanesulfonic acid), 0.8% agar, pH 5.7 with KOH) containing the appropriate antibiotic selection.

Transient assay was performed as follows: expression clones were transformed into Agrobacterium strain GV3101 and incubated in YEB media containing the antibiotic selection at 28°C and 221 rpm. Bacterial culture was pelleted after overnight cultivation and rinsed twice with an infiltration media (10mM MES, 10mM $MgCl_2$, 200µM Acetosyringon – 3,5-dimethoxy-4-hydroxy-acetophenone). Finally, bacterial pellet was resuspended in the infiltration media to an OD_{600} =0.1 and the mixture was incubated at room temperature in the dark for 3h. Bacterial suspension was then infiltrated into abaxial epidermis of tobacco leaves using a syringe. Plants were grown at normal conditions for 36h and infiltrated leaf discs were subjected to confocal laser scanning microscopy.

Microscopy

Pollen for phenotype analysis of individual transgenic lines was collected from freshly opened mature flowers into DAPI (4'-6'-diamino-phenylindole) solution according to Park *et al*., 1998 and observed by Nikon Eclipse TE 2000-E inverted microscope. Fluorescence microscopy was applied to reveal possible cell-division defects and male germ unit disorganization and BF microscopy was used to explore pollen morphology. For transient assay, *Nicotiana benthamiana* transformed leaf discs were observed using Zeiss LSM 5 DUO confocal laser scanning microscope. Fluorescence and confocal laser scan microscopy were used for the observation of transgenic lines harboring AtbZIP18 genomic sequence fused to GFP.

Statistical evaluation

Statistical evaluation (the percentage of seed gaps in siliques of SALK_111120 and Col-0 plants was performed using Number Cruncher Statistical System (NCSS software, Kaysville, UT). Statistical significance was analyzed by non-parametric Kruskal-Wallis test. Statistical evaluation of the transmission efficiency of progeny arisen from reciprocal test crosses was performed by Chi-squared test using MS Excell 2010 (Microsoft Corp., Redmont, WA). A P value<0.05 was considered statistically significant.

qPCR and RT-PCR

Pollen, stem, leaf and inflorescence RNA was isolated from Col-0 and a*tbzip18/-* homozygous plants as described in Honys and Twell (2003). RNA in total amount of 1 500 µg was DNase-treated (Promega, Madison, WI) and subsequently reverse-transcribed using ImProm ImProm-II Reverse Transcription System (Promega). For PCR amplifications, 1µL of 20x diluted cDNA was used. Quantitative real-time PCR was carried out on a Light-Cycler 480, (Roche Applied Science, Mannheim, Germany) using GoTaq® qPCR Master Mix (Promega). The reaction was performed in 96-well plastic plate (Roche). Real-time PCR data were collected on the light cycler with following cycling conditions: 5 min of initial denaturation at 95°C, then 45 cycles of 10s at 95°C, 10s at 58°C and 15s at 72°C. C_p values were normalized with the reference gene eEF1α (eukaryotic elongation factor 1α 4, At5g60390). The ratio of the relative amount of the target and reference gene was calculated as follows: E_R^{CpR}/E_T^{CpT} (ET, E R: efficiency for target or reference gene qRT PCR assay; CpT, CpR: a crossing point for target or reference genes).

Yeast two Hybrid assay

Small-scale yeast transformation was performed according to Matchmaker Gal4 Two Hybrid System3 manual (Clontech) based on PEG/Lithium acetate using yeast strain AH109 [MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS}$-$GAL1_{TATA}$-HIS3,$GAL2_{UAS}$-$GAL2_{TATA}$-ADE2,URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ]. All bZIP genes were first transformed as full-length versions (bZIPs in pDEST32-baits) together with pDEST22 empty prey. In auto-activation test all full-length baits promoted self-activation of reporter genes. For that reason NΔ versions, negative for self-activation were used together with the respective preys to include all combinations. Yeast cells were grown on –LEU-TRP selection media and interaction were screened on –LEU-TRP-ADE; -LEU-TRP-HIS and –LEU-TRP-ADE-HIS selections. Single colonies were resuspended in 1mL of water and OD_{600} was adjusted to 0.5 for all interactions tested. Interactions were dropped out on 3 types of selection media and on –LEU-TRP as a growth control and incubated at 28°C. Transformations and interaction tests were repeated 4 times.

1. RESULTSSELECTION OF AtbZIP CANDIDATES FOR DIMER FORMATION

To establish the bZIP regulatory network active in mature male gametophyte, the crucial step was to select the candidate genes for dimerization assays. There are several major mechanisms limiting the dimerization potential of individual transcription factors: protein localization and local concentration, their posttranslational modifications, binding to DNA, intracellular transport as well as their structure. We applied a combination of two selection criteria: AtbZIP gene family co-expression data and the composition of dimerization domains, namely the presence of specific amino acids (aa) in leucine domains (LD) favourable for dimerization (Deppmann *et al*., 2004). First, we have compared the expression profiles of 75 *AtbZIP* genes, in the publicly available databases: Genevestigator, (www.genevestigator.ethz.ch); Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi); Arabidopsis Gene Family Profiler (http://agfp.ueb.cas.cz/, Dupľáková *et al*., 2007) and selected only those candidates having the mean expression signal in pollen over 400 (for each bZIP gene, Affymetrix Arabidpsis ATH1GeneChip). Finally, 17 genes were selected and highlighted in green boxes (Fig.1 A).

To narrow down the number of candidate genes and to increase the specificity of the putative bZIP network, we have analysed the amino acids composition of leucine dimerization domains. For this, we adopted the method of Deppmann *et al*. (2004) who predicted the dimerization potential of 67 AtbZIP proteins out of 75 members of the AtbZIP family. The authors excluded eight TFs because these proteins did not meet their selection criteria. However, two out of eight excluded bZIP factors are present in our study, AtbZIP34 (At2g42380) and AtbZIP61 (At3g58120), both belonging to the same subgroup E.

The bZIP domain consists of two structural features located on contiguous α helices. These contain basic region with nuclear localization signal and a heptad repeat of leucines or similar bulky hydrophobic amino acids positioned exactly nine amino acids towards the C-terminus creating amphiphatic helix (Jakoby *et al*., 2002). Based on multiple amino-acid alignment of 17 AtbZIP proteins, we have identified the basic region and adjacent leucine domains for each candidate protein (Fig.1 B). Positions in every leucine heptade were marked as "**g a b c d e f",** where positions "**a e g"** represented amino acid residues determining the attraction or repulsion of the two proteins. Charged amino acids in position "**a"** inhibited homodimer formation, while lysine at the same position was

favourable for heterodimer formation. Charged amino-acids in positions **"g e"** allowed the electrostatic attraction of α helices (adopted from Deppmann *et al.*, 2004). Therefore, formation of electrostatic interaction between R and E localized at positions **"g e",** and/or the presence of N at position **"a a"** enhance the probability of dimerization. Based on this analysis we hypothesize that interaction partners of of AtbZIP34 in pollen are AtbZIP18 and AtbZIP52 belonging to group I. We can also assume formation of heterodimer between AtbZIP18 with AtbZIP61 and AtbZIP18 forming homodimers. These *in silico* predictions are also corroborated by previously published dimerization results of bZIP family in Arabidopsis (Shen at al. 2007).

Finally, we have selected eight candidate proteins for the dimerization study: AtbZIP1 (At5g49450; group S), AtbZIP18 (At2g40620; group I), AtbZIP25 (At3g54620; group C), AtbZIP28 (At3g10800; group B), AtbZIP34 (At2g42380, group E), AtbZIP52 (At1g06850; group I), AtbZIP60 (At1g42990; group S) and AtbZIP61 (At3g58120; group E). As the putative interactions among six of these proteins have been reported by Deppmann *et al.* (2004) *in silico*, we have focused on two originally excluded genes using Deppmann analysis. We found out, that the heterodimerization was favourable between bZIP34/bZIP52, bZIP18/bZIP61 and bZIP61/bZIP52. On the contrary, the interaction between bZIP34 and bZIP61, possessing proline residue in the third leucine zipper domain, resulted in repulsion of the two proteins. However, when proline was replaced by alanine, bZIP34 and bZIP61 could interact (Shen *et al*., 2007).

1.1 AtbZIP NETWORK CANDIDATES ARE WIDELY EXPRESSED IN *ARABIDOPSIS*

The gene expression patterns obtained from the transcriptomic datasets were verified by RT-PCR for all eight selected genes among sporophytic (root, stem leaf) and reproductive tissues (mature pollen and inflorescences) indicating their putative regulatory function in this specific haploid cell lineage (Fig. 2). Semi-quantitative RT-PCR confirmed the expression profiles observed in the microarray data showing the strongest signal in mature pollen for *AtbZIP18*, followed by *AtbZIP52* and *AtbZIP34*. The expression patterns of selected bZIP TFs were indeed not tissue specific, as it is known also for other genes in

the bZIP family. Specificity of their function lies rather in post-translational mechanisms and protein dimerization, amplifying the effect of alternative splicing.

1.2 SEVERAL POLLEN EXPRESSED AtbZIP TFs ARE DIMERIZING IN Y2H ASSAY

From our preliminary results, eight candidate genes were selected for yeast-two-hybrid (Y2H) assay: *AtbZIP1, AtbZIP18, AtbZIP25, AtbZIP28, AtbZIP34, AtbZIP52, AtbZIP60, and AtbZIP61.* The candidates were cloned as full-length coding sequences (CDS) into bait and prey Y2H vectors using Gal4 system**.** Due to high probability of self-activation of transcription factors in the Y2H system, the auto-activation test was performed before the actual protein-protein interaction tests. All tested Gal4 DNA-binding domain (DBD) fusion proteins were activating the transcription of selection marker genes without the presence of activation domain (AD) fusion protein. For that reason, expression clones lacking the auto-activation domains were prepared. Individual protein domains were identified using SMART database (http://smart.embl-heidelberg.de). Accordingly, we have cloned C–terminal fragments of candidate proteins starting several amino acids upstream of the beginning of basic region leucine zipper domain (BRLZ). After the auto-activation test, all N-flanking expression clones, except three (bZIP1, bZIP25, bZIP52), didn't show any auto-activation potential and therefore were suitable for direct interaction tests. bZIP1 and bZIP25 were excluded due the impossibility to design their further truncated forms without avoiding part of the BRLZ domain important for dimerization. In case of bZIP52, we prepared another even shorter protein deletion fragment and tested its self-activation potential. Despite this, the second truncated form was driving the expression of histidine and adenine in the absence of AD fusion protein. We attempted the titration of yeast growth on –W-L-H media at the increasing concentration of 3-Amino Triazole (3-AT). Despite of the higher concentration of 3-AT in the -W-L-H media (up to 100 mM), we could still detect yeast growth 5 days post inoculation. Nevertheless, we kept this highly pollen-expressed TF in our Y2H assay and considered only those interactions where bZIP52 was used as a prey. From the spotting of individual pairs of bait and prey colonies with the adjusted OD_{600} to 0.1, we identified three homodimerization events: bZIP18/bZIP18; bZIP28/bZIP28 and bZIP60/bZIP60. Heterodimerization occurred between bZIP61/bZIP18;

bZIP34/bZIP18; bZIP28/bZIP60 in reciprocal manner and proteins bZIP61, bZIP34 and bZIP18 were positive for the interaction with bZIP52 (Fig. 3A, B). These results are in agreement with our *in silico* prediction for dimerization (according to Deppmann *et al*., 2004).

2. FUNCTIONAL CHARACTERIZATION OF AtbZIP18

2.1 CHARACTERIZATION OF AtbZIP18 T-DNA INSERTIONAL MUTANT

To further confirm the Y2H data, we characterized the most abundantly expressed candidate gene, *AtbZIP18*. Moreover, the previously characterized transcription factor *AtbZIP34* showed reproducible but not severe phenotypic defects together with incomplete penetrance of the mutant allele through both gametophytes. Therefore, the functional redundancy of these two physically interacting transcription factors was presumptive.

The reverse genetic approach was applied to characterize the *AtbZIP18* gene (At2g40620). The appropriate T-DNA insertion line SALK_111120 was used and the T-DNA insertion was confirmed in 5' UTR, at the position -266 nt upstream of the ATG start codon (Figure S 1A). The absence of the *AtbZIP18* transcript was verified in mature pollen cDNA by quantitative RT-PCR (Figure S 1B) confirming that SALK_111120 line represents a knockout allele of *AtbZIP18.* Our aim, supported by the *AtbZIP18* abundant expression in mature pollen, was to uncover the involvement of *AtbZIP18* in male gametophyte and therefore we focused on this cellular lineage in the phenotypic screen. We have analysed mature pollen isolated from 40 *atbzip18* plants covering T1, T2 and T3 generations. Mature pollen was analysed by bright field and fluorescence microscopy to evaluate the morphological appearance of pollen grains and the position and number of nuclei (Reňák *et al*., 2012). Our results showed no significant phenotype changes in pollen isolated from *atbzip18/-* plants when compared to the wild type. Consequently, we have focused on the events following the progamic phase of the male gametophyte development and assessed the frequency of seed gaps in siliques of *atbzip18/-* homozygous plants that reached 4 ± 6% (mean ± SD; n=45) representing slight but significant increase (P-value 0,02) when compared to the frequency of abolished seed set in wild type plants (1 ± 2%; n=35).

As Mendelian segregation ratio distortion is a good indicator of transmission defects and gametophytic gene function (Lalanne *et al*., 2004), transmission efficiency of the mutant allele through both gametophytes was assessed as well as the analysis of selfing progeny. Reciprocal test crosses showed an apparent decrease of the mutant allele transmission through the male gametophyte by 18%, while the transmission through the female gametophyte remained unaffected. After self-pollination, the progeny of heterozygous *Atbzip18* plants showed 1: 1,51 : 1,27 ratio diverging from Mendelian ratio 1: 2: 1, further supporting the reduced gametophytic transmission (Tab. 1.).

2.2 AtbZIP18 IS WIDELY EXPRESSED IN GAMETOPHYTIC AND SPOROPHYTIC TISSUES

To obtain deeper insight into the *AtbZIP18* expression pattern, we extended our semi-quantitative RT-PCR analysis and showed the presence of AtbZIP18 mRNA in four stages of pollen development and four sporophytic tissues (Fig. 4A). Furthermore, we have observed the activity of GUS reporter gene under the *AtbZIP18* promoter in a broad range of gametophytic and sporophytic tissues (Fig. 4B-D). In the male gametophyte, a weak GUS signal was observed at the microspore stage graduating towards pollen maturity (Fig. 4B, I-III). In inflorescences, the GUS signal was present in young buds with the localized activities of the *AtbZIP18* promoter in tapetum and sepals (Fig. 4C, I, II, IV). In mature flowers, we observed GUS signal in carpels, petals and anther filaments (Fig. 4C, IV). In the sporophyte, GUS activity was detected in distal parts of the cotyledons (Fig. 4D, I, II) and later in vascular tissues of five days old seedlings. In true leaves, the *AtbZIP18* promoter activity was detected in vasculature and in areas surrounding hydatodes (Fig. 4D, III). Stems and roots of normally grown and etiolated seedlings also showed GUS signal in vascular tissues (Fig. 4D, IV, VI). In siliques, we observed specific GUS staining only in proximal and distal parts (Fig. 4D, V). Taken together, analysis of *AtbZIP18* expression pattern by three independent approaches corroborated our results demonstrating a wider expression profile of *AtbZIP18* without apparent tissue specificity. These results emphasize what has been previously reported on bZIPs function to be modulated post-translationally and through protein-protein interaction rather than at the transcriptional level.

2.3 AtbZIP18-GFP FUSION PROTEIN IS LOCALIZED IN TWO COMPARTEMENTS

All Arabidopsis bZIP genes were annotated as transcription factors containing DNA-binding domain and their localization in the nucleus was therefore prerequisite. AtbZIP18 protein localization was verified by transient expression of GFP N- and C-terminally fused to *AtbZIP18* coding sequence. Localization patterns were comparable for both fusion proteins (Fig. 5). We observed the AtbZIP18 protein localized in the perinuclear region (Fig. 5A-C) fully co-localizing with the ER marker and a portion of the fusion protein in the cytoplasm, partially or fully associated with the endoplasmic reticulum (ER; Fig. 5D-F). As a control, we tested localization of free GFP co-infiltrated with the ER-marker. We did not detect such co-localization in all observed cells (Figure S4).

We obtained independent verification of AtbZIP18 localization from stable transgenic lines expressing *AtbZIP18* genomic sequence fused to GFP. In the male gametophyte, GFP signal was not detected in microspores (Fig. 6A) but there was a weak GFP signal in bicellular pollen (Fig. 6B) that significantly increased in mature pollen grains (Fig. 6C) and pollen tubes (Fig. 6D). After fertilization, GFP signal was observed in whole mature embryo restricted to the nuclei (Fig. 6 right panel). In shoots of 6-days old seedlings, the GFP signal was localized in cotyledons and guard cells nuclei (Fig. 7A), later also in the true leaves (Fig. 7B), stems (Fig. 7C) and nuclei of the trichomes (Fig. 7D). Further analysis of roots showed the GFP signal in primary (Fig. 7E) and secondary root primordia (Fig. 7F). GFP expression was observed further at the elongation zone (Fig. 7G) and in the nuclei at the root tip (Fig. 7H). Apart of the predominant nuclear localization, we occasionally detected the AtbZIP18-GFP protein in the cytoplasm (Fig. 6 and 7- zoomed windows).

2.4 OVEREXPRESSION OF AtbZIP18

As the SALK_111120 knockout allele didn't show severe phenotypic aberrations in mature pollen, we explored the *AtbZIP18* biological function by means of overexpression to help to identify pathways that might remain undetected by traditional los-of-function

analysis (Prelich, 2012). Therefore, we have examined OEx lines driving the expression of *AtbZIP18* specifically in a vegetative cell of mature pollen by strong pollen-specific LAT52 promoter. Wild type Col-0 plants were transformed with the LAT52::AtbZIP18 construct and microscopic analysis was performed on pollen covering T1 and T2 generations. Our observation showed an increased proportion of aborted pollen grains, the only phenotype category. After Agrobacterium transformation we obtained 18% of antibiotic-resistant plants (T1) showing the presence of aborted pollen reaching 20 ± 3% (mean ± SD) on average (n=3). To verify the stability and genetic identity of individuals with the observed phenotype, we have selected plants harbouring disturbed pollen grains and analysed the progeny of T2 generation after selfing. As a control we sown wt seeds as well as seeds of T1 plants showing no phenotype. Seeds from six T1 plants in total were sown individually and 15 segregating plants from each parent were selected for microscopic observation. Our results showed the reoccurrence of the aborted pollen in two out of three parent T1 plants, counting 23 ± 4% on average (n=15) among heterozygous plants, 6 ± 10% in average (n=15) among homozygous plants and absence of the phenotype in the control set of plants (n=60). To further study the regulatory role of AtbZIP18 together with its binding partner AtbZIP34, we have performed overexpression analysis of *AtbZIP34* in the same way as for *AtbZIP18*. We have scored the number of aborted pollen grains of LAT52:AtbZIP34 plants; it occurred in 26% of T1 plants (n=7), where 22 ± 4% aborted pollen were observed. In T2 generation, 26 ± 4% on average (n=16) of aborted pollen grains of heterozygous plants and 1 ± 1% on average (n=13) of disturbed pollen of homozygous plants were observed.

All together, these results show a significant incidence of pollen abortion in heterozygous individuals and a decrease of phenotypic defects of pollen isolated from homozygous plants of *AtbZIP34* and *AtbZIP18* OEx lines.

DISCUSSION

IDENTIFICATION OF PUTATIVE bZIP NETWORK IN POLLEN

We have explored the transcriptional regulatory mechanisms active during male gametophyte development focusing on late pollen developmental program, to better

understand how the haploid cell lineage is established and which regulatory mechanisms are participating in reproductive tissues. Up to date, few examples of complex regulatory networks have been identified, underlying certain developmental processes important for the production of both male (Verelst *et al*., 2007; Xu *et al*., 2010) and female gametes (Brambilla *et al*. 2007; Colombo *et al*., 2008; Galbiati *et al*., 2013). However, with respect to the size of the pollen transcriptome counting approximately 7000 transcripts, with large proportions of pollen-enriched (26%) and presumably pollen-specific (11%) genes (Honys and Twell, 2004) it still represents only small fraction of characterized genes. To describe novel transcription factor gene families potentially important for pollen development, we used previously published transcriptomes of *Arabidopsis thaliana* pollen developmental stages (Honys and Twell, 2004) with special interest to transcription factors as crucial. In Arabidopsis genome, there are over 1595 putative transcription factors distributed among 50 gene families (Reňák *et al*., 2012). Transcription factor genes possessing reliable expression signals in developing male gametophyte represented candidates for our further analysis. One of the selected gene families of TFs putatively involved in male gametophyte development is bZIP, the second largest family of dimerizing TFs. With this functional feature, bZIPs became an excellent model to study how TFs modulate cellular physiology with respect to the actual environmental state. As these transcriptional regulators dimerize in all eukaryotes and are involved in a wide range of cellular functions, such as metabolism, development, response to stress, hormone signaling and many others, it is presumed that they also play important role in the male gametophyte. Several *AtbZIP* genes were selected for their putative involvement in pollen development.

Our selection criteria were based on microarray expression data together with the prediction of their homo- and heterodimerization potential according to Deppmann *et al*. (2004). However, this study did not cover several AtbZIP proteins in the interaction profile, including AtbZIP34 and AtbZIP61. Remaining bZIPs in our selection, bZIP18, bZIP28, bZIP52 and bZIP60 were predicted to favor homodimerization whereas, heterodimerization was predicted between bZIP18/bZIP28, bZIP60/bZIP28 and bZIP52/bZIP18. These predictions were confirmed by our Y2H results. We identified three homodimers between bZIP18/bZIP18; bZIP28/bZIP28 bZIP60/bZIP60 and three heterodimer combinations bZIP18/bZIP61; bZIP18/bZIP34; bZIP28/bZIP60. The Y2H assay also showed that proteins bZIP18, bZIP34 and bZIP61 are able to interact with bZIP52. Out of all proteins tested,

bZIP18 possesses the highest dimerization capacity as it interacts with most of the tested proteins including the formation of homodimers. From this point of view and from the supporting evidence based on microarray data, semi-RT-PCR and promoter-GUS assay showing the highest expression signal in late pollen development, AtbZIP18 represented a good candidate to be involved in the regulation of male gametophyte development.

Although bZIP dimerization domains are stereotyped, they are also involved in many diverse functions. This is achieved by so called combinatorial control of gene expression, where different heterodimers show intermediate effects, depending on the monomers combined. Such system was described in the regulation of late embryogenesis by A group bZIPs ABA-insensitive 5 (ABI5) and Enhanced Em Level (EEL). These two bZIPs compete for the same binding site, conferring antagonistic transactivation functions: ABI5 homodimers activate gene expression whereas EEL homodimers and ABI5/EEL heterodimers function as repressors (Bensmihen *et al*., 2002). Another regulatory model was identified in tobacco, where BZI-4 homodimers and BZI-1/BZI-2 heterodimers perform opposing functions and act as negative and positive transcriptional regulators during pollen development (Iven *et al.,* 2010). Similarly, the expression of RBCS1a is modulated by HY5, HYH and GBF1, where GBF1 acts as a repressor whereas HYH and HY5 are activators of RBCS1a expression (Singh *et al*., 2012).

We propose similar mode of regulatory control for the newly identified pollen bZIP network, based on the genome wide screen for the presence of ethylene-represponsive element binding factor–associated amphiphilic repression (EAR) motif in Arabidopsis. EAR motif is a small distinct transcriptional regulatory domain present in active repressors that inhibits the activation of transcription either by interacting with components of basal transcription machinery or by recruiting histone deacetylases (HDACs) that modify chromatin structure and prevent other transcriptional activators from binding to their target *cis*-elements (Kagale *et al*., 2010). EAR motif is present in both bZIP18 and bZIP52 TFs, favouring them for the role as putative transcriptional repressors during pollen development. On the contrary, other AtbZIP18 interactors, bZIP28, bZIP34 and bZIP60, do not contain the EAR motif. It has been shown that the progression from proliferating microspores to terminally differentiated pollen is characterized by large-scale repression of early male gametophytic genes and the activation of a unique late gene-expression program in late Arabidopsis pollen development (Honys and Twell, 2004). As *AtbZIP18* was

detected to be expressed from the microspore stage, we cannot exclude the possibility of its involvement in such regulatory machinery.

FUNCTIONAL CHARACTERIZATION OF AtbZIP18

AtbZIP18 represented the most promising candidate for further functional analysis for the following reasons. First, *AtbZIP18* is the most abundantly pollen-expressed gene within the whole bZIP family. Second, it forms dimers with several other pollen-expressed bZIP proteins and possesses the highest dimerization capacity. Third, AtbZIP18 contains EAR domain shown to be important for the regulatory function of other transcriptional repressors (Borg *et al*., 2014; Dong and Liu, 2010; Liu and Karmarkar, 2008). We have conducted a complex functional analysis of AtbZIP18 in order to supplement previous characterization of its binding partner AtbZIP34 and to establish regulatory function of the putative bZIP network in pollen.

At first, we verified the *AtbZIP18* expression profile presumed from the microarray experiments by semi-quantitative RT-PCR and monitored the promoter-GUS activity throughout plant development. *AtbZIP18* promoter showed pollen-enriched expression first detected at the microspore stage with increasing activity towards pollen maturation. In inflorescences, we detected GUS staining in young flower buds, in sepals and tapetum. Later, we observed the activity of *AtbZIP18* promoter in carpels and anther filaments. Apart from the male gametophyte, *AtbZIP18* was also expressed in majority of vegetative organs. In cotyledons and true leaves, the promoter activity was restricted to the vasculature and hydatodes of the true leaves. *AtbZIP18* promoter activity was positive also in vascular tissues of roots and stems, where it was previously shown to overlap with the activity of *AtbZIP52* and *AtbZIP59* promoters (Pyo et al., 2006). Such pleiotropic promoter activity was reported also for other bZIP TFs belonging to the C/S1 bZIP TF network (Weltmeier *et al*., 2009). The expression pattern we observed in *AtbZIP18* promoter:GUS transgenic plants coincided with the microarray data. Moreover, the expression profiles of *AtbZIP18* and *AtbZIP34* were very similar (Gibalová *et al*., 2009). In the male gametophyte, both promoters shared similarly strong late activities. In flowers, the activity of both promoters had similar pattern with the only difference in carpels, where *AtbZIP34*

promoter-driven GUS activity was detected in papillary cells. We observed the *AtbZIP34* promoter activity in young female gametophytes as supported by transmission defects (Gibalová *et al*., 2009). However we did not observe any activity of *AtbZIP18* promoter in female gametophyte, neither reduction of mutant allele transmission through the female gametophyte. Both bZIPs were also expressed in sporophytic tissues where *AtbZIP34* promoter acted similarly to *AtbZIP18* in vasculature of cotyledons, true leaves, roots and stems. On the contrary to *AtbZIP18,* the *AtbZIP34*–driven GUS signal was additionally detected in trichomes and root hairs (Gibalová, unpublished data). In the light of similar expression profiles, protein localization and their confirmed dimerization, we can hypothesize the involvement of *AtbZIP18* and *AtbZIP34* TFs in the regulation of the same metabolic and/or developmental pathway.

Transcription factors usually localize in the nucleus, driving the expression of their target genes. This was the case of AtbZIP34 protein that was present specifically in the nuclei of transiently transformed tobacco leaf epidermal cells (Figure S2). To establish its cell specificity localization pattern, we expressed AtbZIP34 fused with the histone protein H2B and GFP in stably transformed plants under its native promoter. We observed the GFP signal only in vegetative nuclei, implying the vegetative cell-specific expression (Figure S3). However, the cellular localization of AtbZIP18 protein was different. We observed its localization not only in the nuclei but also in the cytoplasm/ER of transiently transformed tobacco leaf epidermal cells. To exclude potential artifacts that could arise from the strong CaMV 35S promoter activities, we generated and analyzed stably transformed Arabidopsis plants expressing whole genomic sequence of *AtbZIP18* fused with GFP. In the male gametophyte, we detected the GFP signal in whole pollen grain. Additionally, in the sporophyte, AtbZIP18-GFP fusion protein was observed in the cytoplasm and nuclei of developing embryo. Such redistribution of TFs between two cellular compartments, nucleus and cytoplasm, was already described in the bZIP family. AtbZIP28 and AtbZIP17 TFs were shown to localize on the ER in orientation towards the cytoplasm by their N-terminal parts containing DNA-binding domains (Gao *et al*., 2008). After stress treatment resulting in the aggregation of unfolded proteins in the ER (unfolded protein response, UPRE), N-terminal parts of both bZIP proteins were released by S1P and S2P proteases and imported to the nucleus, where they activated the expression of chaperons, such as BiP genes. AtbZIP60 and AtbZIP49 represent another examples of ER-resident bZIP

591 transcription factors executing UPRE (Iwata *et al*., 2005; Iwata *et al*., 2008). Also AtbZIP10
592 shuttles between the nucleus and the cytoplasm, retained by Lesions Simulating Disease
593 resistance 1 protein (LSD1; Kaminaka *et al*., 2006). In other cases, bZIPs are actively
594 shuttled out of the nucleus due to the presence of Nuclear Export Signal (NES; Tsugama
595 and Takano, 2012) and they are retained in the nucleus only when the NES is masked (Li *et*
596 *al*., 2005; Llorca *et al*., 2014). Other examples of gene regulation by membrane-tethered
597 TFs belonging to other than bZIP gene families have also been reported (Goldstein *et al*.,
598 2006; Hoppe *et al*., 2001; Makinoshima and Glickman, 2006; Ron and Walter, 2007; Selkoe
599 and Kopan, 2003).

600 In order to uncover the mode of action of *AtbZIP18*, we have carried out the
601 phenotypic characterization of SALK_111120 line harbouring the T-DNA insertion in the
602 5'UTR of At2g40620 gene causing the complete knock-down of *atbzip18* mRNA. As
603 *AtbZIP18* is the most abundantly expressed in mature pollen, we have scored phenotype
604 aberrations in pollen isolated from homozygous *atbzip18/-* plants. Surprisingly, knock-out
605 of *AtbZIP18* did not result in phenotypic differences. Therefore, we adopted the
606 overexpression strategy to uncover the function of *AtbZIP18* in pollen. Our microscopic
607 observations of two generations showed the presence of aborted pollen, which
608 represented the most abundant phenotypic category. Surprisingly, pollen abortion was
609 observed mostly in heterozygous plants and was reverted to a near wild type phenotype in
610 homozygous plants. This phenomenon is still enigmatic particularly in pollen. However,
611 there are few examples of importance of protein equilibrium state represented by
612 overexpression of histone proteins. Overexpression of either histone H2A-H2B or histone
613 H3-H4 gene pairs cause aberrant chromosome segregation (Meeks-Wagner and Hartwell,
614 1986) and gene defects (Clark-Adams and Winston, 1987), yet co-overexpression of all
615 four-core histones reverts the phenotypic effects due to restoration of the normal histone
616 stoichiometry (Prelich, 2012). Moreover, disruption of protein equilibrium has been
617 reported to be relatively common; in systematic overexpression studies to cause ~ 23% of
618 observed phenotypes in cell morphology (Sopko *et al*., 2006). In every case, it is still
619 challenging to bring more light on regulatory function of TF networks due to combinatorial
620 complexity that we have yet to understand.

621 Conclusively, our work outlines the identification of putative Arabidopsis pollen
622 bZIP TF network, and points to the greater impact *AtbZIP18* gene possessing the highest

623 expression in pollen and the highest dimerization potential among other pollen-expressed
624 *AtbZIP* genes in the Y2H analysis. We have examined six pollen-expressed genes for
625 dimerization in reciprocal manner and identified several homo- and heterodimerization
626 events. We aimed to extend our knowledge of the bZIP network in pollen with the long-
627 term goal of using the acquired information together with other pollen studies to better
628 understand the regulatory mechanisms determining the male gametophyte development.
629 Furthermore, we have functionally characterized *AtbZIP18* gene belonging to the putative
630 pollen bZIP regulatory network. *AtbZIP18* is widely expressed in vegetative and
631 reproductive tissues. Not only promoter activity but also protein localization showed the
632 presence of this protein in both types of tissues suggesting the pleiotropic mode of action
633 for *AtbZIP18*. As our goal was to study the *AtbZIP18* TF involvement in male gametophyte,
634 we have provided several genetic and functional studies to examine its importance for
635 pollen development. Actually, only the gain-on-function approach showed the disturbance
636 of pollen phenotype mostly among heterozygous plants, which together with the reduced
637 transmission of the mutant *atbzip18* allele through the male gametophyte and slightly
638 increased number of seed gaps, suggests the involvement of *AtbZIP18* in male
639 gametophyte development through unknown pathway. These data supplement our
640 previously described gene *AtbZIP34* belonging to the same putative bZIP regulatory
641 network. Based on similar expression pattern of both genes and their strong interaction
642 we can hypothesize their involvement in the regulation of male gametophyte
643 development. It appears that there is a clear need to explore more on the function of
644 A*tbZIP18* through putative downstream targets and/or identification of other interactors.
645 So far, this work represents the first insight into the regulatory network of the important
646 family of bZIP TFs in male gametophyte.
647
648 **SUPPLEMENTARY DATA**
649 Supplementary data contain four figures (Figure S1; Figure S2; Figure S3; Figure S4) and
650 one table (Table S1).
651
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Figure legends

Figure 1

Expression profiles of the AtbZIP family transcription factors (www.genevestigator.ethz.ch). 17 candidate genes possessing mean expression signal over 400 (Affymetrix ATH1 GeneChip) in pollen are highlighted in green boxes. Relative level of gene expression is illustrated at the bottom scale **(A)**. Amino acid sequences of 17 bZIP dimerization domains. Individual leucine domains were identified based on the alignment and divided into heptades. Positions in individual leucine heptades are marked "gabcdef" to visualize the putative dimerization, whereas g-e pairs are critical. Amino acid residues predicted to regulate the dimerization specificity are color-coded (positions "g a e"). Positively charged amino acid residues (K, R) in positions "g" and "e" are marked in green. Negatively charged amino acids (D, E) are marked in red. Purple colour is used in case of electrostatic attraction of amino acid residues (g-e positions). N residue at position "a" is marked in blue and in case that the same residue is present in other bZIP at the position "a", dimerization would be favourable **(B)**.

Figure 2

Semi-quantitative RT-PCR of eight candidate genes among sporophytic tissues; ST (stem), RT (root), LF (leaf), INF (inflorescence) and mature pollen grains (MPG). Actin (ACT) was used as a control of expression.

Figure 3

Pairwise interactions of six bZIP candidates. Binding domain (BD-bait) fusions are illustrated in rows, activation domain (AD-prey) fusions are illustrated in columns. bZIP52 was used as a prey only. Tested colonies were resuspended in water to reach OD_{600} = 0.1 and dropped (10µL) on selection media lacking W, L, A, H. Weak interaction between bZIP28 and bZIP60 is framed **(A)**. Graphical illustration of individual bZIP interactions. bZIP proteins in orange circles are also homodimerizing, except of the interactions with other bZIPs. Double lines are representing reciprocal interactions, simple lines are showing interactions carried out in one direction **(B)**.

Figure 4

Verification of *AtbZIP18* expression in four stages of pollen development: microspores (MS), bicellular pollen (BC), tricellular pollen (TC) and mature pollen (MP) and among four sporophytic tissues: mature flowers (FW), stem (ST), leaf (LF) and root (RT), KAPP3 – control of expression **(A).** Activity of the *AtbZIP18* promoter in male gametopyte starts at microspore stage (I) accumulates slightly more at bicellular stage (II) and it is increasing towards to pollen maturity (III). **(B).** In young anthers, GUS signal is present in tapetum (I, II) and later is restricted to the anther filaments, carpels and petals (III). Complex view of the *AtbZIP18* expression in whole inflorescence (IV) **(C)**. In vegetative tissues, GUS signal was observed at the distal parts of cotyledons and their vasculature (I, II), in true leaves GUS staining accumulated in vascular tissues and parts corresponding to hydatodes (III). Stems of the etiolated seedlings showed *AtbZIP18* promoter activity in vascular tissues (IV). Siliques possessed GUS signal at the proximal and distal parts (V), roots showed GUS expression mostly at the vascular tissues and stronger promoter activity was observed at the root tip **(D)**.

Figure 5

Localization of AtbZIP18-GFP fusion protein in tobacco epidermal cells. Fusion protein was localized to nucleoplasm and perinuclear region **(A)** confirmed by co-localization with ER marker **(B)**. Merged image **(C)**. AtbZIP18 is partially or fully localized to the ER and cytoplasm **(D)** ER marker **(E)** merged image **(F)**.

Figure 6

Localization of the whole genomic sequence of *AtbZIP18* fused to the GFP during male gametophyte development. GFP signal was absent in microspores **(A)** and started to be accumulated from bicellular pollen **(B)**, and increases in mature pollen grains **(C)**. During pollen germination, the fusion the GFP signal was also detected in the cytosol of pollen tubes **(D)**. Right panel is showing localization of AtbZIP18_*GFP* fusion protein in mature embryo. The signal was restricted to the nuclei and ER and/or cytoplasm of individual cells – zoomed windows.

Figure 7

AtbZIP18-GFP fusion protein is localized in the cotyledons of six days-old seedlings. Bottom epidermis with guard cells-zoomed window **(A)**. True leaf between cotyledons **(B)** stem, zoomed window showing details of AtbZIP18 localization **(C)** and nuclei of trichomes **(D)**. AtbZIP18 localizes in primary root **(E)**, root primordial cells of secondary roots **(F)** at the elongation zone of the primary root showing nucleoplasm localization **(G)** and at nuclei of the root tip **(H)**.

Table 1

Genetic transmission analysis of *atbzip18* mutation. Numbers of progeny arisen from reciprocal test crosses and selfing progeny are illustrated in the second row together with the calculated transmission efficiencies (TE), through the male (TE^m; P-value 0,81) and the female gametophytes (TE^f; P-value 0,21). Calculated ratio of the self-progeny differs from the Mendelian ratio 1:2:1.

Table 1

SALK_111120

atbzip18/- ♀ x Col-0 ♂			Col-0 ♀ x *atbzip18/-* ♂				*atbzip18/-* ♀ x *atbzip18/-* ♂		
+/-	wt	TE[f]	+/-	wt	TE[f]		wt	+/-	-/-
82	79	103.8	73	89	82.02		55	83	70
						Calculated ratio	1	1.51	1.27

Figure 1.

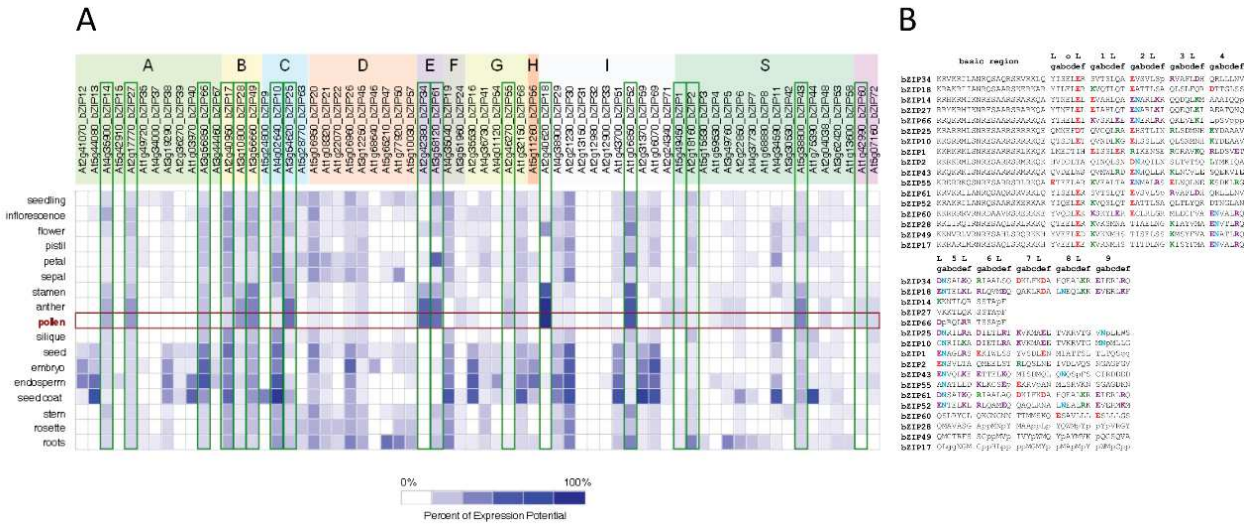
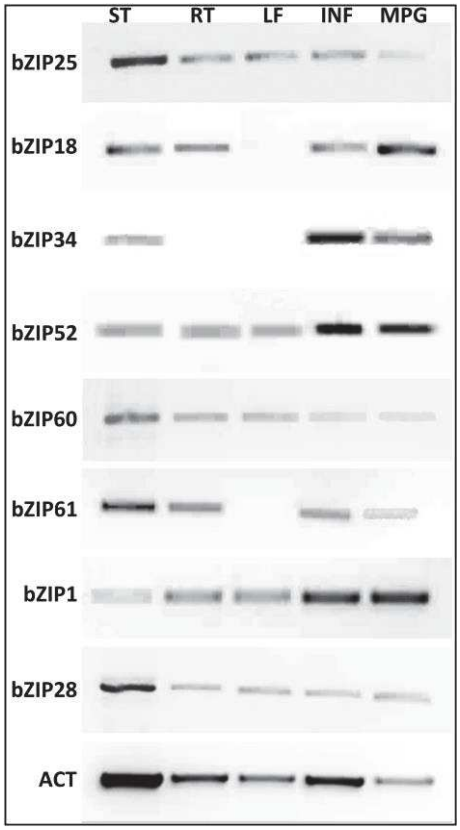


Figure 2.



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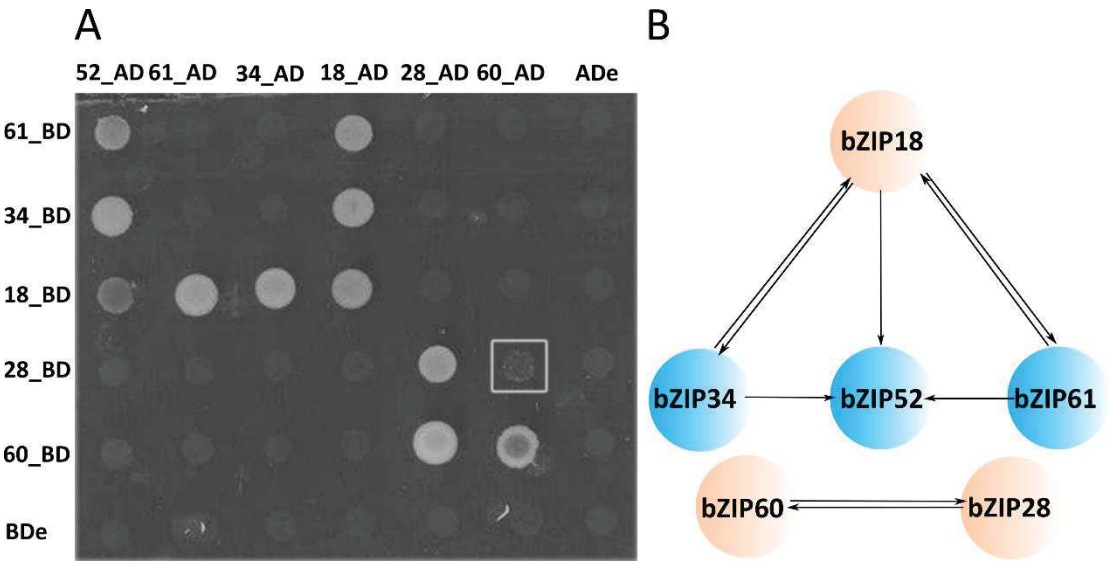

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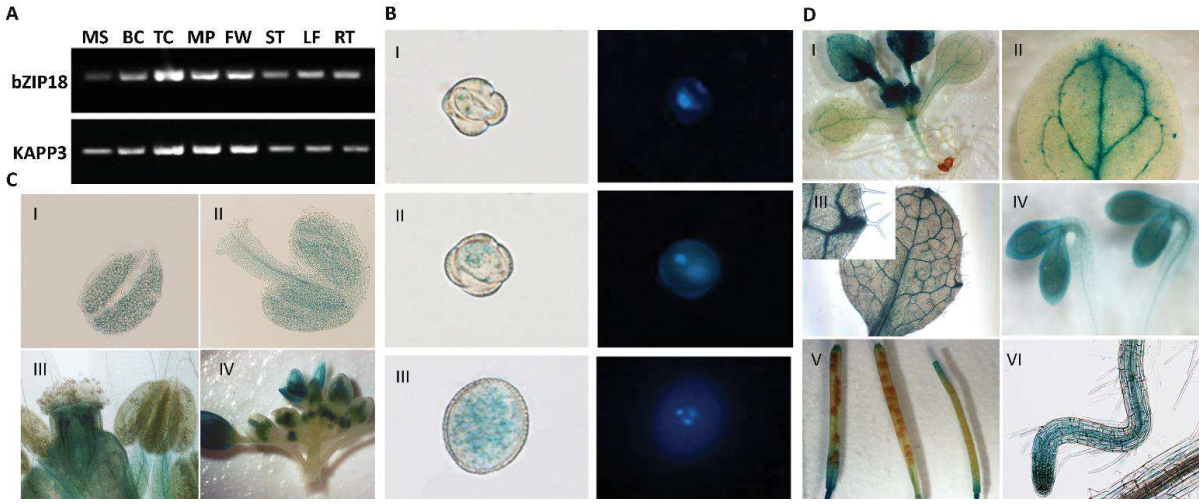

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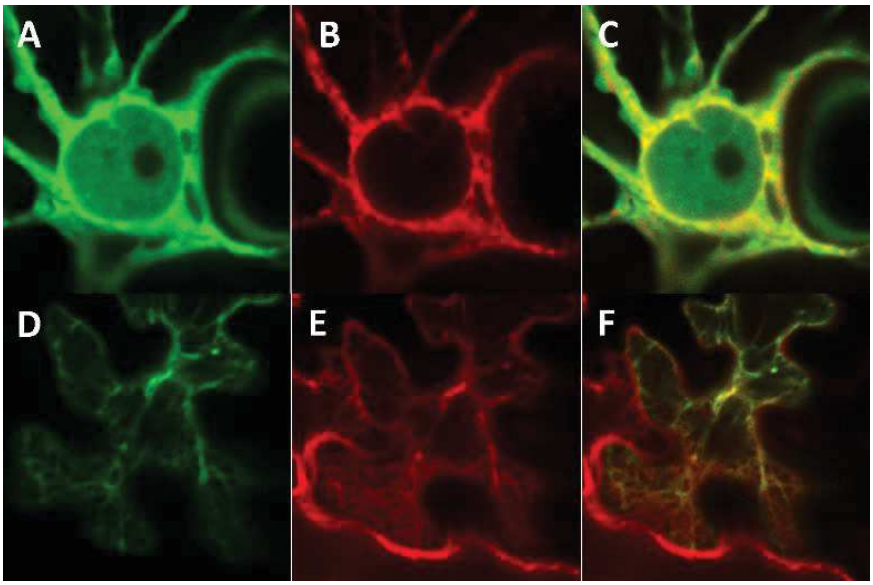

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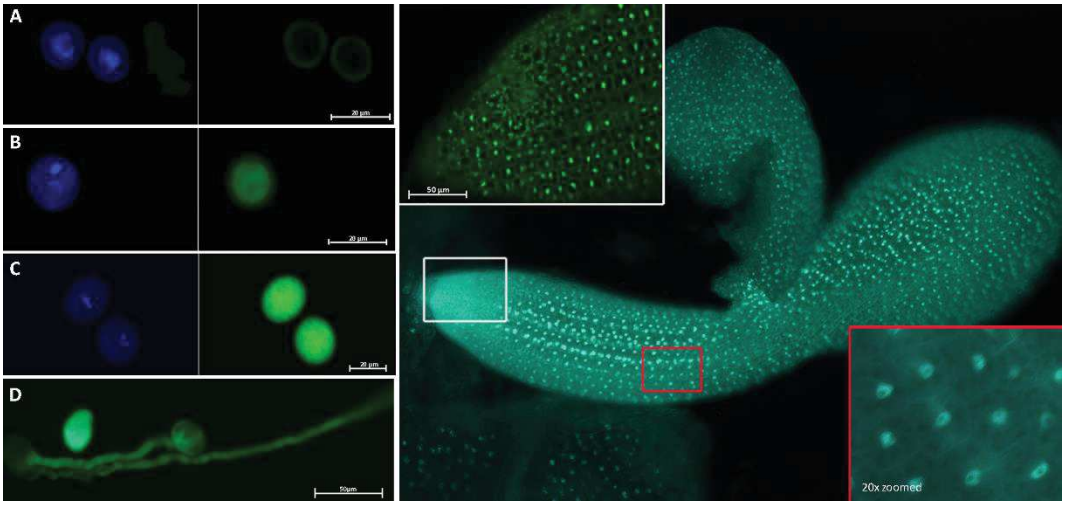
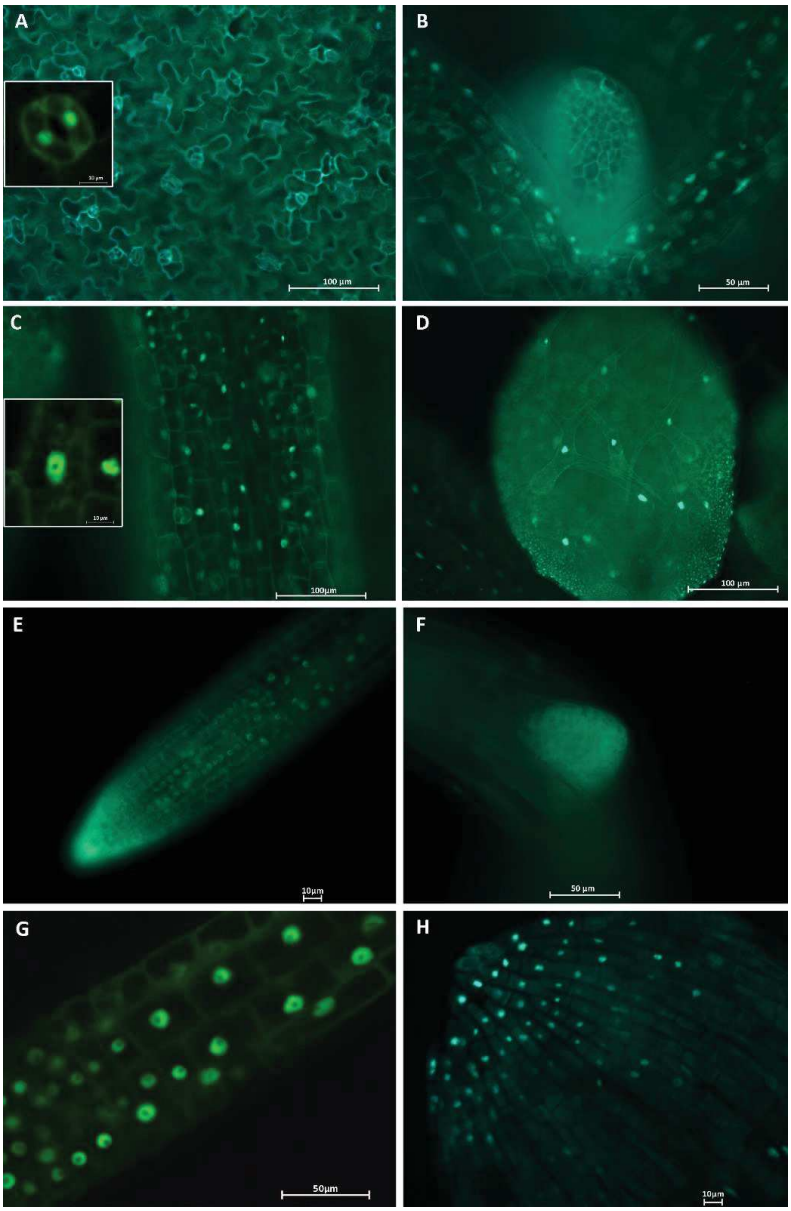


Figure 7.



NETWORKS OF bZIP TRANSCRIPTIONAL REGULATORS ACTING DURING MALE GAMETOPHYTE DEVELOPMENT IN ARABIDOPSIS THALIANA

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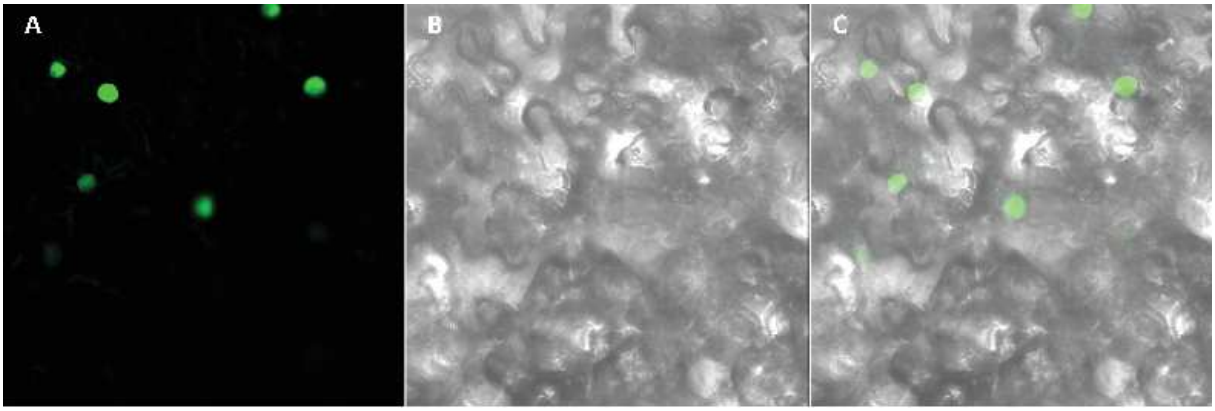
Figure S1

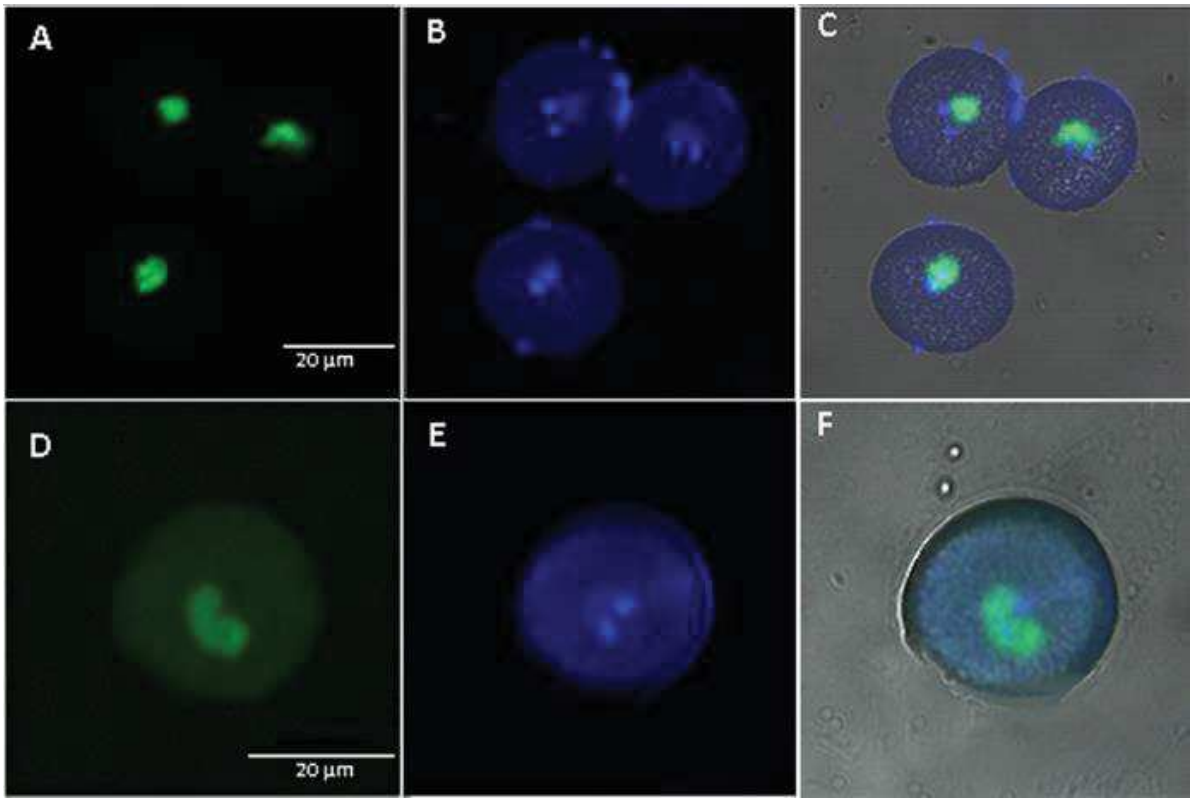
Schematic representation of T-DNA insertion of SALK_111120 localized at the 5´UTR (triangle), starting at -266bp upstream of the ATG start codon. Positions of primers used for genotyping and qPCR are marked by arrows **(A)**. Quantification of the *AtbZIP18* transcript abundance in Col-0 and *atbzip18* pollen cDNA **(B).**

Figure S2



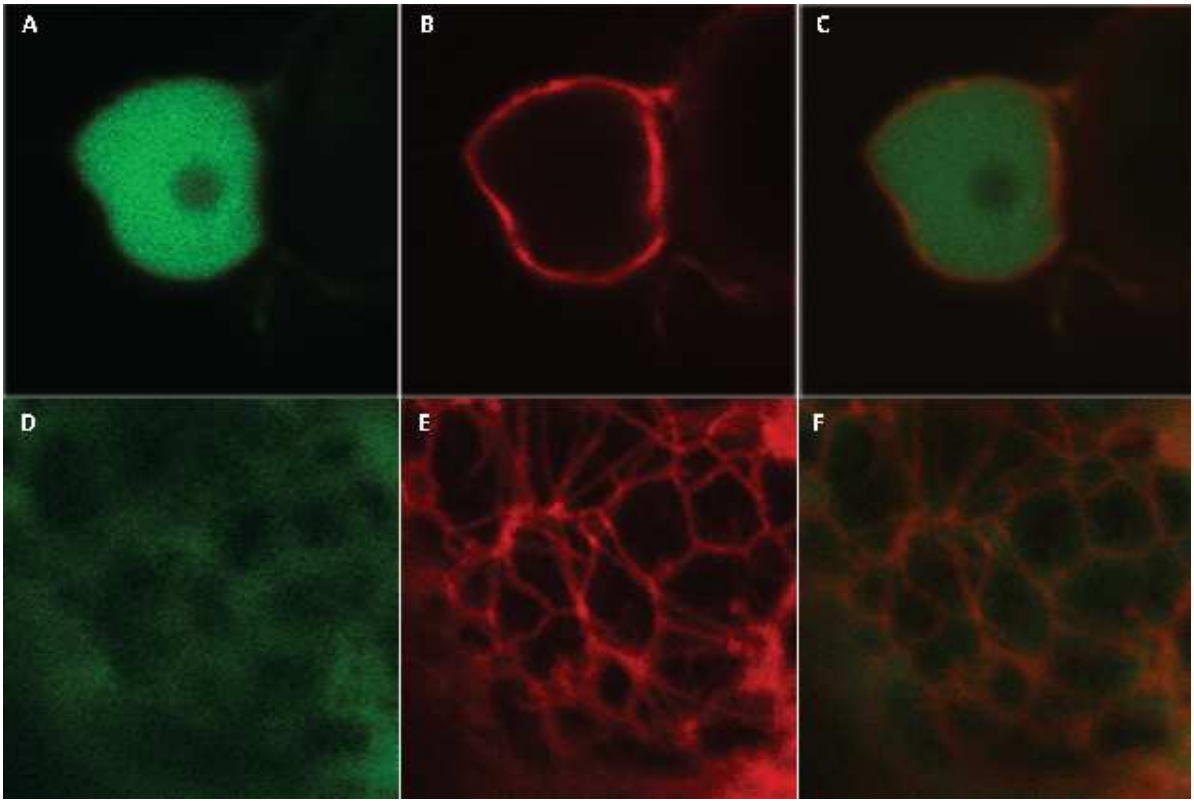
Localization of AtbZIP34_GFP fusion protein in epidermal cells of transiently transformed tobacco leaves is restricted specifically to the nucleus **(A)**, bright field **(B)**, merged image **(C).**

Figure S3



Localization of Lat52 promoter fused to GFP **(A-C)**, AtbZIP34 promoter fused to H2B_GFP **(D-F)**. GFP channel left column, DAPI channel middle column, merged image right column.

Figure S4



Co-localization of free GFP (pGWB6 vector alone) in transiently transformed tobacco leaves. GFP in nucleus and cytoplasm **(A, D)**, ER marker fused to RFP in nucleus and on the ER **(B, E)**, merged images **(C, F)**.

Table S1

Primer name	Primer sequence 5’ - 3’
LP	AGCTCTTTTAGGGTCGACGAC
RP	GAAGCAAATGTGTTTGGATCG
ZIP18_F	TCTCGCAATGCCCGCCTTT
ZIP18_R	AGCTCTTTTAGGGTCGACGAC
EF_F	TGAGCACGCTCTTCTTGCTTTCA
EF_R	GGTGGTGGCATCCATCTTGTTACA