Aperture number influences pollen survival in
Arabidopsis mutants
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Aperture number influences pollen survival in Arabidopsis mutants.  


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• **Premise of the study:** Pollen grains are subject to intense dehydration before dispersal. They rehydrate after landing on a stigma or when placed in a humid environment by absorbing water from the stigma or surroundings. Resulting fluctuations in water content cause pollen grains to undergo significant changes in volume. This suggests that morphological or structural adaptations might exist to help pollen adjust to sudden volume changes, though little is known about the correlation between pollen morphology and its ability to accommodate volume changes. We studied the effect of one morphological feature of pollen grains, the aperture number, on pollen wall resistance to water inflow in *Arabidopsis thaliana*.

• **Methods:** We used three *Arabidopsis thaliana* mutants producing pollen with different aperture numbers (zero, four, and a mix of four to eight) and the wild-type pollen with three apertures. We tested pollen survival in solutions with various mannitol concentrations.

• **Key results:** Our experiments show that the number of intact pollen grains increases with increased mannitol concentration for all pollen morphs tested. At a given mannitol concentration, however, an increase in aperture number is associated with an increase in pollen breakage.

• **Conclusions:** Aperture patterns influence the capacity to accommodate volume variations in pollen grains. When subjected to water inflow, pollen grains with few apertures survive better than pollen with many apertures. Trade-offs between survival and germination are likely to be involved in the evolution of pollen morphology.

Key words: pollen performance, aperture pattern, *Arabidopsis thaliana*, harmomegathy
Competition between individuals is a key feature of all ecosystems. Plants compete for light, space, water and nutrients, as well as for opportunities to reproduce. For vascular plants, studies on competitive abilities usually focus on sporophytes (the dominant phase in the life cycle of most land plants), and rarely concern gametophytes, although they are also involved in ecological interactions.

Pollen, the male gametophyte of all seed plants, is very diverse morphologically, having frequent inter-species variations in its shape, size, wall ornamentation, and aperture patterns (Erdtman, 1952; PalDat, 2015). Apertures are the thinned areas of pollen wall where exine, the extremely resistant outer layer made of sporopollenin, is absent or reduced (Edlund et al., 2004). Aperture patterns are defined by the number, shape and positions of apertures (Walker and Doyle, 1975; Hesse et al., 2009). In many species, apertures are the primary sites through which exchanges between pollen and environment take place – particularly during dehydration, when the grain is released from the anther, and rehydration, when it lands on a stigma and absorbs water from stigmatic cells or surface (Heslop-Harrison, 1979b; Edlund et al., 2004). Apertures also play an important role in pollen tube germination, since in many cases pollen tube exits through these sites (Edlund et al., 2004).

Additionally, apertures are involved in harmomegathy, a pollen-specific process defined by Wodehouse as “volume change accommodation” (Wodehouse, 1935) and manifested, for example, by pollen’s ability to in-fold itself at the time of water loss to prevent complete desiccation (Katifori et al., 2010). Wall structures involved in this process were called harmomegathus by Wodehouse, namely “an organ or mechanism which accommodates a semirigid exine to changes in volume” (Wodehouse, 1935). Harmomegathic properties of pollen grains are expected to mainly depend on apertures (Payne, 1972; Blackmore and Barnes, 1986; Scotland et al., 1990), although as the exine layer has some elastic properties, it may also be able to deform to some extent and accommodate some volume changes (Bolick and Vogel, 1992; Rowley and Skvarla, 2000).

In angiosperms, pollen with a single distal aperture is ancestral for the clade (Furness and Rudall, 2004). This pollen type, called monosulcate, is the main type in early diverging angiosperms and monocots, though derived types with other aperture patterns can be found at the generic or the specific level. In eudicots, a very large clade with approximately 75% of the extant angiosperm species, pollen grains are characterized by three furrow-like equatorial apertures (Doyle and Hotton, 1991; Furness and Rudall, 2004). The tricolpate pollen grain is an evolutionary innovation of the clade and is the only morphological synapomorphy of eudicots known so far. Some authors have suggested that the acquisition of tricolpate pollen could be the key innovation of eudicots that had been essential for the evolutionary success of the clade (Furness and Rudall, 2004).

Previous studies have highlighted the connections between the aperture distribution on pollen surface and characteristics of cell division during the male meiosis. Apertures are usually formed at the last points of contact between the microspores (Wodehouse, 1935; Ressaye et al., 2002) and variations in key aspects of pollen development, such as the type of cytokinesis or the geometrical form of a tetrad, result in differences in aperture patterns (see, for example, Albert et al., 2010, 2011). Although the relationship between pollen development and aperture patterns has been studied for some time, the link between aperture patterns and pollen fitness has received less attention.

When focusing on apertures, some assumptions can be made regarding aperture pattern and pollen performance. Since apertures are involved in pollen tube germination, an increase in aperture
number is expected to accelerate pollen germination on stigma. Moreover, high number of apertures could enable faster pollen rehydration, especially in the case of dry stigmas (an assumption made, for example, by Heslop-Harrison, 1979b). If the presence of many apertures indeed had a positive effect on pollen fitness, then pollen grains with a high number of apertures should be widespread in wild species. However this is not the case, since the vast majority of angiosperms produce pollen with one or three apertures, whereas pollen grains with larger numbers of apertures occur rarely (Erdtman, 1952). Some studies carried out on Viola diversifolia, a species producing both three- and four-colpate pollen, give some clues on this issue. These pollen types have different properties: pollen with three apertures live longer than pollen with four apertures, whereas pollen with four apertures germinate faster on stigma (Dajoz et al., 1991, 1993). It thus seems that increasing aperture number is advantageous for the on-stigma competition between pollen grains, but could provide disadvantage for pollen survival. Aquatic species dispersing pollen directly in water constitute another example of aperture pattern adaptation. These species tend to produce pollen lacking localized apertures; however, the exine layer of these pollen grains is uniformly thin and the entire wall functions as an aperture (omniaperturate pollen; Pettitt and Jermy, 1974; Furness and Rudall, 1999). The reduced thickness of exine is interpreted as an adaptation to water dispersal: pollen grains do not dehydrate before release, thus the protective outer layer becomes unnecessary and tends to disappear (Pettitt and Jermy, 1974). Apart from these few examples, the connection between aperture patterns and pollen fitness remains largely unexplored.

Apertures are involved in harmomegathy, but the precise effects of aperture number or shape on volume accommodation are not completely understood. Theoretical approaches have shown that aperture pattern and exine ornamentation can influence the way in which deformation of the pollen wall takes place during dehydration (Katifori et al., 2010). In particular, that study has shown that pollen grains with pore-shaped apertures are not able to deal with strong volume decrease very efficiently, while in pollen grains with furrow-shaped apertures the wall can adapt to changes in pollen size by folding inwards along the furrows as the volume decreases. Empirical studies have shown that harmomegathy depends on aperture pattern (Payne, 1972; Blackmore and Barnes, 1986; Scotland et al., 1990; Volkova et al., 2013), but it might also be influenced by other characteristics, such as the size or shape of the grain (Halbritter and Hesse, 2004).

The aim of this study was to investigate the connection between aperture patterns and harmomegathy. We are particularly interested in the link between aperture number and accommodation of volume increase during water inflow, which happens during the on-stigma rehydration phase (Heslop-Harrison, 1979a; Edlund et al., 2004) or is brought by variations in humidity during pollen dispersal. In order to study the relationship between the aperture number and the wall mechanical resistance, we used the wild-type Arabidopsis thaliana and three Arabidopsis mutants with different numbers of apertures: this method enabled us to test the influence of aperture number, since other pollen features remained largely the same among the different morphs, except for the size of the grain. The different mutants came from two different genetic backgrounds, this was taken into account when analyzing the results. The different plants used here produce pollen grains with either 0, 3, 4 or a mix of 4 to 8 apertures, largely covering the range of aperture numbers observed in wild species of angiosperms (Erdtman, 1952; PalDat, 2015).

We studied the ability of pollen grains to withstand the effects of water inflow when placed in solutions of variable osmolarity. Pollen grains subjected to water inflow can either survive, if the wall
is resistant to swelling, or die, if the wall or plasma membrane breaks. In our study, we considered pollen grains to be dead if wall and/or plasma membrane were broken, and we assumed that pollen grains survived if they remained intact, regardless of their germination abilities (which can be affected by several intrinsic and extrinsic factors). We evaluated the number of surviving pollen grains for each genotype and for three different levels of osmolarity.

In theory, aperture number can influence pollen resistance to breakage during hydration in several possible ways. Because apertures are more flexible than other parts of the pollen wall, their increased number could potentially enable the wall to accommodate deformations more easily, therefore leading to higher resistance to breakage. On the other hand, since water is absorbed through apertures, more of these sites could result in faster swelling of the grain, compromising its ability to cope with increased volume; also, increased aperture number could weaken the wall. These possibilities are not exclusive. Our aim was to study the effect of aperture number on resistance to osmotic stress and to test whether an increase in aperture number is associated with higher or lower pollen mortality. We show here that the number of intact pollen grains decreases with hydration intensity and that an increase in aperture number is associated with an increase in pollen breakage.

MATERIALS AND METHODS

Arabidopsis lines

We chose several mutants that differ in their pollen aperture number from the wild type, which has tricolpate pollen grains (Fig. 1A). The inp1-1 mutant produces inaperturate pollen (Fig. 1B; Dobritsa et al., 2011; Dobritsa and Coerper, 2012; length=18.07 μm, sd=1.62 μm, n=12). This mutant exhibits well-developed siliques and a normal number of seeds per silique, indicating normal performance of this pollen type during reproduction (Dobritsa et al., 2011). The lsq6 mutant (Dobritsa et al., 2011) produces mostly tetracolpate pollen grains (Fig. 1C), and a few tricolpate grains. Pollen grains of lsq6 are larger (length=27.86 μm, sd=2.02 μm, n=9) than the wild-type Columbia (col) pollen grains (length=23.49 μm, sd=1.12 μm, n=9). The osd1-1 (d’Erfurth et al. 2009) produces a mix of four-to-eight-aperturate pollen grains (Fig. 2). This genotype has a mutation in the OSD1 gene that leads to the production of functional diploid pollen grains due to the absence of the second meiotic division (D’Erfurth et al., 2009). These pollen grains are a little larger (length=24.25 μm, sd=2.00 μm, n=11) than wt (see below) Arabidopsis pollen studied here (length=22.24 μm, sd=0.68 μm, n=9). Apertures in the six- and eight-aperturate pollen grains also differ in their distribution on the surface of the pollen grain compared to tricolpate pollen grains, where apertures are equatorial (Fig. 1A). In the six- and eight-aperturate osd1-1 pollen, apertures form the edges of a tetrahedron (Fig. 2C) and a square-based pyramid (Fig. 2E), respectively. The osd1-1 plants were obtained from the progeny of osd1-1/+ plants. In this progeny, wild-type and osd1 phenotypes segregated. These wild-type plants, called wild type (wt) here, were used as control plants for osd1-1 plants, as they have the same genetic background (Nooseen ecotype). Another wild-type line, called col here, corresponds to the Columbia ecotype (NASC N60000) and was used as a control for the inp1-1 and lsq6 mutants, which both have the Columbia genetic background.

Other mutants presenting wall anomalies were available (for example, tom mutants from Magnard et al., 2001, or the collection of mutants from Dobritsa et al., 2011), but we restricted our study to...
mutants presenting normal exine and having aperture shape and size comparable to those of the wild type, in order to limit the differences between mutants and controls to aperture number.

**Growth conditions**

Plants were grown in a climatic chamber under the following conditions: 8 hours of dark at 16°C, 16 hours of light at 20°C. Experiments were carried out as soon as plants produced flowers (usually 6 weeks after sowing). Seeds were sown during summer 2013 (in July and in August), and experiments were done in September.

**Microscopy**

Each flower line was represented by four to eight different individuals. We used flowers picked from different individuals for the experiments. These experiments lasted three weeks, since after this time plants start to show signs of senescence, which could affect pollen. Open flowers were removed from each plant the day before each experiment, in order to work with freshly open flowers, since a majority of pollen grains are viable at this stage. On the day of the experiment, anthers from freshly opened flowers were dissected and placed in a 20 µL drop of mannitol solution on a glass slide to disperse pollen grains. We used one flower for each slide. Anthers were removed after a few minutes and the drop with the pollen grains was gently sealed with a cover glass.

Mannitol is a non-metabolic sugar, which allowed us to specifically study the effects of osmolarity, without affecting pollen metabolism. Three mannitol concentrations were used in our experiments: 0.2 mol.L\(^{-1}\), 0.45 mol.L\(^{-1}\) and 0.7 mol.L\(^{-1}\). These concentrations were chosen in an attempt to reproduce osmolarity conditions faced by pollen grains *in vivo*. Because almost all pollen grains explode in solutions devoid of mannitol, this condition was not used. As there are no quantitative data available for stigmatic sugar concentrations, it is difficult to precisely match the osmolarity conditions faced by pollen grains on the stigma. Therefore, we chose mannitol concentrations similar to sugar concentrations of the previously used germination media. Since these concentrations are suitable for germinations, they may resemble natural conditions occurring on stigmas. The following mannitol concentrations were taken from the literature: low mannitol concentration (0.2 mol.L\(^{-1}\)) corresponds to sucrose concentration used by Mouline (Mouline et al., 2002), and lies in the optimal range defined by Boavida (5% to 15% or 0.15 mol.L\(^{-1}\) to 0.44 mol.L\(^{-1}\) in Boavida and McCormick, 2007). The intermediate mannitol concentration (0.45 mol.L\(^{-1}\)) is close to the sucrose concentration used by Li (18% or 0.53 mol.L\(^{-1}\) in Li et al., 1999), and the high mannitol concentration (0.7 mol.L\(^{-1}\)) is close to the osmolarity used by Fan (about 0.68 mol.L\(^{-1}\) in Fan et al., 2001).

Solutions were prepared with 3 mL of buffer (see below), 6 g of Ficoll, a variable quantity of mannitol (1.09 g for the 0.2 mol.L\(^{-1}\) solution, 2.46 g for the 0.45 mol.L\(^{-1}\) solution and 3.8 g for the 0.7 mol.L\(^{-1}\) solution), and deionized water (amount necessary to bring up solution to 30 mL). Buffer was adapted from a medium routinely used to germinate pollen grains *in vitro* (Bergamini-Mulcahy and Mulcahy, 1983). This mineral salt solution contained 1.62 mmol.L\(^{-1}\) of H\(_2\)BO\(_3\), 1.27 mmol.L\(^{-1}\) of Ca(NO\(_3\))\(_2\)-4H\(_2\)O, 0.81 mmol.L\(^{-1}\) of MgSO\(_4\)-7H\(_2\)O. The solution was buffered to pH 6 with 0.2 mmol.L\(^{-1}\) of KH\(_2\)PO\(_4\) and 0.05 mmol.L\(^{-1}\) of K\(_2\)HPO\(_4\)-3H\(_2\)O. Ficoll is a polysaccharide that increases the viscosity of the solution without changing the osmolarity. If the solution is not viscous enough, the cytoplasm leaking out of broken pollen grains can disperse in the solution, and pollen with exploded cytoplasm can be wrongly taken for intact pollen grains.
After 2 hours (most pollen grains rehydrate within 30 min according to our observations), 100 pollen grains on each slide were scored as being either intact (having both intact wall and cytoplasm, Fig. 3A), having broken plasma membrane (visible as leaking cytoplasm, Fig. 3B), or having broken wall (Fig. 3C). In Columbia lines (inp1-1, col and lsq6), 14 slides were counted for each concentration and each genotype, which yields a total of 126 slides. In Nooseen lines (wt and osd1-1), 16 to 19 slides were counted for each concentration, for a total of 104 slides.

Statistical analysis

R software was used to carry out statistical analysis (R Core Team, 2014). The number of intact pollen grains over intact and non-intact pollen grains being a binomial variable, we used a generalized linear model (GLM, which is an extension of the linear model to non-normal variable) with Binomial distribution and the classical logit link function. We tested the effect of genotype, osmotic concentration, and genotype-osmotic concentration interaction. To limit the number of tests, we only conducted pairwise comparisons between genotypes for each osmotic concentration, and between osmotic concentrations for each genotype.

RESULTS

Pollen grains with a broken wall were very rarely observed in the conditions tested. Therefore, for each experiment we only present the proportion of intact pollen grains; the rest of the grains being mostly grains with a broken plasma membrane.

The pollen grains of inp1-1 and lsq6 were compared to col, as they all share the Columbia genetic background, while the pollen from osd1-1 was compared to wt, as they share the Nooseen genetic background.

A GLM test compared the number of intact and damaged pollen grains between genotypes with zero (inp1-1), three (col, wt), four (lsq6) or four to eight (osd1-1) apertures; it showed a significant effect of genotype, mannitol concentration, and interaction between the two (Tab. 1). Similarly, a GLM test comparing the number of intact and damaged pollen grains between the lines with three (wt) and four to eight (osd1-1) apertures showed a significant effect of genotype, mannitol concentration, and interaction of both (Tab. 2).

• Influence of mannitol concentration

For pollen grains with zero (inp1-1), three (col, wt), four (lsq6) or four to eight (osd1-1) apertures, an effect of mannitol concentration was detected (Fig. 4 and Fig. 5): the proportion of intact pollen grains increases with increased mannitol concentration, showing that pollen grains tend to survive better in higher mannitol concentrations (p < 0.005, Tab. 3 and Tab.4). This result implies that varying mannitol concentration is a useful way to study pollen harmomegathy.
• *Influence of aperture number*

When we compared the results for the Columbia background lines with zero (*inp1-1*), three (*col*) or four (*lsq6*) apertures, we found that pollen mortality increases with increased aperture number (*p* < 0.005, Tab. 3, except between inaperturate and triaperturate pollen at high mannitol concentration), regardless of mannitol concentration (Fig. 4): the percentage of intact pollen grains was the highest for pollen with no apertures, and the lowest for pollen with four apertures. Triaperturate pollen survival rate was intermediate between those two. Differences among the three genotypes are stronger at low mannitol concentration than at high concentration. In general, pollen grains survive better in high mannitol concentration. Therefore, it is not surprising that differences among pollen types are more obvious at low concentration than at high concentration.

Similarly, pollen from Nooseen with three (*wt*) apertures had a higher survival rate than pollen with four to eight (*osd1-1*) apertures (*p* < 0.005, Tab. 4) and this was true for all three mannitol concentrations tested (Fig. 5). Here again, the contrast in mortality between the two pollen types is stronger at low mannitol concentration than at high concentration: as the overall mortality rate is reduced at high mannitol concentration, the differences between morphs become less pronounced. Taken together, our results suggest that aperture number has a negative impact on pollen survival rate under the osmotic stress conditions.

**DISCUSSION**

In nature, pollen grains are exposed to various environmental conditions, including changing atmospheric humidity and rainfall events, which can affect pollen viability (Lisci et al., 1994). Also, during the rehydration on stigma, pollen grains can either be immediately submerged into stigmatic liquid covering the stigma surface in the case of plants with wet stigmas, or can gradually rehydrate by transferring water from stigma cells in the case of plants with dry stigmas (Heslop-Harrison and Shivanna, 1977; Edlund et al., 2004). Therefore, during both pollen dispersal and pollen-stigma interactions, volume accommodation is a critical issue.

Our experiments reveal that both aperture number and mannitol concentration affect pollen survival. The more apertures the pollen has, the more likely its plasma membrane to break. The intensity of this effect depends on mannitol concentration, and is more pronounced at low concentration. Survival rate of pollen grains is higher at high mannitol concentration than at low concentration for all aperture numbers.

In *Arabidopsis thaliana*, as in most angiosperm species, pollen grains are dispersed in a dehydrated state (Edlund et al., 2004), i.e. their cytoplasmic osmolarity is very high. Therefore, the difference in osmolarity between pollen cytoplasm and the solutions used in our study is likely to be higher with low mannitol concentration than with high concentration. At low mannitol concentration, water inflow is expected to be more pronounced and may occur faster. This is consistent with our observations that at low mannitol concentration plasma membrane of pollen grains tends to break more easily. Moreover, some studies have shown the presence of potassium in the pollen grain cytoplasm (Rehman et al., 2002, 2004), indicating that a potassium gradient might be involved in pollen grain osmolarity. Potassium gradient are present in other plant cells, for example in stomata
guard cells (Talbott and Zeiger, 1996), and this cation is often associated with osmotic water movements.

By comparing lines with zero, three, four, and four to eight apertures, we demonstrated that aperture number has a clear effect on pollen survival at all mannitol concentrations and in both genetic backgrounds: an increase in aperture number is associated with higher vulnerability to osmotic stress. We wanted to find out if an increase in aperture number would weaken the wall, or if more apertures would allow the wall to accommodate volume variations more easily, since apertural areas are more flexible than exine. In most cases, dead pollen grains exhibited a broken plasma membrane without visible exine breakage. Since exine rarely breaks, we can conclude that aperture pattern does not affect the resistance of the exine layer, which might have some elastic properties of its own (as reported in Bolick and Vogel, 1992 and Rowley and Skvarla, 2000). An increase in aperture number, however, increases the number of sites where the plasma membrane can break. Therefore, aperture pattern in *Arabidopsis thaliana* has an effect on the harmomegathic properties of pollen grains, but not directly on the resistance of the exine layer.

Another possible reason for the reduced survival of pollen with higher number of apertures is that more apertures may enable faster water inflow into pollen grains, since water enters pollen through these structures (Heslop-Harrison, 1979a). This may lead to faster cytoplasmic swelling, which in some cases might be too quick to allow the cell membrane to accommodate volume increase. This hypothesis could be tested through observations of water inflow dynamics. Some studies have shown that pollen hydration might be quick, lasting less than a second in some cases (Matsui et al., 1999; Rehman et al., 2002, 2004), though some of these observations were made in vitro. They still point out that quick hydration is not necessarily lethal for pollen grains. Other factors can influence rehydration intensity of the pollen grain, such as the type of stigma (wet or dry: Heslop-Harrison and Shivanna, 1977), or the osmolarity of the stigma solution. We can also expect that rehydration intensity depends on the volume of the pollen grain, since small pollen grains are likely to swell faster than large pollen grains (a small volume being more affected than a large volume with the same water quantity added).

In addition to aperture number, other pollen characteristics, such as shape of pollen grains (Muller, 1979; Halbritter and Hesse, 2004), morphology of apertures (Payne, 1972; Katifori et al., 2010), and presence of pseudocolpori (Scotland et al., 1990; Volkova et al., 2013), have been suggested to influence harmomegathy. However, these studies compared pollen from different species that differed in multiple pollen characteristics, including exine thickness or patterning. In our study, comparisons of *Arabidopsis* mutants with constant wall characteristics enabled us to primarily test the effect of aperture number. We note, however, that we cannot completely rule out the effects of pollen size on harmomegathy. The *lsq6* and *osd1-1* mutants are somewhat larger than the wild-type pollen grains, and such a difference in size could also have an impact on their lower resistance to osmotic stress.

Connection between pollen performance and aperture numbers has been studied previously by looking at germination and longevity of pollen grains in *Viola diversifolia*, a species in which individuals produce both three-aperturate and four-aperturate pollen. That study showed that the four-aperturate pollen grains germinate faster, but possess reduced longevity than the three-aperturate pollen (Dajoz et al., 1991, 1993). In this *Viola* species, there is a trade-off between
longevity (a component of survival) and competitive ability in germination. A study on another heteromorphic Viola species, Viola calcarata (which produces pollen with four and five apertures), has shown that the proportions of the two morphs vary according to altitude (Till-Bottraud et al., 1999). This result has been interpreted as an adaptation to pollinator abundance. Pollinators become scarcer at high altitude, and pollination is thus uncertain. Flowers at high altitude produce a higher proportion of four-aperturate pollen grains, which survive longer than the five-aperturate ones (which, in turn, germinate faster). This strategy is likely to be safer in an environment with few pollinators, where visits are rare and competition between pollen grains to fertilize female gametes is weak. Empirical results on Viola are backed by theoretical approaches: a game theory model, taking into account germination and longevity in a competitive context, has been developed, and the results suggest that heteromorphism is an Evolutionarily Stable Strategy (Till-Bottraud et al., 2001).

Pollen resistance to osmotic stress studied here constitutes another component of survival. We thus point out a new trade-off between germination, longevity, and pollen wall resistance. Triaperturate pollen, the dominant type in eudicots, could result from the trade-off between the germination ability, longevity, and harmomegathic properties. Since the tricolpate pollen is the only morphological synapomorphy of the clade, it has been suggested that this pollen type could be the key innovation of the eudicot clade (Furness and Rudall, 2004). This hypothesis is supported by the studies carried out on the species-rich family Euphorbiaceae (>6700 species) and on a representative set of eudicot species in which the developmental sequence leading to the formation of triaperturate pollen is under strong stabilizing selection (Matamoro-Vidal et al., 2012, 2015).

Some species seem to have evolved particular reproductive strategies to avoid strong volume changes due to intense dehydration and rehydration (Nepi and Pacini, 1993; Nepi et al., 2001). In these species, pollen grains are dispersed in a partially hydrated state: their water content is above 30% (Nepi et al., 2001), whereas in most angiosperm species it is generally between 15% and 30% (Heslop-Harrison, 1979a). These partially hydrated pollen grains do not undergo strong volume decrease before anthesis, and they are able to germinate quickly on the stigma. They are, however, usually short-lived, which means pollination has to occur quickly after anther dehiscence (Nepi et al., 2001; Franchi et al., 2002). This particular reproductive strategy appeared several times independently in angiosperms (Nepi et al., 2001; Franchi et al., 2002) and could be another way to achieve a compromise between the germination and survival. These species almost always have porate apertures (Nepi et al., 2001; Franchi et al., 2002), suggesting a possible link between aperture pattern and reproduction syndrome.

To conclude, we have shown that aperture number in Arabidopsis has an effect on pollen survival under osmotic stress, and that pollen grains with few apertures survive better than pollen with many apertures. Pollen with many apertures is probably maintained in the long run, thanks to trade-offs between the survival rate and the speed of germination. Apertures are involved in several aspects of pollen life, and what is favorable for reproduction is not necessarily good for survival. Many selective pressures might contribute to generation of various morphologies produced during pollen development. As the ancestral state of pollen grains in flowering plants is monoaperturate, the number of apertures has generally increased over time. The great success of the tricolpate pollen of eudicots seems to indicate that it constitutes a good compromise between fast germination and survival. Thanks to the existence of mutants with defective pollen traits, the selective pressures that
have been proposed in broad-scale studies to influence the evolution of pollen morphology can now be studied experimentally.

LITERATURE CITED


R Core Team. 2014. R: A language and environment for statistical computing.


### TABLES

#### Table 1: Summary of GLM results testing the effect of genotype \((\text{col vs inp1-1 vs Isq6})\), mannitol concentration, and the genotype-mannitol concentration interaction, on pollen resistance to osmotic stress. Threshold value: \(\alpha = 0.005\), sample size: \(N = 126\).

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#### Table 2: Summary of GLM results testing the effect of genotype \((\text{wt vs osd1-1})\), mannitol concentration, and the genotype-mannitol concentration interaction, on pollen resistance to osmotic stress. Threshold value: \(\alpha = 0.005\), sample size: \(N = 104\).

<table>
<thead>
<tr>
<th>model</th>
<th>Df</th>
<th>Deviance</th>
<th>Residual Df</th>
<th>Residual deviance</th>
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<td>NULL</td>
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<tr>
<td>genotype</td>
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<td>1864.96</td>
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<tr>
<td>genotype+concentration</td>
<td>2</td>
<td>1049.83</td>
<td>100</td>
<td>815.13</td>
<td>(&lt; 1^{-5})* ***</td>
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</table>
Table 3: Pairwise comparisons between the different plant lines (col, inp1-1, and lsq6) and different mannitol concentrations (0.2 mol.L\(^{-1}\), 0.45 mol.L\(^{-1}\), and 0.7 mol.L\(^{-1}\)). Global threshold value: \(\alpha = 0.005\). Threshold value for each comparison was adjusted using Bonferroni correction. Sample size: \(N = 126\).

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>(X^2)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>col-inp1-1: 0.2</td>
<td>1</td>
<td>90.25</td>
<td>(&lt; 1\times10^{-5}***)</td>
</tr>
<tr>
<td>col-lsq6: 0.2</td>
<td>1</td>
<td>60.41</td>
<td>(&lt; 1\times10^{-5}***)</td>
</tr>
<tr>
<td>inp1-1-lsq6: 0.2</td>
<td>1</td>
<td>282.37</td>
<td>(&lt; 1\times10^{-5}***)</td>
</tr>
<tr>
<td>col-inp1-1: 0.45</td>
<td>1</td>
<td>62</td>
<td>(&lt; 1\times10^{-5}***)</td>
</tr>
<tr>
<td>col-lsq6: 0.45</td>
<td>1</td>
<td>100.45</td>
<td>(&lt; 1\times10^{-5}***)</td>
</tr>
<tr>
<td>inp1-1-lsq6: 0.45</td>
<td>1</td>
<td>294.16</td>
<td>(&lt; 1\times10^{-5}***)</td>
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<tr>
<td>col-inp1-1: 0.7</td>
<td>1</td>
<td>6.75</td>
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<tr>
<td>col-lsq6: 0.7</td>
<td>1</td>
<td>64.61</td>
<td>(&lt; 1\times10^{-5}***)</td>
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<tr>
<td>inp1-1-lsq6: 0.7</td>
<td>1</td>
<td>107.47</td>
<td>(&lt; 1\times10^{-5}***)</td>
</tr>
<tr>
<td>col: 0.2-0.45</td>
<td>1</td>
<td>97.56</td>
<td>(&lt; 1\times10^{-5}***)</td>
</tr>
<tr>
<td>col: 0.2-0.7</td>
<td>1</td>
<td>346.22</td>
<td>(&lt; 1\times10^{-5}***)</td>
</tr>
<tr>
<td>col: 0.45-0.7</td>
<td>1</td>
<td>96.23</td>
<td>(&lt; 1\times10^{-5}***)</td>
</tr>
<tr>
<td>inp1-1: 0.2-0.45</td>
<td>1</td>
<td>67.56</td>
<td>(&lt; 1\times10^{-5}***)</td>
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<tr>
<td>inp1-1: 0.2-0.7</td>
<td>1</td>
<td>152.71</td>
<td>(&lt; 1\times10^{-5}***)</td>
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<tr>
<td>inp1-1: 0.45-0.7</td>
<td>1</td>
<td>21.65</td>
<td>(&lt; 1\times10^{-5}***)</td>
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<tr>
<td>lsq6: 0.2-0.45</td>
<td>1</td>
<td>58.13</td>
<td>(&lt; 1\times10^{-5}***)</td>
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<tr>
<td>lsq6: 0.2-0.7</td>
<td>1</td>
<td>353.68</td>
<td>(&lt; 1\times10^{-5}***)</td>
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<tr>
<td>lsq6: 0.45-0.7</td>
<td>1</td>
<td>139.5</td>
<td>(&lt; 1\times10^{-5}***)</td>
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</tbody>
</table>
Table 4: Pairwise comparisons between the different plant lines (wt vs osd1-1) and different mannitol concentrations (0.2 mol.L\(^{-1}\), 0.45 mol.L\(^{-1}\), and 0.7 mol.L\(^{-1}\)). Global threshold value: \(\alpha = 0.005\). Threshold value for each comparison was adjusted using Bonferroni correction. Sample size: \(N = 104\).

<table>
<thead>
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<th>DF</th>
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<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>osd1-1-wt: 0.2</td>
<td>1</td>
<td>261.04</td>
<td>&lt;1(^{-5})***</td>
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<tr>
<td>osd1-1-wt: 0.45</td>
<td>1</td>
<td>41.25</td>
<td>&lt;1(^{-5})***</td>
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<tr>
<td>osd1-1-wt: 0.7</td>
<td>1</td>
<td>12.47</td>
<td>0.00041 **</td>
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<tr>
<td>osd1-1: 0.2-0.45</td>
<td>1</td>
<td>334.83</td>
<td>&lt;1(^{-5})***</td>
</tr>
<tr>
<td>osd1-1: 0.2-0.7</td>
<td>1</td>
<td>693</td>
<td>&lt;1(^{-5})***</td>
</tr>
<tr>
<td>osd1-1: 0.45-0.7</td>
<td>1</td>
<td>121.11</td>
<td>&lt;1(^{-5})***</td>
</tr>
<tr>
<td>wt: 0.2-0.45</td>
<td>1</td>
<td>65.22</td>
<td>&lt;1(^{-5})***</td>
</tr>
<tr>
<td>wt: 0.2-0.7</td>
<td>1</td>
<td>246.96</td>
<td>&lt;1(^{-5})***</td>
</tr>
<tr>
<td>wt: 0.45-0.7</td>
<td>1</td>
<td>73.44</td>
<td>&lt;1(^{-5})***</td>
</tr>
</tbody>
</table>

FIGURE LEGENDS

Fig. 1: Pollen grains of *Arabidopsis thaliana* with different aperture patterns, in Columbia genetic background. 1-A: Tricolpate pollen with three furrow-like equatorial apertures (col). 1-B: Inaperturate pollen with no apertures (*inp1-1*). 1-C: Tetracolpate pollen with four equatorial apertures (*lsq6*). Confocal images of pollen grains stained with auramine O. Scale bar: 10 µm.

Fig. 2: Pollen of *osd1-1* mutants, in Nooseen genetic background. 2-A: Lower and upper focus of a tetracolpate pollen. 2-B: Lower and upper focus of a pentacolpate pollen, with five furrow-like apertures. The four edging furrows in the lower focus are in continuity with the furrows of the upper focus. 2-C: Lower and upper focus of a six aperturate pollen, the apertures forming the edges of a tetrahedron. 2-D: Lower and upper focus of a seven aperturate pollen. 2-E: Lower and upper focus of an eight aperturate pollen, the apertures forming the edges of a square based pyramid. Epifluorescence microscopy with FITC filter. Scale bar: 10 µm.

Fig. 3: Examples of pollen responses to osmotic stress in our experiments. 3-A: Intact pollen grain. 3-B: Pollen with plasma membrane broken. 3-C: Pollen with exine breakage. Scale bar: 10 µm.

Fig. 4: Percentage of intact pollen for the genotypes *inp1-1*, *col* and *lsq6*, in solutions of different mannitol concentrations (0.2 mol.L\(^{-1}\), 0.45 mol.L\(^{-1}\) and 0.7 mol.L\(^{-1}\)).
Fig. 5: Percentage of intact pollen for the genotypes \textit{wt} and \textit{osd1-1}, in solutions of different mannitol concentrations (0.2 mol.L$^{-1}$, 0.45 mol.L$^{-1}$ and 0.7 mol.L$^{-1}$).
Figure 1
Percentage of intact pollen

- inaperturate
- tri-colpate
- tetra-colpate

Figure 4
Figure 5

Percentage of intact pollen

tri-colpate

wt

four to eight-colpate

osd1-1

0.2

0.45

0.7