Methods

A novel method for efficient *in vitro* germination and tube growth of *Arabidopsis thaliana* pollen

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Summary

- In addition to its importance in studies of plant reproduction and fertility, pollen is as widely employed as a model system of cell growth and development. This work demands robust, reproducible methods to induce pollen germination and morphologically normal growth of pollen tubes *in vitro*. Despite numerous advantages of *Arabidopsis thaliana* as a model plant, such experiments on pollen germination and pollen tube growth have often proved challenging.
- Our new method employs a physical cellulosic membrane, overlying an agarose substrate. By modulating the substrate composition, we provide important insights into the mechanisms promoting pollen growth both *in vitro* and *in vivo.*
- This effective new technical approach to *A. thaliana* pollen germination and tube growth results in swift, consistent and unprecedented levels of germination to over 90%. It can also promote rapid growth of long, morphologically normal pollen tubes.
- This technical development demonstrates that exogenous spermidine and a cellulosic substrate are key factors in stimulating germination. It has potential to greatly assist the study of reproduction in *A. thaliana* and its closest relatives, not only for the study of germination levels and pollen tube growth dynamics by microscopy, but also for biochemical and molecular analysis of germinating pollen.

Introduction

In flowering plants, sexual reproduction involves the formation of the gametophyte – a highly specialized post-meiotic haploid generation, substantially reduced compared with the diploid sporophyte but dependent on it for resources. While the female gametophyte remains enveloped within the maternal sporophytic tissue, the male gametophyte – the pollen – completes only the first phase of its development protected by the sporophyte. Following programmed developmental arrest and dehydration, pollen is released from the anther and transported to the stigma. Assuming compatibility between the pollen grain and the specialized sporophytic surface of the stigma, it undergoes regulated rehydration and mobilizes stored resources (including mRNAs, complex carbohydrates and lipids) to initiate germination and highly polarized tip-based growth of the pollen tube (Taylor & Hepler, 1997). The tube acts as an altruistic 'carrier', transporting the sperms destined to fertilize the female gametes. In species that shed bicellular pollen, sperms are generated by the mitotic division of the generative cell during pollen tube growth, while in pollen from tricellular species, this division takes place before pollen release (Twell, 2011).

Pollen tube growth is unique to flowering plant development, with the tube tip showing exceptionally high vesicular activity and membrane synthesis, and cylindrical callosic and pecto-cellulosic walls being formed behind this focused tip region (Taylor & Hepler, 1997; Zonia, 2010). The active vesicle trafficking that supports pollen tube growth involves both exocytosis of vesicles to supply materials for extension growth, and endocytosis to recycle surplus membrane components and to import materials from the stigma. Through complex interactions with other cellular systems, turgor is a key driver of this rapid growth (Zonia, 2010; Winship *et al.*, 2011; Zonia & Munnik, 2011; Hill *et al.*, 2012) and, once initiated, the tube extends directionally to penetrate the sporophytic tissues of the stigmatic surface. Once within the stigmatic apoplast, the pollen tube uses signals and cues from the external cellular environment to navigate to the female gametophyte and deliver the nonmotile sperm cells to the egg and central cells (Takeuchi & Higashiyama, 2011). Pollen germination and tube growth thus emerge as highly dynamic and co-ordinated processes, integrating many different signals from the local environment to regulate growth and development.

The apparent simplicity and independence of pollen have also resulted in its use as a paradigm for plant cell growth and

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development (Taylor & Hepler, 1997). This, combined with the importance of pollen to scientific and applied studies of plant reproduction, has created a strong demand for systems by which pollen germination and tube growth can be induced *in vitro*. While some species have lent themselves to the development of effective protocols, other groups of plants remain intractable. A general correlation exists between the facility with which pollen germination and tube growth can be induced *in vitro* and stigma structure. Development of *in vitro* methods has generally proved successful in so-called 'wet stigma' plants, such as *Nicotiana* and *Lilium*, where the stigma produces an exudate in which pollen germinates (Allen & Hiscock, 2011). By contrast, taxa with 'dry' stigmas, for instance members of the Poaceae and Brassicaceae, have proved more challenging. On these surfaces, complex interactions take place that mediate highly regulated and perhaps directional uptake of water from the papillar cells of the stigmatic surface (Allen & Hiscock, 2011). It has probably been our inability to parallel these processes *in vitro* that has hampered successful development of protocols for efficient pollen germination and tube growth.

The model plant *Arabidopsis thaliana* is exceptionally practical and versatile in most aspects of experimental study, and is supported by excellent genomic and bioinformatic resources. However, its utility in studies of reproductive biology is impaired by the absence of an easy, reproducible technique for germination of pollen *in vitro*. Numerous papers have been published that describe methods to induce pollen germination and pollen tube growth *in vitro* (e.g. Li *et al.*, 1999; Palanivelu *et al.*, 2003; Boavida & McCormick, 2007; Bou Daher *et al.*, 2009), and some of these have considerable utility. However, a robust and reliable method promoting high levels of germination and morphologically normal tube growth, and in which the material can be recovered for cell and molecular analysis, has remained elusive.

Here, we describe a new method that allows an unprecedented level of germination from *A. thaliana* pollen *in vitro*, followed by rapid growth of morphologically normal pollen tubes with regular callose plugs and correct localization of sperm cells. A significant innovation in our method is the use of a novel physiochemical environment that may more closely mimic the stigma, by employing a cellulose-based membrane as a support for pollen germination. Not only promoting germination and tube growth, this synthetic membrane substrate makes it easier to use the germinating gametophytes in downstream applications. Our method will permit the more effective use of *A. thaliana* pollen tubes in studies using microscopy (for example investigations involving the visualization of dyes and fluorescently tagged proteins), as well as research in cell biology and biophysics (such as studies of pollen tube membrane channels, transporters, wall generation and the processes of exocytosis and endocytosis) and biochemistry (for instance the extraction of RNAs and proteins from this stage of development). Finally, by subtracting from, or altering the proportion of components of the pollen germination medium, new insights are provided into the natural mechanisms that may stimulate and promote the germination and growth *in planta*. We anticipate that this new experimental strategy will facilitate efficient, reproducible studies of the post-hydration development of pollen from *A. thaliana* and its relatives.

Materials and Methods

Plant growth conditions

Seedlings of *Arabidopsis thaliana* (L.) Heynh. Columbia-0 (Col-0) and Landsberg *erecta* (Ler) ecotypes were grown in pots in 4 : 1 ratio of multipurpose peat-based compost to vermiculite mixture and stood in trays. Plants were grown under standard glasshouse conditions at 25°C with supplemental lighting and were kept well watered. To ensure that pollen was not affected by water collecting in the opening flower buds, plants were not watered from above before use in pollen germination assays. Only flowering plants that had fully entered into the flowering state (i.e. had started to set siliques on the first flowers of the main inflorescence axis), but had not started to exit flower production (i.e. always had more unopened flower buds than siliques and mature flowers) were used for study.

Culture of *A. thaliana* pollen *in vitro*

Agarose substrate medium composition Medium was made in a Falcon tube and consisted of 18% or 10% sucrose (see the Results section), 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate (N-Z-Amine A; Sigma C0626), 0.01% myo-inositol (Sigma), 0.01% ferric ammonium citrate (FAC) (Sigma), 0–1 mM spermidine (Sigma) (see the Results section), and 0–10 mM GABA (Sigma) (see the Results section). Components were subtracted from the medium or the levels modified as described in the Results. Sucrose was dissolved first, then the other materials added, and the pH monitored using indicator strips (Paper Dosatest, pH 4.5–10.0, VWR International, Radnor, Pennsylvania, USA) and adjusted to 8.0, or to other values described in the Results, with dilute KOH solution. Finally, agarose (Molecular Grade; Bioline) was added to the solution to 0.5%, then mixed and briefly heated in a microwave until it had dissolved completely. The pH was again measured as previously and adjusted if necessary. The solution was kept molten in a water bath (*c.* 60–65°C) until required.

The cellulose membrane/agarose culture apparatus For germination and subsequent pollen tube growth, an agarose pad was layered onto an uncoated glass microscope slide (VWR). Slides were prepared (see Supporting Information Fig. S1a) by drawing a single c. 1.5 × 2 cm rectangle on the surface using a PAP pen (Liquid Block Super PAP pen; Daido Sangyo Saitama, Japan). The slides were placed on a very flat surface and, within each rectangle, 500 µl of hot solution was added to form a flat agarose pad. The slides were left to cool until the agarose had solidified. For subsequent use, slides were stored vertically, on their sides, in a microscope slide container, with wet tissue paper placed in the container (but not touching the slide surfaces) to ensure a constant saturated humidity (Fig. S1b). When the container was sealed and held at 4°C the slides could be used for up to 7 d after preparation.

Pollen culture Freshly made slides at room temperature were used immediately, but in preparation for use the cold-stored slides

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were first equilibrated in a humid chamber as follows. A humid chamber was prepared by placing a layer of tissue paper wetted with warm (*c.* 40–50°C) water at the bottom of a large square Petri dish. Glass pipettes were placed on top of the paper and used to support the slides. Slides were allowed to equilibrate in the chamber for 10–15 min. Rectangular pieces (*c.* 1.2 cm × 1.7 cm, always smaller than the pad surface) of cellulosic Cellophane membrane (325P Cellulose; AA Packaging Limited, Preston, UK; www.aapackaging.co.uk) were cut out. These were placed carefully, held tensed between two pairs of forceps, on top of the agarose pad, leaving an edge of agarose pad on all sides (see Fig. S1a). This method was employed to ensure a perfectly flat surface and complete contact between the membrane and agarose. Once the cellulose membrane had been layered onto the gel, slides were used without delay.

Pollen was placed on the surface of the cellophane membrane as follows. Flowers were carefully selected to ensure that only those very recently opened and shedding pollen (*c.* day 1 to day 2 stage) (Boavida & McCormick, 2007) were used. It was found that pollen shedding for use on slides was optimal in the morning. Using forceps to hold the pedicel, the flower was lightly brushed on the surface in a limited area. For each *c.* 1.2 × 1.7 cm rectangular cellulose sheet, *c.* 12 different flowers (in 3 × 4 rows) were used. Slides were immediately placed in a vertical position in the same slide box construction used for cold storage, with wet paper used to keep constant saturated humidity. Sealed in boxes, slides were incubated in the dark at 24°C. When taken for examination, slides were not transferred in the open air but were placed in the humid Petri dish system described above to avoid environmental change.

Substrate, medium composition and temperature variation studies In studies where individual components of the germination substrate and germination medium were varied by subtraction, addition or concentration, all steps were performed as previously described except for the altered component. Where the cellulose membrane was omitted from the set-up, great care was taken with spreading of pollen on the surface to ensure that pollen was not pushed below the surface of the agarose.

All experiments were performed at 24°C except for those designed to study the effect of variation in temperature on pollen germination, where slides were incubated in growth chambers set at a range of temperatures.

Microscopy

Slides were examined using a Zeiss Axiophot photomicroscope. Pollen germination was counted using random fields, selected independently across the slide surface in an ordered fashion to prevent duplication. For each reading, 300 pollen grains were counted, and scored as germinated or ungerminated depending on whether an intact pollen tube could be seen emerging. For pollen tube lengths, images were captured in random fields and analysed using ImageJ software. One hundred (or, in the rare cases where germination was minimal and pollen tubes were difficult to locate, 50) pollen tubes were measured for length using ImageJ (NIH, Bethesda, Maryland, USA).

For analysis of pollen germination and pollen tube growth kinetics, slides were examined at a number of time-points after pollen had contacted the cellulosic membrane, up to 24 h.

For study of pollen tube structure, pollen tubes were removed from the cellulose layer following 5 h in culture. This was achieved by displacing pollen tubes from the membrane surface by dropping a small amount of sucrose solution (equivalent in concentration to the pollen germination medium) onto the membrane to form a small pool. A SuperFrost Plus slide (VWR International, Radnor, Pennsylvania, USA) was gently manoeuvred to touch the pollen tubes floating on the pool surface and quickly removed, leaving the displaced pollen tubes adhering to the slide surface. The material was fixed in 3 : 1 ethanol : acetic acid for 10 min, transferred to 70% ethanol, and then rehydrated in distilled water for 15 min. After draining, the material was stained with aniline blue (Linskens & Esser, 1957) and mounted in DABCO (Sigma) containing DAPI (Sigma) (100 ng ml^{-1} DAPI in 2 mg ml^{-1} DABCO, from a DAPI stock solution dissolved in glycerol, containing 10% 1 M Tris buffered to pH 8). Pollen tubes were examined using epifluorescence and images captured using an Optronics (Goleta, California, USA) camera running Magnafire (Melville, NY, USA) software.

Results

Pollen germination, tube development and growth dynamics on a substrate promoting maximal rates of germination

Arabidopsis thaliana Col-0 pollen placed in contact with the cellulose membrane overlying an agarose-based medium (18% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% ferric ammonium citrate, 0.01% myo-inositol and 0.25 mM spermidine; see the Materials and Methods section and Fig. S1) rapidly hydrated and developed visible tubes within 30 min. This rapid hydration and germination mirrors that occurring on the stigma surface of *A. thaliana* (Chapman & Goring, 2010). During subsequent growth the pollen tubes were unbranched and relatively straight and possessed intact and generally unswollen tips. The presence of callose in the tubes was determined using aniline blue, which revealed the regular formation of complete callose plugs, indistinguishable from those seen *in vivo* (Fig. 1). DAPI staining was used to confirm the correct location of the two sperms and the vegetative nucleus close to the tip of the growing tubes (Fig. 1).

Analysis of pollen germination kinetics (Fig. 2a) showed germination to proceed rapidly, with >80% germination seen within 90 min of contact with the substrate. Maximum germination was achieved by 24 h, with approaching 100% of the grains germinated. These data indicate that the medium has the ability to induce germination of all, or almost all viable pollen grains, apparently exceeding the germination levels reported for other *A. thaliana* germination protocols.

Although pollen germination levels were exceptionally high, tube growth was not comparable to growth *in vivo*, or even to

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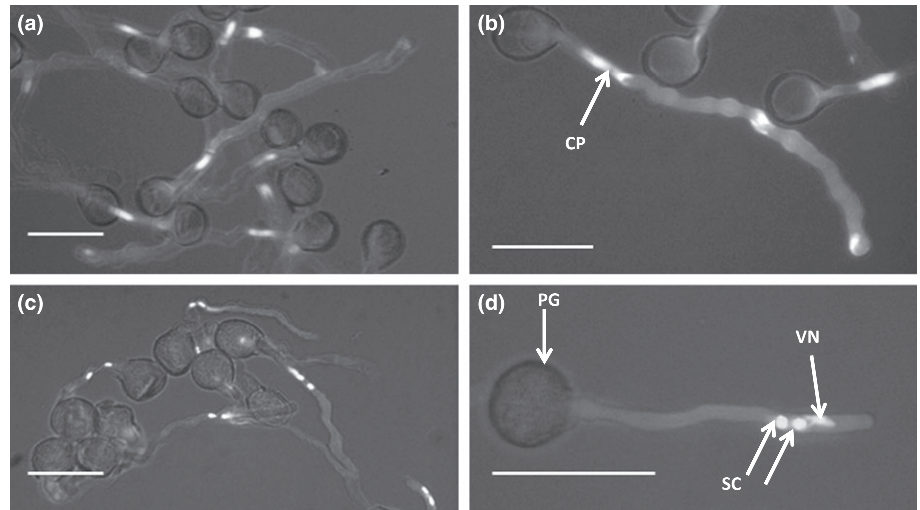

Fig. 1 Pollen tube growth of *Arabidopsis thaliana* Col-0 *in vitro*. Pollen tubes are shown following 5 h culture at 24°C on a cellulosic membrane overlying a 0.5% agarose medium with 18% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% myo-inositol, 0.01% ferric ammonium citrate and 0.25 mM spermidine, pH 8. (a, b) Tubes stained with aniline blue to show callose plugs. (c, d) Pollen tubes stained with DAPI revealing the location of sperm cells (generally spherical) and the vegetative nucleus (more diffuse and elongated). Arrows indicate examples of key features: PG, pollen grain; CP, callose plug; SC, sperm cell; VN, vegetative nucleus. Bars, 50 μm.

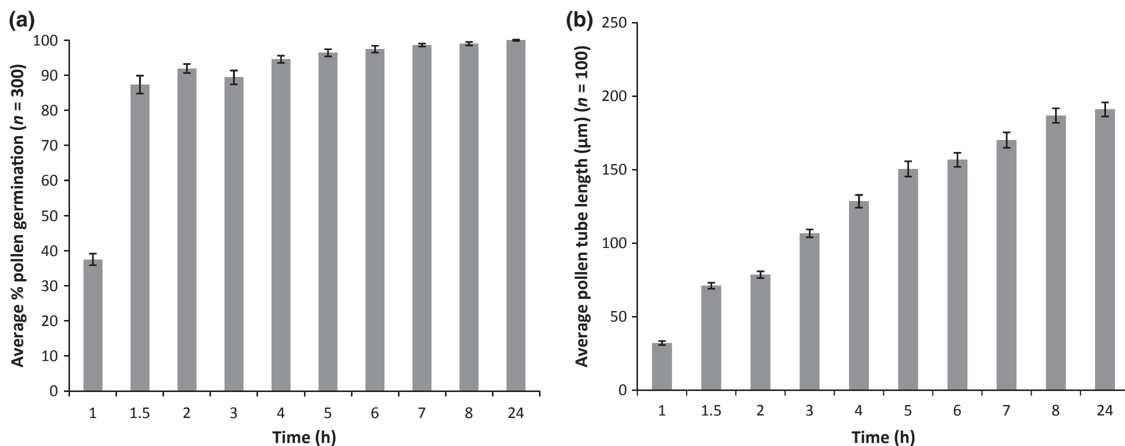

Fig. 2 Pollen tube growth of *Arabidopsis thaliana* Col-0 *in vitro* following culture at 24°C on a cellulosic membrane overlying a 0.5% agarose medium with 18% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% myo-inositol, 0.01% ferric ammonium citrate and 0.25 mM spermidine, pH 8.0. (a) Kinetics of pollen tube germination. (b) Kinetics of pollen tube growth. Error bars indicate standard error.

some other reported *in vitro* methods (Boavida & McCormick, 2007). Investigation of pollen tube growth kinetics showed that, while the tubes grew steadily during an initial 8-h period, their growth rate was slow – 22.5 μm h^{-1}, compared with 150–200 μm h^{-1} reported by Boavida & McCormick (2007) – and little further growth occurred after 8 h (see Fig. 2b). To maximize pollen tube growth, while still retaining high levels of pollen germination, we subsequently modified the composition of the substrate medium, focusing on the primary osmoticum (sucrose) and levels of the key promoter of germination – spermidine (see later).

The effect of temperature and pH on germination

In vitro germination of *A. thaliana* Col-0 pollen has been reported to be unusually sensitive to temperature (Boavida & McCormick, 2007), growth being optimal at 22°C with as little as 2°C divergence from this temperature impacting strikingly on the pollen germination percentage. Using our substrate-based method, we analysed pollen germination percentages at four temperatures (20, 24, 28 and 37°C) after 5 h (Fig. S2). While pollen germination was substantially reduced at 20°C, it remained high compared with the optimal levels seen in other studies, for example, Boavida & McCormick (2007) and Li *et al.* (1999). Likewise, at 28°C pollen germinated using our method only showed a slight fall in germination. Unexpectedly, even at 37°C, a small percentage of pollen was able to initiate germination.

pH has also been reported to have a significant effect on pollen germination (Boavida & McCormick, 2007). Using our standard cellulosic substrate and agarose medium we varied the pH from 6.5 to 8.5. Our data substantiate the reported effect of pH on pollen performance *in vitro*, with pollen germination levels being reduced at pH 6.5 to 7.0 (Fig. 3) after 5 h of culture. Grains on media with pH between 7.5 and 8.5 showed high levels of germination, peaking at pH 8.0.

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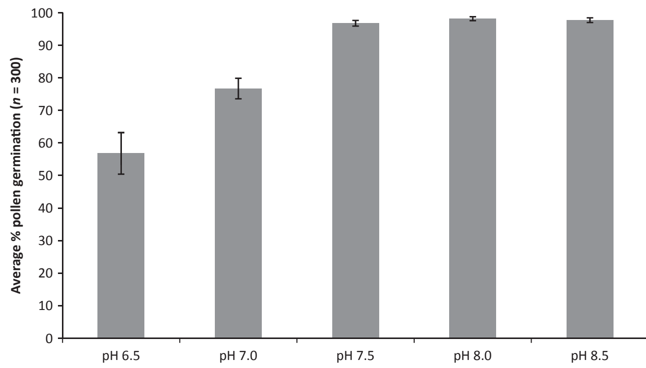

Fig. 3 Effect of variation of pH on pollen germination in *Arabidopsis thaliana* Col-0, following culture on a cellulosic membrane overlying a 0.5% agarose medium with 18% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% myo-inositol, 0.01% ferric ammonium citrate and 0.25 mM spermidine. Germination was measured after 5 h at 24°C. Error bars indicate standard error.

The effect of the cellulosic substrate and agarose medium on pollen germination and tube length

To determine the effect of the composition of the growth medium on *A. thaliana* Col-0 pollen germination and pollen tube growth, we devised a series of experiments eliminating elements of our standard medium including casein enzymatic hydrolysate, myo-inositol, ferric ammonium citrate and spermidine – but retaining the cellulosic membrane. In a parallel set of experiments we removed the membrane and placed the pollen directly on the agarose surface (Fig. 4a,b). Percentage pollen germination and pollen tube lengths were measured at 5 h after contact with the substrate/agarose. These experiments revealed the two most important components to be the cellulose membrane and spermidine. The membrane completely restored high germination rates and pollen tube lengths in the absence of ferric ammonium citrate, casein enzymatic hydrolysate and myo-inositol, but not spermidine (Fig. 4a,b). Spermidine at 0.25 mM has a stimulatory effect on pollen germination in the presence of the membrane (Fig. 4a).

Importantly, in the absence of spermidine and in the presence of the membrane, the lower numbers of pollen grains that did germinate had considerably longer tubes (Fig. 4b). This dramatic stimulatory effect on pollen tube length in the absence of spermidine was only seen in the presence of the cellulose membrane (Fig. 4b). The data thus point to a 'trade-off' between an early stimulatory effect of spermidine on overall germination and a later inhibitory effect on tube growth, and this itself is dependent on the presence of a cellulose-based substrate. This relationship was confirmed by varying spermidine concentration in the presence of the cellulose membrane (Fig. 5a,b). After 5 h the germination was highest with 0.5 mM spermidine, but concentrations at and above 0.25 mM spermidine showed a very significant decrease in pollen tube length after 5 h.

Improving pollen tube elongation by modulating levels of spermidine and sucrose *in vitro*

Our technical approach was focussed initially on generating reliably high rates of pollen germination *in vitro*. While this was achieved, the pollen tube lengths we observed were clearly shorter than those reported using other approaches (e.g. Palanivelu *et al.*, 2003; Boavida & McCormick, 2007; Qin *et al.*, 2011). We interpreted this as resulting from a combination of basing our medium on a published formulation that generated relatively short tubes (Li *et al.*, 1999) and the inhibitory effects of some of the additives we employed, particularly spermidine (Figs 4b, 5b).

Other methods have employed lower concentrations of sucrose and obtained considerably longer tubes *in vitro* (e.g. Boavida & McCormick, 2007). High sucrose concentrations may inhibit tube extension not only through osmotic and hydrodynamic

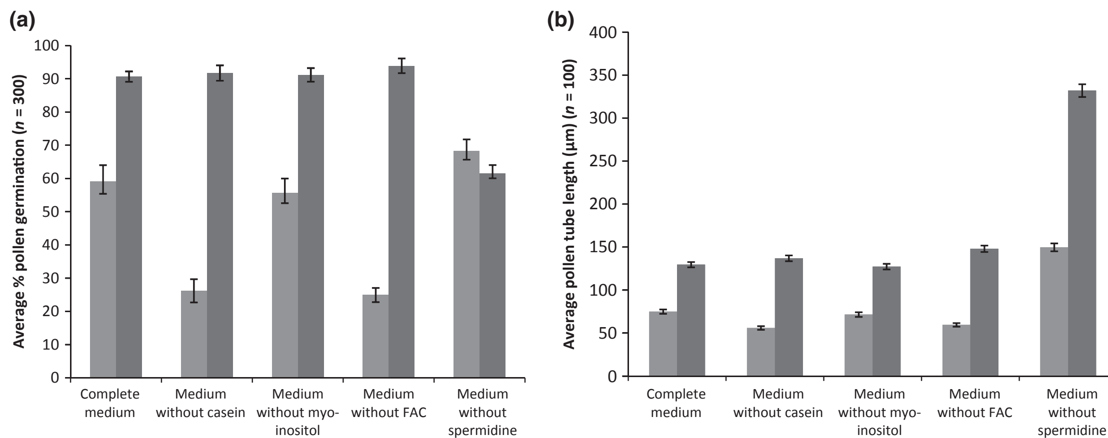

Fig. 4 Effect of agarose medium composition on *Arabidopsis thaliana* Col-0 pollen germination and pollen tube growth *in vitro*, with and without an overlying cellulosic membrane. Measurements were taken after 5 h of incubation at 24°C. Error bars indicate standard error. pH (8.0) and concentrations of sucrose (18%), boric acid (0.01%) and salts – 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$ and 1 mM KCl – were unaltered. (a) Effect of subtracting various agarose medium components, with and without an overlying cellulosic membrane, on pollen germination rates. (b) Effect of subtracting various agarose medium components, with and without an overlying cellulosic membrane, on pollen tube lengths. Light grey bars, without cellulosic substrate; dark grey bars, with cellulosic substrate.

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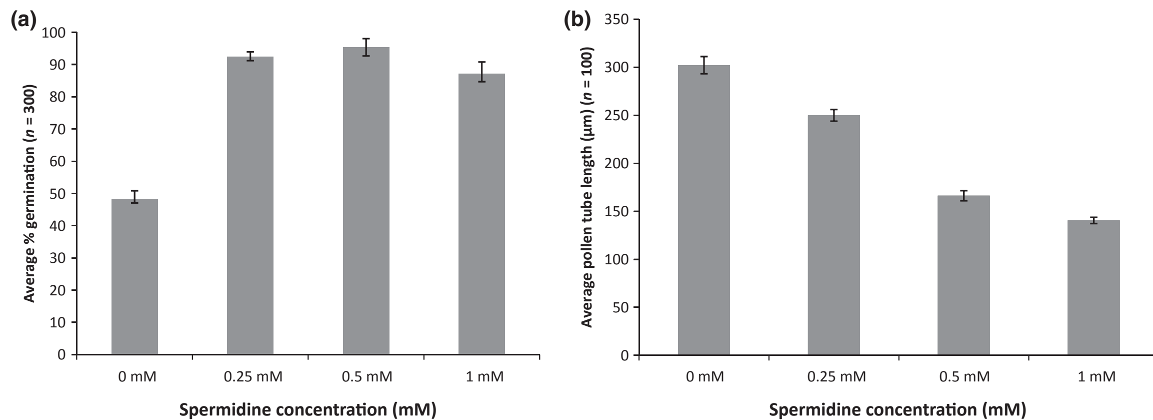

Fig. 5 Effect of spermidine concentration on *Arabidopsis thaliana* pollen germination and pollen tube growth *in vitro* following culture on a cellulosic membrane overlying a 0.5% agarose medium with 18% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% myo-inositol and 0.01% ferric ammonium citrate, pH 8. Measurements were taken after 5 h of incubation at 24°C. Error bars indicate standard error. (a) Effect of spermidine concentration on Col-0 pollen germination. (b) Effect of spermidine concentration on Col-0 pollen tube growth.

effects, but also through inducing physiological and metabolic effects in pollen tubes such as increasing permeability of the pollen tube and inducing ethanolic fermentation (reviewed in Taylor & Hepler, 1997). We thus initiated a series of preliminary experiments using medium containing 0.5 mM spermidine – a concentration chosen because of its more inhibitory effect on pollen tube extension – together with a range of lower sucrose concentrations, to determine whether sucrose concentration alone could overcome this inhibition of pollen tube extension. As anticipated, use of lower sucrose concentrations dramatically increased pollen tube length. Critically, low levels of sucrose compromised germination and induced irregular tube growth and bursting of the tip. In agreement with Boavida & McCormick (2007) these preliminary experiments indicated that a sucrose concentration of 10% represented an acceptable compromise between pollen germination and tube growth.

To investigate the inhibitory effect of spermidine further we repeated assays using lower spermidine concentrations (0–0.1 mM) than employed previously. The data (Fig. 6a–d) showed pollen germination to decrease at lower concentrations but pollen tube length to increase substantially, with the best compromise between pollen germination and tube length being achieved at 0.1 mM spermidine. On the basis of the data from these experiments, we judged a combination of 0.1 mM spermidine and 10% sucrose as promoting maximal levels of pollen germination and tube growth.

GABA enhances pollen tube length

Gamma amino butyric acid (GABA) is produced from maternal tissues in the pistil and acts as a key signalling molecule promoting pollen tube development *in planta* (Palanivelu *et al.*, 2003). Addition of physiological concentrations of GABA to *A. thaliana* pollen in liquid culture has been shown to increase pollen tube growth (Palanivelu *et al.*, 2003). We explored the effect of GABA on pollen germination and tube growth in our system, using a medium containing 0.1 mM spermidine and 10% sucrose. GABA concentrations within the physiological range (1.25, 2.5, 5 and 10 mM) were found to affect pollen germination marginally and pollen tube growth significantly (Fig. 7a,b). Ten millimolar GABA showed maximal promotion of pollen tube growth. Analysis of pollen germination and pollen tube growth kinetics of this medium demonstrated a combination of high levels of germination along with significant tube growth (Fig. 7c,d), attaining over 800 µm after 24 h. The significant improvement in pollen tube growth is also illustrated in Fig. 7e.

Germination of pollen from *A. thaliana* Ler requires different conditions

To determine how effective our system would be for ecotypes other than Col-0, replicate experiments were performed with Landsberg *erecta* (Ler), the other ecotype of *A. thaliana* most commonly used in research. We first used a substrate containing 0.25 mM spermidine and 18% sucrose at pH 8.0 and, although this did induce pollen germination in Ler, rates of germination after 5 h were 30% below those found for Col-0 (Fig. S3).

With such a strong genotype-dependent effect, we co-varied the two properties of the medium shown to most affect pollen germination in the Col-0 ecotype – spermidine concentration and pH. We chose two different pH levels (pH 7.0 and 8.0) and four spermidine concentrations (0, 0.25, 0.5 and 1 mM). Co-varying these properties dramatically improved pollen germination at 5 h (Fig. 8a), revealing an interaction between spermidine and pH. Strikingly, a combination of 0.25 mM spermidine and pH 7.0 gave germination rates of *c.* 90%. Measurement of pollen tube length at 5 h confirmed that this combination of spermidine and pH resulted in the growth of the longest tubes, but, as with Col-0 pollen, tube length in these early experiments was shorter than reported by Boavida & McCormick (Fig. 8b), as a result principally of our use of higher sucrose concentrations (see 'Pollen germination, tube development and growth dynamics on a substrate promoting maximal rates of germination').

These experiments were then repeated incorporating the innovations of lower spermidine concentration, reduced sucrose

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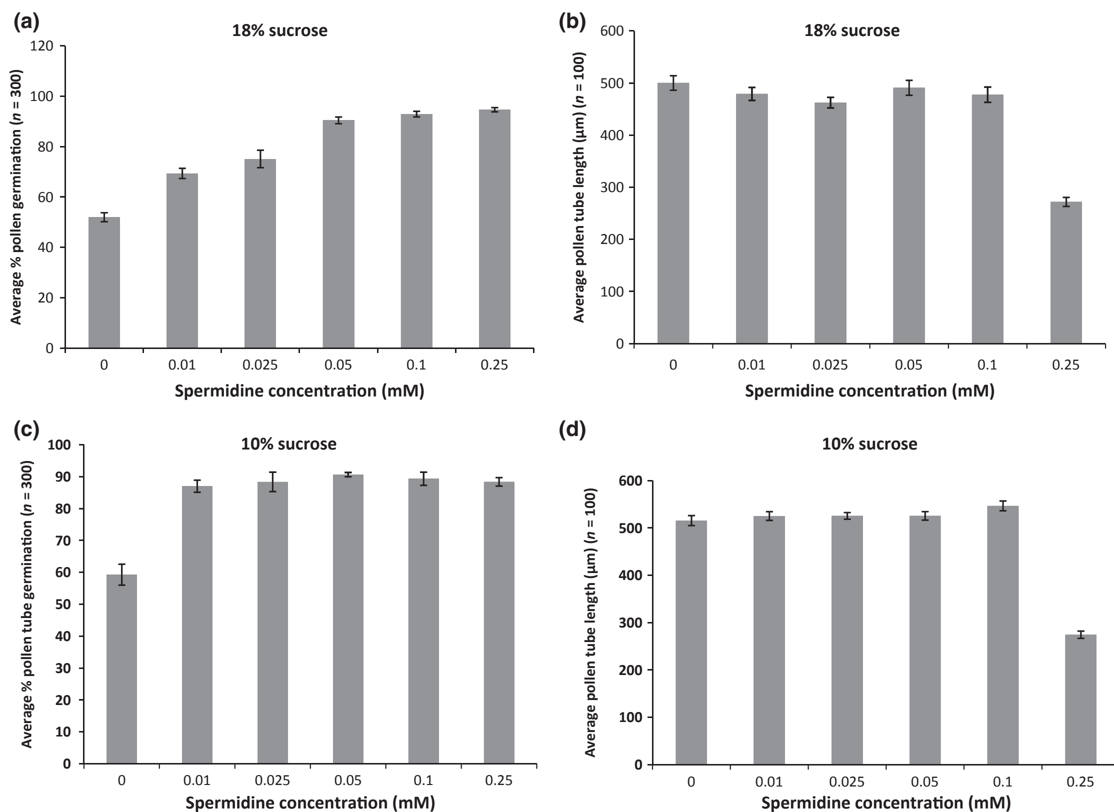

Fig. 6 Effect of co-varying spermidine and sucrose concentration on *Arabidopsis thaliana* pollen germination and pollen tube growth *in vitro* following culture on a cellulosic membrane overlying a 0.5% agarose medium with 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% myo-inositol and 0.01% ferric ammonium citrate, pH 8. Measurements were taken after 5 h of incubation at 24°C. Error bars indicate standard error. (a) Effect of varying spermidine concentration in medium with 18% sucrose on pollen tube germination. (b) Effect of varying spermidine concentration in medium with 18% sucrose on pollen tube length. (c) Effect of spermidine concentration in medium with 10% sucrose on pollen tube germination. (d) Effect of spermidine concentration in medium with 10% sucrose on pollen tube length.

concentration and addition of GABA from work on Col-0 ecotype pollen (see 'GABA enhances pollen tube length'). Using a substrate containing 0.1 mM spermidine, 10% sucrose, 10 mM GABA and pH reduced to 7.0, the germination rates and tube lengths of Ler pollen after 5 h were comparable to those of Col-0 pollen grown over the same period at pH 8 (Fig. 9a,b).

Discussion

We have developed a new protocol for germinating pollen from *A. thaliana* in a synthetic environment independent of female reproductive tissues. The development of this protocol became a necessity as we and others have failed consistently to achieve a useful and dependable level of germination despite exhaustive trials using a range of published methods. Our protocol consistently gives germination levels > 90% with minimal variation when using higher concentrations of spermidine (0.25–0.5 mM). Further, pollen tube growth appears normal with relatively straight tubes containing callose plugs and the correct localization of the male germ unit (sperm cells) within the pollen tubes. Subsequent reduction of spermidine in the medium (to 0.1 mM) permitted the rapid formation of long tubes of normal morphology, while maintaining exceptional germination rates *in vitro*. Spermidine appears to promote initial germination but has an inhibitory effect on subsequent post-germination growth. Importantly, the data we present permit our protocols to be modified for use in experiments where maximal pollen germination is required, or extensive tube growth, or a compromise between the two. Although novel compounds – sulfinylated azadecalins – have been implicated as important stimulants in the germination of *A. thaliana* pollen (Qin *et al.*, 2011), we have purposely restricted the composition of our germination medium to reagents readily obtained.

Key elements of the new system

The use of a cellulosic matrix Our experiments where we have changed the components of the germination substrate components have proved informative. An integral element contributing to the performance of our system is the use of a cellulose-rich membrane layered upon the medium partially solidified by agarose. This probably results from its hydrodynamic impact, with the sheet of membrane preventing pollen grains sinking into the medium and becoming immersed in an aqueous, partially anoxic environment. While the membrane must create a 'semi-dry' environment better suited to development of Brassicaceae pollen which is adapted to a dry stigma, it is probable that other factors

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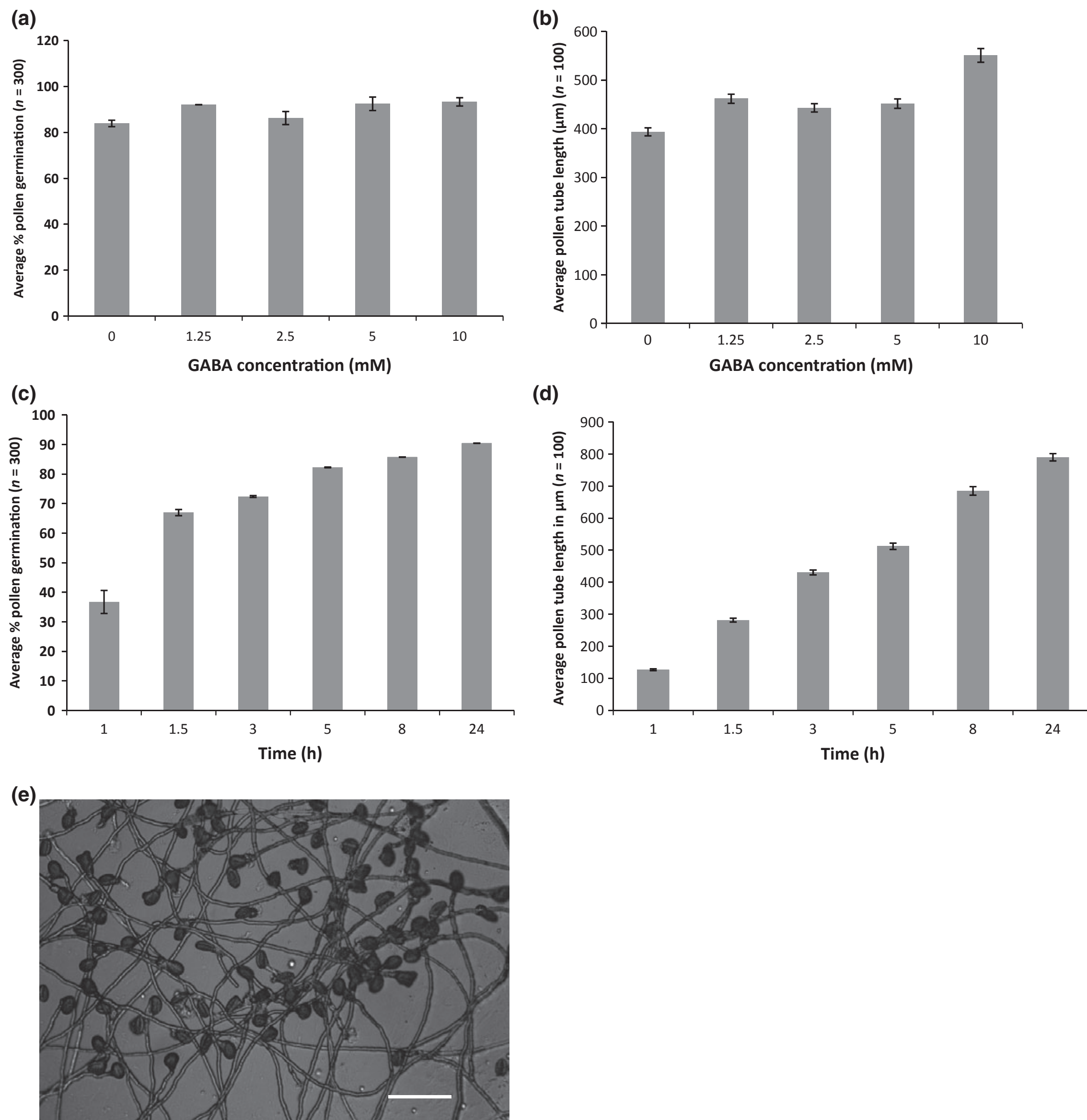

Fig. 7 Effect of gamma amino butyric acid (GABA) on *Arabidopsis thaliana* pollen germination and pollen tube growth *in vitro* following culture on a cellulosic membrane overlying a 0.5% agarose medium with 10% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% myo-inositol, 0.01% ferric ammonium citrate and 0.1 mM spermidine, pH 8. Measurements were taken after incubation at 24°C. Error bars indicate standard error. (a) Effect of varying GABA concentration on pollen germination. Measurements were taken after 5 h of incubation at 24°C. (b) Effect of varying GABA concentration on pollen tube length. Measurements were taken after 5 h of incubation at 24°C. (c) Kinetics of pollen germination grown on medium containing 10 mM GABA. (d) Kinetics of pollen tube length grown on medium containing 10 mM GABA. (e) Pollen tube growth on 10 mM GABA medium after 8 h. Bar, 100 µm. Note the smoother, narrower tubes and more pointed tips of the pollen tubes compared with Fig. 1.

may also be responsible for the high levels of germination involved. For example, pollen may sense the physiochemical properties of this cellulosic polymer-based environment, which may more closely mimic the stigmatic papillar surface than agarose. Thus, enzymes released from the pollen wall and apoplast on hydration may use the cellulosic matrix as a substrate to generate oligosaccharides and simple sugars that could 'feed back' a stimulatory effect on pollen germination. Equally, this heavily processed cellulose-rich membrane itself may already contain low levels of such stimulatory compounds. Our data show that, for promoting germination, the presence of this membrane could substitute for the absence of casein enzymatic hydrolysate, myo-inositol and ferric ammonium citrate from the medium, but not spermidine. The cellulose-rich membrane also promotes extended pollen tube growth. Xyloglucans (XyGs) are known to be enriched at the tip of *A. thaliana* pollen tubes and the acetyl groups of XyG may promote hydrophobic interactions with the female extracellular matrix *in planta* (Dardelle *et al.*, 2010). Similar interactions between extracellular matrix (ECM) components (including XyGs) and this artificial surface may stabilize the rapidly growing tip and hence promote more rapid, morphologically normal tube growth.

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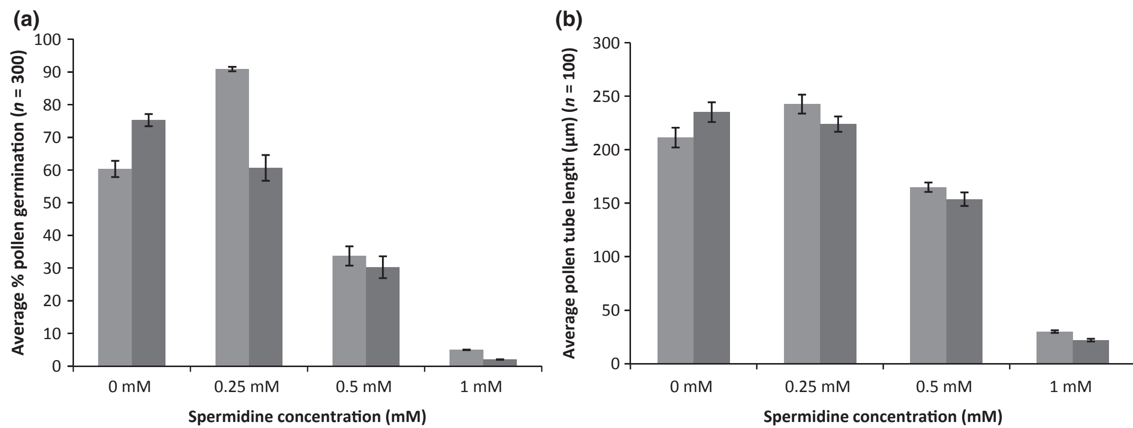

Fig. 8 Effect of co-varying pH and spermidine concentration on *Arabidopsis thaliana* Ler ecotype pollen germination and pollen tube growth *in vitro* following culture on a cellulosic membrane overlying a 0.5% agarose medium with 18% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% myo-inositol and 0.01% ferric ammonium citrate. Measurements were taken after 5 h of incubation at 24°C. (a) Effect of spermidine concentration and pH on Ler pollen germination. (b) Effect of spermidine concentration and pH on Ler pollen tube growth. Light grey bars, pH 7; dark grey bars, pH 8.

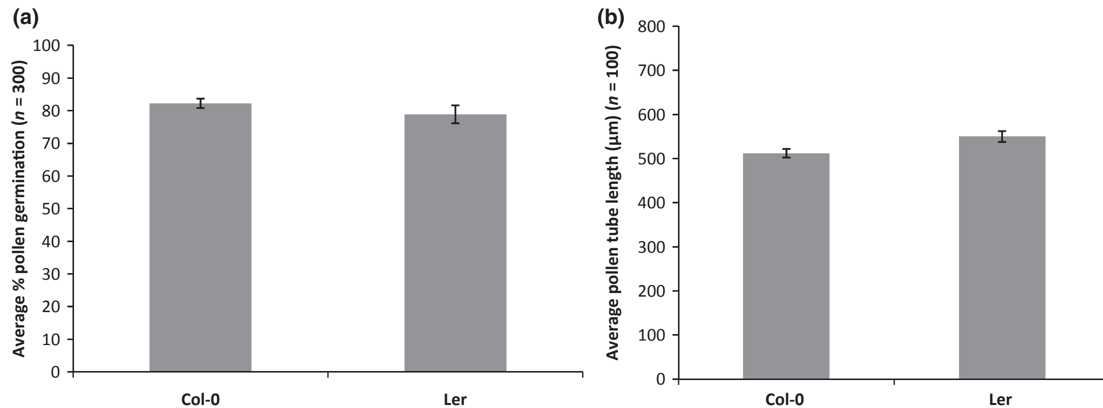

Fig. 9 *Arabidopsis thaliana* Col-0 and Ler ecotype pollen germination and pollen tube growth *in vitro* following culture on a cellulosic membrane overlying a 0.5% agarose medium with 10% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% myo-inositol, 0.01% ferric ammonium citrate and 0.1 mM spermidine. Col-0 pollen was grown at pH 8 and Ler pollen at pH 7. Measurements were taken after 5 h of incubation at 24°C. Error bars indicate standard error. (a) Germination percentage; (b) pollen tube growth.

The inclusion of myo-inositol in the substrate medium Myo-inositol has also been used in our growth medium, and its overlap with the cellulosic membrane in stimulating pollen growth may result from it being a precursor to UDP-glucuronic acid, itself the principal precursor to galacturonic acid, xylose, apiose and arabinose, all of which are required for cell wall biosynthesis (Kanter *et al.*, 2005). Myo-inositol oxygenases catalyse its conversion to UDP-glucuronic acid and are encoded by a small gene family (*MIOX*) in *A. thaliana,* two of which (*MIOX4* and *MIOX5*) are highly expressed in pollen (Kanter *et al.*, 2005). Interestingly, experimental work suggests that uptake of exogenous myo-inositol pools is physiologically important during pollen germination and tube growth *in planta* (Schneider *et al.*, 2006). *Arabidopsis thaliana* pollen highly expresses *INOSITOL TRANSPORTER4*, a plasma membrane-localized protein functioning as a high-affinity H^+ symport of myo-inositol across the membrane (Schneider *et al.*, 2006). *INOSITOL TRANSPORTER4* is expressed not only in developing pollen grains but also in germinating pollen tubes in the style.

The role of spermidine in promoting pollen germination *in vitro* The use of the polyamine spermidine is novel and proved very effective in stimulating pollen germination in our *in vitro* system. Indeed, higher concentrations ensured almost complete germination of pollen. Our observations during experiments varying spermidine concentrations suggest that part of this increase in germination may result from the ability of spermidine to eliminate local effects of pollen density, which have previously been shown to affect germination of pollen *in vitro* (Boavida & McCormick, 2007). In kiwifruit (*Actinidia deliciosa*), Falasca *et al.* (2010) have shown that polyamines, in particular spermidine, have a major role in normal pollen function and development, and that polyamine inhibitors *in planta* exerted a substantial negative effect on kiwifruit pollen germination. Loss of tomato (*Lycopersicon esculentum* Mill.) pollen viability in storage is correlated with degradation of transcripts involved in polyamine biosynthesis, with exogenous applications of polyamines having a restorative effect on pollen germination (Song & Tachibana, 2007).

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The pivotal role of spermidine in pollen germination is more easily interpreted in the light of recent data from Wu *et al.* (2010), who report a role for spermidine in *A. thaliana* pollen tube growth, with exogenous spermidine acting to increase the cytosolic free calcium concentration in pollen tubes. Influx of calcium from the external environment is critical for pollen tube growth and a tip-localized cytosolic free calcium gradient is required for normal pollen tube elongation. Spermidine does not directly induce hyperpolarization of plasma membrane Ca^{2+}-permeable channels, but rather spermidine oxidation by peroxisomal polyamine oxidase (PAO) generates hydrogen peroxide which acts as a second messenger to activate these channels. Interestingly, excessive extracellular spermidine concentrations activate these channels, and concomitant calcium ion influx beyond optimal levels, resulting in the inhibition of pollen tube growth. It is thus perhaps significant that our data also show that the higher concentrations of spermidine are inhibitory and there is a trade-off between stimulation of pollen germination and reduction in pollen tube growth.

The mode of action of spermidine suggests that other interacting factors are important in regulating germination and tube growth. Recently, the antioxidant glutathione has been shown to play an essential role in *A. thaliana* pollen germination (Zechmann *et al.*, 2011). These findings suggest that detoxification of reactive oxygen species (such as H_2O_2 generated by spermidine oxidation) and redox signalling by endogenously generated glutathione may be in delicate balance, as an artificial application of high external concentrations of glutathione can inhibit pollen germination (Zechmann *et al.*, 2011), while high external concentrations of spermidine repress pollen tube extension.

Polyamines are multifunctional molecules and spermidine may have other roles in the gametophyte. For example, work on development of the male gametophyte of *Marsilea vestita,* a heterosporous fern, has shown that, as in flowering plant pollen, numerous transcripts become stored and translationally inhibited during gametophyte dehydration (Boothby & Wolniak, 2011), only to be reactivated upon hydration. Importantly, spermidine plays a part in unmasking these translationally inhibited stored mRNAs (Deeb *et al.*, 2010). It seems reasonable to speculate that spermidine plays a similar role in pollen of flowering plants in activating translation of stored transcripts when appropriate conditions for germination are encountered. Significantly, the inhibition of pollen germination in tomato by the protein synthesis inhibitor cycloheximide can be overcome by treatment with exogenous spermidine and spermine (Song *et al.*, 2002).

Spermidine may also serve to 'buffer' the pollen germination *in vitro* against temperature variations. Boavida & McCormick (2007) report pollen germination *in vitro* to have a remarkably narrow optimum temperature of 22°C, a situation clearly not replicating the reproductive biology of *A. thaliana in vivo*. We failed to find this sensitivity to temperature using our technical approach, with low levels of pollen germination even being initiated at 37°C. The sensitivity of tomato pollen germination to high temperatures is held to result from the inhibition of S-adenosylmethionine decarboxylase, a key enzyme in spermidine synthesis (Song *et al.*, 1999, 2002); importantly, this sensitivity could be overcome by exogenous addition of spermidine (Song *et al.*, 1999, 2002).

The omission of spermidine from other growth media shows that germination of *A. thaliana* pollen, albeit at a lower level, can occur in its absence. This may be because pollen contains endogenous spermidine and related polyamines, for Handrick *et al.* (2010) have shown that conjugated spermidines are particularly diverse in *A. thaliana* pollen as a result of a complex enzymatic system. Of course, spermidine is unlikely to be the only regulator of calcium influx into the pollen cytoplasm. For example, Michard *et al.* (2011) have identified glutamate receptor-like proteins in *A. thaliana* pollen that form calcium channels regulated by exogenous D-serine supplied by the pistil. Further, in addition to providing amino acids for protein synthesis, the casein enzymatic hydrolysate may supply amino acids or small peptides which act as substitute agonists to these channels in this medium.

The role of GABA in promoting pollen tube growth We have explored the use of GABA to increase the length of pollen tubes. GABA is a signalling molecule produced by maternal tissues to attract and guide pollen tubes to the female gametophyte (Palanivelu *et al.*, 2003). Previous studies showed that the addition of physiological concentrations of GABA to liquid media dramatically increased pollen tube length (Palanivelu *et al.*, 2003) and we found a similar effect when incorporated in our system. Although the molecular mechanism of the effect of GABA on pollen tubes remains to be fully elucidated, GABA appears to bind to a receptor in the cell membrane and regulates downstream Ca^{2+} oscillation in the cells, ultimately affecting actin organization and vesicle trafficking (Yu & Sun, 2007).

Ecotypes have different requirements for *in vitro* pollen germination and tube growth

Intraspecific differences exist between ecotypes of *A. thaliana* in their requirement for synthetic pollen germination media. Our data reveal response to pH to be an important factor distinguishing pollen from the Col-0 and Ler ecotypes in our *in vitro* system. The molecular basis of this pH sensitivity may result from differences in sucrose-H^+ symporters between ecotypes (Sauer *et al.*, 2004), a view supported by more recent data from Feuerstein *et al.* (2010), where the AtSUC1 (ARABIDOPSIS THALIANA SUCROSE-PROTON SYMPORTER 1) symporter, highly expressed in the male gametophyte and important for pollen germination and tube growth (Stadler *et al.*, 1999; Sivitz *et al.*, 2008), was shown to be reduced in expression in Ler compared with Col-0.

Users of our protocol wishing to apply it to other ecotypes are therefore advised to perform exploratory work to optimize media for their particular study. Our work has also underscored the importance of performing controls in parallel with test material. We found this essential for studies involving measuring pollen tube length, as, although variance within experiments is low, experiment-to-experiment differences can be considerable, even under seemingly identical experimental conditions. A major contributing factor is almost certainly batch variation in plants; for

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use in pollen germination studies, *A. thaliana* plants remain in peak condition for only a few days and differences may exist between ecotypes. For example, pollen viability of Ler has been reported to decline more rapidly than that of Col-0 (Boavida & McCormick, 2007).

Summary

We have developed an easy yet robust technology for germinating pollen from *A. thaliana in vitro* that employs an artificial cellulose membrane overlying an agarose layer. This system is well adapted for microscopic studies, and can also be used for rapid bioassays (for example, by incorporating toxins or signalling molecules in the medium). Many of the most interesting questions in plant reproduction and pollen tube biology – such as those involving control of pollen tube architecture, metabolic and osmotic regulation and the response to external signals – can now be explored with greater facility in this model species. The great benefits of *A. thaliana* as a model organism, such as the collections of mutants and its ease of transformation (for instance with constructs encoding fluorescently tagged proteins), can now be exploited fully at this unique developmental stage. The facility with which the pollen tubes may be removed from the cellulose membrane suggests that the technique will prove highly effective for rapid, clean extractions of DNA, RNA and protein from growing pollen tubes.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Diagrams to show the set-up of the apparatus for pollen germination.

Fig. S2 Effect of incubation temperature on pollen germination of Col-0.

Fig. S3 Comparison of germination of Ler and Col-0 pollen on the same medium (optimized for Col-0).

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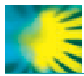

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