TECHNICAL ADVANCE

# Temperature as a determinant factor for increased and reproducible *in vitro* pollen germination in *Arabidopsis thaliana*

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**Summary**

**Despite much effort, a robust protocol for *in vitro* germination of *Arabidopsis thaliana* pollen has been elusive. Here we show that controlled temperatures, a largely disregarded factor in previous studies, and a simple optimized medium, solidified or liquid, yielded pollen germination rates above 80% and pollen tube lengths of hundreds of microns, with both Columbia and Landsberg *erecta* (L*er*) ecotypes. We found that pollen germination and tube growth were dependent on pollen density in both liquid and solid medium. Pollen germination rates were not substantially affected by flower or plant age. The *quartet1* mutation negatively affected pollen germination, especially in the L*er* ecotype. This protocol will facilitate functional analyses of insertional mutants affecting male gametophyte function, and should allow detailed gene expression analyses during pollen tube growth. *Arabidopsis thaliana* can now be included on the list of plant species that are suitable models for physiological studies of pollen tube elongation and tip growth.**

**Keywords: pollen tube growth, pollen grain, tip growth, gametophyte function, Columbia ecotype, Landsberg *erecta* ecotype.**

## Introduction

The male gametophyte, the pollen grain, is composed of three cells: two sperm cells within a larger vegetative cell. Pollen grains of most species are regarded as anhydrobiotic, as they are extremely desiccated at anther dehiscence (Crowe *et al.*, 1992). After landing on the receptive stigmatic surface of the pistil, pollen grains undergo rehydration, a tightly controlled process: the integrity of the plasma membrane is reorganized, metabolism is activated and a pollen tube forms (Taylor and Hepler, 1997). The pollen tube grows through the transmitting tract of the pistil, transporting the two sperm cells to the embryo sac, where they are released and double fertilization occurs. Molecular, biochemical and physiological analyses of male gametophyte development, pollen grain rehydration, germination and pollen tube growth are therefore important for fundamental studies in plant reproduction.

*In vitro* pollen germination rates are considered the best indicator of pollen viability (Shivanna *et al.*, 1991) – the ability of the pollen grain to perform its function of delivering the sperm cells to the embryo sac following compatible pollination. *In vitro* pollen germination can be useful to detect alterations in germination or tube growth performance (Cole *et al.*, 2005; Hashida *et al.*, 2007; Procissi *et al.*, 2003; Steinebrunner *et al.*, 2003), as assays for defects in these parameters are difficult to perform *in vivo*.

Pollen tubes are considered one of the best model systems to study cellular processes involved in polarity and tip growth. Cytoskeletal reorganization, endocytosis and exocytosis, maintenance of polarized ion gradients and fluxes, and periodic reorientation occur during pollen tube growth (reviewed by Cheung and Wu, 2007; Feijo *et al.*, 1995, 2001; Hepler *et al.*, 2001; Mascarenhas, 1993). Most

studies of these processes have been performed in species with bicellular pollen, such as lily and tobacco. In these species, pollen can easily germinate and grow long tubes *in vitro* when placed in a simple medium containing boric acid, sucrose, calcium and some salts (Brewbaker, 1967). *Arabidopsis thaliana* is an excellent model plant (The Arabidopsis Genome Initiative, 2000), but it clearly lags behind in analyses of pollen germination and pollen tube growth. Several attempts have been made to develop a reliable pollen germination protocol for *A. thaliana* (e.g. Johnson-Brousseau and McCormick, 2004), but in general *in vitro* pollen germination is extremely problematic, as is true for other species with tricellular pollen (Preuss *et al.*, 1993; Taylor and Hepler, 1997). Many different recipes for *A. thaliana* pollen germination have been described, from complex (Azarov *et al.*, 1990) to more simple compositions (Fan *et al.*, 2001; Li *et al.*, 1990; Mouline *et al.*, 2002; Thorsness *et al.*, 1993). Most of these references reported 60–80% germination rates when different ecotypes were tested. Although these values are acceptable, the results are often poorly reproducible in other laboratories (Johnson-Brousseau and McCormick, 2004; Scholz-Starke *et al.*, 2003). Moreover, if the utmost objective is to mimic *in vivo* pollen tube growth, pollen tube lengths *in vitro* are far from optimal. The problem of poor pollen germination can be partially overcome by analyzing a mutant phenotype in a *quartet* background (Copenhaver *et al.*, 2000; Johnson-Brousseau and McCormick, 2004). In a *quartet* background the number of affected pollen grains within a tetrad can be determined, but the high variability in the length of pollen tubes may introduce further problems if the objective is to perform a time-course analysis of pollen tube growth or to detect differences between wild-type and mutant pollen tube growth rates. The need for a robust method for *in vitro* pollen germination and tube growth analysis is therefore of extreme importance for *A. thaliana*.

In an attempt to optimize growth conditions for an *in vitro* pollen-germination medium, we were also faced with the problem of reproducibility. If the results of pollen germination assays could be so variable with the same germination medium from day to day, we hypothesized that external factors, to our knowledge not considered in previous studies, might influence the reproducibility of *in vitro* pollen germination assays. To evaluate the effects of external factors on the kinetics of pollen germination and pollen tube growth, we used an optimized artificial medium under strictly controlled incubation conditions. We demonstrate that pollen germination and tube growth show a temperature-dependent performance, and that under optimal temperatures *in vitro* pollen germination rates for both the Columbia and Landsberg *erecta* (L*er*) ecotypes of *A. thaliana* are high, and highly reproducible. Pollen collected from various ages of plants and flowers germinates well, although L*er* is more sensitive to these

parameters. The *quartet1* mutation has a negative effect on pollen germination rate that is more pronounced in L*er*. We also present additional conditions or factors that can be taken into consideration to obtain maximum pollen germination and pollen tube growth rates, or to help synchronize pollen germination for time-course analyses.

## Results and discussion

### Optimization of an A. thaliana *pollen germination medium*

For optimization of the germination medium, all experiments were performed using pollen from flowers that had opened that day (day 0). Pollen germinations were performed on agarose medium on microscope slides that were incubated in moisture chambers constructed from empty pipette tip boxes (Johnson-Brousseau and McCormick, 2004). Pollen from single flowers was spread on the surface of an agarose pad by inverting the flower with the help of a tweezers and gently bringing it to the surface of the agarose (Figure 1). The whole flower can be used as a 'brush' to spread the pollen uniformly on the surface of the agarose. We tested different *A. thaliana* pollen germination media (Fan *et al.*, 2001; Li *et al.*, 1999; Mouline *et al.*, 2002), which reportedly yielded germination rates of 60–80%. However, the results we obtained with these recipes were unpredictable, and clearly suboptimal under our conditions. Some days germination rates were 50–70%, but often they were around 5–20%. Johnson-Brousseau and McCormick (2004), while testing different germination conditions and media, pointed out that success with these assays requires practice, and they recommended the agar pad method and moisture incubation chambers, to maintain humidity, in order to achieve germination rates above 70%. But even with these suggestions, reproducibility was
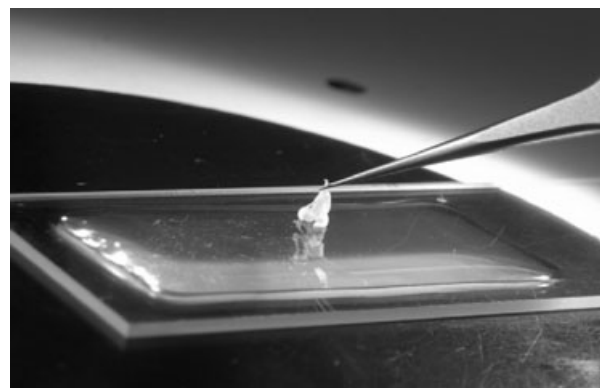


**Figure 1.** Transfer of pollen to solid medium.
*Arabidopsis thaliana* pollen is transferred to an agarose pad by holding an inverted flower with tweezers and gently brushing the flower across the surface.

not acceptable. We assumed that the variability might be due to the physiological conditions of the growing plants, so we decided to optimize a germination medium that would give consistent results with plants grown in our glasshouses.

A modified medium based on that of Li *et al.* (1999), and containing the basic components required for pollen germination (0.01% $H_3BO_3$, 1 mm $CaCl_2$, 1 mm KCl, 5% sucrose pH 7.5, 1% low-melting agarose) was used as starting point to optimize an *in vitro* medium. The optimization approach consisted of varying the concentration of the chemical components, one at a time, followed by fine readjustments of all other components regarding the factor primarily tested. Additional components were considered based on other recipes, and their ability to increase pollen germination rates or pollen tube growth was tested.

Boric acid is known to be crucial for pollen germination and tube growth and is required at concentrations of 0.01% for most species (Brewbaker and Majumder, 1961), therefore no variations of it were considered *a priori*. In preliminary experiments, we found that significant differences in pollen germination were associated with sucrose, calcium concentrations and pH (data not shown). We tested a range of sucrose concentrations (0–20%) and found that pollen germination was maximal at 10% sucrose, whereas below 5% or above 15%, germination did not occur or was reduced. Calcium concentration was important for pollen germination *per se*, but more critical for normal pollen tube growth. Calcium concentrations above 10 mm or below 2 mm gave reduced germination and abnormal tubes, with thick walls and stunted growth. Pollen germination was highest at pH 7.5–8; below these values pollen grains appeared incompletely hydrated and germination rates were reduced. Fan *et al.* (2001) tested several components and reported pH as an important factor contributing to pollen germination success, and that [$K^+$] was important for tube growth. Indeed, we found that pollen germination and tube growth improved drastically when the potassium concentration was increased to 5 mm when in combination with an equivalent calcium concentration. Our final optimized medium (0.01% boric acid, 5 mm $CaCl_2$, 5 mm KCl, 1 mm $MgSO_4$, 10% sucrose pH 7.5, 1.5% low-melting agarose) yielded germination rates of 80% and pollen tubes longer than 1 mm after overnight incubation. We found that using 1.5% agarose, instead of 1%, made it less likely to damage the agarose pad, which could interfere with subsequent imaging, and did not change the germination rate. However, reproducibility was still a problem, even with the optimized medium. Some days germination rates attained the expected 80%, while on other days germination rates were poor (5–10%) or none – pollen grains would burst during hydration. We therefore tested external factors.

## A narrow temperature window is optimal for pollen germination and pollen tube growth and increases reproducibility

Reproduction is one of the most temperature-vulnerable stages of plant development, and negative effects in seed yield (Clarke and Siddique, 2004; Herrero and Johnson, 1980; Morrison, 1993; Prasad *et al.*, 2006; Sato *et al.*, 2006), gametophyte viability, pollen germination and tube growth (Cerovic *et al.*, 2000; Jakobsen and Martens, 1994; Kakani *et al.*, 2005; Kozai *et al.*, 2004; Kuo *et al.*, 1981; Lardon and Triboiblondel, 1994) have been reported for several species. However, the effect of temperature on pollen germination and pollen viability, to the best of our knowledge, was never systematically evaluated in *A. thaliana*. It seemed plausible that temperature variations could contribute to the variability of *in vitro* pollen germinations performed at 'room temperature', a fairly relative measure.

To test the effect of temperature, pollen from both Columbia and L*er* ecotypes was collected from day 0 flowers. Pollen viability, as estimated by a positive fluorochromatic reaction (Heslop-Harrison *et al.*, 1984), was around 95% (data not shown). Pollen was incubated on solidified optimized medium under controlled temperatures ranging from 18–32°C, at 2°C intervals (Figures 2a–f and 3a). We found that Columbia and L*er* ecotypes had defined temperature optima at 22°C, where pollen germination rates were 80%. Above and below 22°C, pollen germinations dropped to <50% (Figure 3a).

The effect of temperature was also evident when the length of pollen tubes was evaluated (Figure 3b). Under optimal conditions, pollen tubes reached lengths of more than 1 mm after 16 h incubation (Figures 2g,h and 3b), lengths that are markedly longer than those in other reported studies. For example, the Fan *et al.* (2001) medium has been used widely to characterize mutants affecting pollen germination and tube growth, but it is worth noting that, with this medium, the mean tube length reported after 16 h germination was 200–400 μm. Similar results were reported by other studies (Derksen *et al.*, 2002; Hashida *et al.*, 2007; Singh *et al.*, 2002; Taylor *et al.*, 1998; Wang *et al.*, 2004), and in some studies (Fan *et al.*, 2001; Thorsness *et al.*, 1993) the pollen tube morphologies appear abnormal, with thicker walls or stunted growth.

At the optimal temperature, pollen tubes remained stable without rupturing for more than 16 h germination, showing regular growth and periodic callose plug deposition. Temperatures >24°C or <20°C had adverse effects on pollen tube growth as well as on pollen germination. At 24°C and higher, although pollen tubes seemed to grow more quickly, pollen germination was reduced and approximately 50% of the pollen tubes showed abnormal morphologies or polarity defects (Figure 2e,f). At temperatures below 20°C, germination was strongly inhibited and many pollen grains burst
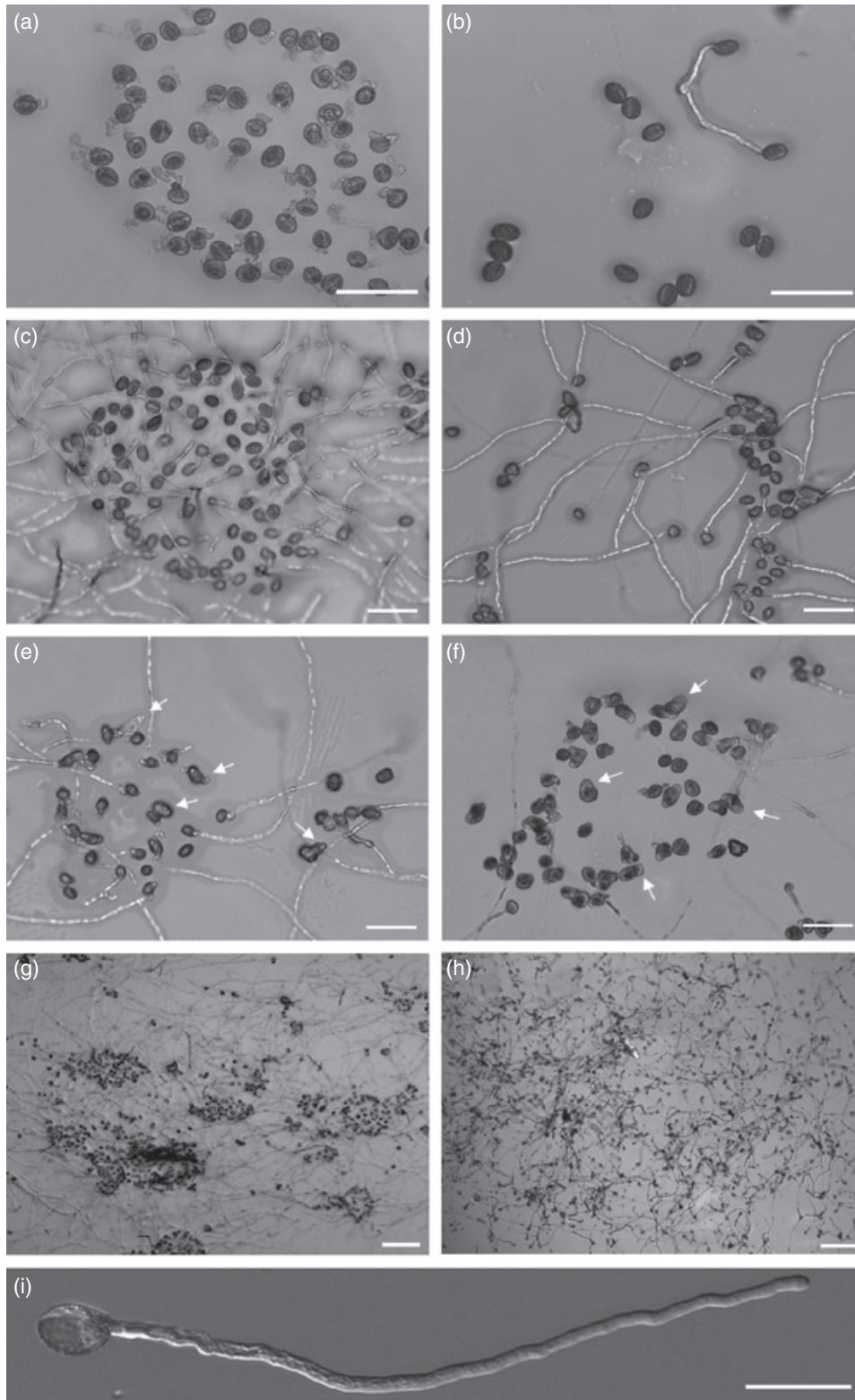
**Figure 2.** Effect of temperature on *Arabidopsis thaliana* pollen germination.
(a) 18°C, pollen grains burst during hydration; (b) 20°C; (c) 22°C; (d) 24°C; (e) 28°C, a high proportion of pollen tubes showed disturbed polarity and growth; (f) 32°C, a high proportion of pollen tubes inflated and blew up, and pollen protoplasts were seen frequently; (g, h) lower-magnification views of pollen germination at 22°C after 16 h incubation on solidified medium (g) and in liquid medium (h); (i) a pollen grain after 2 h germination in liquid medium. White arrows, abnormal tubes. Scale bars: (a–f) 100 μm; (g, h) 500 μm; (i) 50 μm.
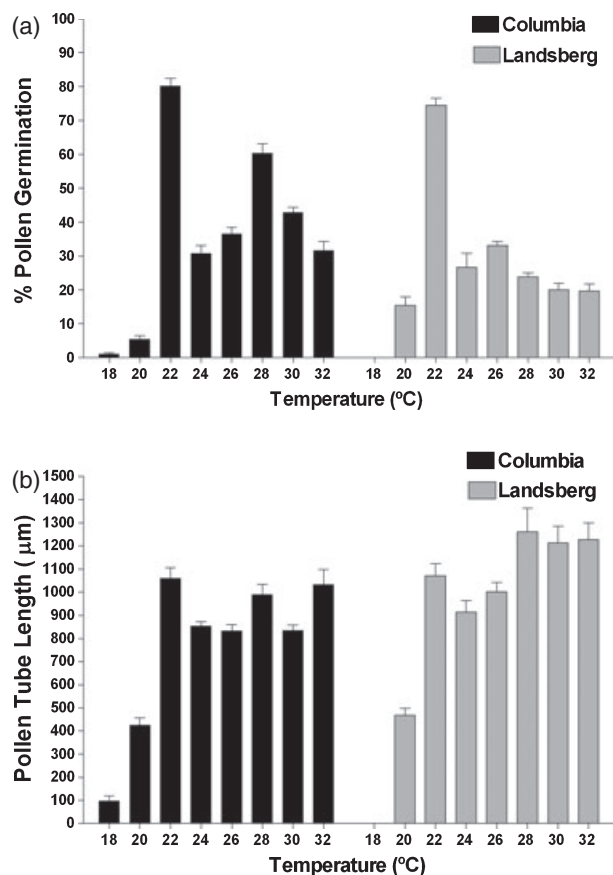
(a)



(b)



**Figure 3.** Effect of temperature on pollen germination rate and pollen tube length.
(a) Pollen germination rate; (b) pollen tube length. Pollen from both Columbia and Landsberg *erecta* ecotypes was germinated on solidified pollen-germination medium. Bars represent means ± SE, n = 2.

(Figure 2a). It is important to maintain the temperature throughout the course of the experiment, as temperature variations at any time led to pollen tube growth arrest or abnormal morphologies (data not shown).

Columbia and L*er* ecotypes showed different behaviors above and below the optimal temperatures, suggesting that these two ecotypes have slightly different temperature tolerances (Figure 3a). The Columbia ecotype showed higher germination rates above the optimal temperature, with a slight increase in germination rate at 28°C, but pollen tube length was reduced, while at higher temperatures the L*er* ecotype showed germination rates lower than Columbia but had longer pollen tubes. It is interesting that the optimal temperature for pollen germination falls in the range of temperatures suggested for *A. thaliana* growth conditions in glasshouses. Temperatures above 24°C or below 18°C have negative effects on plant growth and fertility [Arteca and Arteca, 2000; Arabidopsis Biological Resource Center (ABRC), http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/handling.htm]. A recent study with *Brassica napus*

suggested that the major cause of low pollen fertility under high temperatures was reduced pollen germination (Young *et al.*, 2004).

In addition to the positive effect on pollen germination rates at optimal temperatures, a considerable increase in the reproducibility of results was evident. The variations generally reported (Johnson-Brousseau and McCormick, 2004) between different flowers or experiments on different days were considerably minimized, and the germination rates were routinely homogeneous with different day 0 flowers, as observed by the typical 'spaghetti-like' pollen tubes from different flowers incubated for 16 h in controlled conditions (Figure 2g,h).

Although our experiments were routinely performed in an incubator without lights, we tested whether light affected pollen germination. Pollen germination in light yielded values >80% for the Columbia and L*er* ecotypes, similar to the germination rates obtained in the dark (85%; data not shown). Thus researchers who have access to a 22°C incubator with lights should still achieve successful pollen germination.

It is a common assumption that *A. thaliana* pollen germinates better in solid medium as it has a dry stigma. The inability to use liquid medium has held back the use of *A. thaliana* as a model system for physiological studies on the regulation of pollen tip growth. We therefore tested whether pollen germination was also attainable in liquid medium. To perform this test, we collected pollen from flowers in pollen germination medium, using a procedure similar to that described by Becker *et al.* (2003). Pollen was germinated in glass vials, without agitation, at 22°C. In these conditions, pollen tends to settle on the bottom of the vial, suggesting that oxygen availability is not crucial for pollen germination. After just 2 h, long tubes with thin walls, showing apparently regular growth and producing callose plugs (Figure 2i), were observed floating in the liquid medium. The results after 16 h incubation (Figure 2h,i) were equivalent to those obtained on solidified medium (Figure 2g). Given these results, and a method to collect large amounts of pollen using a vacuum cleaner (Johnson-Brousseau and McCormick, 2004), it should be feasible to isolate enough growing pollen tubes for biochemical assays or proteomics, and for gene expression profiles during different stages of pollen tube growth.

To consolidate the view that temperature was the critical factor determining reproducibility of *in vitro* pollen germination, several *A. thaliana* pollen germination media were selected from the literature and tested under our optimal incubation conditions. The results were evaluated and compared with those reported by the authors. Pollen germination rates varied between 0 (medium A) (Azarov *et al.*, 1990) to 75–80% (Figure 4a). Among all media tested, the best results for pollen germination and tube growth (Figure 4a,b) were obtained with our optimized medium
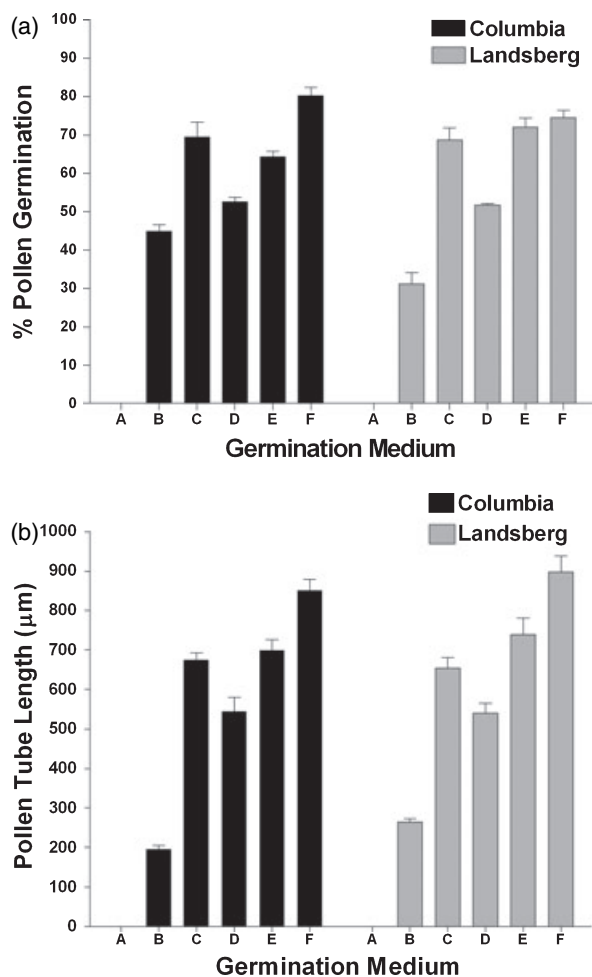
**Figure 4.** Pollen germination rate and pollen tube length with different pollen germination media.

Pollen was germinated on solidified medium at the optimal temperature (22°C). (a) Pollen germination rate; (b) pollen tube length. Pollen germination media recipes were from: (A) Azarov *et al.* (1990); (B) Fan *et al.* (2001); (C) Li *et al.* (1999); (D) Mouline *et al.* (2002); (E) Boavida (2005); (F) our optimized medium. Bars represent means ± SE, *n* = 2.



**Figure 5.** Effects of pH and buffered medium on pollen germination rates. Analysis of pollen germination rates in solid media of different pH or after addition of buffer. Bars represent means ± SE, *n* = 2.

(medium F) followed by those optimized for the C24 ecotype (medium E, Boavida, 2005) and by Li *et al.* (1999; medium C). Medium B (Fan *et al.*, 2001) and medium D (Mouline *et al.*, 2002), two of the most cited pollen germination media for *A. thaliana*, showed the lowest germination rates (50–60%) and shortest pollen tube lengths (600–700 μm after 16 h incubation) (Figure 4a,b). Nonetheless, the pollen tube lengths we obtained with these media were greater than those reported by the authors, and the results were consistent in separate experiments.

*Importance of pH and use of buffered medium for physiological studies of pollen tube growth*

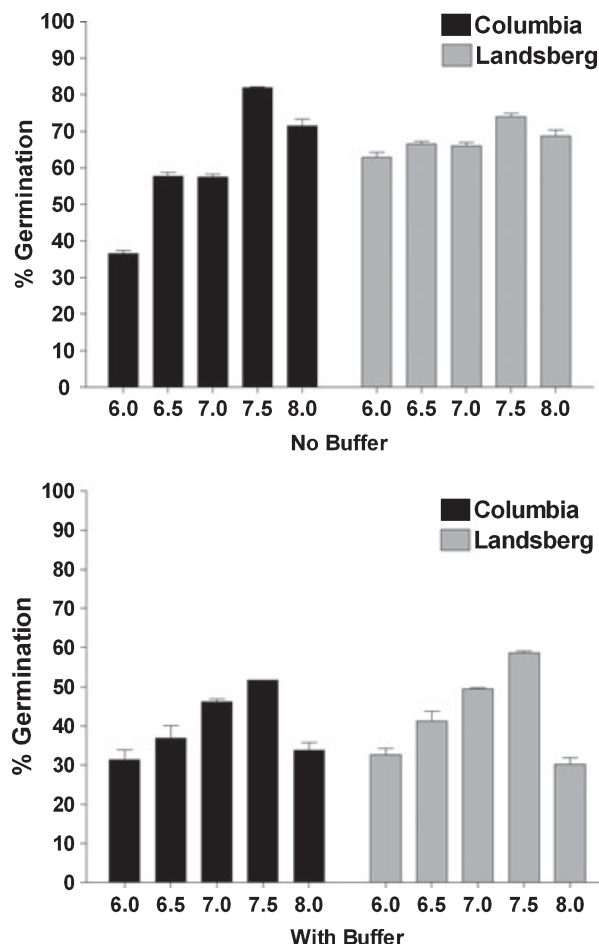Most physiological analyses of pollen germination require the manipulation of ions in the medium, and sometimes the medium is buffered. We therefore tested if adding buffer to our medium would improve pollen germination rates. Variations of pH with buffered and non-buffered medium were tested. We found that adding buffer to the medium decreased the germination rate in both Columbia and L*er* (Figure 5), indicating that pollen tubes grow better and germination rates are higher in a non-buffered medium. L*er* was more tolerant to pH variations in non-buffered conditions, but both ecotypes behaved similarly when buffer was added to the medium. The germination rates were reduced to 50–60% in buffered conditions at pH values of 7.0–7.5, while in non-buffered medium, germination rates of 70–80% were obtained at pH values of 7.5–8.0. In pollen tubes, a proton influx is localized at the extreme apex, whereas the rest of the tube (including the immediate subapical region) experiences an efflux (Feijo *et al.*, 1999; Messerli and Robinson, 1998; Messerli *et al.*, 1999; Robinson and Messerli, 2002). These fluxes are important for the establishment and maintenance of pollen tube tip growth (Messerli and

Robinson, 1998). The efflux of protons from the pollen tube to the extracellular medium may contribute to a gradient of acidification in the surrounding environment, probably promoting pollen germination and tube growth.

*Determining the best flowers and plant ages with which to perform pollen germination assays*

We presumed that *Arabidopsis* pollen would lose viability quickly, and that this could have been one of the main reasons for our initial variable germination rates. Therefore, for the experiments reported so far, we had used day 0 flowers (Figure 6a,b). Given that we had an optimized protocol, we then tested whether flower age or plant age was important. Young plants produce, on average, two newly opened flowers per day, but as plants get older the number of flowers per day is reduced and new flowers do not open every day (Figure 6a,b). We assumed that vacuum-collected pollen is a mixture from flowers of different ages and different viabilities. Germination was evaluated with pollen collected from flowers of different ages and from plants at different developmental stages (Figure 6c,d). Although pollen maintained high viability over several days, the best pollen was from day 0 flowers, and viability in the Columbia ecotype dropped approximately 10% for each day a flower was older (Figure 6c). Columbia pollen collected by vacuum showed germination rates of 70%, in liquid or solid medium (data not shown), confirming that pollen viability was not drastically reduced in the Columbia ecotype as flowers grew older. However, pollen viability in Ler declined more rapidly, with a decrease from an 80% germination rate with day 0

flowers to a rate of 50% with day 1 flowers (Figure 6c). Pollen from older flowers (day 4) for both ecotypes yielded a 40–50% germination rate. Pollen viability was also evaluated throughout plant development (Figure 6d). Germination rates above 80% were achieved for the first bolting flowers of young plants, from flowers the first week after bolting, and from mature plants (approximately 3 weeks after bolting), with only a slight decrease, to about 75%, with pollen from the last flowers of an old plant. However, a considerable reduction in germination (65%) was observed with day 0 flowers from plants that had been cut back to stimulate the production of new bolts. Pollen viability at all these stages, as assessed by the fluorochromatic reaction test, remained at about 95% (data not shown). Although the decrease in performance was not drastic, it is advisable to use day 0 flowers to obtain the best results.

*Pollen germination rates are dependent on pollen density*

The phenomenon of density-dependent pollen germination and tube growth, also known as the pollen population effect, is important in several species (Brewbaker and Majumder, 1961; Chen *et al.*, 2000; Pasonen and Kapyla, 1998). This effect probably involves pollen–pollen interactions, as addition of conditioned medium can promote pollen germination at low densities. Chen *et al.* (2000) demonstrated that the small peptide phytosulfokine was responsible for the conditioned medium effect, suggesting that this compound, and perhaps other molecules, released from pollen grains, promote pollen germination. In the experiments reported so far, pollen from approximately six flowers was
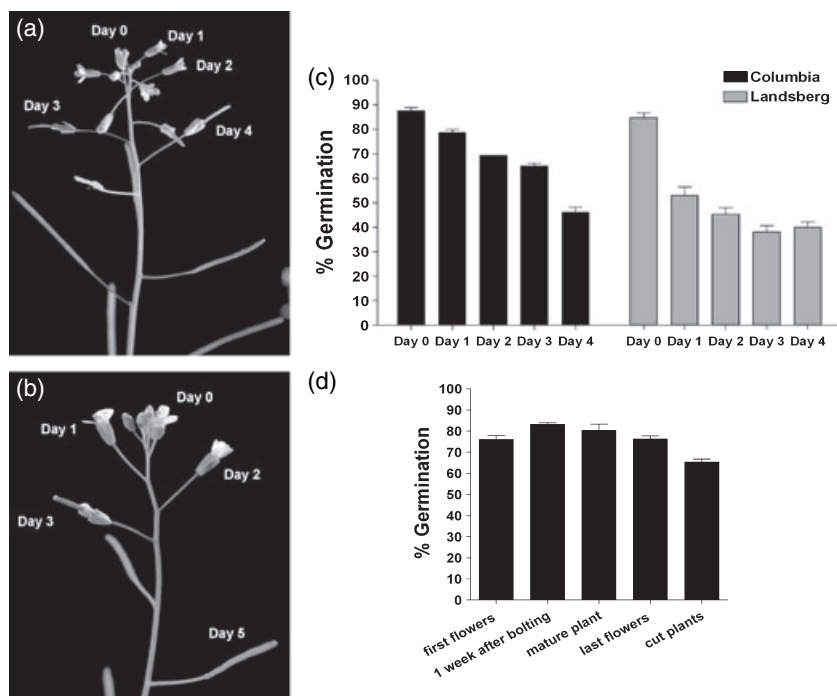


**Figure 6.** Effect of flower and plant age on germination rates.
(a) Mature plants showing position of flowers relative to the inflorescence buds; in general, two flowers open per day.
(b) In old plants, in general, only one flower per day opens.
(c, d) Pollen germination rates in Columbia and Landsberg *erecta* ecotypes relative to flower age (c) and plant age (d). First flowers of a young plant, flowers 1 week after bolting, mature plant (approximately 3 weeks after bolting), last flowers produced by an old plant, and day 0 flowers from a new bolting of a plant that had been cut back. Bars represent means ± SE, *n* = 2.
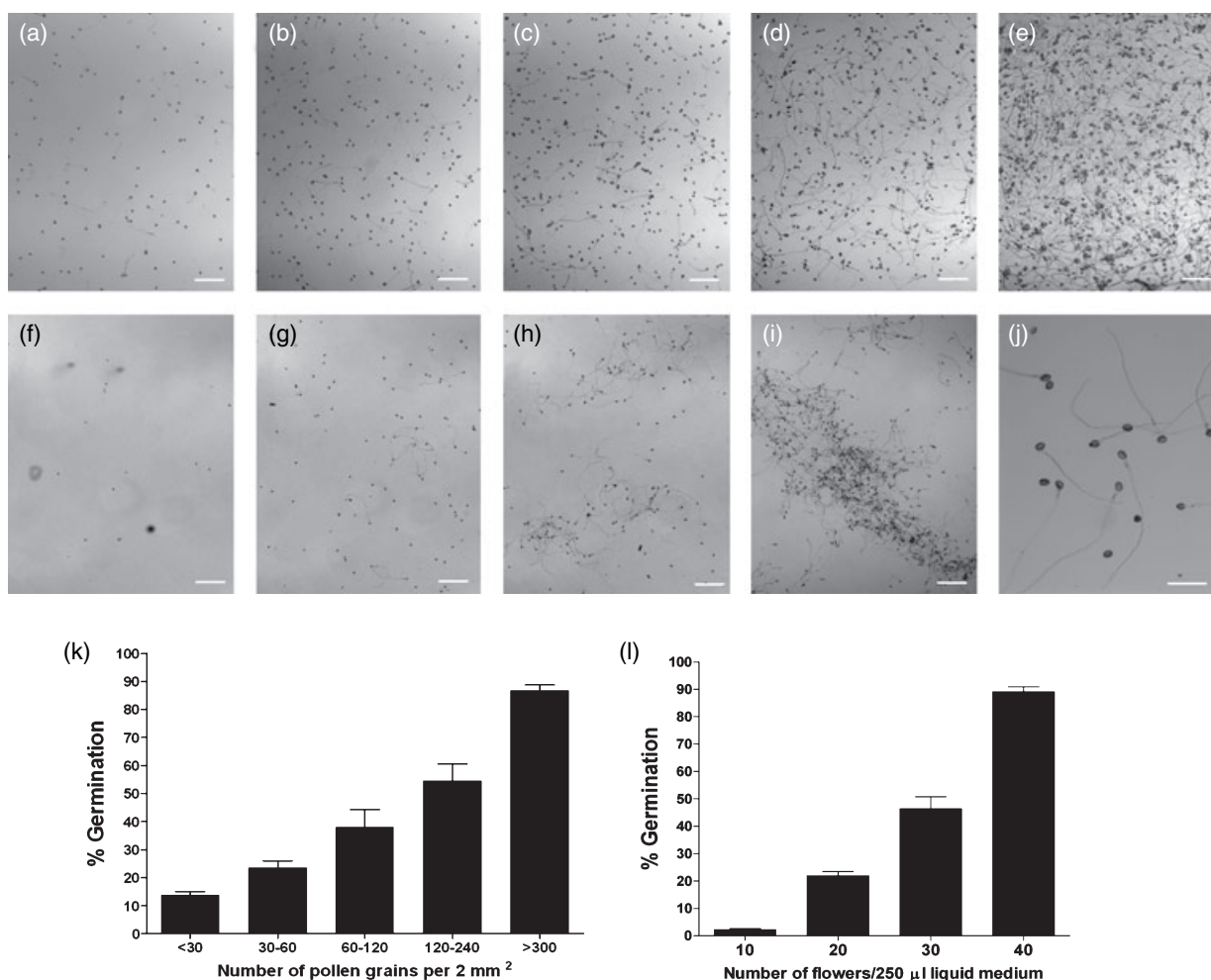
**Figure 7.** Effect of pollen density on germination rate.
Assays were performed with solid (a–e, k) and liquid medium (f–j, l) using Columbia pollen.
(a–e) Pollen from different numbers of anthers was spread in a 5-mm² area: (a) 0.5, (b) one, (c) two, (d) three, (e) eight anthers.
(f–i) Pollen from different numbers of flowers was germinated in 250 µl medium: (f) 10, (g) 20, (h) 30, (i) 40 flowers.
(j) Higher-magnification view from a 40-flower density assay after 3 h incubation.
(k) Pollen germination rates in solid medium; statistical classes were defined according to the number of grains/area present in each density assay.
(l) Pollen germination rates in liquid medium. Bars represent means ± SE, *n* = 3. Scale bars: (a–i), 500 µm; (j), 100 µm.

spread onto the area defined by the agarose pad, to achieve a reasonable pollen density. To develop guidelines for pollen density in liquid medium, pollen from different numbers of flowers was diluted in a defined volume of medium. For guidelines for solid medium, pollen from different numbers of anthers was spread on a defined area (5 mm²) of the agarose pad. Figure 7 shows that pollen density promotes pollen germination and tube growth. At lower pollen densities, in either solid or liquid medium, the germination rates were poor and tubes were shorter (Figure 7a–j). As pollen density increased, the germination rate improved (Figure 7k,l). For solid medium, the highest pollen germination rates (90%) were observed when more than 300 pollen grains were spread in a 2-mm² area (Figure 7k). This pollen density is achieved by spreading pollen from approximately

six to eight anthers on a 5-mm² area, or by dusting pollen from approximately 10 flowers onto the surface of the agarose pad. For liquid medium, maximal pollen germination rates (90%) were achieved with 40 flowers per 250 µl medium (Figure 7l). Liquid germination can also be performed in single wells of 96-well plates, using 50 µl medium and pollen from 8 to 10 flowers (data not shown). Vacuum-collected pollen germinated in liquid medium (at a concentration of 2 mg ml⁻¹) gave a 70% germination rate (data not shown).

*The* quartet1 *mutation negatively affects pollen germination*

The *Arabidopsis quartet (qrt)* mutation (Preuss *et al.*, 1994) has been used extensively (reviewed by Johnson-Brousseau and McCormick, 2004) to facilitate phenotypic analysis of
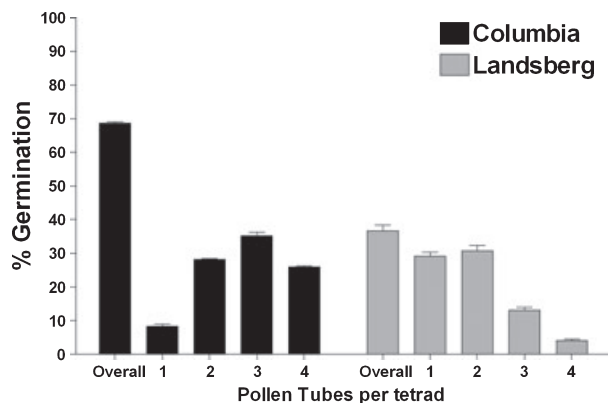
**Figure 8.** Effect of the *quartet1* mutation on pollen germination rates. Overall germination rate (%) represents germination/total number of grains analyzed. 1–4 refer to the distribution of tetrads with 1, 2, 3 or 4 pollen tubes. Bars represent means ± SE, *n* = 3.

male gametophytic mutants, because a male gametophytic mutant will have two normal and two affected pollen grains in each tetrad. To determine if *quartet1* affected pollen germination rates, *quartet1* pollen in either Columbia or L*er* ecotypes was germinated on agarose (Figure 8) and germination rates were compared with those for the corresponding wild-type background. The *quartet1* mutation had a negative effect on pollen germination, which is more pronounced in L*er*, where the overall percentage of germination (considering the total number of grains) was reduced to 40%. In the Columbia ecotype, the overall germination rate was still acceptable (70%). However, if the percentage of germination is evaluated per tetrad, the results are not ideal. In Columbia, tetrads containing two, three or four pollen tubes were present, but in L*er* most of the tetrads had only one or two pollen tubes. Thus using *qrt1* in L*er* requires analysis of a large number of tetrads to find some containing three or more pollen tubes, and therefore usable for scoring the phenotypic effects of a male gametophytic mutant on pollen germination or tube growth.

*Pre-hydration and heat treatment reduce the time required for* in vitro *metabolic activation and synchronize pollen germination*

Pollen grains and seeds are considered anhydrobiotic organisms as they are able to survive under extreme desiccation conditions. They are also extremely sensitive to rapid imbibition in cold medium, particularly when extremely dehydrated (Crowe *et al.*, 1992). In hydrated conditions, membranes are generally in a liquid crystalline phase, while during desiccation the phospholipid bilayers pass into a gel phase. The initial uptake of water by dehydrated pollen grains, when placed directly on germination medium, can be very fast due to their small size, inducing a rapid phase

transition of membranes and potentially compromising pollen viability due to disruption of membrane integrity. This probably explains why pollen grains burst when they were incubated directly on pollen-germination medium at 18°C (Figure 2a). If, however, a brief period of hydration is done at warmer temperatures, damage is avoided as phase transitions in membranes are minimized (Crowe *et al.*, 1992). The initial reorganization of the membranes from dry to hydrated state is therefore a critical cellular process (Crowe *et al.*, 1989).

Pollen germination percentages for many species can be increased considerably if pollen grains are equilibrated in a moisture environment before incubation in a suitable *in vitro* germination medium (Shivanna and Heslop-Harrison, 1981). In an attempt to increase pollen vigor, assessed as the time taken before *in vitro* pollen germination starts, the effects of different conditions of pre-incubation were tested using pollen from the Columbia ecotype. Pollen dehydration, pre-hydration, a short incubation at higher temperatures, or combinations of these treatments were tested. The results were evaluated with respect to the improvement in percentage of germination in the first hours of incubation (synchronization) and the uniformity of pollen tube lengths within the sample.

Dehydration of pollen for 1 h before incubation on pollen germination medium did not improve germination rates relative to the control (data not shown). Dehydration was reported to increase subsequent pollen viability before storage at low temperatures (Pickert, 1988), and may not be relevant for pollen harvested directly from fresh flowers. Similarly, pre-incubation of pollen at 30°C for 30 min to 1 h did not improve pollen germination. However, pre-hydration for 30 min at room temperature (22–24°C) followed by 30 min incubation at 30°C increased the germination rate upon transfer to solidified medium at optimal temperature (22°C). Higher temperatures can have a positive effect on metabolic activation when pollen is exposed to them for short periods (Figure 9a,b), but are detrimental to pollen germination rates if applied for long periods (Figure 2a).

Pre-hydration and incubation at 30°C during pollen hydration appeared to minimize damage during imbibition, and accelerated metabolic activation. *In vitro* pollen grain germination rates increased from 80% to >90%, and pollen tube growth was increased in the first hours of incubation after pre-treatment (Figure 9a,b). The number of germinated grains in the first 2 h after pre-treatment was five-fold higher when compared to the control. With pre-treatment, about 30% of the pollen grains started germinating within the first hour, with another 20% initiating germination between the third and fourth hours. In the control, only 10% of the grains germinated in the first hours of incubation, with an increase to 30% after 6 h incubation. The higher pollen germination percentage and an apparent regular pollen tube growth rate in the first 4 h of incubation contributed to uniformity of tube
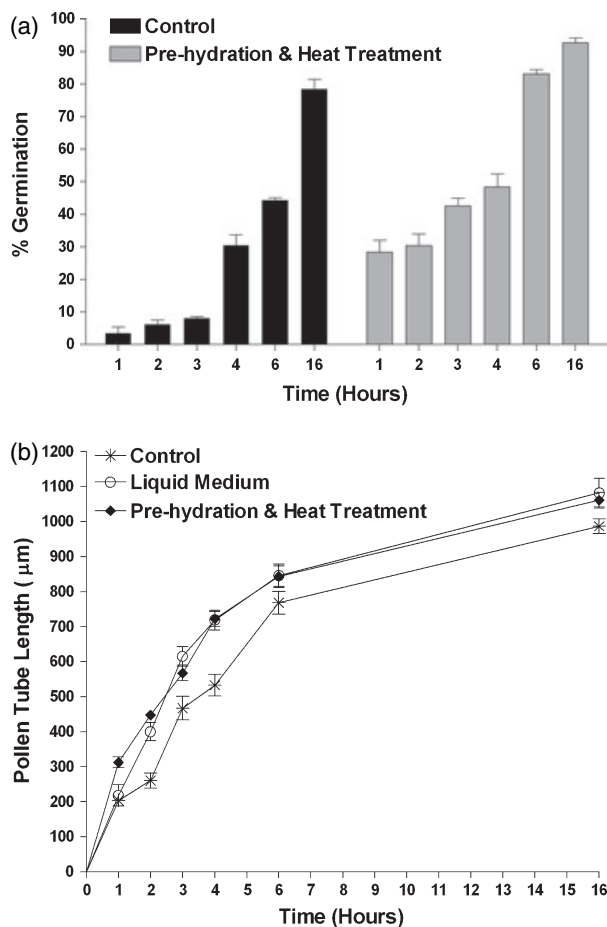
**Figure 9.** Effects of pre-hydration and heat treatment on pollen germination rates and pollen tube length.
(a) Pollen germination rate; (b) pollen tube length. Control: Columbia pollen was placed immediately onto solidified pollen-germination medium and incubated at 22°C. Liquid medium: Columbia pollen was placed immediately into liquid medium and incubated at 22°C. Pre-hydration and heat treatment: flowers were incubated in a moisture chamber for 30 min, then pollen was transferred to solidified germination medium and incubated at 30°C for 30 min before incubation at 22°C. Bars represent means $\pm$ SE, $n = 2$.

the growth rates estimated for fast pollen tube growth species such as lily (approximately 12 $\mu$m min$^{-1}$), the average growth rates we obtained for *A. thaliana* (5 $\mu$m min$^{-1}$) pollen tubes were comparable with those reported for tobacco pollen tubes (6–12 $\mu$m min$^{-1}$).

Transcriptome analyses of the *A. thaliana* male gametophyte (Becker *et al.*, 2003; Honys and Twell, 2003, 2004; Pina *et al.*, 2005) contributed significantly to our knowledge of gametophyte gene expression. The development of a robust method that can be routinely applied for *in vitro* pollen studies is no less important. Our method substantially improves the reliability and reproducibility of *in vitro* *A. thaliana* pollen germination and tube growth, in both liquid and solid media, and will allow detailed analyses of knockout mutants affected in male gametophyte function and time-course analyses of pollen tube growth. *Arabidopsis thaliana* is no longer a challenging organism for physiological and genetic studies of pollen tip growth.

## Experimental procedures

### Plant growth conditions

Seeds of *A. thaliana*, Columbia and Landsberg *erecta* ecotypes, were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). Seeds were surface-sterilized with 20% sodium hypochlorite solution for 10 min, washed three times with sterile water and plated onto Petri dishes containing basal MS medium (Murashige and Skoog, 1962) supplemented with vitamins (1 mg l$^{-1}$ thiamine, 0.5 mg l$^{-1}$ pyridoxine, 0.5 mg l$^{-1}$ nicotinic acid, 0.1 mg l$^{-1}$ myoinositol), 0.5 g l$^{-1}$ 2-($N$-morpholino)ethanesulfonic acid (MES, Sigma, http://www.sigmaaldrich.com), 1% (w/v) sucrose (Fisher Scientific, http://www.fisher.co.uk), 0.8% agar (Fisher Scientific) and adjusted to pH 5.7. Seeds were stratified for 3 days at 4°C in the dark and then grown in short-day conditions (8 h light/16 h dark at 21°C) for 14 days in a Percival growth chamber. After transfer to soil, plants were grown in glasshouse conditions, with 16 h daylight with a light intensity of 75–100 $\mu$mol m$^{-2}$ sec$^{-1}$, day/night temperatures of 21°C/18°C, and approximately 50% relative humidity.

### Optimization of pollen germination medium and incubation conditions

Pollen germination medium (final volume 20 ml) was always prepared fresh from 100$\times$ stock solutions of the main components (KCl, H$_3$BO$_3$, CaCl$_2$, MgSO$_4$) using autoclaved MilliQ water (Millipore, http://www.millipore.com). Sucrose was added and dissolved, then the pH was adjusted to 7.5 using NaOH (with our laboratory water, approximately 30 $\mu$l 0.1 M NaOH is enough to bring the pH to the desired value). It is extremely important that the pH adjustment is done with minimal volumes to avoid variations in the concentration of the main components. For solidified medium, 1% low-melting agarose (Nusieve GTG agarose, Biowhittaker Molecular Applications, http://www.biowhittaker.com) was added and briefly heated in a microwave oven, on medium power, just long enough for the agarose to melt. After optimization, we found that 1.5% agarose was more useful for imaging, as 1% agarose is more easily damaged. All subsequent experiments used 1.5% agarose.

lengths within the sample, and facilitated time-course analyses of pollen tube growth. Mouline *et al.* (2002) reported 500-$\mu$m-long pollen tubes after only 6 h incubation, but these values are far from *in vivo* pollen tube growth rates (300–500 $\mu$m h$^{-1}$, Mansfield and Briarty, 1991). For the Columbia ecotype, during the first hour of incubation, the average pollen tube growth rate for the control was estimated as 200 $\mu$m h$^{-1}$ (Figure 9b), while for pre-treated pollen grains this rate was 300 $\mu$m h$^{-1}$ for the same period of incubation, followed by a relatively steady growth rate of 150–200 $\mu$m h$^{-1}$ in the following hours. Although the influence of pre-treatment was not tested for liquid medium, the average growth rate was equivalent to that obtained for pre-treated pollen grains, suggesting that higher pollen tube growth rates can be achieved for *A. thaliana* pollen tubes when incubated in liquid medium. Although not reaching

A rectangle of $20 \times 45$ mm was drawn on a glass microscope slide using a Super HT PAP pen (Research Products International Corp., http://www.rpicorp.com). This well was filled with 500 µl melted germination medium, to build a flat agarose pad where pollen could be spread after the agarose was solidified. The slides were immediately placed inside a moisture incubation chamber to avoid dehydration of the medium. The incubation chambers were empty micropipette tip boxes, assembled as described by Johnson-Brousseau and McCormick (2004). All experiments were performed using pollen from freshly opened flowers, defined here as day 0 if not otherwise indicated. To minimize biological variation, pollen from six flowers from different plants was spread on the surface of the agarose pad to achieve an appropriate pollen density. Bulk pollen collection with a vacuum was performed as described by Johnson-Brousseau and McCormick (2004). In one experiment, buffers (MES or HEPES) were added to solid medium by addition from 0.5-M stock solutions, for final concentrations of 5 mM. For germination assays and pollen density effects in liquid medium, pollen from a given number of flowers (10, 20, 30, 40 flowers) was collected in germination medium, agitated briefly on a vortex mixer, then the flower parts were removed with a forceps and the pollen was pelleted for 30 sec at 800 *g*. For density assays using pollen collected by the vacuum method, the pollen was weighed and different pollen dilutions were used for germination (0.5, 1, 1.5, 2 mg ml$^{-1}$). In both cases, pollen was resuspended in 250 µl fresh pollen germination medium and placed in small ($12 \times 45$ mm) glass vials with their screw caps loosely attached. Germination was performed without agitation, as a rotating shaker in the incubator would produce heat, which might have disturbed the temperature of the incubator. After germination a 50-µl aliquot was removed, using a 1000-µl pipette tip, and placed on a microscope slide for observation. For pollen density analysis on solid medium, 5-mm$^2$ area squares were defined on the agarose pad, then pollen from different numbers of anthers (1, 2, 3, 4, 8 anthers) was spread evenly on each square to establish different pollen densities. As the number of grains in each assay could not be controlled accurately (the number of pollen grains deposited per anther was variable), we grouped categories by the number of grains present in a defined area. The numbers of pollen grains in a 2-mm$^2$ area were counted and the germination rates were determined for each category.

For pre-hydration assays, flowers were collected and immediately placed into moisture incubation boxes (the same as used for pollen germinations) for 30 min before pollen was transferred to germination medium and incubated under controlled conditions. Pollen hydration can also be performed in 96-well plates by putting four to five flowers in each well and placing a moistened piece of paper wipe between the wells and the lid. For the dehydration assay, flowers were collected and maintained in a parafilm-sealed $100 \times 15$-mm Petri dish containing 30 g of Drierite (magnesium perchlorate hydrate, Acros Organics, http://www.acros.com) for 1 h before pollen was transferred to germination medium. To transfer pollen to solid germination medium, the flower was inverted with the help of tweezers and, using a stereoscope for observation, brought close to the surface of the agarose pad and pollen was dusted upon it. The slides were placed in a moisture-incubation chamber and taken immediately to a controlled-temperature incubator. Pollen germination rates and tube growth were scored after 16 h incubation in the dark, unless otherwise indicated. It is important that the temperature inside the incubator remains stable, as slight variations have negative effects. Therefore repeated opening of the incubator door should be avoided.

*In vitro* pollen germination was examined under a Zeiss Axioskop 2 (Carl Zeiss, http://www.zeiss.com) microscope using DIC optics (2.5×, 0.075 NA; 10×, 0.25 NA; 20×, 0.50 NA). Slides can be observed at these magnifications without a coverslip. Images were captured with AXIOVISION ver. 4.3 software using an AxioCamMR camera, and processed with Jasc PAINT SHOP PRO ver. 9. Pollen germination counts and tube lengths were performed manually with the IMAGEJ software (http://rsb.info.nih.gov/nih-image/index.html) using the cell counter and measure and label plug-ins, respectively. Two replicates (two slides incubated in different pipette boxes under the same conditions) and a minimum of two independent experiments were performed for each assay. Images were collected from at least four different microscopic fields, from each replicate. At least 500 pollen grains were scored for pollen germination rates and 20–30 pollen tubes were measured for analysis of pollen tube growth in each replicate. A pollen grain was classified as germinated if the pollen tube length was equal to or greater than the pollen grain diameter. Statistical analysis of results and further processing was performed using GRAPHPAD PRISM ver. 4.00 for WINDOWS (http://www.graphpad.com).

Pollen viability was estimated by the fluorochromatic reaction test (Heslop-Harrison *et al.*, 1984). Pollen was collected as described for liquid pollen germination, incubated in a 5-mM solution of fluorescein diacetate with 17% sucrose, and observed under the fluorescent microscope using the fluorescein isothiocyanate FITC filter (490/520 nm).

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