LAP3, a novel plant protein required for pollen development, is essential for proper exine formation

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Abstract We isolated *lap3-1* and *lap3-2* mutants in a screen for pollen that displays abnormal stigma binding. Unlike wild-type pollen, *lap3-1* and *lap3-2* pollen exine is thinner, weaker, and is missing some connections between their roof-like tectum structures. We describe the mapping and identification of *LAP3* as a novel gene that contains a repetitive motif found in *β*-propeller enzymes. Insertion mutations in *LAP3* lead to male sterility. To investigate possible roles for *LAP3* in pollen development, we assayed the metabolite profile of anther tissues containing developing pollen grains and found that the *lap3-2* defect leads to a broad range of metabolic changes. The largest changes were seen in levels of a straight-chain hydrocarbon nonacosane and in naringenin chalcone, an obligate compound in the flavonoid biosynthesis pathway.

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Introduction

The ornate and diverse patterns that decorate the surface of pollen grains have long been admired for their beauty. They have also been utilized for taxonomy because of their distinctness between, and consistency within, taxa. Pollen grains owe these properties to their outer wall, termed the exine. Although exine patterns vary widely between species, its overall structure is usually conserved. Exine can generally be described as having two layers: an outer sculpted layer, the sexine, and an inner base, the nexine. This layered pattern is often interrupted by apertures, spaces in the exine that guide pollen tube germination in many species. It is the placement of the apertures, along with the pattern formed by the sexine that

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provides immense taxonomic specificity to pollen grains. Exine is composed of sporopollenin, a biopolymer unsurpassed for its strength, elasticity and chemical durability.

Sporopollenin provides an exceptionally strong protective barrier for the microgametophyte against UV irradiation, dehydration, and pathogen attacks. The characteristics that make sporopollenin excel as a protective barrier make it hard to empirically decipher its composition and structure. Sporopollenin is difficult to chemically degrade into defined components that can be qualitatively and quantitatively analyzed. Indeed, solubilization of sporopollenin is often considered in reverse… it is what's left after you boil pollen grains in acetic anhydride or concentrated sulfuric acid (Southworth 1974). In addition, a possibility exists that sporopollenin is not a single substance, but instead varies chemically between species and even between different stages of development (Hemsley et al. 1993; Meutergerhards et al. 1995). Despite these difficulties, tracer experiments, NMR and spectroscopic/spectrometric studies have yielded a model of sporopollenin composed of polyhydroxylated unbranched aliphatics and phenolics covalently coupled with ether and ester linkages (Guilford et al. 1988; Kawase and Takahashi 1995; Ahlers et al. 1999, 2000, 2003; Dominguez et al. 1999; Meuter-Gerhards et al. 1999; Bubert et al. 2002).

Recently, genetic analysis in the model plant *Arabidopsis thaliana* has been brought to bear upon the problems of sporopollenin synthesis, exine development and pattern formation. A number of mutants have been identified with defects in these processes (Aarts et al. 1997; Paxson-Sowders et al., 1997; Paxson-Sowders et al. 2001; Ariizumi et al. 2003; Ariizumi et al. 2004; Nishikawa et al. 2005; Morant et al. 2007; Guan et al. 2008; Suzuki et al. 2008). While the genetic network that controls exine development is far from understood, some players and trends are emerging. For instance, genetic studies provide support for the role of fatty acids as precursors in the construction of sporopollenin. *Male sterility 2* (*ms2*) plants exhibit a lack of exine, with the *MS2* gene showing sequence similarity to fatty acyl reductases (Aarts et al. 1997). The *FACELESS POLLEN 1* (*FLP1*) gene is similar to genes involved in wax synthesis, and the *flp1* mutation results in pollen grains with weakened exine (Ariizumi et al. 2003). The *defective in exine formation 2* (*dex2*) mutation causes impaired exine construction and *DEX2* encodes a cytochrome P450, CYP703A2, that catalyzes in-chain hydroxylation of saturated medium-chain fatty acids (Morant et al. 2007).

We have previously performed a genetic screen that resulted in isolation of several mutants that have exine defects (Zinkl and Preuss 2000; Nishikawa et al. 2005). Here, we describe the identification and characterization of *LAP3*, a novel gene essential for proper pollen development and exine structuring. Insertional mutations in *LAP3* result in male sterility. Less invasive mutations result in pollen grains with abnormally patterned and thin exine. A mechanical assay of pollen wall strength demonstrates that the *lap3* pollen walls are decidedly weaker than those of wild-type plants. Analysis of sequence structure suggests that LAP3 is a β-propeller enzyme. To explore its possible enzymatic functions, metabolomics analyses were carried out.

Methods

Arabidopsis strains and growth conditions

lap3-1 and *lap3-2* were isolated from *cer6-2* (Landsberg *erecta* background, CS6242) mutagenized with 1.25 mM ethyl nitrosourea (ENU) overnight at 22°C (Nishikawa et al. 2005). Mutants were backcrossed three times to Landsberg *erecta*, and the *cer6-2* mutation was outcrossed. *lap3-3*, *lap3-4* and *lap3-5* are T-DNA insertion mutants obtained from the SIGnAL project (Genbank: BH756760, BH613173, and BZ381150, respectively) (Alonso et al. 2003) via the Arabidopsis Biological Resource Center (ABRC). Plants were grown in Shultz premium potting soil, were watered daily, and fertilized (18-18-21 at 200 ppm) twice per week. Plants were grown under 12 or 16 h 130 µE fluorescent lighting at 22°C, or in ambient greenhouse conditions.

Mapping of mutations

LAP3 was genetically mapped in a population generated by crossing *lap3-2* to wild-type Columbia. Analysis of F2 plants localized the mutation to chromosome 3 between markers F24M12-TGF (19 Mbp) and NGA6 (23 Mbp) (http://www.arabidopsis.org). The mutation interval was further narrowed to a 88 kb region on BAC T16L24, between marker F25L23A2 (F-TTCACCATTACGGGC TTCTTCT; R-GCCCAAACCACAATTCTACTATGAT restriction digestion cuts Landsberg products) and marker T16L24C2 (F-AAGAAGAATACCGAGGAGATGACA; R-CAAAGCTGCAGTGGGAAAAC restriction digestion cuts Columbia products).

For complementation, a genomic *LAP3* fragment was PCR amplified with F primer, 5′-GGATCCCCTCTT AGTGGAGTGGAAGTTGTCT-3′, and R primer, 5′-CC ATGGAGTCCAAACTCACACGTTCTGG-3′. The PCR product was recovered in pGEM-T EASY (Promega, Madison, WI, USA) and inserted into *Bam*HI and *Nco*I sites of a modified binary vector pGreenII02229 (Hellens et al. 2000; von Besser et al. 2006). The complementation plasmid was introduced into *Agrobacterium* (strain

GV3101) and transformed into *lap3-1/lap3-1* plants as described (Clough and Bent 1998). Transformed plants were selected using BASTA herbicide. Presence of the transgenic constructs was verified by PCR using gene-specific and vector-specific primers.

Microscopy

For scanning electron microscopy, pollen grains were coated with 10 nm gold particles (Denton Desk II sputter coater) and viewed with a JEOL JSM-840A electron microscope. Transmission electron microscopy of mature pollen was performed as described (Preuss et al. 1993). For laser-scanning confocal microscopy (LSCM), pollen was stained with 0.001% Auramine O (first dissolved to 0.1% in 50 mM Tris–HCl, pH 7.5, and then diluted to 0.001% in 17% sucrose), and examined under LSCM (Olympus Fluoview FV1000, 100X objective, FITC settings). Anther and pollen phenotypes were assessed using standard dissection stereomicroscopes (Nikon C-FMC).

Atomic force microscopy (AFM) uses a physical probe to survey the surface of a sample. Images were collected using an MFP-3D-BIO instrument (Asylum Research, Santa Barbara, CA, USA) in AC mode with a AC160TS probe (Olympus, Tokyo, Japan). The final images were created using the MFP-3D software to render the raw data as three-dimensional surfaces. For mounting, pollen was applied directly from a fresh anther to a double-sided tape on a standard glass slide. AFM imaging was performed immediately, and was completed within 6 h of sample preparation. Approximately 10% of the mounted pollen grains were not held firmly by this method—moving when approached by the AFM probe—and were thus not analyzable. Contact between the AFM probe and the sticky surface of the tape frequently lowered the resolution of the probe, presumably because of adhesive material coating the tip.

Pollen wall structural strength assay

Flowers were harvested from wild-type Landsberg and *lap3-2* plants at principle growth stage 6.00–6.30 from 1 to 3 days after anther dehiscence (Boyes et al. 2001). Eighteen flowers were swirled in 1 ml DI water. Pollen was concentrated into 50 μl, and then ground for 50 rotations at constant pressure (0.22 ± 0.1 MPa) using a mortar and pestle (CoorsTek, Golden, CO, USA) on a 142KL mechanical dial scale (HOM professional, Alsip, IL, USA). The samples were then scored on a Nikon YS2-T compound microscope (Nikon Precision INC, Belmont, CA, USA). One hundred pollen grains were counted per trial and pollen grains were scored as broken if more than 50% of the grain was present and contained visible exine breaks. Data were analyzed using a two-tailed Student's *t* test.

Bioinformatic analysis

Protein sequences for members of the strictosidine synthase family were obtained through Entrez Protein Database (accession numbers): *Rauvolfia serpentina* (CAA44208), *Rauvolfia verticillata* (AAY81922), *Catharanthus roseus* (CAA71255), *Ophiorrhiza pumila* (BAB47180), *A. thaliana* (NP974462). Predicted proteins were aligned using ClustalW (Larkin et al. 2007).

Metabolomics

Three replicates of anther tissue from stage 9 and 10 (Smyth et al. 1990) wild-type and *lap3* flowers were collected on dry ice. Anthers from 50 buds per replicate were lyophilized, homogenized and extracted in 50 μl of 80% methanol containing 2 μg umbelliferone (an internal standard) for 2 h. After this period, 25 μl of extract was collected and directly analyzed by ultraperformance liquid chromatography (UPLC)/quadrupole time-of-flight mass spectrometry (QTOFMS) (Waters Corp., MA, USA). The remaining extract was added to 500 μl of 80% MeOH containing 2.5 μg ribitol (an internal standard). The sample was thoroughly mixed and incubated for 1 h at 50°C. After equilibrating to room temperature, 500 μl of chloroform containing 1 μg of docosanol (an internal standard) was added to each sample. The samples were mixed and incubated for 1 h period at 50°C. At the end of incubation, 400 μl of H_2O was added. The biphasic solvent system was then centrifuged at 2,900g for 30 min at 4°C to separate the layers. An equal amount of each layer was collected and transferred into individual 2.0 ml autosampler vials. The chloroform layer (non-polar) was dried under nitrogen and the aqueous layer dried in a vacuum centrifuge at ambient temperature. The samples were stored at −80°C prior to gas chromatography/mass spectrometry (GC/MS) analysis.

Gas chromatography/mass spectrometry

A scaled-down version of a published GC/MS metabolomics method (Broeckling et al. 2005) was used for anther analyses. Briefly, an Agilent 6890 GC coupled to a 5973 MSD was used for GC-MS analysis of polar and non-polar extracts. Samples were injected at a 1:1 split ratio, and the inlet and transfer line were held at 2,800°C. Separations were achieved with a temperature program of 80°C for 2 min, then ramped at 50°C/min to 315°C and held for 12 min, on 60 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 μm film thickness) and a constant flow

of 1.0 ml/min. Mass spectra were collected over a range of m/z 50–800. For each compound, a Student's t test was performed to assess statistical difference.

Ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry

Ultraperformance liquid chromatography was performed using a Waters ACQUITY UPLC^{TM} system (Waters), equipped with a binary solvent delivery system and an autosampler. Separations were optimized using a 150 mm × 2.1 mm Waters ACQUITY C18 1.7 mm column. The mobile phase consisted of (A) 0.1% acetic acid in distilled water and (B) acetonitrile. UPLC separations were achieved using a Waters Acquity UPLC 2.1 × 100 mm, BEH C18 column with 1.7 μm particles, a flow of 600 μl/min, and a linear gradient of 95%:5%–30%:70% eluents A:B. Eluent A was 0.1% acetic acid in water and eluent B was acetonitrile. Mass spectra were acquired using a QTOF Premier^{TM}, an orthogonal acceleration TOF mass spectrometer (Waters) operating in negative ion electrospray mode. The nebulization gas was set to 850 l/h at a temperature of 350°C. The cone gas was set to 50 l/h and the source temperature set to 120°C. The TOFMS data were collected between m/z 50 and 2,000. Data were analyzed using a two-tailed Student's t test.

## Results

lap3 mutants produce exine-deficient pollen

Previously, we described a simple binding assay that was used to identify *A. thaliana* mutants with *less adherent pollen* (*lap*) (Zinkl et al. 1999; Zinkl and Preuss 2000; Nishikawa et al. 2005). Among the mutants recovered from an M2 population of ENU-mutagenized plants were two non-complementing *lap3* mutants. *lap3* mutant plants have noticeable pollen surface defects apparent with scanning and transmission electron microscopy, confocal microscopy and AFM (Figs. 1, 2). When viewed under a dissection stereomicroscope, anthers and pollen in *lap3* mutants appear glossy, and pollen is not released easily from the anthers. Both *lap3-1* (Fig. 1b) and *lap3-2* (Fig. 1c) pollen display structurally weakened exine that leads to easy collapse of the pollen grains. Unlike pollen from some previously characterized exine mutants that show irregular aggregates of sporopollenin and a severe disruption of exine wall pattern (Nishikawa et al. 2005), pollen from *lap3-1* and *lap3-2* plants exhibit more subtle exine defects. Although their exines retain a semblance of the reticulate pattern seen in wild-type, the remaining patterns are less regular and distorted. The connections between columellae are discontinuous and elements of the roof-like tectum structures are generally lost (Fig. 1). In addition to this, TEM and AFM demonstrates that the *lap3* exine walls are thinner than in wild-type plants (Fig. 2).

### Mutations in *LAP3* produce pollen with weakened exine walls

To investigate whether the thinner exine walls seen in *lap3-1* and *lap3-2* by TEM (Fig. 2b and data not shown) resulted in weaker pollen, pollen wall strength was assessed using mechanical disruption. Pollen from wild-type and *lap3-2* were ground under a constant pressure using a mortar and pestle. Broken pollen grains were counted. *lap3-2* pollen were five times more likely to be crushed, with the mean

Fig. 1 Pollen from *lap3* plants have defective exine structure. Scanning electron micrographs of pollen grains from wild-type (**a**), *lap3-1* (**b**), and *lap3-2* plants (**c**). Confocal images of pollen grains from wild-type (**d**), *lap3-1* (**e**) and *lap3-2* (**f**) plants. *White bars* 10 μm
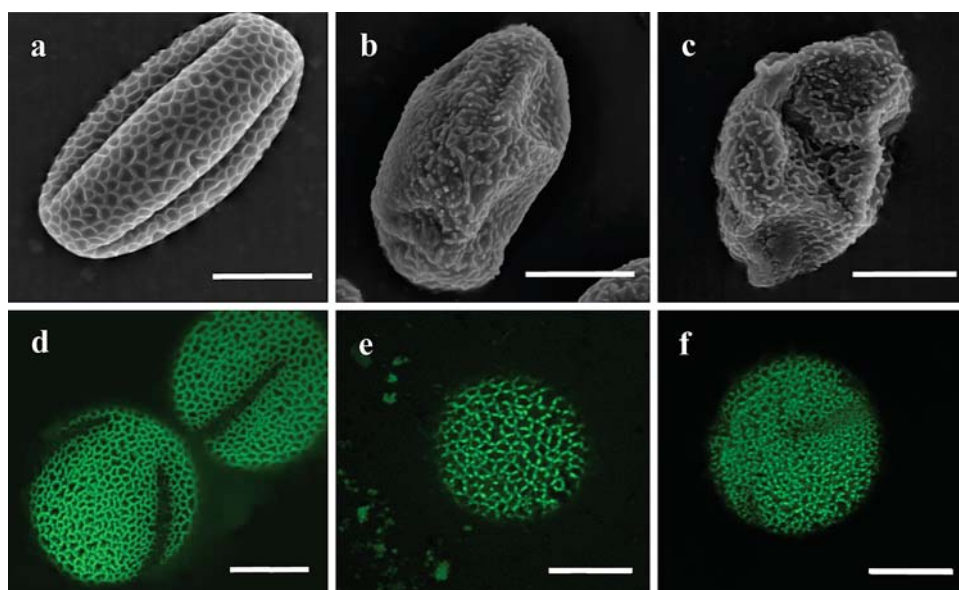
Fig. 2 Exine from *lap3* pollen is thin and missing some tectum. Transmission electron micrographs of pollen from wild-type (**a**) and *lap3-2* (**b**) plants (*arrows* indicate electron dense sporopollenin). Atomic force microscopy imaging from wild-type (**c**) and *lap3-2* (**d**) pollen. *Bars* 1 μm
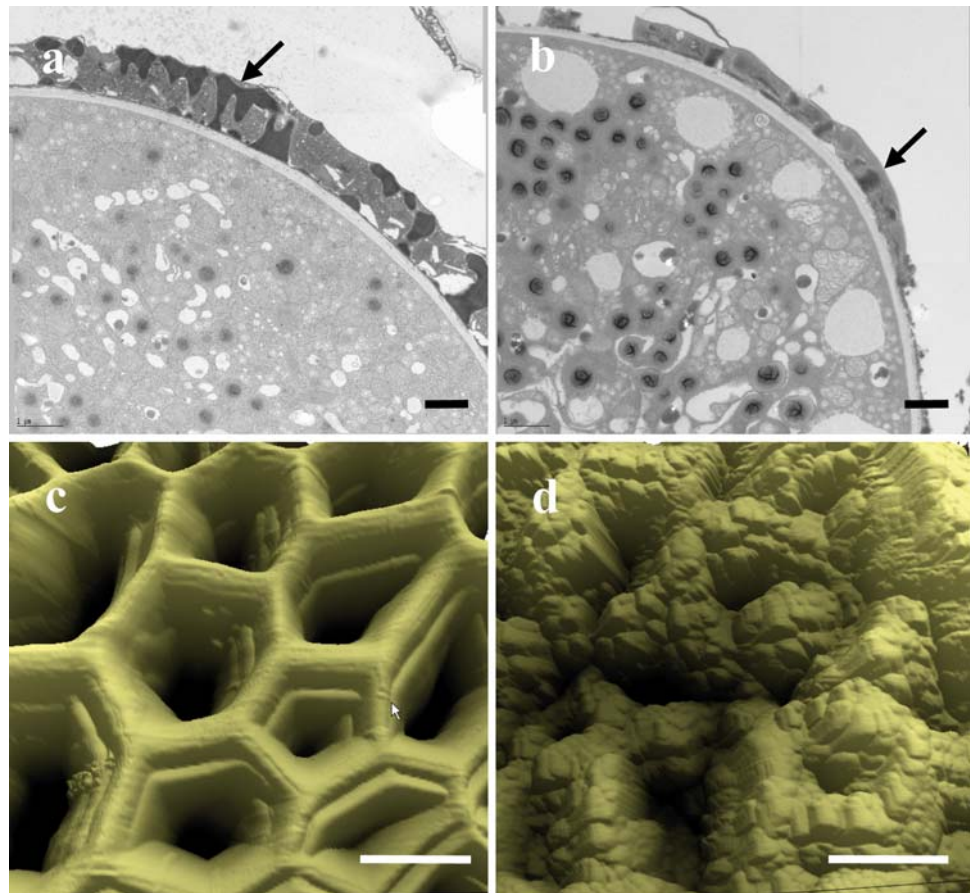
number of crushed pollen grains per 100 being 5 ± 2 for *wild-type* compared to 25.5 ± 10.6 for *lap3-2* (see Table 1). These differences have statistical significance [$t(10) = 4.66$, $P = .001$].

Mapping of *lap3* defects

Using bulked segregant analysis, we mapped *LAP3* to an area on chromosome 3 (linkage to F24M12 and NGA6 markers). Using additional PCR-based markers, we further narrowed the interval to 88 kb on BAC T16L24. DNA sequencing of the predicted genes in this region revealed mutations in the gene At3g59530 that resulted in non-conservative amino acid changes. In *lap3-1*, Glu89 was substituted with Lys, and in *lap3-2* Gly395 was replaced with Glu (Fig. 3). To verify that these mutations were the cause of the exine phenotype, we constructed a T-DNA with a full-length genomic copy of *LAP3*. Transformation of *lap3-1* plants with this construct resulted in plants that manufactured wild-type pollen, confirming the role of *LAP3* in the formation of proper exine structure. The SIGnAL T-DNA insertion collection contained three additional alleles of *LAP3* (Columbia ecotype, Fig. 3). Unlike the point mutations isolated in our screen, which

resulted in aberrant exine phenotypes but functional pollen, the T-DNA insertions led to male sterility, with homozygous plants having brown, shriveled anthers and no visible pollen (Fig. 4). The sterility is not due to the defective female structures, as *lap3* flowers pollinated with wild-type pollen produced normal seed sets (data not shown). All *lap3* mutations tested were recessive.

Sequence analysis of LAP3

LAP3 protein is currently annotated as a strictosidine synthase (TAIR 2009). Database searching reveals a number of proteins with high sequence identity to LAP3 from a variety of different plant families: *Brassica napus* with 88% identity (AAX38236), *Vitis vinifera* with 73% identity (CAO64830), *Oryza sativa* with 65% identity (EAZ26352), *Triticum aestivum* with 63% identity (CAG38622) and *Zea mays* with 65% identity (AAK52489). Interestingly, *Z. mays* AAK52489 is encoded by the *Male-sterile 45* (*Ms45*) gene, a mutation in which leads to poor formation of microspore cell wall (Cigan et al. 2001; Fox et al. 2001) indicating that sequence orthologs of *LAP3* from different species likely play similar roles in exine development. No close paralogs of *LAP3* exist in *Arabidopsis*.

Table 1 Pollen wall structural strength

Trial		Broken pollen/100
1	Ler-0 wild-type	4
2	Ler-0 wild-type	4
3	Ler-0 wild-type	9
4	Ler-0 wild-type	4
5	Ler-0 wild-type	4
6	Ler-0 wild-type	5
1	lap3-2	42
2	lap3-2	26
3	lap3-2	22
4	lap3-2	27
5	lap3-2	9
6	lap3-2	27

We searched publicly available expression databases, such as Genevestigator (Zimmermann et al. 2004), http://www.genevestigator.ethz.ch/) and MPSS (Meyers et al. 2004), http://mpss.udel.edu/at/) to determine tissue expression patterns of *LAP3*. The results of this search suggested that it is predominantly expressed in young anthers and in flower buds, and its transcripts are greatly reduced in flower buds from the stamen-lacking *ap3* and *ag* mutants. This is similar to *Ms45*, the *LAP3* ortholog from maize, which is expressed in anther tapetum, a secretory tissue lining anther locules where microspore development takes place (Cigan et al. 2001). In addition, a search in plant EST databases (http://ncbi.nlm.nih.gov) identified multiple ESTs from 21 different species. Analysis of ESTs revealed a strong bias for expression of *LAP3* orthologs in flower buds, male organs, and inflorescences. Even an EST
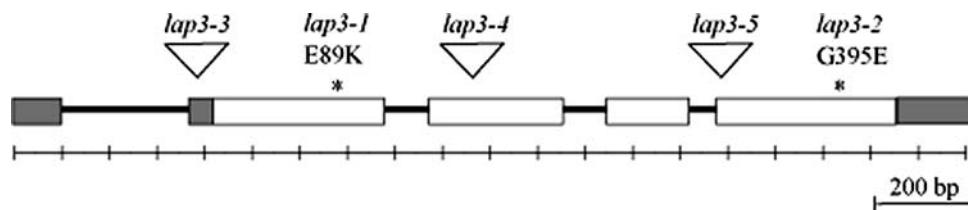
Fig. 3 *lap3-1* maps to the At3g59530 gene. The LAP3 open reading frame encodes a 413 amino acid predicted protein. *lap 3-1* is a E89K substitution. *lap3-2* is a G395E substitution. *lap3-3*, *lap3-4* and *lap3-5* are T-DNA insertions. *Open boxes* represent coding regions; *shaded boxes* represent untranslated regions; *triangles* T-DNA insertion points; *stars* amino acid substitution points. Note: the Col-0 genomic sequence (TAIR 2009) predicted At3g59530 protein contains a ten amino acid deletion in comparison to the Ler-0 protein (Ler-0 amino acids 382–391 VSEVREVQGK), Col-0 cDNAs, as well as At3g59530 homologs. The above map uses the Ler-0 gene and amino acid numbering

Fig. 4 *LAP3* is essential for fertilization. Comparison of inflorescences for **a** Col-0 and **b** *lap3-3* (Genbank: BH756760). Inability to fertilize, and thus no elongation of siliques (compare **c**, **d**) is due to male sterility, as wild-type pollen fertilize *lap3-3* females normally
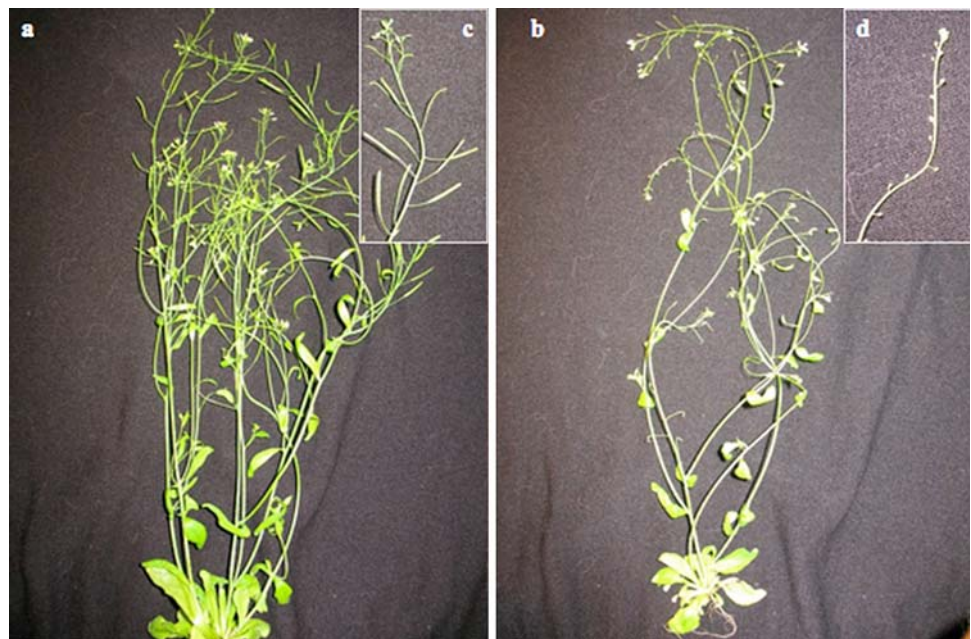
from the evolutionarily distant gymnosperm *Ginkgo biloba* (EX934056) was derived from a microsporophyll tissue and shared extensive regions of homology with LAP3 (71% identity over 235 translated amino acids), indicating a strong evolutionary conservation of LAP3 structure and suggesting a similar conservation of expression pattern.

LAP3 is one of 15 *Arabidopsis* proteins that have been annotated as strictosidine synthase family proteins based upon relatively low (26–39%) sequence identity to STR1, a protein from *R. serpentina* with demonstrated enzymatic activity (TAIR 2009). Strictosidine synthase catalyzes the Pictet–Spengler condensation of tryptamine and secologanin; in higher plants this reaction is central for the biosynthesis of a large number of alkaloids of the monoterpenoid indole family (Stockigt et al. 2008). Recent structural characterizations of strictosidine synthase has led to a sophisticated understanding of the structure of the active site of STR1 and its catalytic mechanism (Ma et al. 2004, 2006; Koepke et al. 2005; Loris et al. 2007). STR1 is a 6-bladed β-propeller, the first one to be found in Kingdom Plantae (Ma et al. 2006). LAP3 contains the common repetitive motif found in β-propellers (Ma et al. 2006) (see supplemental Fig. 1). LAP3 also contains conserved cysteines that form a disulfide bridge in STR1 (Ma et al. 2006), supporting the hypothesis that LAP3 encodes a β-propeller enzyme. But similarly to β-propellers that act on a variety of different substrates, LAP3 lacks the key substrate-binding residues and the conserved catalytic residue found in all functionally active strictosidine synthases (see supplemental Fig. 1), casting substantial doubt on whether LAP3 encodes an enzyme with strictosidine synthase activity.

Metabolomics analysis of *lap3-2*

In order to investigate possible roles for LAP3 in pollen development, we assayed whether mutations in *LAP3* affect the production of metabolic compounds in anthers during the time when pollen grains are developing. We collected developing anthers from the wild-type and *lap3-2* flowers during the time that exine was being produced (stage 9/10, Smyth et al. 1990). We then performed metabolomic analysis on anther extracts. Metabolic profiles were collected using an optimized ultra performance liquid chromatography/time-of-flight mass spectrometry (UPLC/TOFMS) followed by GC/MS. A broad spectrum of primary and secondary metabolites were separated, detected and characterized.

Fifty metabolites were detected with UPLC/TOFMS analysis. Using authentic standards or comparisons with previously published results (Broeckling et al. 2005), we annotated 33 of these metabolites that primarily belonged to three chemical groups: phenolpropanoids (mainly flavonoids), glucosinolates and lipids (see supplemental Table 1). Lipid profiles in the mutant and wild-type tissue exhibited very modest differences. While for some of the lipids (α-linolenic acid, 1-18:3-lysophophatidylethanolamine, 1-16:0-lysophophatidylethanolamine, α-eleosteric acid, and 10E,12Z-octadecadienoic acid) reduction in the *lap3-2* plants was statistically significant, it only ranged from 1.3 to 1.5-fold. Lipids identified with statistically significant higher amounts in *lap3-2* plants included heptadecanoic acid and oleinic acid that showed a 1.5 and 1.3-fold increase, respectively. The phenolic profile was largely unchanged between *lap3-2* and wild-type anthers, with the exception of naringenin chalcone and apigenin-6-C-glucoside, which were undetectable in *lap3-2* anther samples. Naringenin chalcone is the first committed chemical in the flavonoid biosynthesis pathway. Finally, glucosinolate profiles were unchanged between *lap3-2* and wild-type with the exception of 3-hydroxypropyl-glucosinolate, which shows a 1.6-fold increase in *lap3-2* tissue.

Using GC/MS analysis, 83 molecules of known chemical structure were measured. A substantial number of metabolites were altered in *lap3-2* anthers compared to wild-type anthers. Statistically significant changes were observed in the levels of several amino acids, including L-asparagine, L-proline, L-tryptophan and β-alanine, that are 3-fold increased, 7-fold decreased, 2-fold decreased and 1.5-fold decreased, respectively. Levels of several detected lipids were decreased in *lap3-2*. Those included linoleic acid (2.3-fold), palmitic acid (3.1-fold), a fatty alcohol 1-hexadecanol (almost 7-fold), and nonacosane (50-fold). Nonacosane is a straight-chain hydrocarbon and a predominant component of plant cuticular wax. For a complete list of the compounds detected by GC/MS and their changes in developing mutant anthers, see Table 2 in the supplementary material.

Discussion

LAP3 is essential for pollen development and proper exine formation

LAP3 joins a growing number of genes demonstrated to play a role in exine formation. Thus far, three processes have been shown to be vital for the proper formation of exine [reviewed in Ariizumi and Toriyama (2007)]. First, the construction of a protective callose wall around microsporocytes and microspores is critical for exine development. This wall persists until after completion of meiosis and initiation of exine wall formation, when it is degraded by the tapetally derived β-1,3-glucanase (Scott et al. 1991; Piffanelli et al. 1998). Impaired construction (due to mutations in the callose synthase *LAP1/CALS5*) or

premature dissolution (due to overexpression of a β-1,3-glucanase) of this wall can result in aberrations in exine patterning of developing microspores and male sterility (Worrall et al. 1992; Dong et al. 2005). In instances where callose wall construction is only moderately impaired, the pollen develops, but randomly deposits sporopollenin in clumps, losing all semblance of the reticulate exine pattern (Nishikawa et al. 2005).

The second process demonstrated to be vital for proper formation of exine is the biosynthesis of sporopollenin. Thus far, three genes have been suggested to be involved in sporopollenin synthesis. They include *MALE STERLITY 2* (*MS2*) that shows similarity to fatty acyl reductases (Aarts et al. 1997), *FLP1* that is similar to genes catalyzing biosynthesis of very-long-chain fatty acids (Ariizumi et al. 2003), and *DEFECTIVE IN EXINE FORMATION 2* (*DEX2/CYP703A2*) that catalyzes the conversion of medium-chain saturated fatty acids to monohydroxylated fatty acids (Morant et al. 2007). Mutations in all of these genes lead to either lack of exine or development of weakened exine and associated problems with fertility.

Finally, the primexine, which forms between the pollen plasma membrane and the callose wall, acts as a scaffold for proper sporopollenin deposition. Mutants of *DEFECTIVE IN EXINE FORMATION 1* (*DEX1*) show random deposition of sporopollenin that results in pollen degeneration, and has been suggested to be involved in the structuring or assembly of the primexine (Paxson-Sowders et al. 1997; Paxson-Sowders et al. 2001). Mutants in the *NO EXINE FORMATION 1* (*NEF1*) gene synthesize sporopollenin, but fail to deposit it on the membrane, likely due to a poorly developed primexine (Ariizumi et al. 2004). The *RUPTURED POLLEN GRAIN1* (*RPG1*) gene encodes a seven transmembrane domain protein that localizes to plasma membrane of microsporocytes (Guan et al. 2008). Mutations in this gene affect plasma membrane structure and primexine deposition, and lead to irregular exine structure and microspore collapse (Guan et al. 2008). In mutants of the *MALE STERILITY* (*MS1*) gene, thin and abnormal primexine precedes abnormal deposition of sporopollenin (Ariizumi et al. 2005). It is evident, however, that some disruption or delay of primexine formation is acceptable for the proper formation of exine. Mutants in the *TRANSIENT DEFECTIVE EXINE 1* (*TDE1*) gene delay the development of primexine formation, and the primexine that does form is thin. The mature pollen does, however, form proper exine, albeit its development is delayed (Ariizumi et al. 2008).

LAP3 now joins the ranks of *LAP1/CALS5*, *MS1*, *MS2*, *FLP1*, *DEX1*, *NEF1*, and *RPG1* as a gene, which, when disrupted, causes male sterility, and also alters exine formation. Our isolation of point mutations in *LAP3* allowed us to visualize pollen constructed with a less impaired version of *LAP3*. These pollen grains have thinner exine, but retain a semblance of exine patterning while displaying discontinuous collumnae and missing tectum. This is a novel phenotype that is not represented among the known exine mutants.

lap3-2 pollen exhibits a weakened pollen wall

Interest in sporopollenin from a materials science perspective comes from various properties attributed to it: strength, adhesive capability, as well as elasticity. There have been various methods devised to mechanically assay pollen wall properties. Rowley and Skvarla pressed a piston into a close fitting cylinder (with a clearance of about 20 μm) containing pollen from ten different species to assay the elasticity of pollen (Rowley and Skvarla 2000). As a testament to the elasticity and strength of the exine, even when the diameters of pollen grains were much larger than the clearance for the piston, most pollen survived the treatment intact, showing only slight cracks in the exine wall, or in some cases, pollen grains within pollen grains. Bolick and Vogal more quantitatively measured pollen wall strength using a calibrated vise to assay the 50% breaking point pressure for pollen from eleven different species, with most species tested having an average wall strength between 2 and 90 MN/m^2 (Bolick and Vogel 1992). Finally, some aberrantly constructed pollen walls have shown chemical sensitivity to acetolysis treatment (Scott 1994; Aarts et al. 1997; Ariizumi et al. 2003). When tested, *lap3-2* pollen showed no sensitivity to this treatment (data not shown).

We have devised a simple and consistent assay to measure relative mechanical pollen wall strength by grinding pollen under a constant pressure. Under these conditions, *lap3-2* pollen is five times more likely to rupture than wild-type pollen, consistent with TEM results showing *lap3* pollen having thinner exine walls.

LAP3 is likely not a strictosidine synthase

Strictosidine synthase catalyzes the Pictet–Spengler reaction between tryptamine and secologanin. In organic chemistry, this reaction is used to synthesize tetrahydroisoquinoline skeletons (Cox and Cook 1995). In plants, this reaction plays a central role in the biosynthesis of a number of different classes of alkaloids, including the monoterpenoid indole alkaloid family (for excellent reviews, see Kutchan 1993; Stockigt et al. 2008).

Recent structural characterizations of strictosidine synthase of the Indian medicinal plant *R. serpentina* (STR1), alone and in complex with its substrates (tryptamine and secologanin) and product (strictosidine), has led to an understanding of the structure of the active site of STR1

and its catalytic mechanism (Ma et al. 2004, 2006; Koepke et al. 2005; Loris et al. 2007). STR1 is a 6-bladed β-propeller (Ma et al. 2006). Ma et al. (2006) identified a common repetitive motif in the β-propeller structures related to STR1: three hydrophobic residues, followed by a small residue (Ser, Thr, Val or Gly) and then a hydrophilic residue, frequently Asp or Glu. LAP3 contains this repetitive motif (see supplemental Fig. 1). Two of the three α-helices in the STR1 structure are located between the first and second blade of the propeller, and are pulled together via a disulfide bridge between STR1 residues Cys-89 and Cys-101. These residues play a vital role in the integrity of the substrate-binding site and are conserved throughout the STR family (Ma et al. 2006). LAP3 contains these conserved cysteines (see supplemental Fig. 1). LAP3 shares 35% identity with STR1, and although this sequence identity is low, low similarity is characteristic among different β-propellers (Jawad and Paoli 2002).

However, the differences in the substrate-binding pocket, and the absence of the essential catalytic residue makes LAP3's role as a strictosidine synthase doubtful. The hydrophobic substrate-binding pocket is located at the top of the propeller, at the pseudo sixfold symmetry axis. A number of residues demonstrated to position strictosidine and secologanin in this active site are conserved in *bona fide* strictosidine synthases from a variety of plant families (Stockigt et al. 2008). None of these residues are conserved in LAP3 (see supplemental Fig. 1). Indeed, the conserved catalytic residue of strictosidine synthases, Glu309 is replaced by a valine in LAP3. These observations lead us to a hypothesis that LAP3 may be a β-propeller enzyme, but one that is different from strictosidine synthase. This begs the question then, what is the function of LAP3?

Mutations in *LAP3* result in a variety of metabolic consequences

In order to investigate possible roles for LAP3 in pollen development, we assayed whether mutations in *LAP3* affect the production of metabolic compounds in anthers during the time when pollen grains are developing. *lap3-2* leads to a wide variety of metabolic consequences in developing anthers. Given the model of sporopollenin composed of fatty acid and phenolic compounds (Guilford et al. 1988; Kawase and Takahashi 1995; Ahlers et al. 1999, 2000, 2003; Dominguez et al. 1999; Meuter-Gerhards et al. 1999; Bubert et al. 2002), it is of interest to note that some changes were detected in the levels of lipids (such as α-linolenic acid, 1-18:3-lysophophatidylethanolamine, 1-16:0-lysophophatidylethanolamine, α-eleosteric acid, and 10E,12Z-octadecadienoic acid, linoleic acid, nonacosane and palmitic acid) and of at least one phenylpropanoid (naringenin chalcone).

Of all the compounds identified, the two largest differences were in nonacosane and naringenin chalcone, with a 50-fold reduction in nonacosane and undetectable levels of naringenin chalcone. Naringenin chalcone is the first committed chemical in the flavonoid biosynthesis pathway. Flavonoids function in a variety of processes in plants; this is reflective of their structural diversity, with over 8,000 different flavonoids identified in vascular plants (Pietta 2000). In many plants, pollen grains contain large amounts of flavonoids (Shirley et al. 1995; Hsieh and Huang 2007), and those have been shown to be important in normal pollen function. Inhibition of chalcone synthase, which synthesizes naringenin chalcone, in maize and petunia results in male sterility and the development of white pollen grains that are unable to germinate and grow pollen tubes (Coe et al. 1981; Franken et al. 1991; Vandermeer et al. 1992; Napoli et al. 1999). In *Arabidopsis*, however, a *tt4* mutant in chalcone synthase lacks flavonoids in mature stamens, but demonstrates normal pollen structure and function (Burbulis et al. 1996), leading Burbulis et al. to a conclusion that flavonoids are not essential for plant fertility in that species. Nonacosane has not been previously implicated in pollen structure or function, and is best known as a component of plant cuticular wax (Hannoufa et al. 1993; Peschel et al. 2007).

In summary, our work leads to the hypothesis that LAP3 is an enzyme involved in exine development. Metabolomic analysis of *lap3-2* plants identifies broad changes in the anther tissue. It is presently unclear, however, which of the observed effects are directly due to the loss of the enzymatic activity, as opposed to general changes cascading from altered exine development. Future investigations of the enzymatic activity of LAP3, including in vitro identification of potential substrates, will clarify its role.

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