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 humid environment by absorbing water from the stigma or surroundings. Resulting fluctuations in water content cause pollen grains to undergo significant changes in volume. Thus, 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 infl ow in *Arabidopsis thaliana*.

**METHODS:** We used three *Arabidopsis thaliana* mutants that differ in the number of apertures in their pollen (zero, four, or a mix of four to eight, respectively) and the wild type with pollen with three apertures. We tested pollen survival in solutions with various mannitol concentrations.

**KEY RESULTS:** The number of intact pollen grains increased with increasing mannitol concentration for all pollen morphs tested. At a given mannitol concentration, however, an increase in aperture number was associated with an increase in pollen breakage.

**CONCLUSIONS:** Aperture patterns, i.e., number, shape, and position, influence the capacity to accommodate volume variations in pollen grains. When subjected to water infl ow, 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** aperture number; *Arabidopsis thaliana*; Brassicaceae; harmomegathy; pollen performance
of aperture number or shape on volume accommodation are not
Apart from these few examples, the connection between aperture
comes unnecessary and tends to disappear (Pettitt and Jermy, 1974).

Exine is interpreted as an adaptation to water dispersal: pollen grains
do not dehydrate before release; thus, the protective outer layer be-
for pollen survival. Aquatic species dispersing pollen directly
in water constitute another example of aperture pattern adaptation.

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. To study
the relationship between the aperture number and the wall mechanici-
性质, we used the wild-type Arabidopsis thaliana and three
Arabidopsis mutants that differed in the number 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, which was taken into ac-
count when analyzing the results. The different plants used here pro-
duce pollen grains with 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. Pol-
len 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 the wall and/
or plasma membrane were broken, and we assumed that pollen
grains survived if they remained intact, regardless of their germina-
tion abilities (which can be affected by several intrinsic and extrin-
sic factors). We evaluated the number of surviving pollen grains for
each genotype and for three levels of osmolarity.

In theory, aperture number can influence pollen resistance to
breakage during hydration in several possible ways. Because aper-
tures are more flexible than other parts of the pollen wall, their in-
creased 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 aper-
tures, more of these sites could result in faster swelling of the grain,
comprising its ability to cope with increased volume; also, in-
creased 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 mor-
tality. We show here that the number of intact pollen grains de-
creases 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 pol-
len aperture number from the wild type, which has tricolpate pollen
grains (Fig. 1A). The inpl-1 mutant produces inaperturate pollen

(Wodehouse, 1935, p. 542). Harmomegathy properties of pollen
grains are expected to mainly depend on apertures (Payne, 1972;
Blackmore and Barnes, 1986; Scotland et al., 1990), although, because
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 one distal aperture is ancestral for the
clade (Furness and Rudall, 2004). This pollen type, called mono-
sulcate, is the main type in early-diverging angiosperms and mono-
cots, though derived types with other aperture patterns can be
found at the generic or the specific level. In eudicots, a very large
clad comprising approximately 75% of the extant angiosperm spe-
cies, 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 was 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 divi-
sion during the male meiosis. Apertures are usually formed at the last
points of contact between the microspores (Wodehouse, 1935; Ressayre
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 dif-
fences in aperture patterns (see, for example, Albert et al., 2010,
2011). Although the relationship between pollen development and ap-
erture patterns has been studied for some time, the link between aper-
ture patterns and pollen fitness has received less attention.

When focusing on apertures, some assumptions can be made re-
garding 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
has a positive effect on pollen fitness, then pollen grains with a high
number of apertures should be widespread in wild species. They are
not, however; the vast majority of angiosperms produce pollen with
one or three apertures, and 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
cues on this issue. These pollen types have different properties: pol-
len with three apertures live longer than pollen with four apertures,
whereas pollen with four apertures germinate faster on the stigma
(Dajoz et al., 1991, 1993). It thus seems that increasing aperture num-
ber is advantageous for on-stigma competition between pollen grains,
but not 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; how-
ever, the exine layer of these pollen grains is uniformly thin, and the
entire wall functions as an aperture (onmiaperturate 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 be-
comes 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 aper-
ture 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 inward 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).

MATERIALS AND METHODS

Arabidopsis lines—We chose several mutants that differ in their pol-
len aperture number from the wild type, which has tricolpate pollen
grains (Fig. 1A). The inpl-1 mutant produces inaperturate pollen
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 4- to 8-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 the wild-type (*wt*, described later) *Arabidopsis* pollen studied here (length = 22.24 μm, SD = 0.68 μm, *n* = 9). Apertures in the 6- and 8-aperturate pollen grains also differ in their distribution on the surface of the pollen grain compared with tricolpate pollen grains, where apertures are equatorial (Fig. 1A). In the 6- and 8-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

![FIGURE 1](image1.png)
Pollen grains of *Arabidopsis thaliana* in the Columbia genetic background with different aperture patterns. (A) Tricolpate pollen with three furrow-like equatorial apertures (*col*). (B) Inaperturate pollen with no apertures (*inp1-1*). (C) Tetracolpate pollen with four equatorial apertures (*lsq6*). Images are from Dobritsa (2012) with kind permission of the author. Scale bar: 10 μm.

![FIGURE 2](image2.png)
Lower and upper focal plane of pollen of *osd1-1* mutants of *Arabidopsis thaliana* in the Nooseen genetic background. (A) Tetracolpate pollen. (B) Pentacolpate pollen, with five furrow-like apertures. The four edging furrows in the lower plane are in continuity with the furrows of the upper plane. (C) Six-aperturate pollen, with apertures forming the edges of a tetrahedron. (D) Seven-aperturate pollen. (E) Eight-aperturate pollen, with apertures forming the edges of a square-based pyramid. Epifluorescence microscopy with a DAPI filter (excitation at 345, emission at 425 nm long pass). Scale bar: 10 μm.
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, tam 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 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 h of dark at 16°C, 16 h of light at 20°C. Experiments were carried out as soon as plants produced flowers (usually 6 wk after sowing). Seeds were sown during summer 2013 (in July and in August) for experiments in September.

**Microscopy**—Each flower line was represented by four to eight individual plants. We used flowers picked from different plants for the experiments. These experiments lasted 3 wk, 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, 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 nonmetabolic 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 et al. (2002) and lies in the optimal range defined by Bouvida and McCormick (2007) (5–15% or 0.15 mol·L⁻¹ to 0.44 mol·L⁻¹). The intermediate mannitol concentration (0.45 mol·L⁻¹) is close to the sucrose concentration used by Li et al. (1999) (18% or 0.53 mol·L⁻¹), and the high mannitol concentration (0.7 mol·L⁻¹) is close to the osmolarity used by Fan et al. (2001) (about 0.68 mol·L⁻¹).

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 H₃BO₃, 1.27 mmol·L⁻¹ of Ca(NO₃)₂·4H₂O, 0.81 mmol·L⁻¹ of MgSO₄·7H₂O. The solution was buffered to pH 6 with 0.2 mmol·L⁻¹ of KH₂PO₄ and 0.05 mmol·L⁻¹ of KH₂PO₄·3H₂O. 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 mistaken for intact pollen grains.

After 2 h (most pollen grains rehydrate within 30 min according to our observations), 100 pollen grains on each slide were scored as being either intact (having intact wall and cytoplasm, Fig. 3A), having a broken plasma membrane (visible as leaking cytoplasm, Fig. 3B), or having a broken wall (Fig. 3C). In Columbia lines (inp1-1, col, and lsq6), 14 slides were used 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 used for each concentration, for a total of 104 slides.

**Statistical analysis**—R software was used for statistical analyses (R Core Team, 2014). The number of intact pollen grains over intact and nonintact pollen grains being a binomial variable, we used a generalized linear model (GLM, which is an extension of the linear model to nonnormal variable) with a 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 were mostly grains with a broken plasma membrane.

The pollen grains of inp1-1 and lsq6 were compared with col, as they all share the Columbia genetic background, while the pollen grains from osd1-1 were compared with 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), or four (lsq6) apertures; it showed a significant effect of genotype, mannitol concentration, and interaction between the two (Table 1). Similarly, a GLM test comparing the number

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**FIGURE 3** Examples of pollen in response to osmotic stress in our experiments. (A) Intact pollen grain. (B) Pollen with plasma membrane broken. (C) Pollen with exine breakage. Scale bar: 10 μm.
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 (Table 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 (Figs. 4, 5): the proportion of intact pollen grains increased with increased mannitol concentration, showing that pollen grains tended to survive better in higher mannitol concentrations ($P < 0.005$, Tables 3, 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 ($P < 0.005$, Table 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 were stronger at low mannitol concentration than at high concentration. In general, pollen grains survived better in high mannitol concentration. Therefore, it is not surprising that differences among pollen types were 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 ($P < 0.005$, Table 4) in all three mannitol concentrations tested (Fig. 5). Here again, the contrast in mortality between the two pollen types was stronger at low mannitol concentration than at high concentration; because the overall mortality rate was lower at high mannitol concentration, the differences between morphs was 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 pollen dispersal and pollen–stigma interactions, volume accommodation is a critical issue.

Our experiments revealed that both aperture number and mannitol concentration affect pollen survival. The more apertures the pollen has, the more likely its plasma membrane will 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 expectation is consistent with our observations that at low mannitol concentration, the plasma membrane of pollen grains tended 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 gradients are present in other plant cells, for example, in stomata guard cells (Telbott 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 was associated with higher vulnerability to osmotic stress. We wanted to find out whether an increase in aperture number would weaken the wall or whether more apertures would allow the wall to accommodate volume variations more easily, since apertural areas are more flexible than the exine. In most cases, dead pollen grains exhibited a broken plasma membrane without visible exine breakage. Since the exine rarely breaks, we can conclude that aperture pattern does not affect

**Table 1.** Summary of GLM results testing the effect of genotype (col vs. inp1-1 vs. lsq6), mannitol concentration, and the genotype × mannitol concentration interaction on pollen resistance to osmotic stress ($\alpha = 0.05$, $N = 126$).

<table>
<thead>
<tr>
<th>Model</th>
<th>df</th>
<th>Deviance</th>
<th>Residual df</th>
<th>Residual deviance</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NULL</td>
<td>125</td>
<td>2452.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>2</td>
<td>685.67</td>
<td>123</td>
<td>1766.55</td>
<td>$&lt;10^{-5}$***</td>
</tr>
<tr>
<td>Genotype + concentration</td>
<td>2</td>
<td>936.18</td>
<td>121</td>
<td>830.37</td>
<td>$&lt;10^{-5}$ ***</td>
</tr>
<tr>
<td>Genotype + concentration + genotype ×</td>
<td>4</td>
<td>17.18</td>
<td>117</td>
<td>813.19</td>
<td>$&lt;0.005$ **</td>
</tr>
<tr>
<td>concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Summary of GLM results testing the effect of genotype (wt vs. osd1-1), mannitol concentration, and the genotype × mannitol concentration interaction on pollen resistance to osmotic stress ($\alpha = 0.05$, $N = 104$).

<table>
<thead>
<tr>
<th>Model</th>
<th>df</th>
<th>Deviance</th>
<th>Residual df</th>
<th>Residual deviance</th>
<th>$P$-value</th>
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<td>103</td>
<td>2108.64</td>
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<tr>
<td>Genotype</td>
<td>1</td>
<td>243.68</td>
<td>102</td>
<td>1864.96</td>
<td>$&lt;10^{-5}$***</td>
</tr>
<tr>
<td>Genotype + concentration</td>
<td>2</td>
<td>1049.83</td>
<td>100</td>
<td>815.13</td>
<td>$&lt;10^{-5}$ ***</td>
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<tr>
<td>Genotype + concentration + genotype ×</td>
<td>2</td>
<td>66.82</td>
<td>98</td>
<td>748.31</td>
<td>$&lt;10^{-5}$ ***</td>
</tr>
<tr>
<td>concentration</td>
<td></td>
<td></td>
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</tbody>
</table>
of pollen hydration might be more apertures is that more apertures 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 more apertures is that more apertures can enable faster water infl ow into pollen grains, since water enters pollen through these structures (Heslop-Harrison, 1979a). This faster uptake 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 infl ow 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. The results still indicate 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.

Pollen performance as it relates to 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 3-aperturate and 4-aperturate pollen. That study showed that the 4-aperturate pollen grains germinate faster, but possess reduced longevity than the 3-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 4-aperturate pollen grains,

![FIGURE 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).](image)

![FIGURE 5 Percentage of intact pollen for the genotypes wt and osd1-1 in solutions of different mannitol concentrations (0.2 mol·L⁻¹, 0.45 mol·L⁻¹, and 0.7 mol·L⁻¹).](image)

### 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) (α = 0.05, N = 126; threshold value for each comparison was adjusted using Bonferroni correction.).

<table>
<thead>
<tr>
<th>Line: Mannitol mol·L⁻¹</th>
<th>df</th>
<th>X²</th>
<th>P-value</th>
</tr>
</thead>
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<td>1</td>
<td>90.25</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>col-lsq6: 0.2</td>
<td>1</td>
<td>60.41</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>inp1-1-lsq6: 0.2</td>
<td>1</td>
<td>282.37</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>col-inp1-1: 0.45</td>
<td>1</td>
<td>62</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>col-lsq6: 0.45</td>
<td>1</td>
<td>100.45</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>inp1-1-lsq6: 0.45</td>
<td>1</td>
<td>294.16</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>col-inp1-1: 0.7</td>
<td>1</td>
<td>6.75</td>
<td>0.0094</td>
</tr>
<tr>
<td>col-lsq6: 0.7</td>
<td>1</td>
<td>64.61</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>inp1-1-lsq6: 0.7</td>
<td>1</td>
<td>107.47</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>col: 0.2-0.45</td>
<td>1</td>
<td>97.56</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>col: 0.2-0.7</td>
<td>1</td>
<td>346.22</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>col: 0.45-0.7</td>
<td>1</td>
<td>96.23</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>inp1-1: 0.2-0.45</td>
<td>1</td>
<td>67.56</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>inp1-1: 0.2-0.7</td>
<td>1</td>
<td>152.71</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>inp1-1: 0.45-0.7</td>
<td>1</td>
<td>21.65</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>lsq6: 0.2-0.45</td>
<td>1</td>
<td>58.13</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>lsq6: 0.2-0.7</td>
<td>1</td>
<td>353.68</td>
<td>&lt;1·5***</td>
</tr>
<tr>
<td>lsq6: 0.45-0.7</td>
<td>1</td>
<td>139.5</td>
<td>&lt;1·5***</td>
</tr>
</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) (α = 0.05, N = 104; threshold value for each comparison was adjusted using Bonferroni correction.).

<table>
<thead>
<tr>
<th>Line: Mannitol mol-L⁻¹</th>
<th>df</th>
<th>X²</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>osd1-1-wt: 0.2</td>
<td>1</td>
<td>261.04</td>
<td>&lt;1·10⁻⁵***</td>
</tr>
<tr>
<td>osd1-1-wt: 0.45</td>
<td>1</td>
<td>41.25</td>
<td>&lt;1·10⁻⁵***</td>
</tr>
<tr>
<td>osd1-1-wt: 0.7</td>
<td>1</td>
<td>12.47</td>
<td>0.00041 **</td>
</tr>
<tr>
<td>osd1-1: 0.2–0.45</td>
<td>1</td>
<td>334.83</td>
<td>&lt;1·10⁻⁵***</td>
</tr>
<tr>
<td>osd1-1: 0.45–0.7</td>
<td>1</td>
<td>693</td>
<td>&lt;1·10⁻⁵***</td>
</tr>
<tr>
<td>osd1-1: 0.7</td>
<td>1</td>
<td>121.11</td>
<td>&lt;1·10⁻⁵***</td>
</tr>
<tr>
<td>wt: 0.2–0.45</td>
<td>1</td>
<td>65.22</td>
<td>&lt;1·10⁻⁵***</td>
</tr>
<tr>
<td>wt: 0.45–0.7</td>
<td>1</td>
<td>246.96</td>
<td>&lt;1·10⁻⁵***</td>
</tr>
<tr>
<td>wt: 0.45–0.7</td>
<td>1</td>
<td>73.44</td>
<td>&lt;1·10⁻⁵***</td>
</tr>
</tbody>
</table>

which survive longer than the 5-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 newly discovered trade-off among germination, longevity, and pollen wall resistance. Triaperturate pollen, the dominant type in eudicots, could result from the trade-off among the germination ability, longevity, and hamromegathic properties. As we noted earlier, since tricolpate pollen is the only morphological synapomorphy of the clade, this pollen type could be the key innovation of the eudicot clade (Furness and Rudall, 2004). This hypothesis is supported by studies 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 (Matamor-Vidal et al., 2012, 2016).

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 pollen of most angiosperm species are more dehydrated, generally between 15 and 30% water (Heslop-Harrison, 1979a). Partially hydrated pollen grains do not undergo a 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 are probably selected 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 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.

ACKNOWLEDGEMENTS

The authors thank L. Saunois and A. Dubois for plant care and A. Ressayre and C. Dillman for helpful discussions on statistics. They also thank S. Nadot for useful comments on the text.

LITERATURE CITED


