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Sulfinylated Azadecalins act as functional mimics of a pollen germination stimulant in *Arabidopsis* pistils

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SUMMARY

Polarized cell elongation is triggered by small molecule cues during development of diverse organisms. During plant reproduction, pollen interactions with the stigma result in the polar outgrowth of a pollen tube, which delivers sperm cells to the female gametophyte to effect double fertilization. In many plants, pistils stimulate pollen germination. However, in *Arabidopsis*, the effect of pistils on pollen germination and the pistil factors that stimulate pollen germination remain poorly characterized. Here, we demonstrate that stigma, style, and ovules in *Arabidopsis* pistils stimulate pollen germination. We isolated an *Arabidopsis* pistil extract fraction that stimulates *Arabidopsis* pollen germination, and employed ultrahigh resolution ESI FT-ICR and MS/MS techniques to accurately determine the mass (202.126 daltons) of a compound that is specifically present in this pistil extract fraction. Using the molecular formula (C₁₀H₁₉NOS) and tandem mass spectral fragmentation patterns of the *m/z* (mass to charge ratio) 202.126 ion, we postulated chemical structures, devised protocols, synthesized *N*-Methanesulfinyl 1- and 2-azadecalins that are close structural mimics of the *m/z* 202.126 ion, and showed that they are sufficient to stimulate *Arabidopsis* pollen germination *in vitro* (30 μM stimulated ~50% germination) and elicit accession-specific response. Although *N*-Methanesulfinyl 2-azadecalin stimulated pollen germination in three species of Lineage I of Brassicaceae, it did not induce a germination response in *Sisymbrium irio* (Lineage II of Brassicaceae) and tobacco, indicating that activity of the compound is not random. Our results show that *Arabidopsis* pistils promote germination by producing azadecalin-like molecules to ensure rapid fertilization by the appropriate pollen.

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INTRODUCTION

Morphogens initiate polarized elongation of cells in a diverse set of processes including mating in yeast (Jackson and Hartwell, 1990) and axon outgrowth in animals (Sanchez-Camacho and Bovolenta, 2009). In flowering plants, polar extension of the pollen tube is critical for fertilization. During flowering plant reproduction, after a pollen grain lands on the female organ (pistil), pollen forms a tube containing two sperm cells, migrates past several pistil tissues (stigma, style, transmitting tract, septum), enters the ovule and delivers both sperm cells to the female gametophyte within an ovule to effect double fertilization (Higashiyama and Hamamura, 2008).

Upon contacting a stigma papilla cell, a strong and highly selective adhesion between pollen and stigma is established (Zinkl *et al.*, 1999). Subsequently, proteins and lipids in both the pollen cell surface and extracellular matrix of the stigma strengthen the adhesion between the pollen and stigma [reviewed in (Edlund *et al.*, 2004; Chapman and Goring, 2010)]. Following pollen adhesion to stigma, the lipid and protein-rich extracellular pollen coating becomes mobilized (termed, 'coat conversion') and migrates to the pollen-stigma interface, forming a "foot" (Elleman *et al.*, 1992). The pollen and stigma components control the water flow from the stigma to pollen through the "foot" and allow the pollen grain to hydrate [reviewed in (Edlund *et al.*, 2004; Chapman and Goring, 2010)].

Influx of water leads to physiological activation of the pollen grain and results in the emergence of a tube from the grain; this is termed pollen germination. Prior to pollen tube emergence, several physiological changes occur in the activated pollen grain (Johnson and McCormick, 2001). Live imaging of calcium dynamics in *Arabidopsis* showed that in the hydrated pollen grain, the calcium concentration increases at the potential germination site and remains elevated until tube emergence (Iwano *et al.*, 2004). These results are consistent with earlier studies that demonstrated an influx of calcium in hydrated pollen grains (Holdaway-Clarke and Hepler, 2003). Distinct cytoplasmic reorganizations accompany the calcium influx and these include the positioning of the vegetative nucleus so that it could enter the tube ahead of the sperm cells (Lalanne and Twell, 2002), cytoskeleton modifications causing formation of filamentous structures that wrap around the nuclei, and actin cytoskeleton polarization toward the site of tube emergence (Heslop-Harrison and Heslop-Harrison, 1992a; Heslop-Harrison and Heslop-Harrison, 1992b). These changes culminate in the formation of a cytoplasmic gradient of calcium beneath the tube emergence site in the pollen grain (Heslop-Harrison and Heslop-Harrison, 1992b; Iwano *et al.*, 2004).

Besides providing water for pollen hydration, the pistil also produces pollen germination stimulants. An unidentified water-, ether- and methanol-soluble factor in *Chrysanthemum* floral organs significantly increased *in vitro* pollen germination (Tsukamoto and Matsubara, 1967). In tobacco and tomato, an unidentified heat-, acid-, base-, DTT- and protease-resistant component from styles, STIL (STyle Interactor for *Lycopersicum esculentum* protein receptor kinases) promotes pollen tube growth, including *in vitro* pollen germination, in a dose-dependent manner (Wengier *et al.*, 2010). A flavonol (kaempferol) in petunia stigma extract stimulates petunia (Mo *et al.*, 1992) and tobacco (Ylstra *et al.*, 1992) pollen germination *in vitro* and biochemically complements germination and growth defects in petunia pollen lacking chalcone synthase (CHS), the enzyme that catalyzes the first step in flavonoid biosynthesis (Mo *et al.*, 1992). It is unclear whether flavonoids stimulate pollen

germination in all plants. For example, pollen of the flavonol-deficient maize *chs* mutant germinates readily *in vitro* and *in vivo* without addition of flavonols (Pollak *et al.*, 1995). Additionally, a null mutation in the single *CHS* gene in Arabidopsis does not affect pollen germination, growth or its ability to fertilize ovules (Burbulis *et al.*, 1996; Ylstra *et al.*, 1996).

In Arabidopsis, pollen germination is rapid *in vivo*; tubes emerged from ~10% and >60% of pollen within 10 and 30 minutes, respectively (Mayfield and Preuss, 2000). Swift germination *in vivo* is strikingly different from the noticeable delay in *in vitro* pollen germination and points to pistil factors that promote pollen germination. However, in Arabidopsis, such factors are unknown. Identification and analysis of such stimulants in Arabidopsis would facilitate molecular-genetic dissection of the pathways that regulate their production and perception. Here we show that stigma, style, and ovules in Arabidopsis pistils stimulate pollen germination. We employed ultra-high resolution mass spectrometry to determine the formula ($C_{10}H_{19}NSO$) and mass spectrometric fragmentation patterns of a compound [m/z (mass to charge ratio) 202.126 ion] specific to an Arabidopsis pistil extract fraction that stimulates pollen germination. We synthesized *N*-Methanesulfinyl 1- and 2-azadecalins that are close structural mimics of the m/z 202.126 ion and showed that they are sufficient to stimulate Arabidopsis pollen germination *in vitro*.

RESULTS

Diffusible pistil factor(s) stimulate Arabidopsis pollen germination

To test if stigma papillae, the first pistil cells that pollen contacts, can stimulate pollen germination, we placed Arabidopsis pollen and excised unpollinated stigmas in close proximity to each other on pollen growth medium. The pollen proximal to stigmas germinated at a significantly higher frequency than pollen without stigmas (Figures 1a,b and Table 1), indicating that a pollen germination stimulant(s) is present in the unpollinated stigmas. To examine if germination stimulation is restricted to the stigma, we also tested other pistil tissues. Pollen placed proximal to an excised stigma and style portion of a pistil (referred hence forth as 'cut pistil') or ovules germinated at a significantly higher frequency than controls without these tissues (Figures 1c,d and Tables 1 and 2). Additionally, there was no stimulation of germination using stems or leaves (Figures 1e,f), indicating that the germination stimulant(s) is either abundant and/or specifically present in pistils.

In these experiments, there was no cell-cell contact between stigma and pollen, indicating that the pollen germination stimulant diffuses from the pistil tissues into the growth medium. To test if the pollen germination stimulant(s) is indeed secreted from the pistil tissues, we excised unpollinated stigmas, placed them on the pollen growth medium for 16 hours, removed the stigmas and then deposited pollen on the same area of the growth medium. Pollen placed on stigma imprints germinated at a significantly higher frequency than pollen placed on medium without any stigma imprint (Figure 1g and Table 1), indicating that pollen germination stimulant diffused from the stigma. Next, we collected pistil tissue secretions (hence forth referred as 'exudate') by incubating excised pistil tissues in liquid pollen growth medium, and tested if the exudate can stimulate pollen germination. Pollen placed proximal to stigma exudate (not shown), or cut pistil exudate or ovule exudate germinated at significantly higher frequency than pollen placed adjacent to blank liquid pollen growth medium (Figures 1h,i and Tables 1 and 2). Additionally, the cut pistil and ovule exudates stimulated pollen germination in a concentration-dependent manner (Figure 1j). Together, these results demonstrate that factor(s) diffusible from unpollinated pistils can stimulate pollen germination *in vitro*.

A small molecule in *Arabidopsis* pistils stimulates pollen germination

To understand the basic properties of the germination stimulation activity and to begin the process of purification, we subjected the cut pistil exudate to a variety of treatments and tested if they affect the stimulation of pollen germination *in vitro* (Table 1). The pollen germination stimulant was heat stable and resistant to Proteinase K and *N*-glycosidase F treatments (Table 1). Additionally, the pollen germination stimulant was active even after a sequential treatment with *N*-glycosidase F and Proteinase K (not shown). These results indicated that the pollen germination stimulant is likely not a protein or a glycoprotein. The pollen germination stimulant was readily soluble in methanol and also active after treating with base and acid. Since the pollen germination stimulant could not be extracted with organic solvents such as ethyl acetate, it is likely hydrophilic, consistent with results showing it is freely diffusible in the pollen growth medium (Figure 1 and Table 1).

To determine the size of the pollen germination stimulant, we separated the cut pistil exudate into >30 kDa and <30 kDa fractions or >3 kDa and <3 kDa fractions and tested them for pollen germination stimulation. The pollen germination stimulant was present in the <30 kDa and <3 kDa fractions (Table 1), demonstrating that the pollen germination stimulant in the cut pistil exudate is a small molecule.

We next evaluated if the pollen germination stimulant in the ovule exudate is similar to the pollen germination stimulant in the cut pistil exudate. The pollen germination stimulant in ovules is also stable after heat exposure, resistant to trypsin and *N*-glycosidase F treatments and was enriched in the <30 kDa fraction (Table 2). These results indicated that the pollen germination stimulant in the ovule exudate is similar to that in the cut pistil exudate.

Arabidopsis pistils lacking flavonols can stimulate pollen germination *in vitro*

Flavonoids are small molecules that function in various plant developmental processes including pollen germination (Taylor and Grotewold, 2005). *Petunia* stigmatic extracts contain flavonols, especially Kaempferol, that can induce pollen germination (Mo *et al.*, 1992). Maize and *petunia* chalcone synthase (*chs*) mutant stigmas lack flavonols and cannot support efficient *in vivo* pollen germination (Mo *et al.*, 1992). To test whether the pollen germination stimulant from *Arabidopsis* pistils is Kaempferol, or a closely-related flavonol, we used the pollen germination bioassay to test the cut pistil exudate from *chs* null mutants, which lack nearly all flavonols (Burbulis *et al.*, 1996; Ylstra *et al.*, 1996). The germination stimulant in the cut pistil exudate from *chs* mutants was indistinguishable from wild type (Table 1), suggesting that it is not a flavonol. These results are consistent with observations that *in vivo* pollen germination was unaffected in *Arabidopsis chs* mutants, which are fertile (Burbulis *et al.*, 1996; Kim *et al.*, 1996; Ylstra *et al.*, 1996). Additionally, the *Arabidopsis* pollen germination stimulant is ethyl acetate-insoluble (Table 1), but flavonols are ethyl acetate-soluble (Han *et al.*, 2007). Together, these observations suggest that the pollen germination stimulant identified in this study is not a flavonol; instead, it is a small molecule with distinct chemical properties.

Isolation of an *Arabidopsis* pistil fraction containing a pollen germination stimulant

To isolate the pollen germination stimulant, we sought to use an extract that can be more readily prepared than the cut pistil exudate. Stigma, style and ovules constitute most of the pistils and these tissues contain a pollen germination stimulant (Tables 1 and 2). Therefore, we prepared an extract from unpollinated, mature pistils. Guided by the properties of the pollen germination stimulant (Table 1), we prepared an ethyl acetate-insoluble and methanol-soluble fraction of the pistil extract and tested if it can stimulate pollen germination. This partially-purified pistil extract stimulated *Arabidopsis* pollen germination

(Figure 1k) in a concentration-dependant manner (Figure 1l), similar to cut pistil and ovule exudates (Figure 1j).

We next separated the partially-purified pistil extract on a reverse-phase C₁₈ column (Figures S1a,b), collected the column flow through and determined whether it can stimulate germination. To facilitate rapid evaluation of pollen germination, we modified our standard method of scoring pollen germination, which incorporated an incubation time of at least seven hours. We determined that germination differences between control and treatment could be readily distinguished even three hours after spotting pollen on the growth medium. Therefore, in subsequent experiments, we scored the pollen germination three hours after spotting pollen on the growth medium. Using this modified assay, we determined that column-eluted material can stimulate pollen germination, with an activity recovery rate of ~33% (not shown). Subsequently, we separated the pistil extract on a reverse-phase C₁₈ column and collected fractions every five minutes. We found that the germination activity eluted in the 15–20 minute and 20–25 minute fractions (Figures S1c). Additional experiments with 1-minute fractions demonstrated that the fractions with a retention time of 18–20 minutes contained the pollen germination stimulant (Figures S1d).

Identification of a m/z 202 ion that is only present in the pollen germination bioassay-positive fraction of an *Arabidopsis* pistil extract

The pollen germination stimulant is present in the <3 kDa fraction; therefore, we compared the bioassay-positive fraction with all the bioassay-negative fractions using Electrospray ionization (ESI) mass spectrometry (MS). This analysis identified in the bioassay-positive fraction an m/z 202 ion (Figure S2a) that was absent in all other bioassay-negative fractions (Figure S2b). Importantly, in the 0–2000 Da mass range, m/z 202 was the only ion that was present in the bioassay-positive fraction and not in any of the bioassay-negative fractions (not shown). Additional analysis of the partially-purified pistil extract by liquid chromatography-mass spectrometry (LC-MS) in Multiple Reaction Monitoring mode indicated that the m/z 202 ion eluted only between 18–20 minutes (Figure S2c), similar to the fractions with a retention time of 18–20 minutes contained the pollen germination stimulant (Figures S1d). Finally, to gain insight into the structure of m/z 202 ion, we fragmented it by tandem mass spectrometry. The MS-MS fragmentation of the m/z 202 ion led to the appearance of a m/z 138 fragment (Figure S2d), indicating that a m/z 64 ion is readily lost from the m/z 202 ion.

Determination of the molecular formula of m/z 202 ion in the pistil extract

These low-resolution MS experiments were not sufficient to determine the elemental composition of m/z 202 and 138 ions. Therefore, we used electrospray ionization (ESI) Fourier-transform ion cyclotron resonance (FT-ICR) measurements. ESI generated the protonated molecule $[M+H]^+$ in the positive ionization mode and FT-ICR determined the molecular weight of the m/z 202 ion with ultrahigh resolution and precision. As observed in low-resolution MS analysis, FT-ICR analysis also detected an ion with a mass of 202.126 Da only in the bioassay-positive fraction and not in the bioassay-negative fractions or buffer control (Figures S3a–c and Figure 2a). This mass corresponds to a chemical formula of C₁₀H₂₀NSO for the $[M+H]^+$ ion (measured: 202.12618, calculated: 202.126011; 0.8 ppm error) and C₁₀H₁₉NSO for the neutral compound (Figure 2b). The fragmentation of the $[M+H]^+$ ion released a m/z 138.12781 fragment (Figure 2b), which is consistent with the m/z 138 ion detected in the low-resolution LC-MS/MS analysis (Figure S2d). The measured loss associated with the m/z 138.12781 fragment is 63.99804, which is consistent with a loss of a methanesulfenic acid (CH₃SOH) moiety from C₁₀H₂₀NSO.

Synthesis of compounds with a molecular formula of $C_{10}H_{19}NSO$

A search of the SciFinder chemical database failed to identify compounds with a molecular formula of $C_{10}H_{19}NSO$ that also have a structure consistent with the MS/MS fragmentation patterns of the m/z 202.126 ion. For example, neither of the two compounds with a sulfoxyl bond ($S=O$) and a molecular formula of $C_{10}H_{19}NSO$ (Figures S4a,b) (Chemla and Ferreira, 2004; Chen *et al.*, 2006) is expected to produce a m/z 138 ion in MS/MS analyses. These findings raised the possibility that the m/z 202.126 ion identified in this study is likely novel.

Spectroscopic methods such as ^{13}C NMR and proton NMR are useful in identifying the chemical structure of unknown organic compounds (Silverstein *et al.*, 1991). However, owing to the small size of Arabidopsis pistils and loss of activity in purification columns, obtaining sufficient amounts of pure material for NMR was not feasible. For example, only $\sim 10\mu g$ of the bioassay-positive fraction was obtained from an extract that was prepared from $>2,000$ pistils and separated on a reverse-phase C_{18} column (not shown) and typically $\sim 1000\mu g$ is required to solve the structure using NMR. Therefore, we used the characteristics of the m/z 202.126 ion such as chemical formula ($C_{10}H_{19}NSO$) and MS/MS fragmentation patterns to postulate chemical structures [*N*-Methanesulfinyl 1-azadecalin (inset in Figure 2c) and *N*-Methanesulfinyl 2-azadecalin (inset in Figure 2e)] for molecules that could be the m/z 202.126 ion, devised protocols to synthesize these compounds (Figures S4c,d and Data S1) and determined their pollen germination stimulant activity *in vitro* (see below).

The synthesized sulfinylated azadecalins (S-azadecalins) were essentially pure (CHN analysis; Data S1) and yielded spectral data consistent with the proposed structures (GC/MS and NMR, not shown). They possessed a molecular weight of 202.126 (FT-ICR; Figures 2c and 2e), and released a m/z 138.127 ion after a loss of the CH_3SOH fragment (MS/MS fragmentation analyses; Figures 2d and 2f), similar to the m/z 202.126 ion in the pistil extract. We synthesized two additional compounds (10-(*N*-Methanesulfinyl)-10-azabicyclo[4.3.1]decane and 3,3-dimethyl-1-(thiomorpholino)butan-1-one; Figures S4e,f and Data S1) with the same chemical formula ($C_{10}H_{19}NSO$) but different structures than that of S-azadecalins. The NMR (not shown), ESI FT-ICR (Figures S5a and S5c) and MS/MS (Figures S5b and S5d) analyses of these compounds were consistent with their postulated chemical structures. The cellular effects of these four compounds are not known.

Sulfinylated azadecalins stimulate pollen germination *in vitro*

The S-azadecalins stimulated *in vitro* pollen germination in a concentration-dependent manner (Figures 3a,c–e), similar to the cut pistil and ovule exudates (Figure 1) and pistil extract (Figures 1 and 3b). The quinoline precursors used to synthesize S-azadecalins (Figures S4c,d) did not stimulate pollen germination at any concentration (not shown). Unlike the S-azadecalins, the other two synthesized compounds elicited a germination response only at very high concentrations ($\geq 500\mu M$; Figure 3f). The S-azadecalins triggered pollen germination about two hours earlier than the no compound control (Figure 3g and Movie S1). The S-azadecalins did not affect pollen tube growth rate (Figure 3g), and unlike LURE proteins (Okuda *et al.*, 2009) and chemocyanin (Kim *et al.*, 2003), did not attract pollen tubes (not shown). Additionally, tubes germinated in the presence of S-azadecalins did not exhibit polarity defects such as bulbous or branched tubes (Malhó *et al.*, 2006; Sousa *et al.*, 2008) (not shown). These results indicated that the S-azadecalins primarily promote pollen germination and are sufficient to trigger pollen germination *in vitro*.

We tested if the stimulatory effect of S-azadecalins is specific to the pollen growth medium formulation we used (Li *et al.*, 1999). The S-azadecalins enhanced pollen germination

(Figure 4) in two other growth medium protocols optimized for *Arabidopsis* pollen (Boavida and McCormick, 2007; Bou Daher *et al.*, 2008). The S-azadecalins also enhanced the germination frequency of *quartet* pollen, which exhibits low *in vitro* germination rate (Boavida and McCormick, 2007), in a concentration-dependent manner (Figure 5). Finally, the S-azadecalins stimulated pollen germination in liquid growth medium without agarose (Figure 4), ruling out the possibility that a component in agarose interacted with S-azadecalins to stimulate pollen germination.

Sulfinylated Azadecalins are close structural mimics of the m/z 202.126 ion in the *Arabidopsis* pistil extract

We next tested whether the endogenous m/z 202.126 ion in pistils is an S-azadecalin by comparing in the C_{18} column retention times of each S-azadecalin with that of the m/z 202.126 ion in the pistil extract. When eluted from reverse-phase C_{18} column individually, the retention times of both *N*-Methanesulfinyl 1-azadecalin and *N*-Methanesulfinyl 2-azadecalin were 25–27 minutes (not shown). These retention times are longer than m/z 202.126 ion in the pistil extract, which eluted after 18–20 minutes (Figure S2c). The m/z 202.126 ion is a component of a partially purified extract; on the contrary, S-azadecalins are relatively pure. To rule out the possibility that the retention time of m/z 202.126 ion in the pistil extract is affected by other components in the partially purified extract, we spiked the pistil extract with either *N*-Methanesulfinyl 1-azadecalin or *N*-Methanesulfinyl 2-azadecalin and then separated the mixtures on the C_{18} column. Mixed samples also showed that the retention times of both S-azadecalins on a C_{18} column were 25–27 minutes, which is longer than that of the m/z 202.126 ion in the pistil extract (Figure S6a,b), suggesting that S-azadecalins are perhaps less hydrophobic than the m/z 202.126 ion in the pistil extract. These observations indicated the S-azadecalins are distinct from the m/z 202.126 ion in the pistil extract. However, because they shared structural similarities (Figure 2), we concluded that the S-azadecalins are close structural mimics of the m/z 202.126 ion in the pistil extract. Additionally, because they primarily act to stimulate pollen germination, we considered the S-azadecalins to be functional mimics of a pollen germination stimulant in *Arabidopsis* pistils.

Sulfinylated azadecalins show accession-specific effects on pollen germination stimulation

Despite structural isomerism, both azadecalins stimulated pollen germination. These results raise the possibility that pollen germination stimulants are highly divergent and consequently, the pollen response to the germination stimulants is also highly variable. To determine if there is natural variation in response to pollen germination stimulation, we exposed pollen to S-azadecalins and compared the germination response to these compounds between Columbia and Landsberg accessions of *Arabidopsis*. Similar to their effect on Columbia pollen, both S-azadecalins stimulated Landsberg pollen germination (Figure 6a–e and Movie S2). However, we observed accession-specific pollen germination response to the two azadecalins. Whereas *N*-Methanesulfinyl 2-azadecalin elicited maximal germination in Columbia pollen (Figure 3e), *N*-Methanesulfinyl 1-azadecalin induced maximal germination in Landsberg pollen (Figure 6e and Movie S2). The S-azadecalin-specific effect on the time elapsed before the first Columbia pollen tube emerged was not detected in Landsberg pollen (Figures 3h and 6g). Finally, the modest increases in growth rate and terminal length of Landsberg pollen tubes in response to S-azadecalins were not detected in Columbia (Figures 3g and Figure 6f). These results point to a diversity in pollen response to S-azadecalins and present an opportunity to use genetic variation between accessions to map and identify the pollen-expressed genes that mediate these responses.

Sulfinylated azadecalins stimulate germination of pollen from specific species

In two divergent plants such as petunia and tobacco, flavonols stimulated *in vitro* pollen germination (Mo *et al.*, 1992; Ylstra *et al.*, 1992). However, flavonols are not required for Arabidopsis pollen germination (Burbulis *et al.*, 1996; Ylstra *et al.*, 1996) and the pollen germination stimulation was at wild type levels in the absence of flavonols (Table 1). Also, a ~3.5kDa molecule (STIL), that is partially peptidic in nature, promotes tomato pollen germination in a dose-dependent manner (Wengier *et al.*, 2010). These observations point to the diversity among pollen germination stimulants in plants. To explore the species-specificity of pollen germination stimulant function, we examined the response of pollen from other plants to *N*-Methanesulfinyl 2-azadecalin. We tested pollen from four species within the Brassicaceae [Lineage I represented by Arabidopsis (*Arabidopsis thaliana*), *Olimarabidopsis pumila* and *Capsella rubella* and Lineage II represented by *Sisymbrium irio*; (Beilstein *et al.*, 2006)] and tobacco (*Nicotiana tabacum*) from Solanaceae. Similar to Arabidopsis, in *O. pumila*, at all concentrations tested, a significant increase in germination frequency was observed (Table 3). In *C. rubella*, a significant increase in germination frequency was observed in two (50 and 100 μ M) of the three concentrations tested. However, *N*-Methanesulfinyl 2-azadecalin did not stimulate *S. irio* or tobacco pollen germination (Table 3) at any of the concentrations tested. While the compound activity is not species-specific, the specificity is apparent in Lineage I, but not in Lineage II species (*S. irio*), suggesting *N*-Methanesulfinyl 2-azadecalin-like stimulants may have evolved after the split of these two lineages ~42 MYA (Beilstein *et al.*, 2010). Alternatively, *N*-Methanesulfinyl 2-azadecalin may induce germination in pollen that typically exhibits low germination frequency *in vitro*. Additional analysis involving more plants from all three lineages in Brassicaceae will be required to distinguish between these possibilities.

DISCUSSION

Pistil tissues promote pollen germination in Arabidopsis

Using the *in vitro* pollen germination bioassay, we defined a role for Arabidopsis pistils in promoting pollen germination, finding that the Arabidopsis pistils contain a germination stimulant that significantly hastens tube emergence from the pollen grain. The pollen germination stimulant accumulates in unpollinated pistils and thus may mediate the rapid pollen germination observed in Arabidopsis pistils (Mayfield and Preuss, 2000). Besides Arabidopsis, other plants also produce pollen germination stimulants (Mo *et al.*, 1992). Pollen germination stimulation may have evolved to ensure rapid fertilization within the narrow developmental window during which ovules are receptive. In this model, the acceleration of pollen tube emergence increases the likelihood of fertilization, as subsequent steps such as pollen tube growth and guidance to ovules, require several hours to complete (Tsukamoto *et al.*, 2010).

Besides the stigma, the site of *in vivo* pollen germination, the pollen germination stimulant is present in other pistil tissues, raising the possibility that it has additional functions in the pistil. One possibility is that the germination stimulant also induces growth of pollen tubes and its secretion from the pistil cells may help the pollen tube sustain its growth over the considerable length of the pistil. Indeed, the terminal length of pistil-grown pollen tubes is longer than those grown *in vitro* (Palanivelu and Johnson, 2010) and the terminal length of Landsberg pollen tubes is significantly longer in the presence of S-azadecalins (Figure 6f).

The *m/z* 202.126 ion with a chemical formula of $C_{10}H_{19}NSO$ is a candidate pollen germination stimulant in Arabidopsis

Our experiments with *chs* mutants demonstrated that flavonols are not the major pollen germination stimulant in Arabidopsis pistils. Instead, we found that a small hydrophilic

molecule in the pistils is a potent stimulator of pollen germination in *Arabidopsis*. We isolated a germination bioassay-positive pistil fraction that contains a pollen germination stimulant. By mass spectrometry analysis, we identified a m/z 202 ion in this positive fraction and using FT-ICR analysis, we determined that $C_{10}H_{19}NSO$ is the molecular formula of the m/z 202.126 ion. Based on the following observations, we consider the m/z 202.126 ion in the pistil extract a candidate pollen germination stimulant. First, the m/z 202.126 ion is the only ion (in the mass range 0–2000 daltons) that is present in the pollen germination bio-assay positive fraction of a pistil extract but not in any of the bioassay-negative fractions. Second, the m/z 202.126 ion is also present in the stigma exudate that is capable of stimulating pollen germination (not shown). Third, S-azadecalins, structural mimics of the m/z 202.126 ion in the pistil extract, were sufficient to stimulate pollen germination *in vitro*. Taken together, we propose that the m/z 202.126 ion in the *Arabidopsis* pistil contains pollen germination stimulation activity.

The uncommon stability and solubility properties of a pollen germination stimulant in the pistil are also reflected in the chemical structural characteristics of the S-azadecalins, which also exhibit pollen germination stimulant activity. The S-azadecalins contain a sulfinamide group (with a sulfur-oxygen double bond and a sulfur-nitrogen single bond; Figure S4c,d). Sulfinamides are known to cross link peptides (Raftery and Geczy, 2002). However, they are not prevalent in small molecules with bioactivity or those that elicit a cellular response, perhaps because of their hydrolytic lability (Piggott and Karuso, 2007).

Putative roles of sulfinylated azadecalins in stimulating pollen germination *in vitro*

We showed that S-azadecalins stimulate *Arabidopsis* pollen germination *in vitro*. In *Arabidopsis* pistils, pollen hydration is completed in ~5 minutes (Mayfield and Preuss, 2000) and *in vitro*, *Arabidopsis* pollen hydrates almost instantaneously after coming in contact with the pollen growth medium (not shown). Therefore, S-azadecalins likely stimulate pollen germination *in vitro* at some step after hydration, such as regulating calcium dynamics or actin cytoskeletal reorganization (Johnson and McCormick, 2001).

In vivo, the pollen grain is hydrated through the ‘foot’ at the pollen-stigma interface, which perhaps serves as a conduit for a pollen germination stimulant from the pistil and contributes to a swift establishment of the pollen germination site. However, pollen grains placed on the growth medium do not form a foot, and instead hydrates by an isotropic water influx throughout the pollen grain surface. These differences could delay the establishment of the tube emergence site *in vitro* and delay pollen germination. The S-azadecalins could stimulate *in vitro* pollen germination by facilitating a quick establishment of the tube emergence site in the hydrated pollen grain.

In *Arabidopsis*, physiological and molecular analyses have identified markers that specify the future pollen germination site and the S-azadecalins may function by affecting these regulators of pollen germination. The concentration of calcium increases at the potential germination site of a hydrated pollen grain *in vivo* and *in vitro* (Heslop-Harrison and Heslop-Harrison, 1992b; Iwano *et al.*, 2004). In *Arabidopsis* pollen and pollen tubes, ROP1 (Rho-related GTPase) preferentially localizes to the apical plasma membrane of the pollen germination site and the pollen tube tip (Gu *et al.*, 2004). This polar distribution of ROP1 is dependent on ROP1 interacting protein (RIP1), which is localized in the nucleus of mature pollen grain but redistributes to the future pollen germination site after hydration (Li *et al.*, 2008). We propose that perception of S-azadecalins by a receptor on the pollen surface mimics stigma-induced activation of a signaling pathway that either regulates calcium gradient establishment at the germination site or affects polar RIP1 localization.

Sulfinylated azadecalins are useful tools to study the molecular mechanisms that mediate pollen germination response to an external stimulus

In this study we determined the chemical properties of a small molecule in the pistil extract, synthesized structural mimics of this compound, and tested if they elicit a biological response. We suggest this targeted chemical biological approach to identify biologically active small molecules if material for structural studies is limiting. Additionally, the functional mimics will allow characterization of the molecular and physiological mechanisms that underlie their biological activity even before the *in vivo* compounds are identified. For example, the S-azadecalins will serve as a useful tool to synchronize and elicit rapid pollen germination in Arabidopsis and begin identification of molecular mechanisms underlying stimulation of pollen tube emergence in response to a specific signal using genetic (Lalanne and Twell, 2002; Johnson *et al.*, 2004; Johnson-Brousseau and McCormick, 2004), genomic (Qin *et al.*, 2009), physiological (Iwano *et al.*, 2004) and cell biological (Heslop-Harrison and Heslop-Harrison, 1992a) approaches, even before the pollen germination stimulant in pistils is identified.

EXPERIMENTAL PROCEDURES

Plant and growth conditions

Arabidopsis plants were grown as described (Qin *et al.*, 2009). Pollen was from Columbia accession unless indicated otherwise. The *male sterile 1* mutant, *ms1* (CS75) and three other species of Brassicaceae were from the Arabidopsis Biological Resource Center. Pistil extract, exudate and pistil tissues were from *ms1*. Chalcone synthase null mutant alleles used in this study are *tt4-2yy6* and *tt4-SALK_020583*. Tobacco (Petit Havana SR1) was grown under greenhouse conditions.

Isolation of Arabidopsis tissues

Stigmas: stage 12b flowers were emasculated and 24 hours later, unpollinated pistils were excised and placed on a double-sided sticky tape; then, with the aid of a dissecting scope, stigmas (four/experiment) were excised with a syringe needle (27.5 gauge) and were placed upside down on the pollen growth medium (PGM), so that only the stigmatic papillae contacted the PGM.

Stigma imprint: sixteen hours after placing stigmas as described above, the stigmas were removed and pollen was placed on the stigma imprint area.

Other tissues: Cut pistils [obtained as described (Palanivelu and Preuss, 2006)], cut stems and leaf discs were excised (five each/experiment) and placed vertically on the PGM. Ovules were excised from five pistils/experiment as described (Palanivelu and Preuss, 2006).

Exudate preparation

Fifteen unpollinated cut pistils or ovules from fifteen unpollinated pistils were placed in 50µL of liquid PGM [prepared as described in (Li *et al.*, 1999)] and incubated for 12 hours in a humid chamber (30–40% relative humidity) at room temperature (~24°C). The liquid PGM containing the exudate was separated from tissues by micropipetting and was either tested immediately or stored for later use at –20°C.

Exudate treatments

The exudates from cut pistils or ovules were subjected to different treatments and then tested in the pollen germination bioassay. Details of various treatments of exudates are provided in the Supporting Information (Methods S1).

Pistil extract preparation

Thirty-six pistils were ground in liquid nitrogen and 150 μ L of liquid PGM without sucrose. Following centrifugation (5 minutes @ 13,000 rpm), the supernatant was extracted three times with two volumes of ethyl acetate and each time, the ethyl acetate-insoluble fractions were pooled together in a fresh Eppendorf tube and air dried in a fume hood at room temperature for at least six hours. The ethyl acetate-insoluble material was resuspended in 900 μ L of methanol, vortexed (few seconds), and centrifuged (2 minutes @ 13,000 rpm). The methanol-soluble fraction was air-dried, resuspended in 30 μ L of liquid pollen growth medium and tested in *in vitro* pollen germination bioassay with solid PGM plates. For liquid chromatography, the extract from ~36 pistils was separated on C₁₈ columns.

In vitro pollen germination bioassay with tissues, exudate and extract

Pollen growth medium: Unless indicated otherwise, the PGM was prepared as described (Li *et al.*, 1999). To liquid PGM (pH 7.0), agarose (Bio-Rad) was added (0.5% final concentration), boiled in a microwave for 3 minutes and 30 seconds or until the agarose is completely dissolved, with intermittent swirling to mix the contents, poured into nunc plastic Petri dishes (35mmX10mm, Thermo Fisher Scientific; 2mL/Petri dish) and cooled for 30 minutes before use.

Dispensing exudate and pistil extract: 30 μ L of exudate or fractions of exudate after different treatments or pistil extract or pistil extract fractions from C₁₈ column were dissolved in 30 μ L of liquid PGM and dispensed into a dry, empty hole (3mm diameter and 2mm deep) in the solid PGM.

Pollen placement: Pollen grains (~50–200) from anthers of a stage 13 or 14 flower (Smyth *et al.*, 1990) were directly placed no farther than 1 mm from the tissue or the hole containing exudate, extract and fractions from C₁₈ column. To avoid flower-to-flower variation, pollen from the same flower was placed in control (hole containing 30 μ L of liquid PGM) and test plates. The plates were incubated at 22°C in a humid chamber (~80% relative humidity).

Pollen germination frequency: At indicated time after pollen placement, pollen germination in the plates was imaged in Axiovert 100 microscope (Carl Zeiss, Germany) using Metamorph image acquisition software (www.metamorph.com). Pollen germination frequency and tube lengths were determined using ImageJ 1.44f (<http://rsb.info.nih.gov/ij/download.html>) and Metamorph softwares. A pollen grain with a tube length equal to or greater than the diameter of the grain was considered to have germinated. Pollen germination percentage was calculated using the formula [(# of grains germinated/ # of grains deposited) X 100].

In vitro pollen germination bioassay with synthesized compounds

Germination bioassay with solid PGM: Appropriate amounts of methanol-dissolved compounds were completely air-dried at room temperature, resuspended in 30 μ L of liquid PGM, and dispensed into the hole in the solid PGM. Pollen placement and germination frequency measurements were performed as described above. Pollen tube growth rate was determined by calculating the regression coefficient from linear regression of mean pollen tube length and time over a period beginning at the initiation of pollen tube elongation and until maximum pollen tube length is reached.

Bioassay with liquid PGM: The liquid PGM without any compounds (control) or PGM-dissolved compounds (treatment) was spotted on a glass slide (Thermo Fisher Scientific). The PGM liquid meniscus was directly touched by dehiscing anthers to release the pollen and the slide was inverted (Hicks *et al.*, 2004). 2–10 flowers were used to provide pollen for each experiment. The slides were incubated at 22°C in a humid chamber. Three hours after spotting pollen, PGM was removed and mounted with 60% glycerol. Pollen germination frequency was determined as described above.

Statistical analysis

Mean germination percentages for each assay were used as primary data for analysis of germination percentage. Shapiro-Wilk statistics (Shapiro and Wilk, 1965) were calculated for germination percentage using PROC UNIVARIATE in SAS/STAT Version 9.1 of the SAS System for Windows (SAS Institute). Pollen germination percentage was arcsine transformed and subjected to mixed-model analysis of variance in which replicate was considered a random effect and treatment a fixed effect. Analysis was done using PROC MIXED in SAS/STAT and nontransformed least squares means are reported. Pairwise comparisons (two-sided) of least squares means were accomplished using the PDIF option within PROC MIXED. In experiments examining exudate treatments, independent *in vitro* assays and various treatments of the exudate were considered as replicates and treatment factors, respectively. In experiments examining the effects of concentrations of synthesized *N*-Methanesulfinyl 2-azadecalin on other plant species, independent *in vitro* assays were considered replicates and concentrations were treatments with separate analysis for each species.

Liquid Chromatography

Pistil extract (36 pistils) were separated via HPLC, using a Paradigm MS4B (multi-dimensional separations module; Michrom BioResources) equipped with a RP VYDAC C₁₈ column. Details on the HPLC analysis are provided in the Supporting Information (Methods S1).

Mass Spectrometric Analysis

Low-resolution MS analysis of pistil extract was performed using ABI/SCIEX 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer (Applied Biosystems). Details of low-resolution MS analysis are provided in the Supporting Information (Methods S1).

Molecular weight determination and MS/MS analysis using FT-ICR

Mass determination and fragmentation experiments were performed on a 9.4 T Bruker ApexQh FT-ICR (Bruker Daltonics). Detailed descriptions of FT-ICR analysis are provided in the Supporting Information (Methods S1).

Synthesis of compounds with a chemical formula of C₁₀H₁₉NSO

Detailed descriptions of synthesis protocols, assignment of identity and quality control results (¹H NMR and ¹³C NMR data and elemental analysis) on the purity of four compounds reported in the study are provided in the supporting information (Data S1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of a pollen germination stimulant in Arabidopsis pistils

Light micrographs of *in vitro* pollen germination in the presence of (a) stigmas, (b) no tissue, (c) the stigma and style portion of a pistil, (d) ovules, (e) inflorescence stems, (f) leaves, (g) imprint of four stigmas (SI) within the dashed line, (h) cut pistil exudate (CPE), (i) ovule exudate (OE), and (k) ethyl acetate-insoluble and methanol-soluble fraction of entire pistil extract (PE).

(j,l) Graphs of concentration-dependent stimulation of pollen germination by CPE and OE (j) and PE (l); error bars represent the standard deviation of at least three replicates.

(a–j) Pollen germination was recorded seven hours after depositing pollen on the growth media.

(h,i,k) The hole in the growth medium containing CPE, OE and PE (h,i,k, respectively) is on the top left of each image and is beyond the field of view.
Scale bars, 100 μm .

Figure 2. FT-ICR analysis of m/z 202.126 ion in the pistil extract and sulfinylated azadecalins
 (a,b) ESI FT-ICR (a) and QCID MS/MS fragmentation spectra (b) of m/z 202.126 ion present only in the bioassay-positive fraction of pistil extract. * in (b), unrelated to pistil extract peaks due to electrical noise.
 (c-f) ESI FT-ICR (c,e) and QCID MS/MS fragmentation mass spectra (d,f) of m/z 202.126 ion in *N*-Methanesulfinyl 1-azadecalin [inset (c)] and *N*-Methanesulfinyl 2-azadecalin [inset (e)].

Figure 3. Stimulation of Arabidopsis pollen (Colombia accession) germination by sulfinylated azadecalins

(a–d) Light micrographs of *in vitro* pollen germination in the presence of pollen growth medium (PGM) only (a), pistil extract (PE) from 12 unpollinated pistils (b), *N*-Methanesulfinyl 1-azadecalin (*N*-m 1-A; c), or *N*-Methanesulfinyl 2-azadecalin (*N*-m 2-A; d). Scale bars, 100 μm .

(e) Pollen germination stimulation by *N*-Methanesulfinyl 1-azadecalin and *N*-Methanesulfinyl 2-azadecalin. Error bars represent the standard deviation of data from three replicates. Letters indicate least squares means that are significantly different ($P \leq 0.05$, from mixed-model analysis of variance) in pairwise comparisons of germination percentage at

different concentrations of either *N*-Methanesulfinyl 1-azadecalin (a–c; filled squares) or *N*-Methanesulfinyl 2-azadecalin (d–h; open squares).

(f) A table showing the minimum concentration of $\geq 10\%$ pollen germination stimulation by synthesized compounds.

(g) Graph of pollen tube growth. Error bars represent standard deviation of lengths of at least ten pollen tubes grown in liquid pollen growth medium (PGM) only or with the addition of 50 μM of *N*-Methanesulfinyl 1-azadecalin (*N*-m 1-A) or *N*-Methanesulfinyl 2-azadecalin (*N*-m 2-A).

(h) Table of time elapsed prior to emergence of the first pollen tube grown in liquid pollen growth medium (buffer control) only or with the addition of 50 μM of *N*-Methanesulfinyl 1-azadecalin or *N*-Methanesulfinyl 2-azadecalin.

(a–g) Pollen germination was recorded three hours after depositing pollen on the growth media.

a

Compound	Media ^a	Media type	Con ^b (μM)	PG ^c (fraction)	PG (%)	SD ^d	LSM ^e	P Value ^f
<i>N</i> -Methanesulfinyl 1-azadecalin	BM	solid	0	1/709	0.08	0.14	0.03	-
	BM	solid	25	21/759	2.59	2.15	2.27	0.1072 (NS)
	BM	solid	50	50/715	7.32	7.45	6.21	0.0164
	BM	solid	100	65/833	10.43	5.43	9.93	0.0053
	DCG	solid	0	1/358	0.16	0.28	0.05	-
	DCG	solid	25	74/574	14.08	18.97	10.48	0.0603 (NS)
	DCG	solid	50	93/329	27.06	9.78	26.67	0.0078
	DCG	solid	100	89/378	23.75	10.97	23.21	0.0112
<i>N</i> -Methanesulfinyl 2-azadecalin	BM	solid	0	1/358	0.30	0.52	0.10	-
	BM	solid	25	74/574	3.07	1.43	2.97	0.0036
	BM	solid	50	93/329	5.54	2.77	5.34	0.0006
	BM	solid	100	89/378	34.78	10.10	34.53	<0.0001
	DCG	solid	0	0/568	0.00	0.28	0.00	-
	DCG	solid	25	16/480	3.68	18.97	3.62	0.0249
	DCG	solid	50	226/536	33.57	9.78	33.19	<0.0001
	DCG	solid	100	246/481	55.26	10.97	55.30	<0.0001
<i>N</i> -Methanesulfinyl 1-azadecalin	BM	liquid	0	126/1244	10.13	3.42	9.90	-
	BM	liquid	25	303/1177	25.74	2.45	25.98	0.0009
	BM	liquid	50	374/1231	30.38	10.97	29.91	0.0002
	BM	liquid	100	462/1139	40.56	2.66	40.78	<0.0001
<i>N</i> -Methanesulfinyl 2-azadecalin	BM	liquid	0	60/1090	5.50	3.00	5.07	-
	BM	liquid	25	148/979	15.12	12.97	15.50	0.0108
	BM	liquid	50	214/951	22.50	7.14	20.92	0.0016
	BM	liquid	100	250/918	27.23	3.69	27.37	0.0003

^aComposition of pollen growth media as described by BM (Boavida and McCormick, 2007) or DCG (Bou Daher *et al.*, 2008).

^bConcentration (Con) of compounds tested.

^cNumber of pollen germinated (PG) three hours after spotting in four *in vitro* assay replicates and germination rate of ≥ 80 pollen grains was measured in each replicate.

^dSD, standard deviation.

^eLeast squares mean of germination percentage (LSM).

^f*P* values for pairwise comparisons of LSMs from mixed-model analysis of variance of pollen germination in no compound control (0 μM) and each of the three concentrations (25, 50 and 100 μM) of the compound within a grouping. *P* values ≤ 0.05 were considered to indicate statistically significant differences between the indicated treatment and no compound control (0 μM) within a grouping. NS, differences between LSMs of the indicated treatment and no compound control (0 μM) within a grouping were not statistically significant.

Figure 4. Sulfinylated azadecalins stimulate Arabidopsis pollen germination in liquid and two other pollen growth media formulations

(a) Arabidopsis pollen germination stimulation by synthesized azadecalins in solid or liquid pollen growth media.

(b–i) Micrographs of *in vitro* pollen germination in liquid pollen growth media as described in (Boavida and McCormick, 2007) in the presence of indicated concentrations of *N*-Methanesulfinyl 1-azadecalin (b–e) or *N*-Methanesulfinyl 2-azadecalin (f–i).

(a–i) Pollen germination was recorded three hours after depositing pollen on the growth media.

Scale bars, 100 μm.

Figure 5. Sulfinylated azadecalins stimulate *Arabidopsis quartet* mutant pollen germination (a–h) Micrographs of *in vitro* germination of *quartet* pollen in liquid pollen growth media (Boavida and McCormick, 2007) in the presence of indicated concentrations of *N*-Methanesulfinyl 1-azadecalin (a–d) or *N*-Methanesulfinyl 2-azadecalin (e–h). Scale bars, 100 μ m.

(i) Table of *Arabidopsis quartet* pollen germination stimulation by synthesized azadecalins in liquid pollen growth media (Boavida and McCormick, 2007).

(a–i) Pollen germination was recorded three hours after depositing pollen on the growth media.

Figure 6. Effect of sulfinylated azadecalins on Arabidopsis pollen (Landsberg accession) germination

(a–d) Light micrographs of *in vitro* pollen germination in the presence of (a) pollen growth medium (PGM) only, (b) pistil extract (PE) from 12 unpollinated pistils, (c) *N*-Methanesulfinyl 1-azadecalin (*N*-m 1-A), or (d) *N*-Methanesulfinyl 2-azadecalin (*N*-m 2-A). Scale bars, 100 μ m.

(e) Pollen germination stimulation by *N*-Methanesulfinyl 1-azadecalin and *N*-Methanesulfinyl 2-azadecalin. Error bars represent the standard deviation of data from three replicates. Letters indicate least squares means that are significantly different ($P \leq 0.05$, from mixed-model analysis of variance) in pairwise comparisons of germination percentage at

different concentrations of either *N*-Methanesulfinyl 1-azadecalin (a-e; filled squares) or *N*-Methanesulfinyl 2-azadecalin (f-h; open squares).

(f) Graph of pollen tube growth. Error bars represent standard deviation of lengths of at least ten pollen tubes grown in liquid pollen growth medium (PGM) only or with the addition of 50 μ M of *N*-Methanesulfinyl 1-azadecalin (*N*-m 1-A) or *N*-Methanesulfinyl 2-azadecalin (*N*-m 2-A).

(g) Table of time elapsed prior to emergence of the first pollen tube grown in liquid pollen growth medium (PGM) only or with the addition of 50 μ M of either *N*-Methanesulfinyl 1-azadecalin or *N*-Methanesulfinyl 2-azadecalin.

(a-g) Pollen germination was recorded seven hours after depositing pollen on the growth media.

Table 1

Characterization of pollen germination stimulant in Arabidopsis organs and cut pistil exudate

Treatment	PG ^a	PG ± s.d. ^b (%)	n ^c	LSM ^d	P value ^e
PGM	1446/3759	37.42 ± 10.49	30	37.18	-
Stigma	462/536	85.56 ± 6.19	3	85.99	< 0.0001 *
Stigma imprint	795/950	81.94 ± 11.63	3	82.87	< 0.0001 *
Cut pistil	1009/1161	86.40 ± 4.59	7	86.68	< 0.0001 *
CPE	405/523	77.45 ± 4.94	5	77.74	-
PGM	210/535	39.15 ± 4.94	5	39.11	< 0.0001 *
<i>chs</i> CPE (<i>tt4-020483</i>)	389/499	77.96 ± 6.09	5	78.22	0.7765 (NS)
<i>chs</i> CPE (<i>tt4-2YY6</i>)	427/538	79.15 ± 5.74	5	79.41	0.4714 (NS)
CPE	2575/3310	77.80 ± 5.62	28	78.08	-
Heat-treated CPE	692/915	75.62 ± 8.45	6	76.63	0.396 (NS)
CPE	433/573	75.39 ± 1.78	5	75.41	-
CPE+ProteinaseK	451/583	77.22 ± 3.80	5	77.31	0.3781 (NS)
PGM+ProteinaseK	177/576	30.74 ± 3.69	5	30.72	< 0.0001 *
CPE	519/649	80.39 ± 7.41	5	80.81	-
CPE+GlycosidaseF	505/635	79.40 ± 7.67	5	79.87	0.2519 (NS)
PGM+GlycosidaseF	306/658	45.56 ± 10.96	5	45.56	0.0029 *
CPE	523/630	83.06 ± 3.72	5	83.2	-
CPE insoluble in methanol	210/555	37.90 ± 5.12	5	37.84	< 0.0001 *
CPE soluble in methanol	447/557	80.08 ± 3.86	5	80.20	0.014 (NS)
CPE	428/534	80.30 ± 5.90	6	80.61	-
CPE+Acid	461/595	77.49 ± 6.67	6	77.79	0.1965 (NS)
CPE+Base	505/597	84.74 ± 7.33	6	85.26	0.1424 (NS)
CPE insoluble in ethylacetate	454/590	76.90 ± 7.49	6	77.29	0.2119 (NS)
CPE soluble in ethylacetate	201/580	34.81 ± 4.81	6	34.75	< 0.0001 *

Treatment	PG ^a	PG ± s.d. ^b (%)	n ^c	LSM ^d	P value ^e
CPE	606/767	79.32 ± 8.97	5	79.93	-
> 30 kDa CPE	277/798	35.08 ± 7.50	5	34.94	0.0015 *
< 30 kDa CPE	576/803	71.34 ± 10.21	5	71.87	0.2428 (NS)
CPE	340/455	74.77 ± 2.85	5	74.81	-
> 3 kDa CPE	183/531	34.67 ± 3.39	5	34.63	< 0.0001 *
< 3 kDa CPE	373/473	78.70 ± 5.40	5	78.90	0.2176 (NS)

^aFraction of pollen grains germinated (PG) seven hours after spotting pollen in *in vitro* assay replicates and germination rate of ≥ 80 pollen grains were measured in each *in vitro* assay.

^bs.d., standard deviation.

^cN, number of independent *in vitro* assays.

^dLeast squares mean pollen germination percentage (LSM).

^eP values for pairwise comparisons of LSMs from mixed-model analysis of variance of pollen germination of indicated tissue with no tissue control (PGM) or between indicated treatment and the corresponding independently collected cut pistil exudates (CPE) from 15 pistils.

P values ≤ 0.01 denoted with an asterisk (*) indicate statistically significant differences between LSMs of cut pistil and cut stem treatments or between LSMs of indicated treatment and CPE within a grouping.

NS, differences between LSMs of indicated treatment and CPE within a grouping were not statistically significant.

chs CPE, CPE collected from indicated null mutant alleles of *CHALCONE SYNTHASE*.

Table 2
Characterization of pollen germination stimulant in Arabidopsis ovule exudate

Treatment	PG ^a	PG ± s.d. ^b (%)	n ^c	LSM ^d	P value ^e
Ovules	643/753	85.23 ± 3.85	9	85.45	
PGM	542/1375	39.52 ± 12.34	9	39.15	0.0128*
OE	1197/1393	85.63 ± 4.48	9	85.91	-
PGM	542/1375	39.52 ± 12.34	9	39.16	< 0.0001*
Heat-treated OE	960/1147	83.78 ± 4.65	6	84.21	0.1204 (NS)
> 30 kDa OE	557/1318	42.84 ± 3.98	8	42.82	
< 30 kDa OE	1012/1241	81.17 ± 3.42	8	81.28	< 0.0001*
OE + Trypsin	254/313	81.15 ± 1.44	2	81.17	
PGM + Trypsin	94/328	28.01 ± 16.79	2	27.18	0.0361*
OE + GlycosidaseF	600/709	84.53 ± 4.09	5	84.71	
PGM + GlycosidaseF	284/671	42.19 ± 2.32	5	42.18	< 0.0001*

^aFraction of pollen grains germinated (PG) seven hours after spotting pollen in *in vitro* assay replicates and germination rate of ≥ 80 pollen grains was measured in each assay.

^bStandard deviation (s.d.).

^cNumber (N) of independent *in vitro* assays.

^dLeast squares mean of pollen germination percentage (LSM).

^eP values for pairwise comparisons of LSMs from mixed-model analysis of variance of pollen germination of cut pistil and cut stem or between indicated treatment and the corresponding independently collected ovule exudate (OE) from ovules excised from 8 pistils in liquid pollen growth medium or heat-treated OE and OE or between indicated pair-wise comparisons.

P values ≤ 0.05 denoted with an asterisk (*) indicate statistically significant differences between LSMs of OE and the indicated treatment or the two treatments within each pairing.

NS, difference between LSM of OE and heat-treated OE was not statistically significant.

Table 3
Effect of synthesized *N*-Methanesulfinyl 2-azadecalin on pollen from other plants

Plant species	Con ^b (μ M)	PG ^c	PG $\pm$ s.d. ^d (%)	LSM ^e	P value ^f
<i>Arabidopsis thaliana</i> ^g	0	0/568	0.00 $\pm$ 0.28	0.00	-
	25	16/480	3.68 $\pm$ 18.97	3.62	0.0249*
	50	226/536	33.57 $\pm$ 9.78	33.19	<0.0001*
	100	246/481	55.26 $\pm$ 10.97	55.30	<0.0001*
<i>Olinarabidopsis pumila</i>	0	55/766	7.18 $\pm$ 2.83	6.83	-
	25	377/1029	36.64 $\pm$ 15.16	39.00	0.0014*
	50	283/741	38.19 $\pm$ 0.99	38.24	0.0015*
	100	391/788	49.62 $\pm$ 12.6	50.75	0.0004*
<i>Capsella rubella</i>	0	211/1018	20.73 $\pm$ 9.39	20.55	-
	25	377/1042	36.18 $\pm$ 6.99	37.22	0.0555 (NS)
	50	470/1180	39.83 $\pm$ 14.66	40.27	0.0325*
	100	519/1267	40.96 $\pm$ 9.68	42.11	0.0238*
<i>Sisymbrium irio</i>	0	402/899	44.72 $\pm$ 6.17	45.11	-
	25	371/919	40.37 $\pm$ 9.44	42.46	0.6572 (NS)
	50	460/901	51.05 $\pm$ 6.20	50.23	0.4036 (NS)
	100	456/939	48.56 $\pm$ 5.01	49.03	0.5176 (NS)
<i>Nicotiana tabacum</i>	0	462/1263	36.60 $\pm$ 3.27	36.51	-
	25	480/1309	36.67 $\pm$ 15.70	36.01	0.9348 (NS)
	50	565/1397	40.44 $\pm$ 5.27	40.46	0.5235 (NS)
	100	451/1234	36.55 $\pm$ 2.42	36.52	0.9981 (NS)

^aData from Figure 4.
^bConcentration (Con) of *N*-Methanesulfinyl 2-azadecalin tested.
^cFraction of pollen grains germinated (PG) three hours after spotting in three *in vitro* assay replicates and germination rate of $\geq$ 120 pollen grains was measured in each assay.

^dStandard deviation (s.d.).

^eLeast squares mean of pollen germination percentage (LSM).

^f*P* values for pairwise comparisons of LSMs from mixed-model analysis of variance of pollen germination in no compound control (0 μ M) and each of the three concentrations (25, 50 and 100 μ M) of the compound.

P values ≤ 0.05 denoted with an asterisk (*) indicate statistically significant differences between LSMs of the indicated concentration and the no compound control (0 μ M) within a species grouping. NS, differences between LSMs of indicated treatment and no compound control (0 μ M) within a species grouping were not statistically significant.

Composition of pollen growth media used in this experiment was as described in (Bou Daher *et al.*, 2008).