BURSTING POLLEN is required to organise the pollen germination plaque and pollen tube tip in Arabidopsis

<table>
<thead>
<tr>
<th>Journal:</th>
<th><em>New Phytologist</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>Draft</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>MS - Regular Manuscript</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
</tr>
</tbody>
</table>
| Complete List of Authors: | Rieu, Ivo; Radboud University, Molecular Plant Physiology, Institute for Water and Wetland Research  
Hoedemaekers, Karin; Radboud University, Molecular Plant Physiology, Institute for Water and Wetland Research  
Derksen, Jan; Radboud University, Molecular Plant Physiology, Institute for Water and Wetland Research  
Hoogstrate, Suzanne; Radboud University, Molecular Plant Physiology, Institute for Water and Wetland Research  
Wolters-Arts, Mieke; Radboud University, Molecular Plant Physiology, Institute for Water and Wetland Research  
Oh, Sung Aeong; Kyungpook National University, Division of Plant Biosciences  
Twell, David; University of Leicester, Biology  
Mariani, Celestina; Radboud University, Molecular Plant Physiology, Institute for Water and Wetland Research |
| Key Words:        | Pollen germination, Germination plaque, Pollen tube tip, GT4-related glycosyltransferase, Pectin, Male fertility, Arabidopsis thaliana |
BURSTING POLLEN is required to organise the pollen germination plaque and pollen tube tip in Arabidopsis

Karin Hoedemaekers¹,†, Jan Derksen¹, Suzanne W. Hoogstrate¹, Mieke Wolters-Arts¹, Sung-Aeong Oh², David Twell², Celestina Mariani¹, Ivo Rieu¹,*

¹ Department of Molecular Plant Physiology, Institute for Water and Wetland Research, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ, Nijmegen, The Netherlands.
² Department of Biology, University of Leicester, Leicester, LE1 7RH, United Kingdom.

* For correspondence (e-mail i.rieu@science.ru.nl)
† Present address: Bayer CropScience Vegetable Seeds, Napoleonsweg 152, 6083 AB Nunhem, The Netherlands

Corresponding author for submission:
Ivo Rieu, Tel: +31 (0)243652769, i.rieu@science.ru.nl

Email list:
Karin Hoedemaekers: karin.hoedemaekers@bayer.com
Jan Derksen: jwmderksen@versatel.nl
Suzanne W. Hoogstrate: suzannehoogstrate@gmail.com
Mieke Wolters-Arts: M.Wolters@science.ru.nl
Sung Aeong Oh: aeongoh@knu.ac.kr
David Twell: twe@leicester.ac.uk
Celestina Mariani: c.mariani@science.ru.nl
Ivo Rieu: i.rieu@science.ru.nl

Running title: BUP function in pollen
Word count: 5675 (Introduction 651; Materials and Methods 1784; Results 1796; Discussion 1525; Acknowledgements 65)

Number of figures: 7 (in colour: Fig. 2, 5)
Number of tables: 3
Supporting information: 7 figures, 2 tables
Summary

- Pollen germination may occur via the so called germination pores or directly through the pollen wall at the site of contact with the stigma. In this study, we address what processes take place during pollen hydration (i.e. prior to tube emergence), in a species with extra-poral pollen germination, Arabidopsis thaliana.
- A T-DNA mutant population was screened by segregation distortion analysis. Histological and electron microscopy techniques were applied to understand the wild-type and mutant phenotypes.
- Within one hour of the start of pollen hydration an intine-like structure consisting of cellulose, callose and at least partly de-esterified pectin was formed at the pollen wall. Subsequently, this ‘germination plaque’ gradually extended and opened up to provide passage for the cytoplasm to the emerging pollen tube. 
  BURSTING POLLEN (BUP) was identified as a gene essential for the correct organization of this plaque and the tip of the pollen tube. BUP encodes a novel Golgi-located glycosyltransferase related to the GT-4 subfamily that is conserved throughout the plant kingdom.
- Extra-poral pollen germination involves the development of a germination plaque and BUP defines the correct plastic-elastic properties of this plaque and the pollen tube tip by affecting pectin synthesis or delivery.

Keywords: Pollen germination; Germination plaque; Pollen tube tip; GT4-related glycosyltransferase; Pectin; Male fertility; Arabidopsis thaliana

Introduction

Pollen development is an important process in the life cycle of a flowering plant and is a major factor that affects the yield and quality of crop seeds. The role of the male gametophytes or pollen grains in seed plants is to deliver the sperm cells to the ovules
where fertilization takes place. Pollen grains are deposited on the stigma, where each germinates to form a pollen tube that grows through the stigma and style toward the egg apparatus in the ovule. Pollen germination and tube growth are complex processes with distinctive features that depend on post-meiotic (or gametophytic) gene expression. Male gametophytic genes can most simply be separated into two major clusters based on their expression profiles throughout development; the ‘early’ genes that are expressed soon after meiosis and decline before pollen maturation and the ‘late’ genes that are expressed after the first mitosis in the pollen grain (Mascarenhas, 1990). The late genes in particular include a predominant class of pollen-specific genes that are enriched in functions associated with cell wall metabolism, signalling and cytoskeleton organization (Becker et al., 2003; Da Costa et al., 2004; Engel et al., 2003; Honys & Twell, 2003; Honys & Twell, 2004; Twell et al., 2006).

Arabidopsis pollen grains differ from those of most other angiosperm species, as they do not germinate via the pre-formed germinal pores; instead, they germinate directly through the pollen wall at the site of contact with the stigma (Edlund et al., 2004; see also Fig. S1). This site is marked by a localised intracellular deposition of callose near the pollen wall prior to emergence of the pollen tube (Johnson and McCormick, 2001; Lalanne et al., 2004). Arabidopsis pollen tubes show a typical wall architecture and tip growth that is commonly observed (Derksen et al., 2002; Geitmann and Steer, 2006; Krichevsky et al., 2007; Zonia and Munnik 2008, 2009; Dardelle et al., 2010; Mollet et al., 2013). A typical pollen tube wall consists of a primary outer wall layer and a secondary inner wall layer. The primary wall consists mainly of pectin, synthesized within the Golgi apparatus and transported by secretory vesicles to the tube tip, where they are gradually de-esterified (Li et al., 1994; Geitmann et al., 1995). There they form a more or less rigid network, probably together with cellulose synthesized at the growing tip (Derksen et al., 2011). The inner, secondary, wall reinforcing the primary wall mainly consists of cellulose and callose, synthesized at the plasma membrane behind the growing tip (Schlüpmann et al., 1994; Ferguson et al., 1998). Both walls are highly porous allowing communication with the environment (Derksen et al., 2011).
This massive production of cell wall components requires the activity of a number of glycosyltransferases (GTs, enzymes forming the glycosylic bonds), including cellulose- and callose-synthases at the cell surface, together with GTs involved in pectin and hemi-cellulose synthesis in the Golgi apparatus (Moscatelli and Idilli, 2009). Arabidopsis has at least 42 GT families comprising 562 members, 99 of which remain unclassified (http://www.cazy.org/GlycosylTransferases.html). Some members have been shown to be active during pollen formation and pollen tube growth (Goubet et al., 2003; Desprez et al., 2007; Persson et al., 2007; Bernal et al., 2008; Liu et al., 2011, Wang et al., 2013). Specific members have important roles in pollen tube growth, as revealed by the cellulose synthase-like mutant atcs-la7 (Goubet et al., 2003) and the rhamnogalacturan II xylosyltransferase mutant mgp4 that both show strong male gametophyte defective phenotypes (Liu et al., 2011).

To better understand male gametophyte function and identify new genetic components involved, an Arabidopsis T-DNA mutant population was screened by segregation distortion analysis, in a strategy that has been described previously (e.g. Howden et al., 1998; Johnson-Brousseau and McCormick, 2004). Here we characterize the germination plaque that is deposited during pollen hydration and report on the isolation and characterization of the male gametophyte defective bursting pollen (bup) mutant, defective in a novel GT4-related glycosyltransferase and necessary for correct plaque formation.

Materials and Methods

Plant material
Seeds of Arabidopsis thaliana L. ecotype Col-0 and Col-6 (Col-0 with glabra1 mutation, ABRC stock number CS8155) were obtained from the Nottingham Arabidopsis Stock Centre (NASC) and Col-0 was used as the wild-type in this study. The mutant line bup-1/+ was isolated from a set of pooled Tom Jack enhancer trap T-DNA lines (line TJ995, ABRC stock numbers CS31086 and CS31087) in the Col-6 background, transformed
with the binary vector *pD991* (Campisi et al., 1999). The plant used for transformation is referred to as the T0 generation. The *bup-2/+* (SALK_033889) is in the Col-0 background and was obtained from NASC. The *qrt1-1* (in Ler) and *qrt1-2* (in Col-3) mutants were kindly provided by Dr G. Copenhaver and used to generate the *bup-1/+ qrt1-1* and *bup-2/+ qrt1-2* double mutants.

Transmission of the mutations through the male gametes was determined by manually pollinating wild-type (Col-0, Col-6) or male-sterile (*ms1*) Lansberg *erecta* plants with *bup-1/+* or *bup-2/+* pollen.

**Cloning of sequences flanking the T-DNA insertions**

Genomic sequences flanking the right and left T-DNA borders in *bup-1* were amplified with thermal asymmetric interlaced (TAIL) PCR or Inverse PCR. TAIL-PCR was performed according to Liu et al. (1995) with minor modifications. T-DNA-flanking sequences were amplified in three rounds of PCR using one of the three degenerate TAIL-PCR primers (AD1: 5'-NTCGA(G/C)T(A/T)T(G/C)G(A/T)GTT-3', AD2: 5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3' and AD3: 5'-(A/T)GT GNAG(A/T)ANCANAGA-3') in combination with three nested right border-specific primers (RB-123: 5'-GCATGCAAGCTTGGCACTGG-3', RB-124: 5'-TGAGACCTCAATTGCGAGC-3' and RB-86: 5'-TCGGGCCTAACTTTTGGTG-3') or three nested left border-specific primers (LB-156: 5'-CCTATAATACGACGGATCG-3', LB-155: 5'-ATAACGCTGCGGACTCTAC-3' and LB-154: 5'-TGATCCATGTAGATTCCCG-3') (Campisi et al., 1999). Fragments of the third PCR reactions that were smaller than those of the second PCR were isolated and sequenced.

Inverse PCR was performed as described by Long et al. (1993). Genomic DNA was digested with *XbaI* and ligated under dilute conditions in order to generate circular fragments. PCR with the RB (RB-86 and RB-96 (5'-AGTGCCAAGCTTGCATG-3') and LB primers (LB-156 and LB-31 (5'-TTGTAACGCCGTTTCCCCACC-3')) was used to amplify DNA flanking the insertion site. Gel-purified PCR product was cloned into *pGEM-Teasy* (Promega, Madison, WI) and sequenced.
The T-DNA insertion sites within gene *At5g04480* in line TJ995 and SALK_033889 were confirmed by PCR amplification using T-DNA border-specific primers for TJ995 and pROK2 respectively, in combination with the *At5g04480* gene-specific primers flanking the TJ995 T-DNA insertion (5’-CTCGTAAACGCCTTTCCAGTGT-3’ and 5’-CTTCTGGACAAGATTAGACATCGATG-3’) or the annotated SALK T-DNA insertion (5’-TCTGATGGCAGCAAAATTTGATACC-3’ and 5’-TTGGCATCCCTATCATAACCTGA-3’), respectively.

**Generation of the complementation construct and Arabidopsis transformation**

To isolate the complete *At5g04480* gene, BAC clone T32M21 DNA was digested with *Agel* and ligated into the *Xmal* site of binary vector *pPZP221*. The ligation product was transformed into *E. coli* DH5α cells and clones containing the *At5g04480* *Agel* fragment were identified by bacterial colony blot hybridization with the *At5g04480* gene-specific probe (as described by Sambrook *et al.*, 1989). The binary vector construct (‘+04480’) was transferred to *Agrobacterium tumefaciens* strain EHA101 (kindly provided by Dr. Stanton Gelvin and Dr. Elisabeth Hood), using freeze transformation (Chen *et al.*, 1994).

Arabidopsis Col-0 plants were transformed by the floral dip method (Clough and Bent, 1998) and T1 seeds were germinated on agar plates containing 110 mg/L gentamycin. Resistant seedlings were transplanted to soil and segregation analysis of the gentamycin resistance was performed to isolate a plant containing a single T-DNA insertion (Col+04480). TJ995 and SALK plants were pollinated by Col+04480 and the segregation of the T-DNA insertion in *At5g04480* (kanamycin resistance) and the complementing gene (gentamycin resistance) was tested in two generations after self pollination and reciprocal crosses with Col-0 plants.

**Generation of fusion protein construct and transient expression in onion cells**

To generate the *BUP:GFP* fusion construct, the 515 bp *BUP*-gene fragment encoding the N-terminal BUP fragment was amplified from the complementation construct ‘+04480’ using the primers 5’-ATCACTAATAGTGACAATTACTGCTCAAGAAAGC-3’ and 5’-
TGAAACTAGTCACGAGAGCTAGCCTGGGCGGACG-3'. The PCR-fragment was digested with Ncol and SpeI and ligated into the Ncol and SpeI sites of pCAMBIA1302 (Roberts et al., 1998) to create 35S::BUP:GFP. The 35S::ST:RFP plasmid was generously provided by I. More and C. Saint-Jore. For biolistic transformation, plasmid DNA (5 μg) was delivered into onion (Allium cepa) epidermal cells using tungsten particle bombardment. For experiments in which BUP:GFP and ST:RFP were expressed simultaneously, 2.5 μg of each plasmid was bound to the tungsten microprojectiles. Inner epidermal onion peels were placed on agar plates containing 1x MS salts, 30 g/L sucrose and 7% select agar, pH 5.7. Peels were bombarded within 1 h of transfer to agar plates. Tungsten microprojectiles (1.1 μm; Bio-Rad) were coated with DNA according to the manufacturer’s instructions. Microprojectiles were bombarded into the onion epidermal cells using a Biolistic PDS-1000/He system (Bio-Rad) with 900-p.s.i. rupture discs under a vacuum of 28 inch Hg. After bombardment, the cells were allowed to recover for 24-48 h on agar plates at 22°C under a 16 h photoperiod regime before being viewed under a fluorescence microscope and confocal laser scanning microscope.

RT-PCR analysis
Total RNA was extracted from pollen, flower, leaf and stem tissue using Trizol reagent (Life Technologies, Rockville, MD). For the isolation of mature pollen, inflorescences from over 100 plants were harvested in ice-cold 0.3 M mannitol and vigorously shaken for 1 min. The pollen suspension was filtered through a 53-μm nylon mesh and pollen grains were concentrated by repeated centrifugation (450 g, 5 min, 4°C) and stored at -80°C. RT-PCR was performed as described by Honys and Twell (2003). 1 μg total RNA was reverse transcribed in a 20 μL reaction using the ImProm-II Reverse Transcription System (Promega) using a 3′-RACE primer (5′-AAGCAGTGGTAACAAACGAGTAC-(T)30VN-3′). For PCR amplification, 1 μL of 10-40x diluted RT mix was used in 25 μL with 0.5 units of Taq, 1.2 mM MgCl2, and 10 pmol of each primer. The PCR program was as follows: 2 min 95°C, 30 or 45 cycles of 15 s 94°C, 15 s 56°C, and 1 min 72°C, followed by 10 min 72°C. The conditions were 1.25 ng cDNA and 30 cycles for the
Rubisco gene and 1.25 ng cDNA and 45 cycles for BUP and APG-like. Intron-spanning primers for BUP (At5g04480) 5’-CTCGTAAACGCCTTTCCAGTGT-3’ and 5’-CTTCTGGACAAGATTAGACATCGATG-3’. For Rubisco (At5g38430), the gene-specific primer 5’-ATTTACCTCTGACCTTTACTGACGT-3’ was used in combination with a nested primer (5’-AAGCAGTGGTAAACACGAGGT-3’) overlapping the 3’-RACE primer.

**Cytological analysis of pollen and pollen tubes**

*In-vitro* germination assays were performed as described in Derksen et al. (2002). Pollen was collected by tapping the open anthers on a solid agar surface covered with a piece of prepared Visking dialysis membrane (Serva; [http://www.serva.de/enDE](http://www.serva.de/enDE)) and then incubated in the dark at 27°C. Before use, the membranes were boiled for 6 h in water, rinsed thrice for 30s in liquid medium and placed on a semi-solid pollen germination medium which consisted of 0.01% H$_3$BO$_3$, 0.7% Bacto-Agar, 0.07% CaCl$_2$·2H$_2$O, 3.0% polyethylene glycol 4,000 and 20% sucrose, pH 7 (Hodgkin, 1983), keeping the film of medium on the membrane as thin as possible.

For analysis of pollen germination and pollen tube growth *in vitro*, membranes with pollen were incubated overnight (16 h). Pollen and pollen tubes were gently washed off using a 50 μL drop of medium. In these preparations, most pollen tubes continued growth and cytoplasmic streaming continued for at least 5 min. Samples were photographed with a conventional camera on a Leitz Dialux 20 MB microscope (Leica Microsystems GmbH, Wetzlar, Germany). The images were digitized and the pollen tube lengths were measured using Metavue software (Universal Imaging Corporation, West Chester, PA).

For staining of pollen tubes, mature pollen grains were stained with 5 μg/mL fluorescein diacetate (FDA) in liquid pollen germination medium or with 0.4 μg/mL 4’,6-diamidino-2-phenylindole (DAPI) in 0.1 M sodium phosphate (pH 7), 1 mM EDTA, 0.1% Triton X-100 (Park *et al.*, 1998).

Samples from pollen incubated for 1-1.5 h were stained for callose, cellulose or pectin using, respectively, a 20x diluted decolorized Aniline Blue solution (Polysciences,
Warrington, Pa.), 0.001% Calcofluor White (Fluorescent Brightener 28; Sigma) or 0.01% Ruthenium Red (Sigma) in liquid pollen germination medium.

For staining with Toluidine Blue, 1-5 µL 0.01% Toluidine Blue in medium was added to the samples and examined within 20 min (Derksen et al., 2011). The walls of living pollen tubes stained pink to light purple, but the cytoplasm remained unstained. In dead pollen tubes, the cytoplasm was dark blue, sometimes obscuring the staining of the wall. Below pH 4.5, no coloration of the wall occurred, which indicates pure carboxylic acid (de-esterified pectin derived) mediated binding (Gurr, 1965). Preparations were made at 1 h intervals for 6 h after start of the incubation and after incubation overnight.

The samples were examined and photographed using a Leitz Orthoplan (Leica Microsystems GmbH) microscope, equipped with illumination and filters for epifluorescence. Images were made using a Colour Coolsnap digital camera (Roper Scientific, Tucson, AZ).

Cryo-scanning electron microscopy

Fresh pollen samples from heterozygous plants (bup-1/+ or bup-2+) or from the qrt1 crossings (bup-1/+ qrt1 or bup-2+/+ qrt1) were mounted on 3 mm aluminium stubs and, either, directly transferred to the preparation cryo-unit, or rapidly frozen in nitrogen slush and transferred to the cryo-unit at ≤ -120°C (Alto 2500 HF high-resolution transfer system; Oxford Instruments Ltd, http://www.oxinst.com). The samples were then sublimated at -85 to -90°C with the anti-contaminator at -120°C for 4-8 min, cooled to -120°C and coated with 1 nm Au-Pt using the internal sputter-coater (Denton Vacuum Inc; http://www.dentonvacuum.com) of the cryo-unit. Then they were transferred into the vacuum chamber of the cryo-Field Emission Scanning Electron Microscope (cryo-FESEM, Jeol-JSM 6330F; Jeol Ltd; http://www.jeol.com), examined and photographed at -140°C.

In addition, pollen was grown on pistils after in vitro pollination. Therefore, pistils were planted into brass rivets (Cryotech; http://www.cryotech.co.th; internal diameter 1.0 mm, thickness 0.25 mm) filled with solidified agar with the stigma resting
on the surface and pollinated. After various time intervals (4, 6 and 12 h) the rivets with pistils were frozen in liquid nitrogen and handled as described for fresh pollen. The cryo-FESEM was operated at an acceleration voltage of 3 kV and a working distance of 15 mm (Derksen et al., 2011).

Transmission electron microscopy
Transmission electron microscopy was carried out as described previously (Derksen et al., 2002). In short, membranes with pollen tubes (see above) grown for about 1-2 h were plunged into liquid propane 3.5 (Air Liquide, Belgium; -175°C), transferred into liquid nitrogen and freeze substituted in a freeze-substitution apparatus (Reichert-Jung, Austria). After substitution at -90°C for 36 h in 1% OsO₄ and 0.1% uranyl acetate in acetone, the temperature was raised (4°C/h) to room temperature. Membranes with pollen tubes were embedded in Spurr’s resin (Agar Scientific) and mounted on Sylon-CT-coated (Supelco, Bellefonte, PA.) slides. The dialysis membrane was removed during the trimming process and pollen with correct orientation were selected. Longitudinal sections, about 10 nm thick, were post-stained with uranyl acetate and lead citrate according to standard procedures, and examined and photographed in a Jeol100CX II transmission electron microscope.

Results

bup is a male gametophytic mutant
The Tom Jack Arabidopsis T-DNA mutant population (Campisi et al., 1999) was screened by segregation distortion analysis, a method described previously to identify gametophytic mutants (Howden et al., 1998; Johnson et al., 2004). We screened 379 mutant lines for segregation of the T-DNA linked kanamycin resistance marker. One of the lines, which we named bursting pollen-1 (bup-1), consistently segregated 1:1 (kan⁺:kan⁻) over several generations and after backcrossing (Table 1), indicative of a gametophytic mutation. Further analysis showed that female transmission was
unaffected and that T-DNA transmission through pollen was severely reduced.

Pollinating wild-type flowers with bup-1 pollen resulted in only 1-2% resistant offspring (Table 2). Further crossing using seven of the rare male transmitted BC1 (MBC1) plants confirmed that these plants contained the bup-1 mutation, because their offspring segregated for the kanamycin marker with the same distorted ratio as the bup-1 parent (Table 2). The observation that the number of seeds per silique was normal in the bup-1 mutant indicated that bup-1 pollen tubes did not efficiently compete with the wild-type for fertilisation.

**BUP encodes a putative glycosyltransferase that is located in the Golgi apparatus**

Southern blot and TAIL-PCR flanking sequence analysis revealed a complex T-DNA insertion pattern, comprising five or six T-DNA copies organized in tandem and inverted repeats at three different insertion sites (Fig. S2). Since no genetic recombination events between the separate T-DNA copies were ever detected, it was concluded that the NPTII-containing T-DNA insertion sites are closely linked. A T-DNA flanking sequence was obtained which showed homology to intron 13 of gene At5g04480 (Table S1). To test whether the male gametophytic phenotype of bup-1 was caused by disruption of this gene, an independent T-DNA insertion in exon 8 of At5g04480 (SALK_033889; bup-2) was analysed (Fig. 1a). Similar to bup-1, bup-2 showed strong segregation distortion, due to reduced male T-DNA transmission (Tables 1 and 2). Final confirmation that BUP corresponded to At5g04480 came from molecular complementation of bup-1 and bup-2 with a 10 kb At5g04480 genomic fragment. In plants homozygous for the At5g04480 transgene and hemizygous for bup-1 or bup-2, the kan⁺:kan⁻ segregation was fully restored to 3:1 and homozygous kan⁺ plants could be recovered (Table 3).

There is no published experimental data on the function of BUP, but a bioinformatic study predicted BUP to encode a novel glycosyltransferase (Egelund et al., 2004). RT-PCR analysis showed that BUP was expressed in samples of pollen, flower, leaf and stem (Fig. 1b). The purity of the pollen sample was confirmed by the lack of expression of Rubisco small subunit 1b gene At5g38430, which is expressed in
green tissues only (Kamada et al., 2003; Honys and Twell, 2003, 2004). Analysis of
publicly available micro-array data in the Genevestigator database (Zimmermann et
al., 2004; Hruz et al., 2008) confirmed that BUP was expressed at low to medium levels
in all tissues and developmental stages tested, including pollen (Fig. S3).

To study the sub-cellular localisation of the BUP protein, a 515 bp 5'-terminal
gene fragment of BUP was fused in frame to the 5’ end of the green fluorescent
protein (GFP) gene under transcriptional control of the CaMV35S promoter (Fig 2a).
This BUP(N) protein fragment contained the cytoplasmic N-terminal tail, the 22 amino
acid predicted trans-membrane domain and part of the stem region, which has been
shown to be sufficient for correct localisation of glycosyltransferases (Paulson and
Colley, 1989). Expression of 35S::BUP(N):GFP in onion epidermal cells gave rise to
fluorescent GFP dots within the cytoplasm (Fig. 2b). Co-bombardment of
35S::BUP(N):GFP and the Golgi marker 35S::ST::RFP (Saint-Jore et al., 2002), showed co-
localisation of the two fluorochromes (Fig. 2b,c,d), confirming that the BUP(N)-GFP
fusion protein is targeted to the Golgi bodies, in vicinity to fibre-like strands that were
also highlighted by the BUP-GFP fusion protein. These fluorescing strands, probably ER,
appeared to be randomly oriented (Fig. 2e).

Taken together, the above data indicate that BUP encodes a novel, Golgi-
localised glycosyltransferase that may function throughout the plant.

**bup is affected in pollen germination and pollen tube growth**
Despite the incomplete penetrance of the phenotype, homozygous bup-1 mutants,
that were expected to have a severe reduction in fertility and produce fully kanamycin-
resistant offspring, were never identified. Because the hemizygous state of bup-1
mutants complicated pollen phenotype analysis, we additionally studied bup-1/+ and
bup-2/+ plants that were crossed with the quartet1 (qrt1) mutant (Preuss et al., 1994).
In this mutant the four products of meiosis remain attached during pollen maturation
and two members of each bup/+ qrt1 tetrad carry a bup allele, while the other two are
wild-type at the BUP locus. To investigate the stage at which male transmission of bup-
1 is affected, we first analyzed mature pollen. Using FDA and DAPI staining of the
mature *bup-1/+ qrt1* tetrads we did not observe defects in pollen viability and the
development of the sperm cell nuclei (Fig. S4). To test whether *BUP* is necessary for
early post-pollination processes, such as pollen germination or pollen tube growth,
*bup-2* pollen were incubated *in vitro* on solid germination medium. No defect in
hydration of *bup-2/+* pollen was observed as all pollen swelled within minutes after
application to the medium, similar to wild-type pollen. Moreover, SEM of dry and
hydrated pollen prior to germination did not show any differences in appearance (Fig.
S5). During pollen germination and tube growth, however, the mutant showed a clear
defect. After incubation overnight, about 50% of the wild-type pollen had germinated,
with the majority of the tubes being longer than five times the pollen grain diameter
(Fig. 3a). In contrast, *bup-1/+* pollen germinated overnight showed the presence of
pollen with short pollen tubes or very short tube-like structures (Fig. 3b,c). Frequency
distribution analysis of the length of the pollen tubes confirmed that the proportion of
short pollen tubes (shorter than 40 μm) was significantly greater in *bup-1/+* than in
wild-type (Fig. 3d). Similarly, analysis of number of pollen tubes growing from *qrt1* and
*bup-1/+ qrt1* tetrads showed a significant difference in the number of normal pollen
tubes versus short pollen tubes/tube-like structures (Table S2). Furthermore, SEM of
germinating pollen of *qrt1* and *bup-1/+ qrt1* tetrads *in vitro*, or after pollination of
explanted stigmas with *bup-2* pollen, often revealed collapsed pollen and occasionally
malformed, cauliflower-like bulges sometimes with damaged tubes (Fig. 4).

Formation of the germination plaque and pollen tube wall development are
defective in *bup*

To explore the *bup* defective phenotype, we analysed the start of *in vitro* pollen
germination in more detail. During pollen germination, polarisation of the pollen grain
is marked by the deposition of callose (Johnson and McCormick, 2001; Lalanne *et al.*, 2004). We looked at this future site of pollen tube emergence in more detail and found
a substantial amount of intine-like wall material (see below). This deposition is
referred to as “germination plaque”. No preferential site of deposition could be
determined. The germination plaque could be stained with the callose dye Aniline
Blue, as well as with Calcofluor White and Ruthenium Red, indicating also the presence of cellulose and pectin, respectively (Fig. S6). Using these dyes, no conspicuous differences between wild-type and bup-1/+ pollen could be detected prior to or at the onset of germination.

This stage of development was studied further using bright field microscopy after Toluidine Blue staining and TEM. In wild-type pollen, plaque formation started following hydration (Fig. S6) and was complete within one hour, by which time the first pollen tubes had emerged. As seen in Toluidine Blue stained preparations, tube formation started by bulging of the plaque, then the bulge extended, was breached by the pollen tube and remained as a ‘collar’ at the base of the tube (Fig. 5a-c). The collar persisted during the entire period studied, up to 24 hours. The plaque was also seen after in-vivo germination (Fig. S7).

In bup-2/+ pollen the germination plaque was always present (Fig. 5d-h, see also Fig. S6), but tube formation was hampered. Mostly, the cytoplasm streamed out and coagulated at the site of the plaque (Fig. 5e,f), giving rise to the tube-like extensions seen in Fig. 3b,c and Fig. 4b,c. A “collar” or collar-like structure was never observed. The remaining cytoplasm was detached from the wall and stained darkly, indicating cell death. A small percentage (< 3%) of the population showed irregularly bulging plaques (see above, Fig. 4c) or, more often, regular tubes leaking at the tip (Fig. 5g,h), indicating that not only plaque formation but also tip-growth was affected. Remarkably, after Toluidine Blue staining, the longer (wild type) pollen tubes often showed a more or less regular banding pattern not unlike that seen in tobacco (Derksen et al., 2011), albeit less regular (Fig. 5i). The darker stained areas coincided with the typical bumps occurring during Arabidopsis pollen tube growth.

TEM analysis of wild-type germinating pollen and pollen tubes (Fig. 6) confirmed the formation of the germination plaque and the collar surrounding the base of the emerged pollen tube observed after Toluidine Blue staining. TEM preparations of germinating bup-2/+ qrt1 mutant pollen (Fig. 6) showed the presence of dead pollen, visible by the rough, electron-dense appearance of the cytoplasm and the plasma membrane that was detached from the pollen (compare also Fig. 5g).
Mostly, instead of forming a pollen tube, the cytoplasm of these germinated pollen seemed to have been forced out of the pollen grain through a relatively small opening, mostly in the centre of the plaque. No wall material or plasma membrane was shown to surround the cytoplasmic extrusions. In these cases the plaques never bulged to form a collar (see also Fig. 5g,h) and they often had a disturbed shape. These observations suggested disturbed plaque formation as the cause of germination failure. Comparison of hydrated but non-germinated pollen of wild-type and bup-2/+ pollen indeed revealed marked differences (Fig. 7). In wild-type pollen plaque formation was regular, resulting in a lens-shaped structure (Fig. 7a,b). The surface at the cytoplasmic side was almost flat (Fig. 7a), but mostly slightly convex (Fig. 7b). The ultrastructure of the plaques (Fig. 7c) was fibrous, without a clear pattern or clear preferential orientation. At the wall sometimes a few dark inclusions were seen. The plaques in pollen from bup-2/+ plants showed a variety of deviations (Fig. 7d-j) and occasionally an almost complete canal was seen (Fig. 5d). The deviations ranged from regularly shaped plaques with cytoplasmic inclusions and a rough appearance (Fig. 7e,f) to odd shaped plaques (Fig. 7d,g-j), that sometimes were seemingly partitioned with cytoplasmic inclusions, irregular swirls of fibrous material and spindle-like, darkly stained depositions at the edges (Fig. 7j).

Altogether, these observations clearly point to a malformed germination plaque as the major cause of germination failure in bup pollen. The rupturing of the occasionally formed pollen tubes indicates additional disturbance of the control of wall deposition or properties at the tip of the pollen tube.

Discussion

The bursting pollen mutant, bup-1, isolated from the Tom Jack Arabidopsis T-DNA insertion population (Campisi et al., 1999) was shown to be specifically deficient in male transmission. Molecular and bioinformatic analyses suggested that BUP is a type II membrane protein with a single trans-membrane domain and an N-terminal
cytoplasmic tail. The C-terminal tail is predicted to contain a UDP-glycosyltransferase/glycogen phosphorylase domain, which assumes GT-B type folding. In accordance with this, the protein contains a sugar-nucleotide-binding DxD motif (Egelund et al., 2004). Although BUP is predicted to contain the glycosyltransferase family 1 domain (GT-1; PFAM00534/ IPR001296), the rather low confidence of the prediction explains why the protein is considered as a "non classified" glycosyltransferase in the Carbohydrate-Active enzyme (CaZy) database, which curates all UDP-glycosyltransferases (http://www.cazy.org/Glycosyltransferases.html; see also: Henrissat and Davies, 2000 and Yonekura and Hanada, 2011). Recently, however, structure-based homology analysis clearly showed that BUP is related to the GT4-subfamily (Nikolovski et al., 2012). No specific substrate has been identified for this subfamily and the members have a broad range of glycosyltransferase activities. The BUP protein shows overall primary sequence homology to one other Arabidopsis protein, encoded by At4g01210, the only other GT4-related Arabidopsis protein (Nikolovski et al., 2012). The two homologous genes belong to an evolutionarily old clade, as both genes have distinct orthologs in both dicots and monocots and the clade is rooted by a single branch of Physcomitrella patens and Selaginella moellendorffii genes (http://plants.ensembl.org/Multi/GeneTree?gt=EPlGT00140000003021). The intron-exon boundary structure of the genes coding for the homologous proteins appeared to be highly conserved (data not shown) and both the GT domain the N-terminal trans-membrane domain are predicted to be present in most of the homologous proteins. Currently, there is no functional information for any of the proteins in this phylogenetic clade.

Two non-targeted proteomics studies using isotope-tagging techniques coupled to density gradient separation of organelles suggested that the At5g04480 (BUP) gene product preferentially resides in the Golgi apparatus and trans-Golgi network (TGN) (Dunkley et al., 2006; Nikolovski et al., 2012). The present fluorochrome studies using particle bombardment of onion cells with the Golgi marker ST-RFP (Wee et al., 1998; Saint-Jore et al., 2002), confirmed this sub-cellular localisation. The marked fibrillar elements observed in Fig. 2e represent ER, as Golgi bodies are known to originate from
the ER (Hawes & Satiat-Jeunemaitre, 2005). Similar distributions were also found for both ST-RFP and the GONST-YFP Golgi marker (Wee et al., 1998; Saint-Jore et al., 2002).

Detailed cytological analysis of Arabidopsis pollen germination and pollen tube growth has been hampered by the relatively inefficient germination, here about 50%, and the lack of synchronization of the various events during germination and growth (e.g. Derksen et al., 2002). In addition, unlike most other pollen, Arabidopsis pollen does not germinate via one of the preformed apertures, but instead germinates directly via the intine at the site of adhesion (Edlund et al., 2004; see also Lalanne et al., 2004). As shown here in vitro, germination involves the formation of an intine-like so called ‘germination plaque’ at the future site of tube emergence, a structure that has not been described before. The germination plaque was deposited after hydration started and completed before germination was initiated, within 1 h. It contained cellulose, callose, pectin and at least partly de-esterified pectin, as demonstrated with histochemical dyes. Though Toluidine Blue is not entirely specific for de-esterified pectin, under the conditions described here it may be confidently regarded as such, as shown for tobacco pollen (Derksen et al., 2011). The germination plaque is clearly differentiated from the intine of the pollen (Fig. 7a-c). Its fibrous aspect may be derived from cellulose connected to and possibly cross-linked by de-esterified pectin (Derksen et al., 2002, 2011). Though cross-links were not observed, their presence may have been obscured by a disorderly fibre arrangement. Alternatively, the fibrous nature may derive from de-esterified pectin alone as pectin may self-organize in fibrous structures (Chanliaud & Gidley, 1999). The germination plaque bears some similarity to the oncus or “Zwischenkorper” seen in many Angiosperm species. However, by definition the oncus is deposited below a germination pore. Comparable depositions may occur at the site of germination in some apertureless Angiosperms such the Lauraceae (Pacini et al., 2014). The situation in Gymnosperm is completely different and is extremely variable or even still unknown. The present situation is quite different as the deposition occur in an aperturate system, which may thus represent a
neotenic or perhaps novel trait (Hesse, 2000; Banks, 2003; Pacini et al., 2014). Besides the failure of the plaque, also the tip of the occasionally emerging bup pollen tubes may fail (Fig. 3b,c and 5g,h). Leaking tips of caffeine-treated lily and flavonol-depleted petunia pollen tubes (Lancelle et al., 1997; Derksen et al., 1999) show spindle-like structures almost identical to those seen at the edges of the bup plaques (Fig. 7j), and also result in ejection of the cytoplasm at that site. As the plastic-elastic properties of the pollen tube tip are ruled by de-esterification of pectin secreted in the tip (Li et al., 1994, Geitmann et al., 1995; Dardelle et al., 2010; Chebli et al., 2012), tip failure and its cytological reflexion must relate to a disturbance in pectin composition and/or deposition. The presence of substantial amounts of (de-esterified) pectin in the plaque and especially the collar, together with the occurrence of comparable cytological aberrations strongly suggest a similar defect of the plaque in bup. Plaque and tip failure cannot be due to faulty cellulose or callose synthesis as these occur at the plasma membrane (Chebli et al., 2012, Mollet et al., 2013) and they are absent from the pollen tube tip (e.g. Derksen et al., 2002; Derksen et al., 2011). Also hemicellulose defects are unlikely to cause plaque and pollen tube failure as the Arabidopsis mur1, mur2 and mur3 mutants cause dwarfed phenotypes, but do not affect pollen formation, germination or pollen tube growth (Zablakis et al., 1996; Vanzin et al., 2002; Madson et al., 2003). Furthermore, the fast deposition of the plaque may only be achieved by a massive secretion, which also points towards Golgi derived pectin as the major component of the plaque. Thus it may be inferred that BUP encodes a novel, Golgi/TGN-localised GT4-related glycosyltransferase that is directly or indirectly involved in pectin synthesis or delivery and thereby, germination plaque formation and wall formation at the pollen tube tip.

No other genes affecting plaque formation have been identified so far, but the bup phenotype bears strong resemblance to that of a mutant of VANGUARD1, a pollen specific pectin-methylesterase gene, which has reduced pectin de-methylation resulting in retardation in pollen tube growth and bursting of the pollen tube tips (Jiang et al., 2005). Double mutants of two pollen-expressed
galacturonosyltransferases (GAUT13 and GAUT14) show a defect in pectin synthesis necessary for pollen tube wall formation, resulting in swollen tubes, though pollen germination itself is unaffected (Wang et al., 2013). The only other glycosyl transferase gene described to be involved in pollen germination, is Cdi, a gene predominantly expressed in pollen and encoding a cytosolic glycosyl transferase (Li et al., 2012). Mutants of the Cdi gene have a defect in pollen germination and pollen tube growth, similar to the bup mutants. Whether in cdi-1 this is also associated with a defect in the formation of the germination plaque has not been described. Another pectin-synthesis-deficient mutant, quasimodo1/gaut8, is a knockout of a GT8 galacturonosyltransferase/xylan syntase and shows defects only in sporophytic growth (Bouton et al., 2002, Orfila et al., 2005).

The BUP gene is expressed in both, gametophytic and sporophytic tissues, suggesting a broad function. A similar phenomenon was recorded for mutants in the cell wall-synthesis-related AtCSLA and MGP4 genes. The atcsla7 mutant has a mildly reduced male transmission, allowing the homozygous embryos to be identified as defective in patterning (Goubet et al., 2003). When the male defective mgp4 mutant was rescued in pollen specifically, an additional sporophytic defect in root growth became apparent in the homozygous mutant (Liu et al., 2011). Such mutants should not be considered true male or gametophytic specific; rather their effects become manifest first in the haploid, male gametophytic stage. Considering the broad expression pattern of the BUP gene and our inability to identify homozygous bup plants, bup may not have a strictly male-gametophyte specific function, and is likely to be important for pectin synthesis and cell wall formation in sporophytic tissues as well.

Further studies like transient complementation studies could reveal whether BUP affects the formation of primary walls that are largely made up of pectin. Also, major effects might be expected for the initial walls of the cell plate as these, like pollen tube tips, are almost entirely formed from Golgi-derived decretory vesicles that provide the wall’s pectin building blocks. Yeast studies could help identify the specific substrates used, which might clarify the role of BUP in pectin synthesis and stabilisation of the germination plaque and the pollen tube tip.
In conclusion, we have identified a novel structure, that we term the germination plaque, which is formed at the site of pollen tube emergence prior to germination and have shown an important role for BUP, one of the two strongly conserved GT4-related glycosyltransferases, in formation of this plaque and the pollen tube tip.

Acknowledgements

We thank Dr Tom Jack for providing seed of T-DNA lines, Dr Liesbeth Pierson for help with the light microscopy, Richard Feron for technical assistance and Dr Koen Weterings for general discussions. We thank Prof. Dr E. Pacini for helpful discussions on the germination plaque. This work was supported in part by grants from the UK Biotechnology and Biological Sciences Research Council (BBSRC) to DT.
References


Supporting Information

**Fig. S1** Extra-poral germination of Arabidopsis pollen. (a) Germination on stigmatic papillae. (b,c,d) Examples of the variability in the position of tube emergence from the pollen.

**Fig. S2** Southern blot analysis of the T-DNA insertions. 10 μg of genomic DNA of Col-0 and *bup-1* was digested with *KpnI, XbaI* or *BglII*, and hybridized with a T-DNA specific probe (KAN or GUS) or with a gene-specific probe. (a) the *KpnI, XbaI* and *BglII* restriction sites and the two T-DNA probes within the T-DNA in *bup-1*. (b) the *BglII* restriction sites and the gene-specific-probe of At5g04480. (c) Southern blot of *bup-1*, hybridized with the GUS or KAN-probe. Asterisk: a 5.4 kb fragment which could indicate a T-DNA repeat. Arrow: putative truncated T-DNA, d. Col-0 and *bup-1* Southern blot. Arrows: T-DNA insertion within gene At5g04480.

**Fig. S3** Expression levels of *BUP* in different organs and at different stages of development. Data was obtained from the Genevestigator “anatomy” tool (Hruz et al., 2008).

**Fig. S4** Mature, hydrated *bup-1/+ qrt1* tetrads. (a) DAPI staining of generative cell and sperm nuclei showing their presence and correct position in all four pollen of the quartet. (b) FDA fluorescence is presence in all four pollen of the quartet indicating an intact cytoplasm. (c) FDA staining controls with (left) and without FDA (right). (d) bright field images corresponding to (c).  

**Fig. S5** SEM micrograph of mature *bup-2/+ qrt1* tetrads. (a) Before hydration. (b) After hydration (1h). No differences in size and morphology were observed between the pollen grains in the quartets. Background in (b) petal fragment.
Fig. S6 Plaque staining of hydrated pollen from wild-type and *bup* pollen. (a, b) Ruthenium Red staining showing the presence of pectin as a dark red color. (c, d) Calcofluor White staining showing the presence of crystalline cellulose as bright fluorescence. (e, f) Aniline Blue staining showing the presence of callose as bright fluorescence. In each case, a time series of 10 (left), 20 (middle) or 30-60 minutes hydration (right) was made. No differences between wild-type (a, c, e) and *bup-1/+* pollen (b, d, f) could be found.

Fig. S7 In-vivo hydrated pollen stained with Toluidine Blue. One hour after pollination (a), pollen were washed from the stigma and photographed (b).

Table S1 Flanking border sequence analysis

Table S2 Tetrad analysis of *bup-1/+* pollen tube growth *in vitro*
Tables

Table 1 Segregation distortion analysis of *bup-1* and *bup-2*

<table>
<thead>
<tr>
<th></th>
<th>Selfing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T3</td>
</tr>
<tr>
<td><em>bup-1</em></td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>(1/409)</td>
</tr>
<tr>
<td><em>bup-2</em></td>
<td>1.14*</td>
</tr>
<tr>
<td></td>
<td>(27/4062)</td>
</tr>
</tbody>
</table>

Kan\(^R\):Kan\(^S\) segregation ratios observed after selfing. In brackets are the number of individual plants / the total of number of seeds tested. \(\chi^2\) R:S=1:1, P>0.05. *, \(\chi^2\) P>0.01.
Table 2 Male and female transmission of bup-1 and bup-2

<table>
<thead>
<tr>
<th></th>
<th>male transmission</th>
<th>female transmission</th>
<th>MBC1&lt;sup&gt;+&lt;/sup&gt; selfing</th>
<th>gl1/ms1 x MBC1&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T4</td>
<td>BC1</td>
<td>T4</td>
<td>BC1</td>
</tr>
<tr>
<td>bup-1</td>
<td>0.01 (33/870)</td>
<td>0.02 (8/863)</td>
<td>1.08 (34/836)</td>
<td>1.01 (8/573)</td>
</tr>
<tr>
<td>bup-2</td>
<td>0.02 (24/1383)</td>
<td>1.18 (7/368)</td>
<td>1.10 (19/931)</td>
<td></td>
</tr>
</tbody>
</table>

Kan<sup>R</sup>:Kan<sup>S</sup> segregation ratios observed after reciprocal crosses with Col-6(gl1) or ms1 plants. In brackets are the number of plants / the total of number of seeds tested. χ<sup>2</sup> R:S=1:1, P>0.05.
Table 3 Complementation of *bup* mutants with the *At5g04480* gene

<table>
<thead>
<tr>
<th></th>
<th>Kan&lt;sup&gt;R&lt;/sup&gt;: Kan&lt;sup&gt;S&lt;/sup&gt; ratio</th>
<th>Expected Kan&lt;sup&gt;R&lt;/sup&gt;:Kan&lt;sup&gt;S&lt;/sup&gt; ratio upon selfing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F2</td>
<td>F3</td>
</tr>
<tr>
<td>- / -</td>
<td>1.02 (14/4468)</td>
<td>0.77 (3/209)</td>
</tr>
<tr>
<td>Gen&lt;sup&gt;R&lt;/sup&gt;/-</td>
<td>1.97 (16/4236)</td>
<td>2.25 (20/1716)</td>
</tr>
<tr>
<td>Gen&lt;sup&gt;R&lt;/sup&gt;/Gen&lt;sup&gt;R&lt;/sup&gt;</td>
<td>2.89 (9/802)</td>
<td></td>
</tr>
<tr>
<td>- / -</td>
<td>1.26 (23/1333)</td>
<td>1.01 (8/706)</td>
</tr>
<tr>
<td>Gen&lt;sup&gt;R&lt;/sup&gt;/-</td>
<td>2.00 (22/1430)</td>
<td>2.00 (10/902)</td>
</tr>
<tr>
<td>Gen&lt;sup&gt;R&lt;/sup&gt;/Gen&lt;sup&gt;R&lt;/sup&gt;</td>
<td>3.01 (115/294)</td>
<td></td>
</tr>
</tbody>
</table>

Kan<sup>R</sup>:Kan<sup>S</sup> segregation ratio of F2 and F3 progeny of *bup-1* and *bup-2* plants crossed with a transformant (Col+4480) containing a single T-DNA with a wild-type copy of *At5g04480*. The plants analyzed were heterozygous for *bup-1* or *bup-2* and carried the complementing T-DNA (Gen<sup>R</sup>) as indicated. In brackets are the number of independent F1s / the total number of seedlings tested. \( \chi^2 P > 0.05 \). n.a., not analyzed.
Table S1 Flanking border sequence analysis

<table>
<thead>
<tr>
<th>Flanking border</th>
<th>Sequence homology</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB1</td>
<td>At5g04480, BAC T32M21, position 29033-29593</td>
<td>566 bp inverse PCR, TAIL-PCR (AD1) and direct PCR</td>
</tr>
<tr>
<td>LB2</td>
<td>At5g04480, BAC T32M21, position 28696-29020</td>
<td>270 bp direct PCR</td>
</tr>
<tr>
<td>LB3</td>
<td>beyond the LB sequence plasmid pD991</td>
<td>225 bp TAIL-PCR (AD2)</td>
</tr>
<tr>
<td>LB4</td>
<td>homology to VirE2 gene</td>
<td>623 bp TAIL-PCR (AD2)</td>
</tr>
<tr>
<td>RB1</td>
<td>tandem repeat LB sequence T-DNA</td>
<td>525 bp TAIL-PCR (AD3)</td>
</tr>
<tr>
<td>RB2</td>
<td>LB vector sequence (no homology to beyond LB sequence pD991)</td>
<td>289 bp TAIL-PCR (AD2)</td>
</tr>
<tr>
<td>RB3</td>
<td>replication of origin and BOM site (no homology to beyond LB sequence pD991)</td>
<td>770 bp TAIL-PCR (AD1)</td>
</tr>
<tr>
<td>RB4</td>
<td>pieces of RB sequence</td>
<td>35 bp TAIL-PCR (AD2)</td>
</tr>
<tr>
<td>RB5</td>
<td>pieces of RB sequence as in RB4, and homology to At1g11280</td>
<td>35 bp 110 bp TAIL-PCR (AD2)</td>
</tr>
</tbody>
</table>

Summary of the flanking border sequences found by Inverse PCR, TAIL-PCR and direct PCR. TAIL-PCR was performed using three different random primers (AD1, AD2 or AD3). The sequence homology was analysed using NCBI nucleotide blast (non-redundant database) and TAIR nucleotide blast (AGI genes database). Sequences with homology to a vector sequence were aligned to part of the binary vector pD991 to test the sequence identity. The total length of the sequence that shows homology (≥95%) is indicated.
**Table S2** Tetrad analysis of *bup-1/+* pollen tube growth *in vitro*

<table>
<thead>
<tr>
<th>Plant genotype</th>
<th># tetrads</th>
<th>Only short tubes (%)</th>
<th>1 tube (%)</th>
<th>2 tubes (%)</th>
<th>3 tubes (%)</th>
<th>4 tubes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>qrt1/qrt1</td>
<td>190</td>
<td>13.6</td>
<td>28.6</td>
<td>32.6</td>
<td>14.6</td>
<td>2.2</td>
</tr>
<tr>
<td>bup-1/+ qrt1/qrt1</td>
<td>113</td>
<td>37.2</td>
<td>47.3</td>
<td>14.6</td>
<td>0.8</td>
<td>0</td>
</tr>
</tbody>
</table>

* Only tetrads that showed pollen germination were included in the analysis
**Figure legends**

**Fig. 1** RT-PCR analysis of BUP. (a) At5g04480 intron-exon structure and T-DNA insertion structures. Bar = 1000 bp. (b) BUP is expressed in samples of pollen, flower, leaf and stem. The vegetative tissue-specific RUBISCO transcript is absent from the pollen sample. P: pollen; F: flower; L: leaf; S: stem.

**Fig. 2** Localisation of BUP protein. (a) BUP protein domain-structure and BUP(N)-GFP fusion protein. (b) Fluorescence of BUP-GFP fusion protein in onion epidermal cell. (c) Bombardment with Golgi marker 35S::ST::RFP. (d) Overlay of C and D showing co-localization of BUP-GFP and ST-RFP. (e) Fluorescence of BUP-GFP fusion protein in onion epidermal cell.

**Fig. 3** LM micrograph after pollen germination overnight. About 50% of the pollen germinated. (a) Wild-type (Col-0) showing pollen tubes with variable lengths. (b) bup-1/+ pollen showing many small pollen tubes and pollen with tube-like extensions. (c) bup-1/+ pollen at higher magnification of the pollen tubes and the tube-like extensions. (d) Comparison of length distributions of wild-type (Col-0) and bup-1/+ pollen tubes shows a significant decrease of the proportion of long pollen tubes in the bup-1/+ pollen population (Fisher’s exact test for 0-40 µm, P<0.001). SD’s are indicated.

**Fig. 4** SEM micrograph of germinated wild-type pollen and bup-2/+ qrt1 tetrads, 8 h after germination. (a) Wild-type (Col-0) pollen tube. (b) Irregular and damaged pollen tube from a bup-2/+ qrt1 tetrad. (c) Irregularly germinated pollen from a bup-2/+ pollen population. (d) Collapsed pollen of a bup-2/+ qrt1 tetrad.

**Fig. 5** Toluidine Blue staining for de-esterified pectin. (a) Wild type pollen showing a bulged plaque, about 1 h after start of germination. (b) Wild type pollen 1-2 h after germination showing the remnants of the plaque as a collar (][; Bracket) at the base of
the pollen tube. (c) Wild type pollen 2-3 h after germination showing the remnants of
the plaque as a collar (\(\rangle\); Bracket) at the base of the pollen tube. (d) Pollen from a \textit{bup-
2/+ qrt1} tetrad 1-2 h after germination. The plaque has not extended into a “collar”.
(e) Pollen from a \textit{bup-2/+} population, 2-3 h after germination. A mutant pollen grain
with extruded cytoplasm is seen next to a wild-type germinated pollen grain with a
clear “collar”. (f) Pollen from a \textit{bup-2/+} population, 3-4 h after germination. A mutant
pollen grain with extruded cytoplasm is seen next to a wild-type germinated pollen
grain with a clear “collar”. (g) Pollen from a \textit{bup-2/+ qrt1} tetrad, 2-3 h after
germination. A germinating pollen grain forming a bulge without extension of the
plaque (\(\rangle;\)arrowheads) is seen near to small pollen tube extruding cytoplasm from the
tip. The cytoplasm (cyt) is clearly contracted. (h) Pollen from a \textit{bup-2/+} population, 3-4
h after germination. The pollen tube is ejecting cytoplasm from the tip. At the base of
the pollen tube a non-extended plaque van be seen (\(\rangle;\)arrowheads). The cytoplasm
(cyt) is contracted. (i) Wild type pollen germinated for 8-9 h. Only the first half of the
pollen tube is shown. At the base of the tube a clear “collar” is visible (\(\rangle\); Bracket). The
pollen tube shows a more or less regular banding pattern indicated by dots.

\textbf{Fig. 6} TEM images of sections from germinating wild-type and \textit{bup} pollen. (a) Wild-type
pollen 1-2 h after germination showing the bulging plaque (\(\rangle;\) pointers). (b) Wild-type
pollen tube after 3-4 h of germination showing a clear “collar” at the base of the pollen
tube (\(\rangle\); Bracket). (c) Pollen from a \textit{bup-2/+ qrt1} tetrad, 1-3h after germination. The
pollen grain on the left side is extruding cytoplasm (cyt) through a channel near to the
center of the large plaque (*, asterisks). The cytoplasm in the pollen grain is
contracted. The pollen grain on the right side shows an intact wild-type plaque (*,
asterisk). (d) Intact small channel through a plaque (*, asterisk). (e) Large plaque with a
broad channel through the plaque (*, asterisk) and extruded cytoplasm (cyt). The
cytoplasm in the pollen grain is contracted.

\textbf{Fig. 7} TEM images of sections from wild-type and \textit{bup} pollen. (a, b) Lens-shaped
plaques of wild type pollen with a respectively flat and convex side at the cytoplasm.
(c) Higher magnification from the plaque in B. The plaque has a fibrous structure; no larger areas with a clear preferential orientations are seen. At the wall a few ark cytoplasmic inclusions, may be seen. (d) Large *bup*-2 plaque with a notch in the center where possibly a channel will be formed. (e, f) Almost wild-type like *bup*-2 plaque, but with a different aspect: rough and with small inclusions. (g, h, i) Large irregular shaped *bup*-2 plaques with seemingly empty spaces more or less parallel to the surface (g) or many irregularly distributed curling spaces (h). Many cytoplasmic inclusions may be seen. (i) high magnification of (h). (j) Especially at the edges of the *bup*-2 plaques, dark, spindle-like inclusions may be seen.
Fig. 1 RT-PCR analysis of BUP. (a) At5g04480 intron-exon structure and T-DNA insertion structures. Bar = 1000 bp. (b) BUP is expressed in samples of pollen, flower, leaf and stem. The vegetative tissue-specific RUBISCO transcript is absent from the pollen sample. P: pollen; F: flower; L: leaf; S: stem.

62x48mm (300 x 300 DPI)
Fig. 2 Localisation of BUP protein. (a) BUP protein domain-structure and BUP(N)-GFP fusion protein. (b) Fluorescence of BUP-GFP fusion protein in onion epidermal cell. (c) Bombardment with Golgi marker 35S::ST::RFP. (d) Overlay of C and D showing co-localization of BUP-GFP and ST-RFP. (e) Fluorescence of BUP-GFP fusion protein in onion epidermal cell.
Fig. 3 LM micrograph after pollen germination overnight. About 50% of the pollen germinated. (a) Wild-type (Col-0) showing pollen tubes with variable lengths. (b) bup-1−/+ pollen showing many small pollen tubes and pollen with tube-like extensions. (c) bup-1−/+ pollen at higher magnification of the pollen tubes and the tube-like extensions. (d) Comparison of length distributions of wild-type (Col-0) and bup-1−/+ pollen tubes shows a significant decrease of the proportion of long pollen tubes in the bup-1−/+ pollen population (Fisher's exact test for 0-40 μm, P<0.001). SD’s are indicated.

160x150mm (300 x 300 DPI)
Fig. 4 SEM micrograph of germinated wild-type pollen and bup-2/+ qrt1 tetrads, 8 h after germination. (a) Wild-type (Col-0) pollen tube. (b) Irregular and damaged pollen tube from a bup-2/+ qrt1 tetrad. (c) Irregularly germinated pollen from a bup-2−/+ pollen population. (d) Collapsed pollen of a bup-2/+ qrt1 tetrad.

61x47mm (300 x 300 DPI)
Fig. 5 Toluylene Blue staining for de-esterified pectin. (a) Wild type pollen showing a bulged plaque, about 1 h after start of germination. (b) Wild type pollen 1-2 h after germination showing the remnants of the plaque as a collar ( bracket) at the base of the pollen tube. (c) Wild type pollen 2-3 h after germination showing the remnants of the plaque as a collar ( bracket) at the base of the pollen tube. (d) Pollen from a bup-2/+ qrt1 tetrad 1-2 h after germination. The plaque has not extended into a “collar”. (e) Pollen from a bup-2¬/+ population, 2-3 h after germination. A mutant pollen grain with extruded cytoplasm is seen next to a wild-type germinated pollen grain with a clear “collar”. (f) Pollen from a bup-2¬/+ population, 3-4 h after germination. A mutant pollen grain with extruded cytoplasm is seen next to a wild-type germinated pollen grain with a clear “collar”. (g) Pollen from a bup-2/+ qrt1 tetrad, 2-3 h after germination. A germinating pollen grain forming a bulge without extension of the plaque (>;arrowheads) is seen near to small pollen tube extruding cytoplasm from the tip. The cytoplasm (cyt) is clearly contracted. (h) Pollen from a bup-2¬/+ population, 3-4 h after germination. The pollen tube is ejecting cytoplasm from the tip. At the base of the pollen tube a non-extended plaque van be seen (>;arrowheads). The cytoplasm (cyt) is contracted. (i) Wild type pollen germinated for 8-9 h. Only the first half of the pollen tube is shown. At the base of the tube a clear “collar” is visible (>; Bracket). The pollen tube shows a more or less regular banding
pattern indicated by dots.
194x222mm (300 x 300 DPI)
Fig. 6 TEM images of sections from germinating wild-type and bup− pollen. (a) Wild-type pollen 1-2 h after germination showing the bulging plaque (>). (b) Wild-type pollen tube after 3-4 h of germination showing a clear “collar” at the base of the pollen tube (I); Bracket. (c) Pollen from a bup-2/+ qrt1 tetrad, 1-3 h after germination. The pollen grain on the left side is extruding cytoplasm (cyt) through a channel near to the center of the large plaque (*, asterisks). The cytoplasm in the pollen grain is contracted. The pollen grain on the right side shows an intact wild-type plaque (*, asterisk). (d) Intact small channel through a plaque (*, asterisk). (e) Large plaque with a broad channel through the plaque (*, asterisk) and extruded cytoplasm (cyt). The cytoplasm in the pollen grain is contracted.

216x391mm (300 x 300 DPI)
Fig. 7 TEM images of sections from wild-type and bup pollen. (a, b) Lens-shaped plaques of wild type pollen with a respectively flat and convex side at the cytoplasm. (c) Higher magnification from the plaque in B. The plaque has a fibrous structure; no larger areas with a clear preferential orientations are seen. At the wall a few ark cytoplasmic inclusions, may be seen. (d) Large bup-2 plaque with a notch in the center where possibly a channel will be formed. (e, f) Almost wild-type like bup-2 plaque, but with a different aspect: rough and with small inclusions. (g, h, i) Large irregular shaped bup-2 plaques with seemingly empty spaces more or less parallel to the surface (g) or many irregularly distributed curling spaces (h). Many cytoplasmic inclusions may be seen. (i) high magnification of (h). (j) Especially at the edges of the bup-2 plaques, dark, spindle-like inclusions may be seen.

174x178mm (300 x 300 DPI)