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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

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

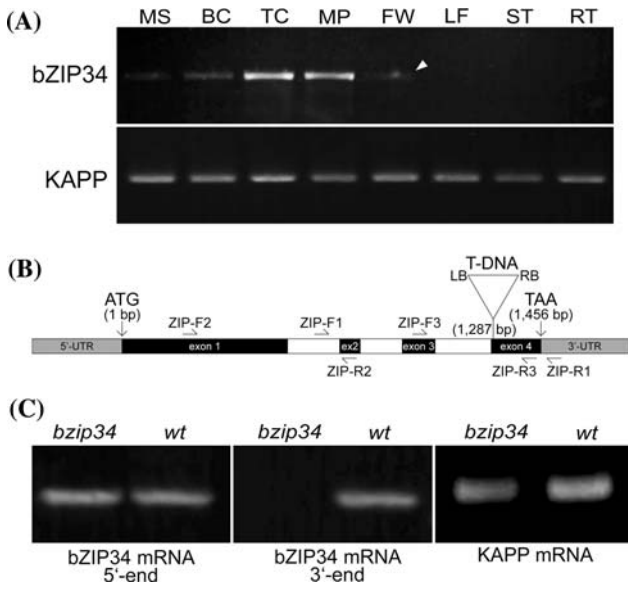

Fig. 1 Verification of At2g42380 expression profile and control KAPP gene expression by RT-PCR (**A**) in microspores (MS), bicellular (BC), tricellular (TC) and mature pollen (MP), whole flowers (FW), leaves (LF), stems (ST) and roots (RT). *White arrowhead* shows expression of At2g42380 in flowers. 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

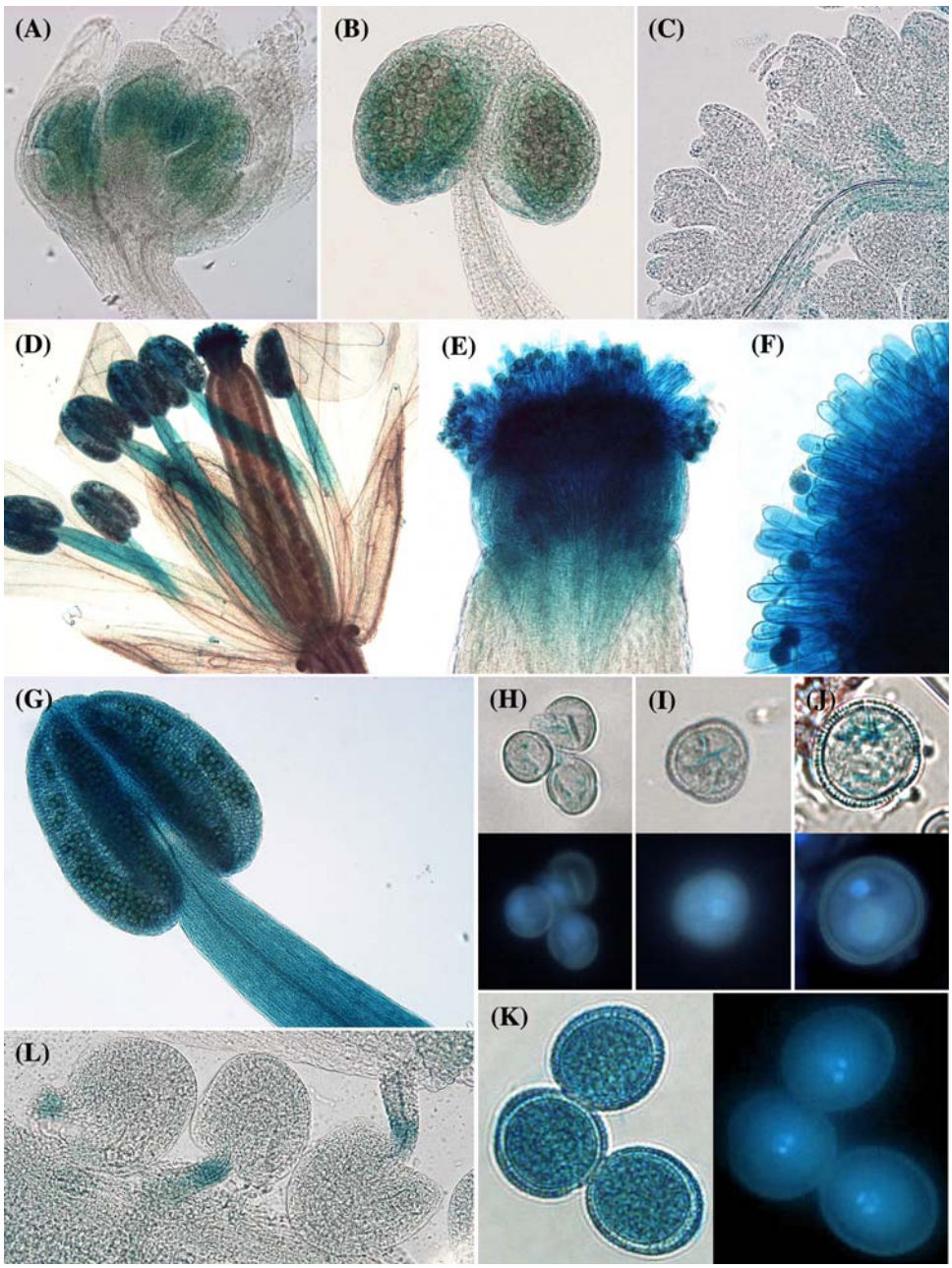

Fig. 2 Activity of the *AtbZIP34* promoter. Bright field microscopy of flower bud (stage 8, **A**) with detailed view of anther (**B**) and ovary (**C**). Later developmental stages are represented by an open flower (stage 15, **D**) with details of anther (**G**), pistil (**E**), papillar cells with 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

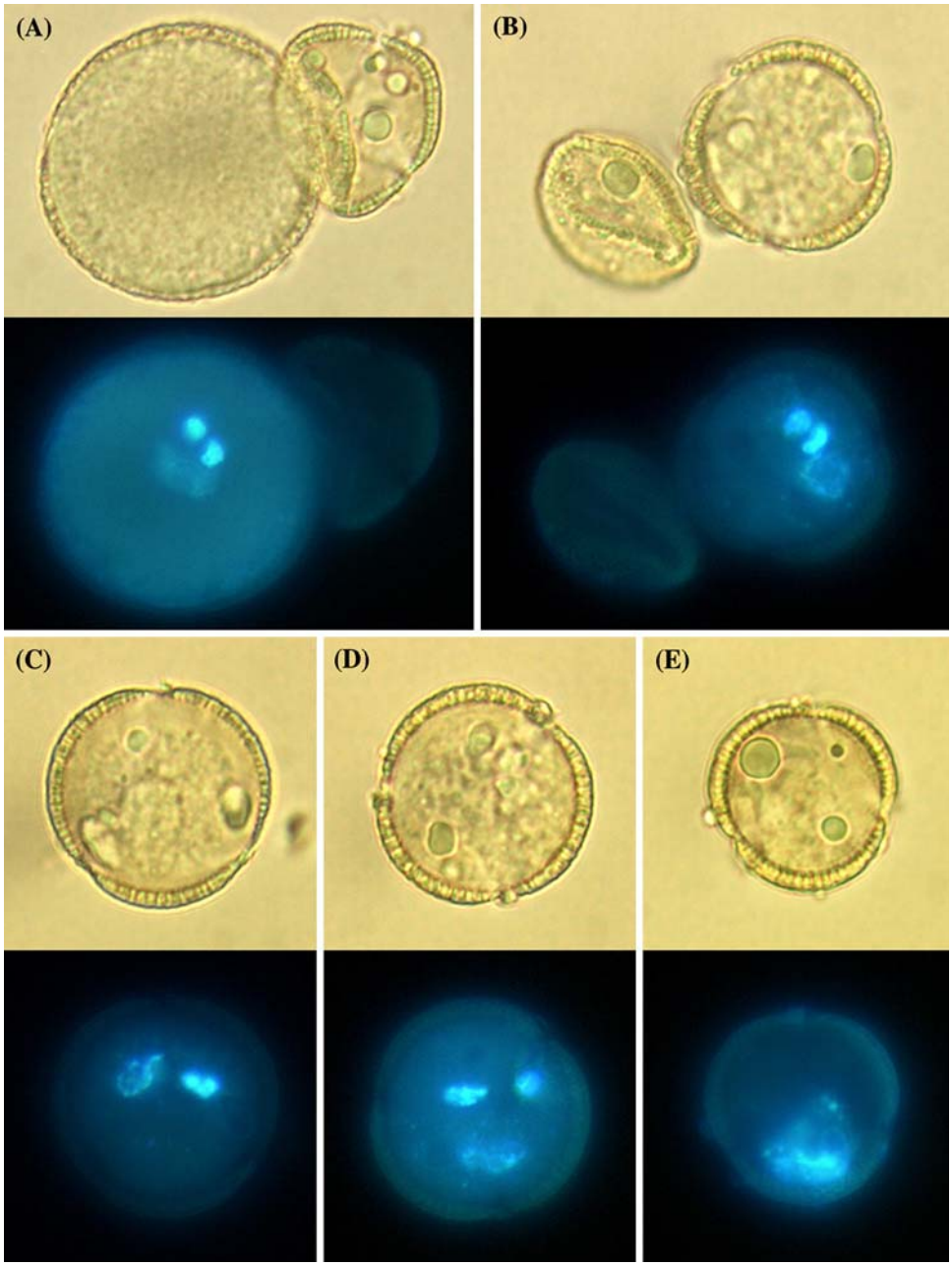

Fig. 3 Phenotypic defects in *atbzip34* pollen. Bright field and fluorescence images after DAPI-staining are shown, (**A**) wild type and *atbzip34* collapsed pollen, (**B**–**E**) *atbzip34* pollen

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).

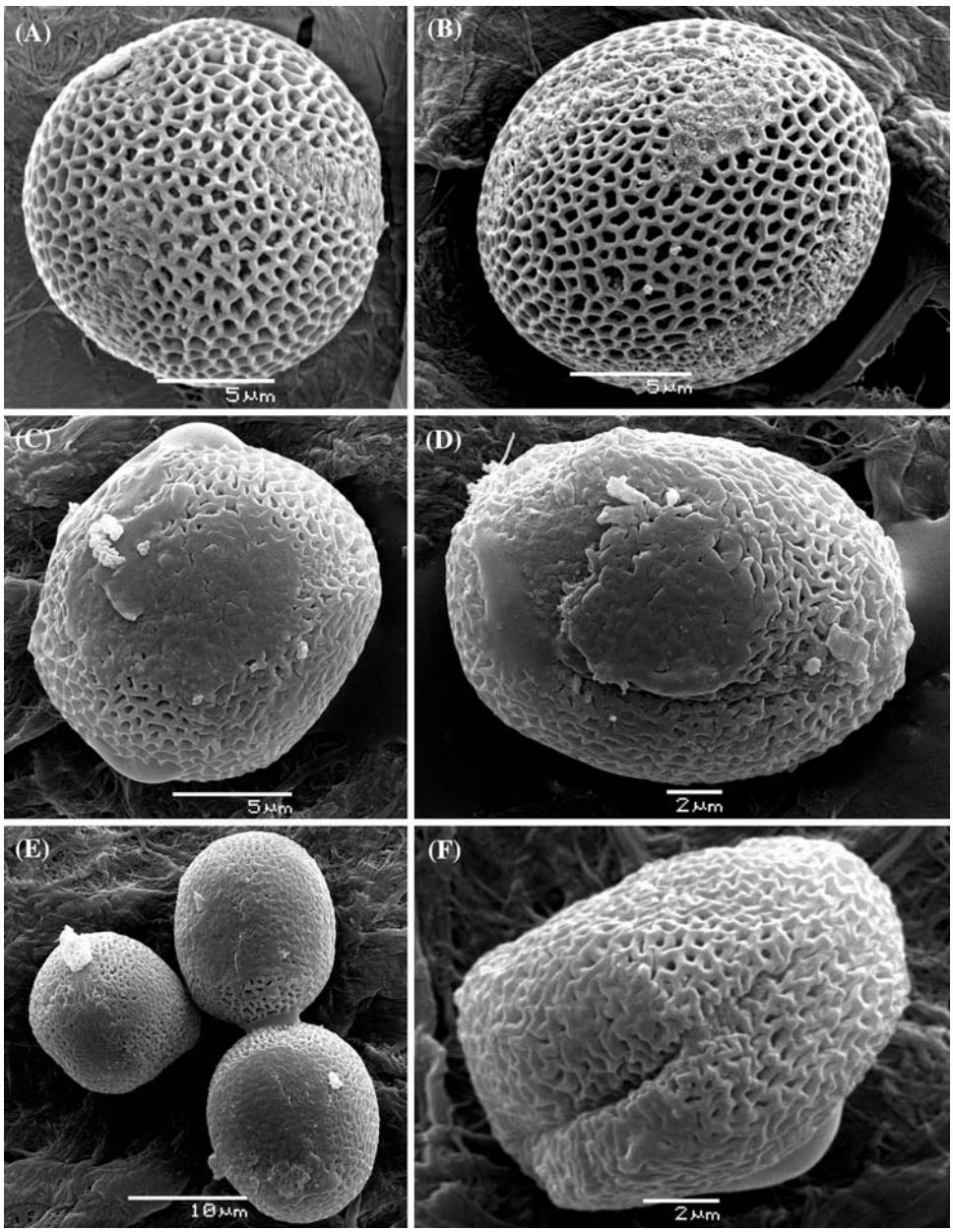

Fig. 4 Scanning electron micrographs of wild type pollen (**A**), *atbzip34* pollen complemented with At2g42380 genomic fragment (**B**) and *atbzip34* pollen grains (**C**–**F**). *atbzip34* pollen is defective in exine pattern formation (**C**–**F**) with often irregular shape (**F**). Pollen grains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

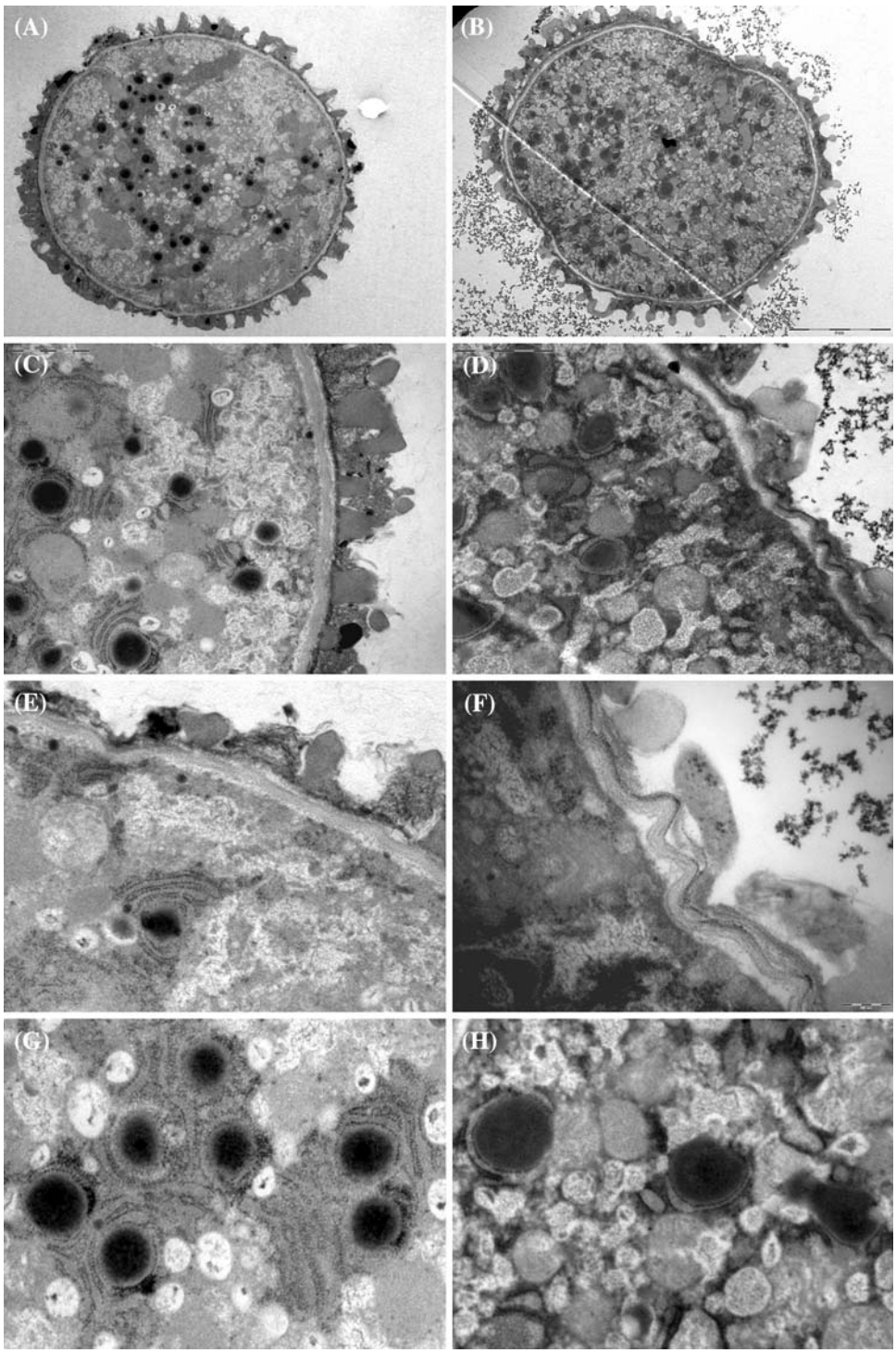

Fig. 5 Transmission electron micrographs of mature wild type (**A**, **C**, **E**, **G**) and *atbzip34* (**B**, **D**, **F**, **H**) pollen grains. *atbzip34* pollen has an irregular, wrinkled intine and exine with misplaced tecta and baculi (**D**, **F**). Mutant pollen has less developed endomembrane 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

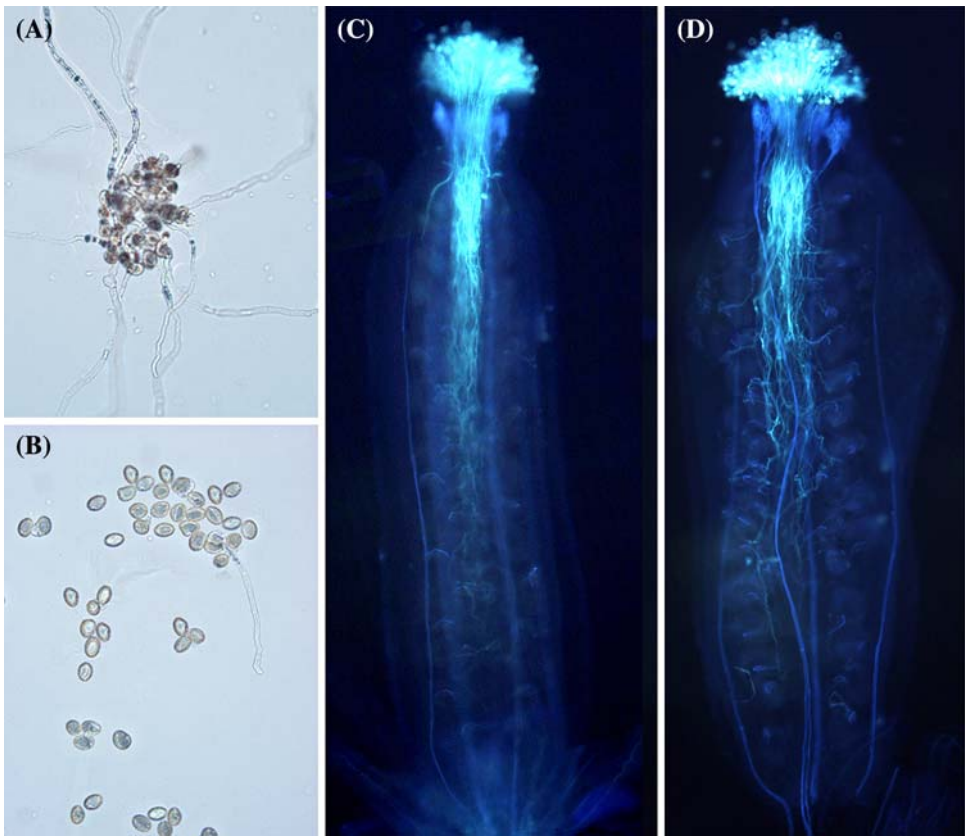

Fig. 6 Pollen tube growth tests. Wild type (**A**) and *atbzip34* (**B**) pollen tubes grown in vitro for 10 h. *atbzip34* pollen tubes were indistinguishable from wild-type pollen tubes, but there was markedly impaired germination. Wild type (**C**) and *atbzip34* (**D**) pollen tubes grown in wild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

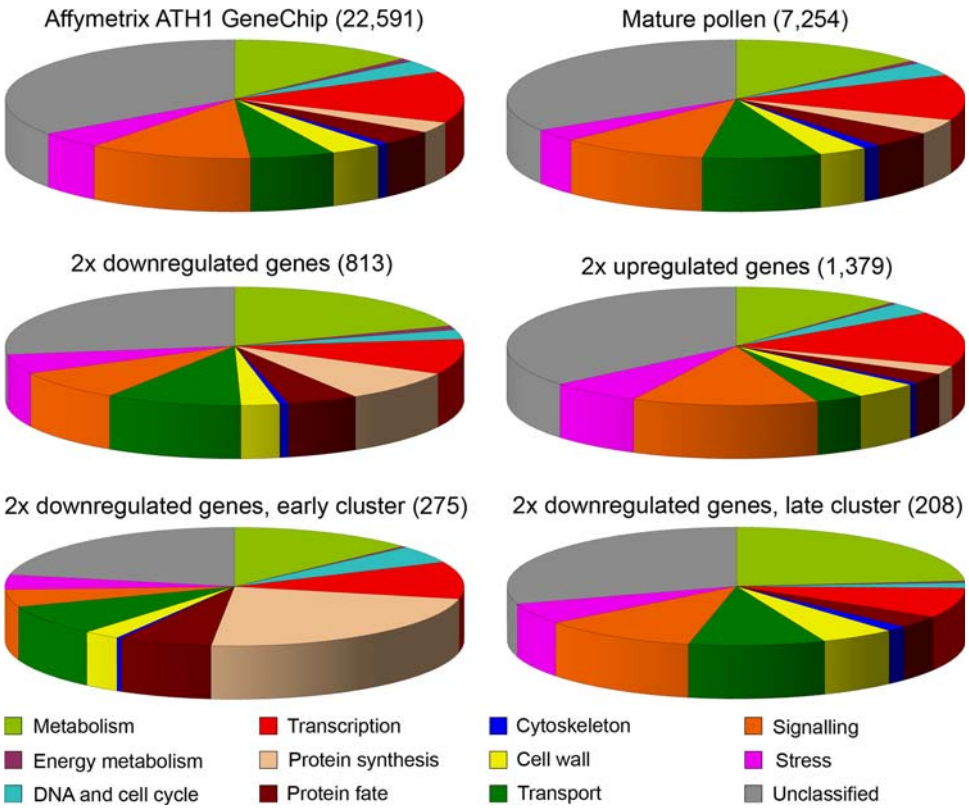

Fig. 7 Proportional representation of expressed mRNA among gene function categories. Data is presented for up and down regulated genes in *atbzip34* pollen in comparison with wt

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.

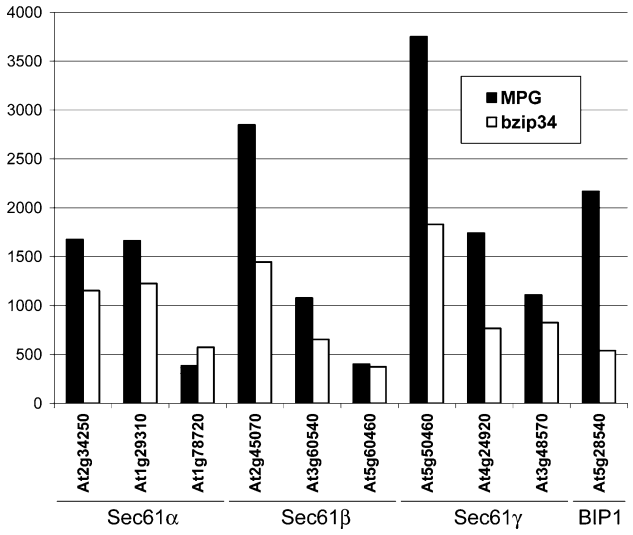

Fig. 8 Relative expression levels of nine genes encoding all Sec61 translocon subunits and gating protein AtBIP1 in wild type and *atbzip34* pollen

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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