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ORIGINAL ARTICLE

Characterization of pollen-expressed bZIP protein interactions and the role of ATbZIP18 in the male gametophyte

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Key message **bZIP TF network in pollen.**

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 effective gene regulation for rapid response to variation in environmental and developmental conditions. Transcription factors (TFs) provide such control ensuring correct gene expression in spatial and temporal manner. Our work reports the interaction network of six bZIP TFs expressed in *Arabidopsis thaliana* pollen and highlights the potential functional role for AtbZIP18 in pollen. AtbZIP18 was shown to interact with three other pollen-expressed bZIP TFs—AtbZIP34, AtbZIP52, and AtbZIP61 in yeast two-hybrid assays. *AtbZIP18* transcripts are highly expressed in pollen, and at the subcellular level, an AtbZIP18-GFP fusion protein was located in the nucleus and cytoplasm/ER. To address the role of AtbZIP18 in the male gametophyte, we performed phenotypic analysis of a T-DNA knockout allele, which showed slightly reduced transmission through the male gametophyte. Some of the phenotype defects in *atbzip18* pollen, although observed at

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low penetrance, were similar to those seen at higher frequency in the T-DNA knockout of the interacting partner, AtbZIP34. To gain deeper insight into the regulatory role of AtbZIP18, we analysed *atbzip18*/– pollen microarray data. Our results point towards a potential repressive role for AtbZIP18 and its functional redundancy with AtbZIP34 in pollen.

Keywords bZIP · Transcription factors · Regulatory network · Male gametophyte · Y2H · Pollen development

Introduction

The mature male gametophyte is a uniquely specialized structure for the delivery of two sperm cells via the pollen tube to the embryo sac within the ovary of the flower where double fertilization takes place (Bedinger 1992; Hafidh et al. 2016). This event is essential for plant fertility and crop production; however, the molecular mechanisms underlying 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. Reports of TF networks in pollen are limited to the MADS TF regulatory network directing pollen maturation, which involves repression of early male gametophytic program and the DUO1-DAZ1/DAZ2 germline regulatory network, which involves activation and repressive components (Verelst et al. 2007; Brownfield et al. 2009; Borg et al. 2011, 2014). Based on our previous characterization of the dynamics of the male gametophyte transcriptomes of Arabidopsis and tobacco (Honys and Twell 2004; Hafidh et al. 2012a, b; Bokvaj et al. 2015), we 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 TFs. They are involved in many crucial processes across eukaryotic organisms (Lee 1992; Jakoby et al. 2002; Corrêa et al. 2008; Degnan et al. 2009). In plants, the complexity of the bZIP family was studied in several species (Jakoby et al. 2002; Corrêa et al. 2008; Zhao et al. 2016) and bZIP TFs were shown to be employed in general development including seed maturation (Alonso et al. 2009), flowering (Abe et al. 2005), reproductive development (Gibalová et al. 2009; Iven et al. 2010; Lozano-Sotomayor et al. 2016), senescence (Smykowski et al. 2010), responses to various environmental cues, such as the unfolded protein response (Liu et al. 2007; Iwata et al. 2008), heat stress response (Ohama et al. 2016; Zhao et al. 2016), abiotic stress signalling (Fujita et al. 2005; Zhu 2016), and energy metabolism (Baena-González et al. 2007). Moreover, AtbZIP18 was recently shown to interact with a member of a novel family of nuclear envelope-associated proteins (AtNEAP1) and proposed to represent a link between NEAP and chromatin in the maintenance of nuclear morphology and chromatin structure (Pawar et al. 2016). In many eukaryotic TF gene families, proteins require physical interaction between identical and closely related proteins within the same family to form a functional DNA-binding dimer (Amoutzias et al. 2008). Dimerization provides a way to deliver 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). The Arabidopsis bZIP gene family consists of 77 members (Corrêa et al. 2008), which in theory can generate 175 possible dimeric combinations based upon bioinformatics analyses (Deppmann et al. 2004). Regulation of dimer formation is achieved by protein affinity, specificity, and local protein concentration (Deppmann et al. 2006). Although there is broad evidence for the importance of bZIP TFs in plants and several interaction studies between bZIP family proteins 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), information about bZIP networks in the male gametophyte is limited. There is only a single published example demonstrating the functional cooperation bZIP TFs during pollen development in tobacco (Iven et al. 2010); therefore, we aimed to extend knowledge of the pollen-expressed bZIP network and to shed more light on transcriptional controls in the male gametophyte.

Previously, we have functionally characterized one member of the bZIP TF network in pollen, AtbZIP34 (Gibalová et al. 2009). Its expression pattern together with the phenotypic defects in *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 TFs together with the mild phenotype defects observed in *atbzip34* pollen suggests redundant action with other bZIP TFs. Here, we show the interactions of bZIP proteins encoded by pollen-expressed genes using Y2H assays and investigate the expression and importance of the AtbZIP34-binding partner AtbZIP18, for its role in 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 16-h 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 (Supplementary 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-day-old and 6-day-old ethiolated seedlings, whole inflorescences and siliques) harbouring *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 48 h. Samples were analysed 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 the progeny from 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 (Supplementary 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 endoplasmic reticulum (ER) marker (ER-rk CD3-959; Nelson et al. 2007) fused to mCherry.

Expression vectors for yeast two-hybrid (Y2H) assay were prepared by PCR amplification of At3g10800, At1g42990, At3g58120, At1g06850, At2g42380, At2g40620 full-length coding sequence (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: AtbZIP25$\Delta N_{227–403}$, AtbZIP28$\Delta N_{161–298}$, AtbZIP60$\Delta N_{138–208}$, AtbZIP61$\Delta N_{200–329}$, AtbZIP52$\Delta N_{149–337}$, AtbZIP34$\Delta N_{175–321}$, AtbZIP18$\Delta N_{146–367}$. 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 and protein localization 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 ½MS 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 selection for each expression vector: pKGWFZ7 (kanamycin, 50 µg/mL), pB7WG,0 (BASTA, 15 µg/mL), pGW5 and pGW6 (kanamycin, 50 µg/mL and hygromycin, 25 µg/mL).

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 220 rpm. Bacterial culture was pelleted after overnight cultivation and rinsed twice with an infiltration media (10 mM MES, 10 mM $MgCl_2$, 200 µM 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 3 h. Bacterial suspension was then infiltrated into abaxial epidermis of tobacco leaves using a syringe. Plants were grown at normal conditions for 36 h, 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, put in 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

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 9 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 analysed 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 were 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 analysed by nonparametric Kruskal–Wallis test.

Statistical evaluation of the transmission efficiency of progeny arisen from reciprocal test-crosses was performed by Chi-squared test using MS Excel 2010 (Microsoft Corp., Redmont, WA). A P value <0.05 was considered statistically significant.

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, C-terminal fragments of candidate proteins were cloned lacking the auto-activation domains starting several amino acids upstream of the beginning of the bZIP domain (BRLZ; Talanian et al. 1990; Hurst 1994). Individual protein domains were identified using SMART database (http://smart.embl-heidelberg.de). These 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 was screened on—LEU-TRP-ADE; -LEU-TRP-HIS, and -LEU-TRP-ADE-HIS selections. Single colonies were resuspended in 1 mL of water, and OD_{600} was adjusted to 0.5 for all interactions tested. Interactions were dropped out on three types of selection media and on—LEU-TRP as a growth control and incubated at 28 °C. Transformations and interaction tests were repeated four times.

qPCR and RT-PCR

RNAs from pollen, stem, leaf, and inflorescence were isolated from Col-0 and *atbzip18/–* homozygous plants as described in Honys and Twell (2003). RNA in total amount of 1.5 µg was DNase-treated (Promega, Madison, WI) and subsequently reverse-transcribed using ImProm ImProm-II Reverse Transcription System (Promega). For PCR amplifications, 2.5 µL of 20 × diluted cDNA was used. Quantitative real-time PCR was carried out on a LightCycler 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 10 s at 95 °C, 10 s at 58 °C, and 15 s at 72 °C. C_p values were normalized with the reference genes eEF1α (eukaryotic elongation factor 1α 4, At5g60390) and PEX4 (PEROXIN4, At5g25760). 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

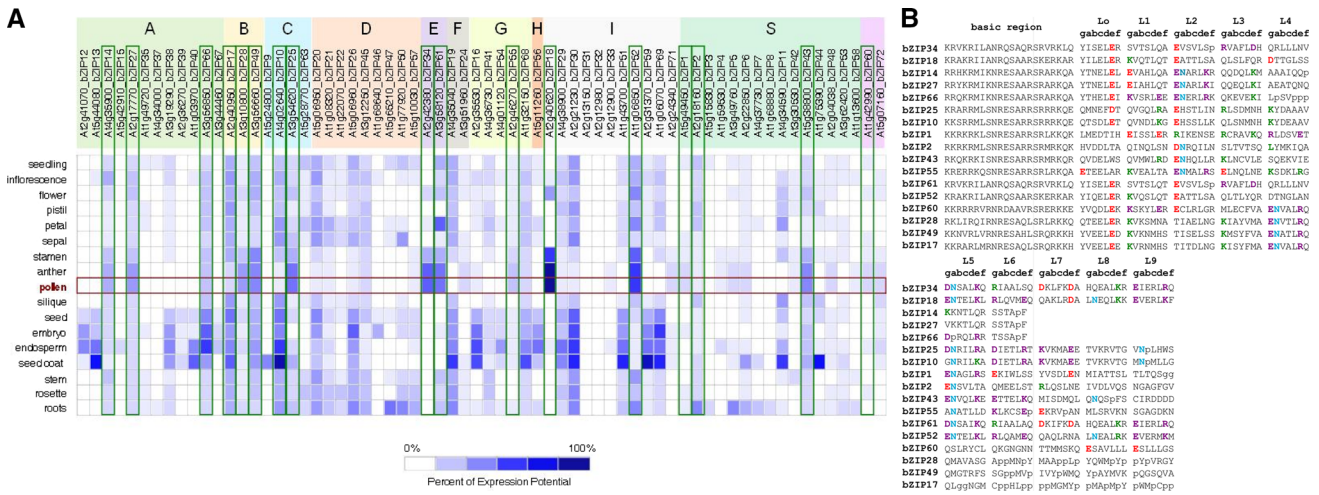

Fig. 1 Expression profiles of the AtbZIP family transcription factors (www.genevestigator.ethz.ch). Seventeen candidate genes possessing mean expression signal over 400 (Affymetrix ATH1 GeneChip) in pollen are highlighted in *green boxes*. Relative level of gene expression is illustrated at the *bottom* 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 *colour-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**)

for target or reference gene qRT PCR assay; CpT, CpR: a crossing point for target or reference genes).

Probe preparation and DNA chip hybridization

Total RNA from 45 mg of mature pollen was extracted using the RNeasy Plant Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Its integrity was verified using Agilent 2100 BioAnalyser (Agilent Technologies, Boblingen, Germany) according to the manufacturer's instructions. RNA hybridization of two *atbzip18/–* pollen biological replicates was performed on ATH1 Affymetrix Genome Arrays platform as described previously (Gibalová et al. 2009).

Microarray data processing

Transcriptomic data sets were processed and normalized using CEL Normalizer Software (Oliveros 2009). Statistical analysis was done in CLC Genomics Workbench (CLC Bio/Qiagen, Aarhus, Denmark). Two biological replicates of *atbzip18/–* mutants were compared to the *atbzip34/* mutants and the WT reference (four available MP transcriptomic data sets as described in Gibalová et al. 2009). Probes with FDR-corrected *P* value ≤ 0.05 were set as differentially expressed. Enriched GO terms were analysed using agriGO (http://bioinfo.cau.edu.cn/agriGO/index.php) online tool (Zhou et al. 2010).

Results

Selection OF AtbZIP candidates for dimer formation

To establish the bZIP interaction network in mature male gametophyte, the crucial first step was to select the candidate genes for dimerization assays. There are several mechanisms limiting the dimerization potential including DNA-binding ability, intracellular transport dynamics, as well as post-translational modifications. We applied a combination of two selection criteria: AtbZIP gene family co-expression data and the composition of dimerization domains, consisting of amino acids favourable for dimerization. First, we compared the expression profiles of 75 AtbZIP genes, in 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/, Dupl'áková et al. 2007), and selected candidates with similar expression profiles possessing a mean expression signal in pollen greater than 400 (for each bZIP gene, Affymetrix Arabidopsis ATH1GeneChip). Finally, 17 genes were selected, and these are highlighted in green boxes in Fig. 1a.

To narrow down the number of candidate genes and to increase the specificity of the putative bZIP network, we analysed the amino acid 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 77 AtbZIP family members. The authors excluded ten TFs because these proteins did not meet their selection criteria. However, two out of these are included in our study, AtbZIP34 (At2g42380) and AtbZIP61 (At3g58120), which belong to subgroup E.

The bZIP domain consists of two structural features located on contiguous α helices. These contain a 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 bZIP proteins, we have identified the basic region and adjacent leucine domains for each candidate protein (Fig. 1b). Positions in every leucine heptad were marked as "g a b c d e f", where positions "a e g" represented amino acid residues that determine the attraction or repulsion of the two proteins. Charged amino acid residues at position "a" are predicted to inhibit homodimer formation, while lysine at the same position is expected to form heterodimer. Charged amino acids in positions "g e" are assumed to allow 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 and the fact that the putative interactions among bZIP proteins were reported by Deppmann et al. (2004) in silico, we focused on two of the previously excluded genes—AtbZIP34 and AtbZIP61. We hypothesized that interaction partners of AtbZIP34 in pollen are AtbZIP18 and AtbZIP52 belonging to group I. We also predicted heterodimer formation between AtbZIP18 and AtbZIP61 and homodimerization of AtbZIP18. On the contrary, the interaction between AtbZIP34 and AtbZIP61, possessing a proline residue in the third leucine zipper domain, resulted in the repulsion of two proteins. However, when proline was replaced by alanine, AtbZIP34 and AtbZIP61 could interact (Shen et al. 2007).

Finally, based on the modified Deppmann analyses presented, we selected eight pollen-expressed proteins for the dimerization studies: 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) using Deppmann et al. (2004) modified analysis. We found that the heterodimerization was favourable between bZIP34/bZIP52, bZIP18/bZIP61, and bZIP61/bZIP52.

AtbZIP network candidates are widely expressed in Arabidopsis

The gene expression patterns obtained from transcriptomic data sets were verified by RT-PCR for all eight selected genes among sporophytic (root, stem, and leaf) and reproductive tissues (mature pollen and inflorescences; Fig. 2). RT-PCR analysis confirmed pollen expression profiles observed in the microarray data, with strong expression signal in mature pollen for AtbZIP18. AtbZIP34 was confirmed to be expressed in reproductive tissues (Gibalová et al. 2009). The expression patterns of other selected bZIP TFs were not tissue-specific (Fig. 2), and specificity of function relies rather on post-translational mechanisms and protein dimerization (see Schütze et al. 2008).

Several pollen-expressed AtbZIP TFs dimerize in Y2H assay

Eight candidate bZIP genes were selected for yeast two-hybrid (Y2H) assays: AtbZIP1, AtbZIP18, AtbZIP25,

Fig. 2 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

AtbZIP28, AtbZIP34, AtbZIP52, AtbZIP60, and bZIP61. Full-length coding sequences were cloned into bait and prey Y2H vectors and tested for auto-activation. All tested Gal4 DNA-binding domain (DBD) fusion proteins were found to activate selectable marker genes in the absence of the activation domain (AD) fusion protein. All N-flanking expression clones, except three (AtbZIP1, AtbZIP25, AtbZIP52), failed to show auto-activation and therefore were suitable for direct interaction tests. AtbZIP1 and AtbZIP25 were excluded as it was not possible to design truncated forms without avoiding part of their BRLZ domains important for dimerization. In the case of AtbZIP52, we prepared a shorter protein deletion fragment, but this was found to support growth on selective media in the absence of the AD fusion protein. We titrated yeast growth on—W-L-H media with increasing concentrations of 3-amino triazole (3-AT), but 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 AtbZIP52 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 a reciprocal manner and proteins bZIP61, AtbZIP34, and AtbZIP18 interacted with AtbZIP52 (Fig. 3a, b). These results are in agreement with our in silico prediction for dimerization (according to Deppmann et al. 2004).

Functional characterization of AtbZIP18

Characterization of AtbZIP18 T-DNA insertional mutant

As a next step to clarify the roles of bZIP TFs in pollen, we characterized the abundantly expressed bZIP gene in pollen, AtbZIP18, which was interacted with the most of the tested prey.

The *AtbZIP18* T-DNA insertion line SALK_111120 was characterized. The T-DNA insertion was confirmed to be localized in the 5′ UTR, at position -266 nt upstream of the ATG start codon (Supplementary Fig. S1A). The absence of the *AtbZIP18* transcript was verified in mature pollen cDNA by quantitative RT-PCR (Supplementary Fig. S1B), confirming that SALK_111120 line harboured a null allele of AtbZIP18. Mature pollen isolated from 40 *atbzip18* homozygous (HM) plants covering T1, T2, and T3 generations was analysed by bright-field and fluorescence microscopy to evaluate the pollen morphology and the positions and number of pollen nuclei (Reňák et al. 2012). As for *atbzip34* pollen (Gibalová et al. 2009), we observed the presence of inclusions as well as aborted pollen grains (Fig. 4a–d). However, the penetrance of these phenotype defects was very low, always below 5%. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were employed to observe exine patterning and the ultrastructure of developing *atbzip18* pollen. Again, we observed exine formation defects similar to those seen for *atbzip34* pollen (Gibalová et al.

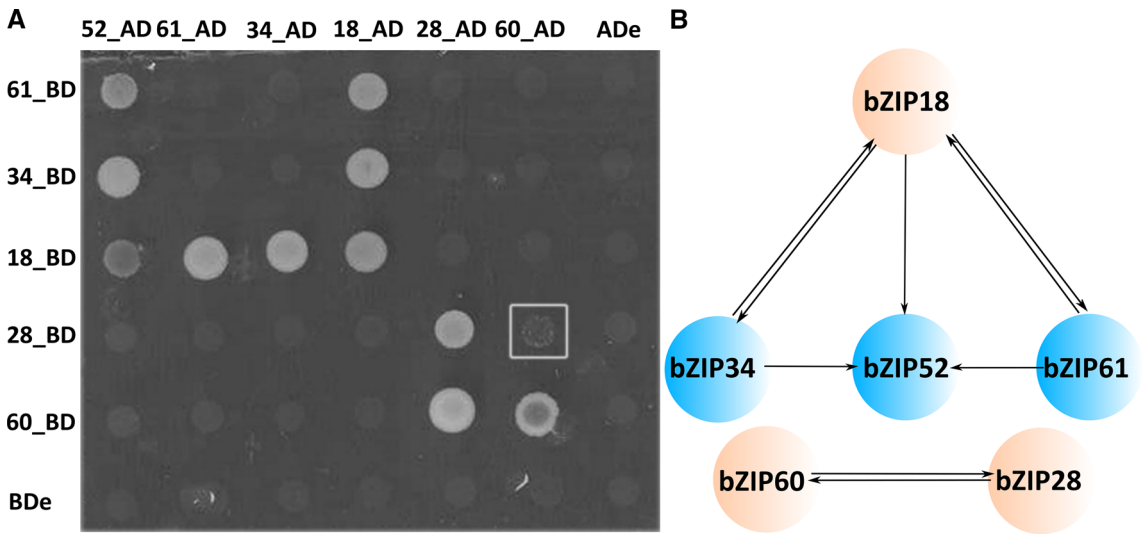

Fig. 3 Pairwise interactions of six bZIP candidates. Binding domain (BD-bait) fusions are illustrated in rows, and activation domain (AD-prey) fusions are illustrated in columns. AtbZIP52 was used as a prey only. Tested colonies were resuspended in water to reach $OD_{600} = 0.1$ and dropped (10 µL) on selection media lacking W, L, A, H. Weak interaction between AtbZIP28 and AtbZIP60 is framed (**a**). Graphical illustration of individual bZIP interactions. bZIP proteins in orange circles are also homodimerizing, except for the interactions with other bZIPs. Double lines are representing reciprocal interactions, and simple lines are showing interactions carried out in one direction (**b**)

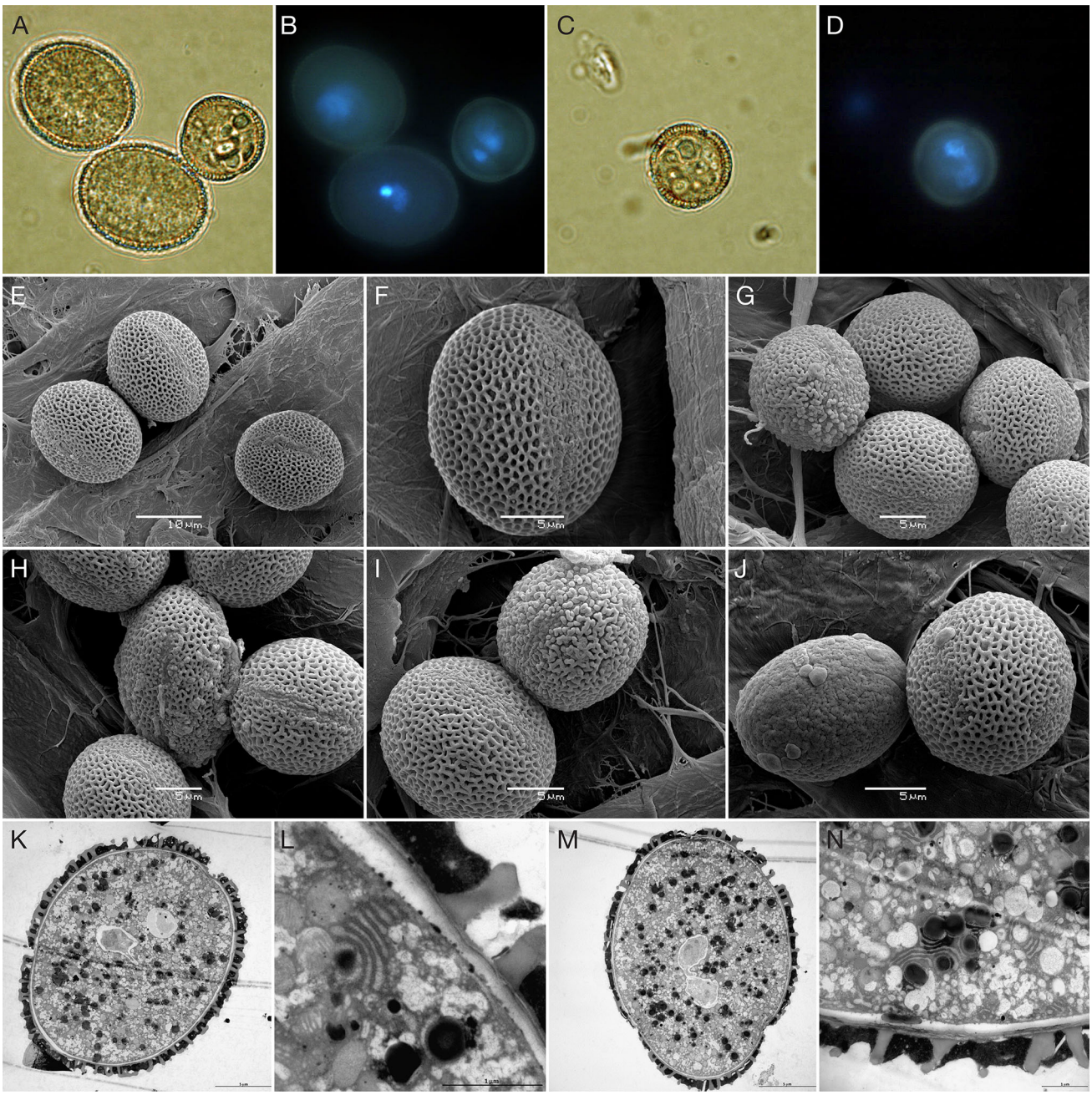

Fig. 4 Morphological characterization of *atbzip18* pollen. Phenotypic defects in *atbzip18* pollen (a**–d**). Bright-field (**a**, **c**) and fluorescence images after DAPI-staining (**b**, **d**) are shown. Scanning electron micrographs of wild-type (**e**, **f**) and *atbzip18* pollen (**g**–**J**). Transmission electron micrographs of mature wild-type (**k,** l) and *atbzip18* (**m**, **n**) pollen

2009), but also at a low (<5%) frequency, (Fig. 4e–j). On the contrary, TEM observations did not show any deviations from wild-type pollen (Fig. 4k–n). Consequently, we assessed the frequency of seed gaps in siliques of *atbzip18* HM plants and observed 4 ± 6% (mean ± SD; $n = 135$) seed gaps, representing slight but significant increase (P value 0.02) when compared to the frequency of failed seed set in wild-type plants (1 ± 2%; $n = 105$).

As Mendelian segregation ratio distortion is a good indicator of transmission defects and gametophytic gene function (Lalanne et al. 2004), the transmission efficiency of the mutant allele through both gametophytes was assessed. Reciprocal test-crosses showed a statistically non-significant decrease in the mutant allele transmission through the male gametophyte by 18%, while the transmission through the female gametophyte remained unaffected (Table 1).

Table 1 Genetic transmission analysis of *atbzip18* mutation

SALK_111120					
atbzip18/– ♀ × Col-0 ♂			Col-0 ♀ × *atbzip18/–* ♂		
wt	±	TE^f	wt	±	TE^m
79	82	103.8	89	73	82.02

Numbers of progeny arisen from reciprocal test-crosses are illustrated 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)

AtbZIP18 is widely expressed in gametophytic and sporophytic tissues

To obtain a deeper insight into the AtbZIP18 expression pattern, we extended our RT-PCR analysis and showed the dynamics of AtbZIP18 mRNA in four stages of pollen development and four sporophytic tissues (Fig. 5a). Furthermore, we observed the activity of GUS reporter gene under the AtbZIP18 promoter in a broad range of gametophytic and sporophytic tissues (Fig. 5b–d). In the male gametophyte, a weak GUS signal was observed at the microspore stage, which gradually accumulated towards pollen maturity (Fig. 5b, I–III). In inflorescences, the GUS signal was present in young buds, restricted to the tapetum and sepals (Fig. 5c, I, II, IV). In mature flowers, we observed GUS signal in carpels, petals, and anther filaments (Fig. 5c, III, IV). In the sporophyte, a gradient of GUS staining was detected in distal parts of the cotyledons further emerging in vascular tissues of five-day-old seedlings (Fig. 5d, I, II). In true leaves, the AtbZIP18 promoter activity was detected in vasculature and in areas surrounding hydatodes (Fig. 5d, III). Stems and roots of normally grown and etiolated seedlings also showed GUS signal in vascular tissues (Fig. 5d, IV, VI). In siliques, we observed specific GUS staining only in proximal and distal parts (Fig. 5d, V). Taken together, analysis of AtbZIP18 expression pattern by three independent approaches demonstrated a wide expression profile of AtbZIP18 without apparent tissue specificity. These results emphasize what has been previously reported that bZIP functions are modulated post-translationally through protein–protein interaction rather than at the transcriptional level (see Schütze et al. 2008).

AtbZIP18-GFP fusion protein is located in two subcellular compartments

All Arabidopsis bZIP genes are annotated as DNA-binding transcription factors, and their presence in the nucleus is expected. AtbZIP18 protein location was verified by

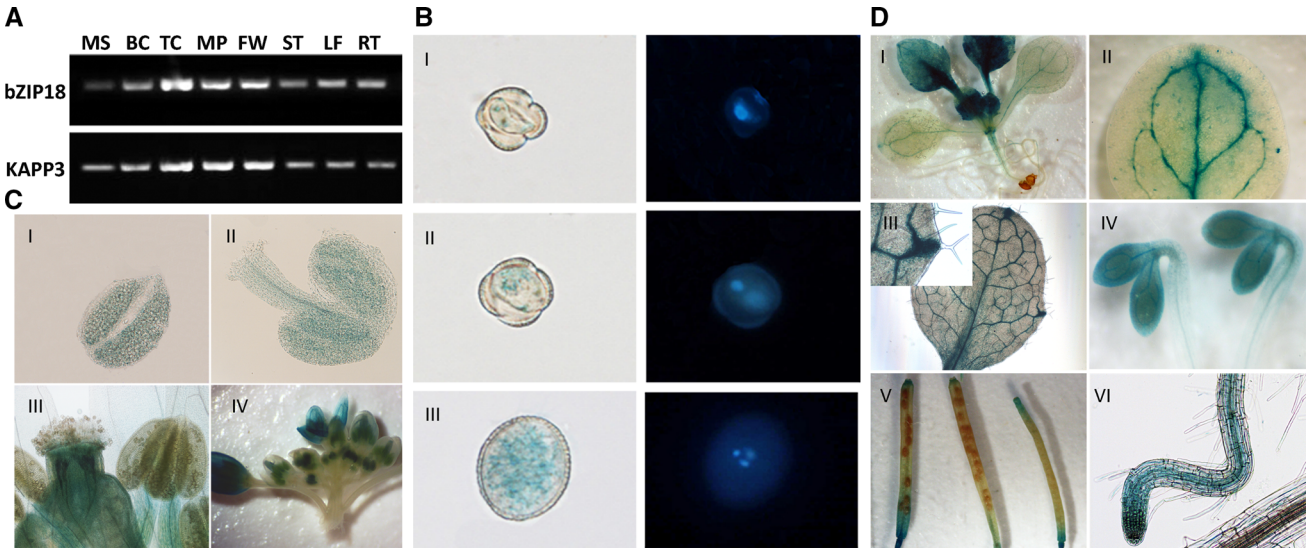

Fig. 5 Verification of *AtbZIP18* expression in four stages of pollen development: microspores (MS), bicellular pollen (BC), tricellular pollen (TC), and mature pollen (MP) and among four sporophytic tissues: mature flowers (FW), stem (ST), leaf (LF), and root (RT), KAPP3—control of expression—kinase-associated protein phosphatase At5g19280 (**a**). Activity of the *AtbZIP18* promoter in male gametophyte starts at microspore stage (*I*) accumulates slightly more at bicellular stage (*II*) and it is increasing towards 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**)

transient expression of GFP N- and C-terminally fused to AtbZIP18 coding sequence under control of the CaMV 35S promoter. We observed comparable location patterns for both AtbZIP18 fusion proteins, in the nucleoplasm and the perinuclear region (Fig. 6a–c), where AtbZIP18 co-localized with the endoplasmic reticulum marker ER-rk CD3-959 (Arabidopsis Biological Resource Center (ABRC), The Ohio State University). A portion of the fusion protein was detected in the cytoplasm, partially associated with the ER (Fig. 6d–f). As a control, we tested the localization of free GFP co-infiltrated with the ER marker. We did not detect such co-localization as for AtbZIP18 in all cells observed (Supplementary Fig. S2). On the contrary, AtbZIP34 protein was present exclusively in the nuclei of transiently transformed tobacco leaf epidermal cells (Supplementary Fig. S3). The fusion of the AtbZIP34 promoter with the histone protein H2B and GFP in stably transformed plants showed GFP signal only in pollen vegetative cell nuclei, implying its vegetative cell-specific expression (Supplementary Fig. S4).

We obtained an independent verification of AtbZIP18 localization from stable transgenic lines expressing AtbZIP18 genomic sequence under its native promoter fused to GFP. In the male gametophyte, GFP signal was not detected in microspores (Fig. 7a), but there was a weak GFP signal in bicellular pollen (Fig. 7b) that significantly increased in mature pollen (Fig. 7c) and in pollen tubes (Fig. 7d). After fertilization, GFP signal was observed in mature embryos restricted to the nuclei (Fig. 7e–g). In shoots of 6-day-old seedlings, GFP signal was seen in cotyledons and guard cells nuclei (Supplementary Fig. 5A), later also in true leaves (Supplementary Fig. 5B), stems (Supplementary Fig. 5C), and nuclei of the trichomes (Supplementary Fig. 5D). Further analysis of roots showed GFP signal in primary roots (Supplementary Fig. 5E) and secondary root primordia (Supplementary Fig. 5F). In detail, GFP expression in roots was observed at the root elongation zone (Supplementary Fig. 5G) and in the nuclei at the root tip (Supplementary Fig. 5H). Apart from the enriched nuclear signal, we also detected weaker AtbZIP18-GFP signal in the cytoplasm (Fig. 7; Supplementary Fig. 5—inset).

AtbZIP18 modulates gene expression in Arabidopsis pollen

Since the *atbzip18* knockout mutant did not produce a significant proportion of pollen grains with morphological or functional abnormalities when compared to WT, we examined gene expression in *atbzip18* pollen to identify potential AtbZIP18 targets and to refine its role in the male

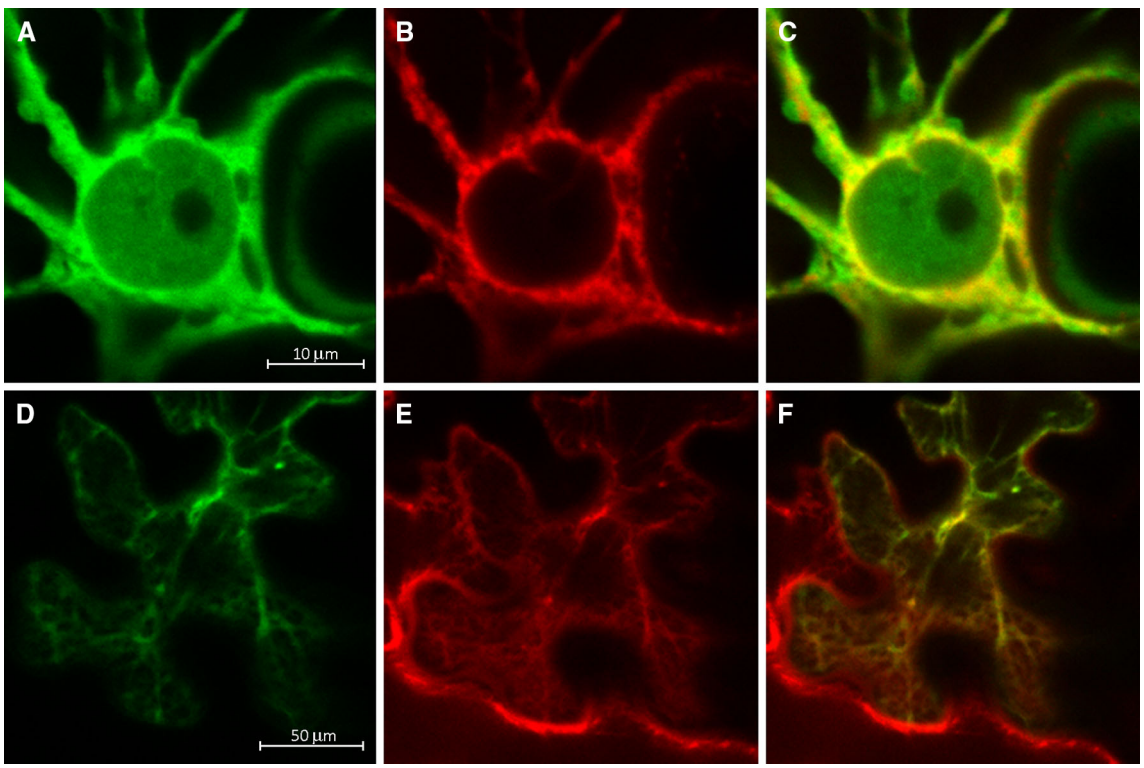

Fig. 6 Localization of AtbZIP18-GFP fusion protein in tobacco epidermal cells. Fusion protein was localized to nucleoplasm and perinuclear region (**a**) confirmed by co-localization with ER marker ER-rk CD3-959 (**b**). Merged image (**c**). AtbZIP18 is partially or fully localized to the ER and cytoplasm (**d**) ER marker (**e**) merged image (**f**)

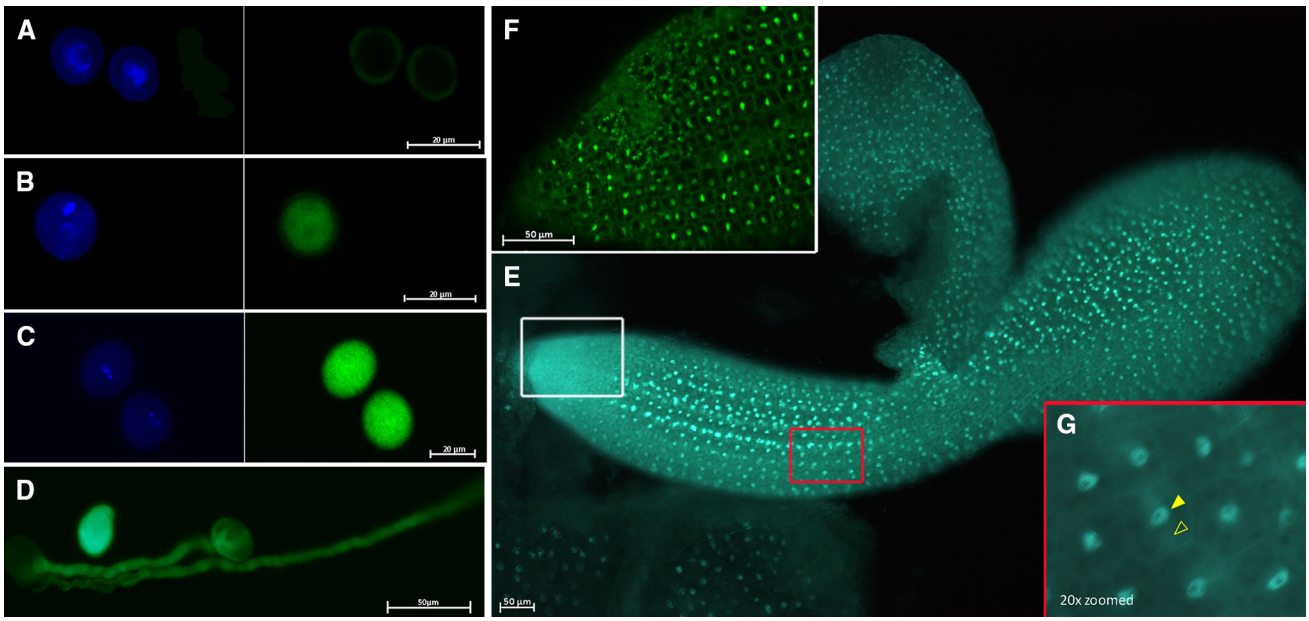

Fig. 7 Localization of the whole genomic fragment of AtbZIP18 containing native promoter, 5′-UTR and ORF fused to the GFP during male gametophyte development. GFP signal was absent in microspores (**a**) and started to be accumulated from bicellular pollen (**b**), and increases in mature pollen grains (**c**). During pollen germination, the fusion the GFP signal was also detected in the cytosol of pollen tubes (**d**). Localization of AtbZIP18-GFP fusion protein in mature embryo (**e**–**g**). The signal was restricted to the nuclei and ER and/or cytoplasm of individual cells (zoomed windows **f**, **g**). *Filled arrowheads* mark AtbZIP18 localization in nucleus and empty arrowheads cytoplasm

gametophyte. Affymetrix ATH1 microarrays were hybridized with cRNA derived from total RNA isolated from HM *atbzip18* pollen. Data sets were compared with previously published data from *atbzip34* pollen. The quality control of the microarray data sets confirmed good reproducibility of biological replicates in all samples (Supplementary Fig. S6; Supplementary Fig. S7A–C) as well as the apparent overall differences between both RNA samples originating from mutant pollen in relation to wild-type controls (Supplementary Fig. S6B–C; Supplementary Fig. S7D–F). Expression profiles of selected genes were validated by quantitative RT-PCR (Supplementary Fig. S8) further confirming reproducibility of the microarray analyses.

We found 403 genes to be differentially expressed in *atbzip18* pollen compared to reference WT samples. The mean signals of 117 probes, which showed a statistical significant increase of at least twofold compared to WT, were considered as upregulated (Supplementary Table S2), while 16 genes under the same criteria were classified as downregulated (Supplementary Table S3). Differentially expressed genes were found to be more related to late pollen developmental stages (111 out of total 117 in upregulated class, and 10 out of 16 in downregulated class), whereas the average expression signal in mature pollen was 336 for upregulated group and 506 for those in downregulated category. The upregulated class contained seven times more genes than downregulated category suggesting a likely predominant repression activity of AtbZIP18. Sets of upregulated and downregulated genes were functionally grouped for singular enrichment analysis (SEA) (http://bioinfo.cau.edu.cn/agriGO/) to identify enriched GO terms within three groups: (1) biological process, (2) cellular component, and (3) molecular function. The most significant GO term within the small subset of downregulated genes was stress response, while lipoprotein biosynthetic processes, peptidyl-amino acid modification, nucleic acid binding, and transcription were the most prominent GO terms in the upregulated set (Supplementary Table S4). The enrichment of these categories suggested the involvement of AtbZIP18 in at least some of the pathways regulated by AtbZIP34 (lipid metabolism) and its role in transcriptional regulation in pollen development. Of the latter, we identified 13 TFs (11%) in the *atbzip18*-upregulated subset falling into several families including C2H2, GATA, GRAS, NAC, and SPL (Supplementary Table S2). Most interestingly, three MYB TFs (At1g01520, At3g25790, At5g05090) and AtbZIP61 (At3g58120), which interacted with AtbZIP18 in the Y2H assay (Fig. 3), were upregulated in *atbzip18* pollen.

Transcription factors were not the only nucleic acid-binding proteins upregulated in *atbzip18* pollen. They were supplemented with several RNA-binding proteins including two DEAD box helicases (At1g20920, At3g06480), three RNA recognition motif-containing proteins (RRM;

At1g67770, At1g73530, At5g19030) and FCA (At4g16280). FCA (Flowering time control protein) is an RNA-binding protein involved in the promotion of reproductive development via RNA-mediated chromatin silencing.

We previously showed that AtbZIP18 interacts with AtbZIP34 TF (Gibalová et al. 2009); therefore, the regulation of the same pathway(s) was expected through intersection of genes differently expressed in both mutants. To evaluate this, up- and downregulated classes of both mutants were compared for possible intersection. Microarray data for *atbzip34* pollen were processed in the same way as *atbzip18* data sets and filtered based on statistically significant up- and downregulated genes showing at least a twofold change in gene expression. However, we found only four probes common for both sets of upregulated genes (i.e. for At3g23260, At2g21920, At2g42000, and probe 252874_at which did not match a gene) and one probe common for AtbZIP18 down- and AtbZIP34 upregulated sets (Supplementary Fig. S9). In the light of these results supported by the phenotype observations (Fig. 4; Gibalová et al. 2009), we can assume the functional redundancy of AtbZIP18 and AtbZIP34 TFs, for example in the control of lipid metabolism and in the metabolic pathway leading to the pollen wall synthesis. More detailed biochemical and immunocytological assays (Dardelle et al. 2010; Lehner et al. 2010) will be further employed to analyse alterations of cell wall composition in *bzip* mutants.

Discussion

Identification of bZIP interaction network in pollen

We explored the transcriptional regulatory mechanisms active during male gametophyte development focusing on the late pollen developmental program. To date, few examples of regulatory networks important for the production of both male (Verelst et al. 2007; Borg et al. 2011; Borg et al. 2014; Xu et al. 2010) and female gametes (Brambilla et al. 2007; Colombo et al. 2008; Galbiati et al. 2013) have been described. However, with respect to the large size of the pollen transcriptome of approximately 6044 transcripts (Rutley and Twell 2015), with large proportions of pollen-enriched (26%) and presumably pollen-specific (11%) genes (Honys and Twell 2004), this still represents only small fraction of characterized genes. To describe transcription factor gene families potentially important for pollen development, we used published transcriptomes of *Arabidopsis thaliana* pollen developmental stages, which included the profiles of 1595 putative transcription factors distributed among 50 gene families (Honys and Twell 2004; Reňák et al. 2012). The bZIP family is the second largest family of dimerizing TFs in Arabidopsis, and with this functional feature, bZIPs represent an excellent model to study how TFs can modulate cellular physiology in response to the internal and external environment. As bZIP proteins dimerize in all eukaryotes and are involved in a wide range of cellular functions, it is presumed that they also play important role in the male gametophyte. Several *At*bZIP 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 bZIP proteins in the interaction map, including AtbZIP34 and bZIP61. Remaining bZIPs in our selection, AtbZIP18, AtbZIP28, AtbZIP52, and AtbZIP60, were predicted to favour homodimerization, whereas heterodimerization was most probable for bZIP18/bZIP28, bZIP60/bZIP28, and bZIP52/bZIP18. These predictions were confirmed by our Y2H results. We identified three homodimers—bZIP18/bZIP18; bZIP28/bZIP28; bZIP60/bZIP60 and three heterodimer pairs—bZIP18/bZIP61; bZIP18/bZIP34; bZIP28/bZIP60. The Y2H assay also showed that AtbZIP18, AtbZIP34, and AtbZIP61 proteins were able to interact with AtbZIP52. Out of all proteins tested, AtbZIP18 possessed the highest dimerization capacity as it interacted with most of the tested proteins and formed homodimers. The presented protein–protein interactions within AtbZIP family, although mostly bidirectional, were demonstrated in the heterologous yeast system. Therefore, it is important in future detailed studies to verify these interactions *in plant*a by means of Bimolecular Florescence Complementation (BiFC; Kerppola 2006) or Arabidopsis Protoplast two Hybrid (P2H; Ehlert et al. 2006), which was successfully used to confirm Y2H interactions among two subgroups of the bZIP proteins.

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) by competing 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). In tobacco, 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. Networks of TFs are indeed flexible and efficient tools reflecting multitude of environmental and developmental stimuli during plant life cycle. Plants as sessile organisms therefore evolved differential strategies of TF regulatory capabilities to sustain and retain plant fitness without excessive multiplication of TF genes. Examples of two different strategies of TF regulation have been described on two TF families: bZIP and WRKY. In the case of the bZIPs, networking by heterodimerization appears to be the preferred tool to fine-tune bZIP function enabling crosstalk between different input signals, compared to WRKY TF family, where controlling transcription of each other stands out as a networking strategy for this family in synergism along with the epigenetic control of their promoters (Llorca et al. 2014). Having such complex system of TF regulatory responses, it is not straightforward to uncover their involvement in studied tissue or cell lineage. Therefore, we aimed to perform follow-up analyses to decipher the function of two strongly pollen-expressed and mutually interacting bZIP TFs, AtbZIP18 and AtbZIP34, with the focus on their regulons.

Proposed AtbZIP18 regulatory mode of action

Based on our phenotype observations (Gibalová et al. 2009; this work), demonstrated interaction, and the lack of dramatic changes in gene expression in *atbzip18* pollen, we can predict the likely partial functional redundancy of AtbZIP18 and AtbZIP34 transcription factors in Arabidopsis pollen. The likelihood that the small differences in gene expression in *atbzip18* pollen were caused by the loss of AtbZIP18 allele and not due to undetected differences in growth conditions was supported by the quality control of the microarray data sets and by quantitative RT-PCR validation of selected genes.

On the contrary, only a limited overlap of genes with expression affected by the absence of *atbzip18* and *atbzip34* might suggest another explanation, for example the hierarchical roles of both TFs, when AtbZIP18 with stronger dimerization potential would be regulating the activity of AtbZIP34. However, the low penetrance of apparent phenotype defects in pollen of *atbzip18/–* knockout plants as well as their similarity to those caused by the absence of *atbzip34* (Gibalová et al. 2009) favours the functional redundancy hypothesis. Moreover, the predominance of the number of upregulated genes in both *atbzip18* and *atbzip34* pollen points out their possible function as repressors. This is further supported by the fact that AtbZIP18 possesses an EAR (ethylene-responsive element binding factor-associated amphiphilic repression) motif involved in transcriptional inhibition through chromatin modifications (Kagale et al. 2010). The link of AtbZIP18 activity with chromatin modifications was recently proposed in relation to the maintenance of nuclear morphology (Pawar et al. 2016). Moreover, there is compelling evidence that TFs containing EAR motifs are involved in transcriptional repression in the male gametophyte. Analysis of the DUO1-DAZ1 male germline-specific regulatory network demonstrated that EAR motifs in DAZ1 are required for the transcriptional repression and for physical interaction with the co-repressor TOPLESS (TPL; Long et al. 2006) as well as for male germline specification (Borg et al. 2014). The identification of bZIP TFs in the TPL interactome using Y2H screen (Causier et al. 2012) and the presence of EAR motif in both AtbZIP18 and AtbZIP52 proteins favour them as putative transcriptional repressors during pollen development. On the contrary, other AtbZIP18 interactors—AtbZIP28, AtbZIP34, and AtbZIP60—do not possess the EAR motif. In Arabidopsis, the progression from proliferating microspores to mature pollen is characterized by large-scale repression of early male gametophytic genes and the activation of a unique late gene expression program (Honys and Twell 2004). Since AtbZIP18 is expressed from the microspore stage, it may contribute to such regulatory machinery.

Functional characterization of AtbZIP18

AtbZIP18 represented the most promising candidate for further functional analysis for its abundant late pollen expression profile, high dimerization capacity, and the presence of repressory EAR domain. We have conducted a complex functional analysis of AtbZIP18 in order to supplement our previous characterization of its binding partner AtbZIP34.

At first, we validated the AtbZIP18 expression profile presumed from the microarray experiments by semi-quantitative RT-PCR and monitored the promoter-GUS activity throughout plant development. The AtbZIP18 promoter showed enriched expression throughout pollen development alongside its activity in other reproductive and vegetative tissues (Figs. 5, 7; Supplementary Fig. S5), partially overlapping with the activity of AtbZIP52 and bZIP59 promoters (Pyo et al. 2006). Another group of bZIP TFs from C/S1 group displayed similar pleiotropic promoter activities (Weltmeier et al. 2009). The expression pattern observed in AtbZIP18 promoter:GUS transgenic plants corresponded to the microarray data for At2g40620 gene (www.genevestigator.ethz.ch; Zimmermann et al. 2004). 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 pollen activities. Unlike AtbZIP18, we observed the AtbZIP34 promoter activity in young female gametophytes as supported by *atbzip34* transmission defects (Gibalová et al. 2009). In the sporophyte, both promoters acted similarly in vasculature of cotyledons, true leaves, roots, and stems; the most prominent difference was the activity of AtbZIP34 promoter in stigmatic papillary cells and trichomes (Gibalová et al. 2009).

However, the cellular localization of AtbZIP18 protein fused to GFP was different. We observed AtbZIP18 not only in the nuclei but also in the cytoplasm/ER of transiently transformed tobacco leaf epidermal cells. To exclude potential artefacts that could arise from the strong CaMV 35S promoter activity, we generated and analysed stably transformed Arabidopsis plants expressing the whole genomic sequence of AtbZIP18 fused with GFP. In the male gametophyte, we detected more uniformly distributed GFP signal in the pollen cytoplasm. We could not specifically distinguish the GFP signal in pollen nuclei, but the signal in the area of both vegetative and generative nuclei appeared weaker at bicellular stage. On the contrary, the AtbZIP18 nuclear localization and ER-co-localization were supported by the observations in the transient assays. Additionally, in the sporophyte, AtbZIP18-GFP fusion protein was distinctively observed in the cytoplasm and nuclei of developing embryos. The variability of the subcellular localization between gametophytic and sporophytic tissues may arise as a result of different mechanisms of AtbZIP18 regulation in these tissues, namely the possible higher level of cytoplasmic sequestration of inactive AtbZIP18 protein in bicellular pollen. 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). Following stress treatment which triggers unfolded proteins response in the ER (UPRE), the 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 further examples of ER-resident bZIP transcription factors executing UPRE (Iwata and Koizumi 2005; Iwata et al. 2008). AtbZIP10 shuttles between the nucleus and the cytoplasm, retained by Lesions Simulating Disease resistance 1 protein (LSD1; Kaminaka et al. 2006). In other cases, bZIPs are actively shuttled out of the nucleus due to the presence of a Nuclear Export Signal (NES; Tsugama et al. 2012), and they are retained in the nucleus only when the NES is masked (Li et al. 2005; Llorca et al. 2014).

Surprisingly, knockout of AtbZIP18 did not result in strongly penetrant phenotypic differences, but phenotype defects were similar to those observed in *atbzip34* pollen, namely pollen abortion, exine formation defects, and the presence of inclusions in the vegetative cell cytoplasm. The high expression of AtbZIP18 in the male gametophyte and interactions with other pollen-expressed bZIP proteins, together with the moderately reduced transmission of the mutant allele through the male gametophyte, are indicative of the functional involvement of AtbZIP18 in pollen development.

Conclusively, our work outlines the interaction network for pollen-expressed bZIP proteins and points to the potential impact of AtbZIP18, which possesses high expression in pollen and the highest dimerization potential among other pollen-expressed AtbZIP proteins tested by Y2H analysis. AtbZIP18 is widely expressed in vegetative and reproductive tissues suggesting its pleiotropic mode of action. We performed genetic and functional studies to examine the functional importance of AtbZIP18 that was indicated by slightly reduced transmission of the mutant *atbzip18* allele through pollen and slightly increased number of seed gaps. The regulatory mode of AtbZIP18 function in the light of available transcriptomic data suggests functional redundancy with its binding partner AtbZIP34 and the presented data supplement our previous characterization of AtbZIP34 in the same bZIP interaction network. Characterization of double, triple, or even quadruple mutants among the AtbZIP18 interactors will be necessary to provide further insight into the stoichiometry and function of the bZIP transcription factors network in the male gametophyte.

Author contribution statement AG, SH, and DH conceived and designed the research, AG, LS, and CM conducted the experiments. VB, ZG, and ND cloned the constructs. KM and RP analysed the data. AG, SH, and DH wrote and edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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