The *Arabidopsis* alkaline ceramidase TOD1 is a key turgor pressure regulator in plant cells

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Turgor pressure plays pivotal roles in the growth and movement of walled cells that make up plants and fungi. However, the molecular mechanisms regulating turgor pressure and the coordination between turgor pressure and cell wall remodelling for cell growth remain poorly understood. Here, we report the characterization of *Arabidopsis TurgOr regulation Defect 1* (*TOD1*), which is preferentially expressed in pollen tubes and silique guard cells. We demonstrate that TOD1 is a Golgi-localized alkaline ceramidase. *tod1* mutant pollen tubes have higher turgor than wild type and show growth retardation both in pistils and in agarose medium. In addition, *tod1* guard cells are insensitive to abscisic acid (ABA)-induced stomatal closure, whereas sphingosine-1-phosphate, a putative downstream component of ABA signalling and product of alkaline ceramidases, promotes closure in both wild type and *tod1*. Our data suggest that TOD1 acts in turgor pressure regulation in both guard cells and pollen tubes.

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A fundamental question in biology is what drives cells to grow and migrate. In animal cells, the cytoskeleton acts as the driving force\(^1,^2\), whereas in the walled cells that make up plants and fungi, turgor pressure is thought to play pivotal roles\(^3\). Cellular turgor pressure is exerted by water moving in response to cellular concentration of ions, sugars and other solutes. Turgor pressure is necessary for cell enlargement, growth and shape maintenance. Pollen tubes and guard cells are two types of highly specialized plant cells, which evolved to allow adaption to life on land\(^5\). In guard cells, turgor pressure is the driving force for stomatal movement. In pollen tubes, turgor pressure is also essential for tip growth. Although turgor pressure is not directly correlated with growth rate, tube growth stops when the turgor pressure is lower than a threshold level\(^8,^9\). Before reaching a plateau, the pollen tube growth rate increases as turgor pressure rises\(^10\). Furthermore, increased external pressure reduces the growth rate\(^8,^10\). Although these in vitro studies demonstrated that turgor pressure is critical for pollen tube growth, the mechanisms of turgor pressure regulation and the coordination between turgor pressure and the cell wall for in vitro growth, that have attracted plant biologists for a long time are still poorly understood.

In angiosperms, after landing on the stigma, pollen grains hydrate and germinate, and each produces a pollen tube—a single-celled structure that delivers the sperm cells to the ovule\(^11\). A pollen tube penetrates into the style, and into the transmitting tract, then emerges onto the surface of the septum and grows along the funiculus and is targeted to the micropyle of the ovule\(^12,^13\). The pollen tube finally bursts when it reaches one of the two synergids of the ovule, and the two sperm cells are released to enable double fertilization\(^14\). During this process, pollen tubes need to pass through different tissues of the pistil including papillar cells of the stigma, the transmitting tract, the septum and the filiform apparatus\(^13\). To penetrate these ‘physical barriers’ of the pistil, pollen tubes must regulate their turgor pressure during in vivo growth.

Sphingolipids are ubiquitously present in all eukaryotic cells and in a few bacteria\(^15\). They are thought to be essential for membrane structure and are also involved in signal transduction, controlling essential cellular processes such as apoptosis, cell migration, differentiation and inflammation\(^15\). Ceramide, sphingosine, sphingosine-1-phosphate (SIP) and their derivatives are examples of a class of well-studied bioactive sphingolipids in animals. Ceramidases hydrolyse ceramide to yield sphingosine and fatty acid. They play a key role in sphingolipid metabolism, being responsible for regulatory activity at the cellular level. Ceramidases are classified as acidic, neutral or alkaline, based on their optimal pH for biological activity. Based on sequence homology, the Arabidopsis genome encodes one alkaline phytoceramidase\(^16\) and three neutral ceramidases\(^17\), but their functions have not been characterized.

Here, we show that TurgOr regulation Defect 1 (TOD1) is a bona fide alkaline ceramidase that is preferentially expressed in pollen tubes and in silique guard cells, where it is required for turgor pressure regulation. Disruption of TOD1 results in partial male sterility, which is due to defects of pollen tube growth in penetrating the pistils. tod1 mutant pollen tubes also show growth retardation inside agamosperm; this retardation is rescued by application of sphingosine or SIP. These data suggest that maintenance of turgor pressure likely controlled by TOD1 is needed for pollen tube growth in pistil. Furthermore, our work provides novel insights into the role of sphingolipid signalling pathways during pollen tube growth in plants.

**Results**

**Isolation of the tod1 mutant.** We screened gene trap lines of Arabidopsis thaliana ecotype Landsberg erecta\(^18\) for mutants defective in pollen tube growth in vivo and thereby identified one mutant, designated TurgOr regulation Defect 1 (tod1). Mutant plants bore short siliques resulting from severe sterility (Fig. 1a). The average silique length of homozygous tod1 was about one-half that of the wild-type (Fig. 1b) and only about 18% of the available ovules were fertilized to produce seeds (Fig. 1c). The F\(_1\) progeny of the heterozygous mutant showed a 1:1 (271:265, \(\chi^2 = \chi^2_{0.01} = 0.1\)) segregation ratio of kanamycin-resistant (Kan\(^R\)) to kanamycin-sensitive (Kan\(^S\)), indicating that the Ds insertion affect the function of either the male or female gametophyte\(^19\). To determine which sex was affected, we conducted reciprocal crosses. We observed that only mutant pollen tubes in Ds \(\chi^2_{0.01}\) segregation ratio of Kan\(^R\):Kan\(^S\) in the F\(_1\) progeny was 0.025:1 (15:601), indicating that NPTII was barely transmitted by the male gametophyte. These data confirmed that the mutation affects the male gametophyte.

**tod1 is defective in in vivo pollen tube growth.** To investigate the cause of male sterility, mature pollen grains were stained with Alexander’s stain and with 4’,6-diamidino-2-phenylindole (DAP) and analysed by microscopy. The cytoplasm of tod1 pollen was red purple after Alexander’s staining (Supplementary Fig. 1a), indicating that the viability of the mutant pollen grains was not affected\(^20\). DAPI staining showed two bright sperm nuclei and one more diffuse vegetative nucleus, as found in wild-type plants (Supplementary Fig. 1b). To determine whether the mutation caused defects in pollen germination and/or pollen tube growth, we checked in vitro pollen germination rates and tube growth. Both wild-type and the mutant pollen yielded above 75% germination after 5 h incubation (Supplementary Fig. 1c). There were also no differences between in vitro pollen tube length of wild type and mutant after 5 h germination (Supplementary Fig. 1d).

To further check whether tod1 had defects in in vivo pollen tube growth, wild-type and mutant pollen tubes growing in Ler pistils were stained with aniline blue. tod1 pollen tubes showed reduced growth potential (Supplementary Fig. 2), and only a few pollen tubes had reached the middle of the pistil 24 h after pollination, whereas wild-type pollen tubes had reached the base of the pistil (Fig. 1d and Supplementary Fig. 2). We also checked the status of pollen tubes 48 h after pollination, when almost all ovules were fertilized and pollen tubes began to degenerate in the wild type (Supplementary Fig. 3); most tod1 pollen tubes were still in the top half of the pistil, and many of them had begun to degenerate (Supplementary Fig. 3). Consistent with the in vivo phenotype of pollen tubes, the seeds distribution in tod1 siliques was distorted, with more seeds at the stigma end (Fig. 1e). It seemed that the mutant pollen tubes had lost the ability to penetrate efficiently through the style.

To confirm whether the low seed set phenotype was also caused by defects in pollen tube guidance in the gametophytic phase, we observed tod1 pollen tubes in detail by aniline blue staining and scanning electron microscopy (SEM). Once the mutant pollen tubes penetrated the septum walls, they grew along the funiculus and then entered the micropyle of the ovule to complete fertilization as seen for wild-type tubes (Supplementary Fig. 4a–d). Limited pollinations showed that only about 10% of tod1 pollen tubes in the transmitting tract could go through the septum wall; more than 95% of septum-penetrating tod1 pollen tubes could enter the micropyle of the ovule.
Supplementary Fig. 4e). This suggests that pollen tube guidance in the gametophytic phase was not affected in the mutant.

**Molecular cloning of TOD1.** We performed thermal asymmetric interlaced PCR to obtain the flanking genomic sequence of the Ds element. Sequencing indicated that the Ds element was inserted into the first exon of At5g46220, 114-bp downstream of the ATG (Fig. 2a). The Ds insertion resulted in an 8-bp duplication (5'-CTCTCAAG-3') at the insertion site. Furthermore, we were unable to detect TOD1 transcripts in inflorescence RNA, indicating that tod1 was a null allele (Fig. 2b).

To confirm that the Ds insertion in TOD1 was indeed the cause of the mutant phenotype, we transformed a 5,557-bp genomic DNA fragment of TOD1, containing the predicted promoter region, open reading frame and 3'-untranscribed region (3'-UTR) into homozygous tod1 plants. Eighty-seven independent T1 transgenic lines were obtained. We randomly selected ten lines for analysis of silique length and seed set. Fertility was restored in complemented mutant plants (Supplementary Table 1), indicating that TOD1 was sufficient to rescue pollen tube growth defect. In addition, SALK_110396, which harboured a T-DNA insertion in the first exon of At5g46220 (Fig. 2a), named tod1-2, also displayed the similar phenotype as observed in tod1-1.

TOD1 is predicted to encode a 462-amino-acid protein, with a transmembrane domain (residues 20–39), and a DUF616 domain (residues 107–409; Fig. 2c), but no other homology to proteins with known functions. However, the PANTHER (Protein ANalysis THrough Evolutionary Relationships) analysis classified TOD1 as a member of a family of alkaline ceramidases, although they have no similarity at the amino-acid level (http://www.pantherdb.org/). We generated a phylogenetic tree using TOD1 orthologues from other plant species and other eukaryotic organisms, all of which belong to the alkaline ceramidase family. Interestingly, these members clustered into two clades: one contains plant-specific proteins and the other includes proteins belonging to other eukaryotic organisms (Supplementary Fig. 5a). The plant-specific clade, including TOD1 from Arabidopsis, contains members from eudicots including grape (Vitis vinifera), soybean (Glycine max), Medicago truncatula, poplar (Populus trichocarpa) and monocots such as rice (Oryza sativa). These members’ functions are currently not known. The other clade contains...
members from yeast (*Saccharomyces cerevisiae*), human (*Homo sapiens*) and mouse (*Mus musculus*). Their functions have been characterized as alkaline ceramidases 24–26, alkaline phytoceramidases16,27 or alkaline dihydroceramidases28,29. The sequence similarity between these two clades is very low, but they share high similarity within their clades (Supplementary Fig. 5b).

The region comprising amino acids 133–458 is predicted as a putative alkaline phytoceramidase domain by PANTHER. (d) Real-time RT-PCR of *TOD1* expression pattern in different tissues of Arabidopsis. Data are mean ± s.e. (*n* = 4).

(e–h) Cell-specific *pTOD1:gTOD1-GUS* expression. (e) Mature pollen grain, (f) pollen tube, (g) guard cells on a silique and (h) magnification of the area indicated in g. Scale bars, 50 μm.

**Figure 2** Detection of mutations causing partial male sterility in *tod1* mutants and expression pattern of *TOD1*. (a) Genomic structure of *TOD1*. Ds and T-DNA inserted into the first exon at 114-bp and 186-bp downstream of start codon in *tod1*-1 and *tod1*-2, respectively. (b) Full-length *TOD1* transcripts are absent in the mutant. Total RNA from inflorescences was reverse-transcribed and amplified by PCR. (c) Scheme of *TOD1*. It contains a transmembrane domain at the N terminus (residues 20–39), and a DUF616 domain (residues 107-409). Amino acids 133–458 are annotated as an alkaline phytoceramidase domain by PANTHER. (d) Real-time RT-PCR of *TOD1* expression pattern in different tissues of Arabidopsis. Data are mean ± s.e. (*n* = 4).

(e–h) Cell-specific *pTOD1:gTOD1-GUS* expression. (e) Mature pollen grain, (f) pollen tube, (g) guard cells on a silique and (h) magnification of the area indicated in g. Scale bars, 50 μm.

**Figure 3** *TOD1* is a *bona fide* alkaline ceramidase localized in the Golgi apparatus. (a) CLSM images showing that *TOD1-mCherry* co-localizes with Golgi marker GFP-Rab2. *pLat52::TOD1-mCherry* and *pLat52::GFP-Rab2* were co-transfected and transiently expressed in tobacco pollen tubes. Scale bar, 20 μm. (b) *TOD1-eGFP* localization in an *Arabidopsis* pollen tube from plants stably transformed with *pTOD1::gTOD1-eGFP*. Scale bar, 20 μm. (c, d) *TOD1* has alkaline ceramidase activity. Expression of *TOD1* was induced in yeast mutant Δypc1Δydc1, which lacks two endogenous yeast ceramidases. Total microsomes from Δypc1Δydc1 containing empty vector pYes2 (Vec) or pYes2-Flag-TOD1 (*TOD1*) incubated with the fluorescent substrate NBD-ceramide. The third lane is the NBD-fatty acid standard. (c) pH 7.0. (d) pH 9.4.
set of the mutant. Neither TOD1A110-462 nor TOD1A11-39 could complement the mutant phenotype. These results indicate that both the transmembrane domain and putative photoceramidase domain are critical for TOD1 function.

**TOD1 is expressed in pollen and silique guard cells.** Real-time reverse transcription (RT)–PCR showed that TOD1 transcripts were present in flowers and siliques, were barely detectable in roots, stems or leaves (Fig. 2d). To further investigate the cellular expression pattern of TOD1, we examined pTOD1::βTOD1-GUS transgenic lines which express β-glucuronidase (GUS); TOD1 was preferentially expressed in pollen grains, pollen tubes and silique guard cells (Fig. 2e–h). To characterize the subcellular localization of TOD1, we co-transformed pLat52::TOD1-mCherry with endomembrane system markers into tobacco (Nicotiana tabacum) pollen grains by bombardment. Confocal laser scanning microscopy (CLSM) showed that TOD1 co-localized only with Golgi marker GFP-Rab2 (Fig. 3a). Moreover, pollen tubes from transgenic Arabidopsis plants expressing TOD1-enhanced green fluorescent protein (eGFP) under its native promoter also showed similar localization pattern (Fig. 3b). Thus, TOD1 is a Golgi-localized protein in pollen tubes.

**TOD1 is a bona fide alkaline ceramidase.** To test whether TOD1 has ceramidase activity, we inserted the full-length TOD1-coding sequence with a FLAG tag at its amino terminus into the yeast expression vector pYES2, under the control of the GAL1 promoter. The resulting construct was transformed into the yeast mutant ΔycplΔycd1, lacking two endogenous yeast ceramidases YPC1p and YDC1p28. The expression of Flag-tagged TOD1 was induced by galactose and confirmed by western blot analysis using an anti-Flag antibody (Supplementary Fig. 6a). Total microsomal extracts were prepared from yeast cells and used for enzyme activity assays with NBD-C12-phytoceramide (with the C-4 hydroxyl group) or NBD-C12-ceramide (with the Δ4-double bond) as substrate. The thin-layer chromatography (TLC) showed that phytoceramide is not a substrate for TOD1 (Supplementary Fig. 6b), but ceramide is (Fig. 3c,d). Furthermore, TOD1 displayed stronger ceramidase activity at pH 9.5 than at pH 7.0 (Fig. 3c,d). Taken together, we conclude that TOD1 is an alkaline ceramidase.

**TOD1 regulates turgor in guard cells and pollen tubes.** As TOD1 is expressed both in pollen tubes and in silique guard cells, we checked whether the stomata on siliques had defects in response to abscisic acid (ABA) or S1P. Stomata on siliques had similar response to ABA as stomata on leaves (Fig. 4a). ABA at concentration of 10 μM promotes pre-opened stomata closing in Ler, but not in tod1 (Fig. 4a), indicating that tod1 is insensitive to exogenous ABA. ABA can activate sphingosine kinases (SphKs), which will phosphorylate sphingosine, the product of the ceramidase reaction, and subsequently increase S1P level30. S1P is known to play a positive role in stomatal closure31. Interestingly, exogenous S1P (10 μM) promoted closure of pre-opened stomata both in Ler and tod1 (Fig. 4b). These data suggested that TOD1 is also involved in ABA-induced guard cell turgor regulation, likely acting downstream of ABA and upstream of S1P.

To confirm whether the in vivo growth defects of tod1 pollen tubes could be caused by altered turgor pressure, we germinated pollen grains inside germination medium with 1.5% agarose, in which the external pressure was higher than normal conditions. We attributed this inability to disregulation of turgor pressure of the mutant pollen tubes. Detailed re-sequencing result, we crossed two T-DNA insertion lines of Arabidopsis plants expressing TOD1-enhanced green fluorescent protein (eGFP) under its native promoter also showed similar localization pattern (Fig. 3b).

**Discussion**

To date, the role of turgor pressure in pollen tube growth is controversial33–36. Here, we identified a Ds insertion mutant, tod1, in which in vivo pollen tube growth was defective. Disruption of TOD1 caused partial male sterility, resulting from the reduced growth potential of mutant pollen tubes. Detailed examination indicated that the ability of tod1 pollen tubes to penetrate pistil tissues was severely compromised. tod1 pollen tubes also showed growth defects inside medium solidified with 1.5% agarose, in which the external pressure was higher than normal conditions. We attributed this inability to disregulation of turgor pressure of the mutant pollen tubes. Pollen tube growth is regulated by the coordination of turgor pressure and cell wall stiffness. Turgor pressure is thought to be the driving force, whereas the cell wall is thought to be the speed setter. For example, application of high concentrations of pectinase caused apical swelling or bursting of pollen tubes, whereas application of moderate concentration pectinase stimulated tube growth37. It was therefore hypothesized that pectinase...
that digest pectins, the main cell wall component of pollen tubes, sets the tube growth rate by regulating the physical characteristics of the tube cell wall. Silencing of the gene encoding tobacco pollen pectin methylesterase involved in the building up of the pollen tube wall strength, resulted in retarded in vivo pollen tube growth, whereas in vitro pollen tube growth was not affected\(^8\). This suggests that the stiffness of pollen tube wall should be kept at an optimum. Turgor pressure also needs to be maintained at an optimal and in a narrow range, neither too high nor too low, because mutation of \(\text{tod1}\) pollen tubes may also affect the building up of the pollen tube wall strength, which might also cause in vitro retarded growth. To resist the increased turgor pressure from bursting, \(\text{tod1}\) mutant pollen tubes might deposit much more wall material, which in turn would display reduced growth potential. Finding that \(\text{gaut13}\) acts as a suppressor of \(\text{tod1}\) supports this hypothesis, because mutation of \(\text{GAUT13}\) has quantitative effects on cell wall deposition.

Sphingolipids are ubiquitous in eukaryotes and some bacteria. The biosynthesis and metabolism of sphingolipids have been well studied for the last two decades. Ceramide is mainly synthesized in the ER (endoplasmic reticulum), and is then transported to the Golgi apparatus for further modifications. \(\text{TOD1}\) shares low sequence similarity with reported alkaline ceramidases from human\(^{25}\) and mouse\(^{24}\). We showed here that \(\text{TOD1}\) possesses
ceramide activity optimally at alkaline pH and it is localized to the Golgi apparatus. Human alkaline ceramidase 2 (hacER2), which shows a pH optimum of 8.0, is also localized to the Golgi apparatus. In eukaryotic cells, different organelles exhibit variable pH to fulfill different requirements for protein function within them. Although the average pH value of the Golgi is about 6.4 (ref. 39), it can be alkalized by Na\(^+\)/H\(^+\) exchangers on its membrane. It is possible that rapid activation of these ion exchangers might generate highly alkalized area in close proximity to Golgi membranes. Furthermore, TOD1 also had weak ceramidase activity at pH 7.0, so it is likely that a basal level of activity is present in the Golgi at neutral pH. The possible coupling between TOD1 activity and the activation of ion exchangers in Golgi membrane is of special interest for further investigation.

In animal cells, ceramide, sphingosine, S1P and their derivatives are attracting considerable interest because of their roles in intra- and extracellular signalling. However, recent studies using lipidomic analyses suggested that the abundance of Δ4-unsaturated sphingolipids is low in Arabidopsis. This is not consistent, because details for fruulipomar and colleagues and our data, several key enzymes involved in metabolism of Δ4-unsaturated sphingolipids metabolism are restrictedly expressed in limited cell types. Therefore, sphingolipids signalling may function only in specific plant cells. S1P had been proposed to act positively in stomatal closure from pharmacological experiments. Stomatal activity is also regulated by turgor pressure. Our results indicate that stomatal closure is defective in guard cells of tod1 siliques, and this can be rescued by exogenous application of S1P, confirming that TOD1 acts upstream of S1P in stomatal closure. In pollen tubes, disruption of TOD1 also results in higher turgor pressure, and the pollen tube growth retardation caused by increased external pressure can be rescued by exogenous application of S1P. These findings suggest that TOD1 acts similarly both in guard cells and pollen tubes, where it is preferentially expressed.

Based on sequence homology, six SpHKS were identified in the Arabidopsis genome, three of which LCBK1 (ref. 45), SpHk1 (ref. 43) and SpHk2 (ref. 44), were confirmed to have kinase activity towards sphingosine. As these three SpHK genes are all expressed in pollen tubes, and SpHK1 and 2 in the cell body, we generated an artificial mRNA-based knockdown allele targeting SpHk1 and 2 in lcbk1 background. However, these transgenic lines did not show tod1-like pollen tube phenotype in pistil. This is most likely due to genetic redundancy, and there may be other genes, acting as SpHk, which have not been characterized yet.

In conclusion, we demonstrated that TOD1 is a bona fide alkaline ceramidase that plays a critical role in turgor pressure regulation during pollen tube growth in vivo and in stomatal closure. This opens up an opportunity to dissect the role of sphingolipids in turgor pressure control in plant cells. Further studies on S1P signalling and sphingolipidomics will likely provide novel insights into turgor pressure regulation in plant cells.

**Methods**

**Plant materials and growth conditions.** Arabidopsis thaliana ecotypes Landsberg erecta (Ler) or Columbia (Col) were used as the wild type. tod1-2 (SALK_110396) was obtained from Arabidopsis Biological Resource Center (ABRC) (SALK_150132) was obtained from Li-Qun Chen (China Agricultural University), YC3.60 transgenic seeds were obtained from Megumi Iwano (Nara Institute of Science and Technology). In animal cells, ceramide, sphingosine, S1P and their derivatives are attracting considerable interest because of their roles in intra- and extracellular signalling. However, recent studies using lipidomic analyses suggested that the abundance of Δ4-unsaturated sphingolipids is low in Arabidopsis. This is not consistent, because details for fruulipomar and colleagues and our data, several key enzymes involved in metabolism of Δ4-unsaturated sphingolipids metabolism are restrictedly expressed in limited cell types. Therefore, sphingolipids signalling may function only in specific plant cells. S1P had been proposed to act positively in stomatal closure from pharmacological experiments. Stomatal activity is also regulated by turgor pressure. Our results indicate that stomatal closure is defective in guard cells of tod1 siliques, and this can be rescued by exogenous application of S1P, confirming that TOD1 acts upstream of S1P in stomatal closure. In pollen tubes, disruption of TOD1 also results in higher turgor pressure, and the pollen tube growth retardation caused by increased external pressure can be rescued by exogenous application of S1P. These findings suggest that TOD1 acts similarly both in guard cells and pollen tubes, where it is preferentially expressed.

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**Mutagenesis and screens for suppressors of tod1.** Approximately 5,000 tod1 seeds were mutagenized by injection of 0.3% ethyl methanesulfonate for 12 h at room temperature, followed by washing once with 100 mM Na2S2O3 and several times with sterilized water. The M2 seeds were collected in 32 pools, each representing approximately 280 M2 plants. We screened approximately 18,000 M2 seeds for suppressors of tod1. Putative suppressors were backcrossed to tod1 before further genetic and phenotypic analyses. Plants homozygous for the tod1 allele were confirmed by PCR analysis. RC-GFP, of putative suppressors were crossed to tod1-2, and the resulting F2 populations were examined for the segregation of fertility. Dominance or recessiveness of putative suppressors was determined by segregation ratio of full seed set to reduced seed set. For recessive mutations, germinated DNA was isolated from individual F2 plants with full seed set. For dominant mutations, seeds from individual F2 plants with full seed set were collected and propagated separately. When all individual F2 plants from the same F1 showed restored seed set, then genomic DNA was isolated from individual F2 plants.

**Whole-genome re-sequencing and data analysis.** DNA was isolated using a DNeasy Plant Mini Kit (Qiagen). Equally pooled genomic DNA was used to prepare a library for Illumina sequencing. The library was sequenced on the HiSeq2000 platform and 100 bp paired-end reads were generated. Reads alignment and variants identification were done according to the references with modifications. Clean reads were mapped against the TAIR10 release of the Arabidopsis genome, using the BWA software with default parameters. Sites with more than 20 reads that, because of sequencing error or different genotyping, were removed from that existed in the variant list (http://1001genomes.org/data/MP/MPISchnieeberger2011/sequences/2012_03_14/Ler-1/Marker/Ler-1.SNPs.TAIR9.txt). SNPs were further filtered with two steps: (i) extraction of SNPs that exhibit G/C-to-A/T transitions, which are the most frequent changes caused by ethyl methanesulfonate mutagenesis. (ii) SNPs with a supporting reads depth less than 20 or more than 100 were removed to reduce mapping errors. Gene mapping analysis was performed according to the reference with default parameters except for SNP filters, which indices more than 0.2 were considered. The SNPs locations within the gene model were annotated using SNPEff V3.3. Genes with ‘HIGH’ impact SNPs were taken as candidates.

**Pollen and pollen tube staining.** Mature pollen grains were stained for DNA with 1 μg ml\(^{-1}\) DAPI for 10 min (ref. 52), and for pollen viability with Alexander’s stain. For pollen tube staining, the pistil carpel was partially dissected using a forceps and a syringe needle, and then was fixed in acetic acid/ethanol (1:9) solution for more than 2 h. The fixed tissue was stained with 0.01% aniline blue in 50 mM potassium phosphate buffer (pH 7.5). The stained samples were then observed with a Zeiss Axioskop microscope (Carl Zeiss) equipped with epifluorescence.

**In vitro pollen germination and pollen tube growth.** Pollen grains from fully opened flowers were spread on pollen germination medium containing 5 mM CaCl\(_2\), 5 mM KCl, 1 mM MgSO\(_4\), 0.01% H\(_2\)BO\(_3\), 10% sucrose (w/v) and 1.5% agarose (for solid medium, pH 7.5, and incubated at 22°C (ref. 34). The germination rate and pollen tube lengths were determined with a Zeiss Axioskop microscope.

Sphingosine and S1P were dissolved in methanol to make stock solution. The complementation experiment was conducted in 1.5% ultra-low gelling agarose (Sigma-Aldrich) germination medium with additions of 10 μM Sphingosine (860490 P, Avanti) or S1P (S9666, Sigma), or the same volume of methanol for the control.

Liquid pollen tube germination was performed in 2 ml Elpendorf tube, using 20 flowers for each 200 μl medium.

**Scanning electron microscopy.** Pistils pollinated with either Ler or tod1-2 were partially dissected 24 h after pollination and immediately fixed with 3% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.0. After an initial fixation for 1 h at room temperature under a gentle vacuum, samples were washed in 0.1 M cacodylate buffer (pH 7.3) overnight. The samples were then rinsed three times in sodium phosphate buffer and dehydrated through an ethanol series. After dehydration, ethanol was replaced by isooamyl acetate. Afterwards, specimens were dried with a critical point drier using liquid carbon dioxide. The dried pistils were mounted on stubs for further dissection and sputter coating with gold. Specimens were observed with a SEM (Hitachi). For stomatal aperture observation, siliques were fixed after ABA or S1P treatment.

**Molecular analysis and genomic complementation.** Genomic DNA of tod1 was extracted by CTAB method. The flanking sequence of Ds element was isolated by thermal asymmetric interlaced PCR. TOD1 genomic DNA containing its promoter sequence was amplified with primers TOD1dP and TODYgR and cloned into pCAMBIA1300 at PstI and BamHI sites. Total RNA was isolated from different tissues of Ler with Trizol (Invitrogen). For first strand cDNA synthesis, 2 μg of total RNA was used for reverse transcription with M-MLV reverse
transcriptase (Promega) according to the manufacturer’s instructions. RT–PCR was performed with primer pair TODY1F and TODY1R. Arabidopsis eIF4A was used as an internal control with primers eIF4AF and eIF4AR. PCR products were checked by 1.2% agarose gel electrophoresis. Uncropped gel images are shown in Supplementary Fig. 8. Real-time RT–PCR reactions containing SYBR Green I were performed on CFX96 real-time system (Bio-Rad). Primers TODY1F and TODY1Q were used for TODY1. Primers ACTZQF and ACTZQR were used for ACT2. The reaction mixture was adjusted to 20 μl containing 10 μl of the assay buffer (10× SYBR Green Master Mix, Bio-Rad). Amplification was performed with an initial step of 10 min at 95 °C, followed by 30 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. All results were analyzed using CFX Manager software (Bio-Rad), and the relative expression levels were calculated by the 2^(-ΔΔCt) method.

Ceramide assay activity assay. Ceramide assay activity was assayed using fluorescent NBD-C2-C2-phytoceramide (810125 P, Avanti), and NBD-C12-ceramide (810111 P, Avanti) as substrates. The substrate was dissolved in reaction buffers with different pHs44. In each reaction, 8 mM substrate in 20 μl reaction buffer was added to 20 μl of yeast microsomes (50 μg protein) in lysis buffer. CaCl2 was added to a final concentration of 5 mM. The reaction was performed at 37 °C for 90 min, and stopped by addition of methanol/chloroform (1:1). The mixture was vortexed for 30 s, kept at room temperature for 5 min and then centrifuged. The organic phase was collected and dried. Lipids were dissolved in 20 μl methanol/chloroform (1:2) then spotted on TLC plates (Merck). The substrate and product were separated by developing the plates in chloroform/methanol/25% ammonium hydroxide (90:30:5) solvent system. The NBD-fatty acid product was identified by comparison with C12-NBD-fatty acid (72963, Sigma) standard. The TLC plates were scanned using PhosphorImager (Typhoon Trio) system set at fluorescence mode. Uncropped TLC images are shown in Supplementary Fig. 8.

Stomatal bioassays. Promotion of stomatal closure by ABA was performed with siliques. Siliques were floated in MES/KCl buffer (5 mM KCl, 10 mM MES and 50 mM CaCl2, pH 6.15) under illumination for 2.5 h (ref. 42), then mock control or 10 mM ABA was added, and siliques incubated in another 2 h, then fixed for SEM observation. SIP-induced promotion of stomatal closure was conducted in a similar way. The aperture ratio (width/length) was calculated.

Turgor pressure measurement. The turgor pressure of pollen tubes was measured by incipient plasmolysis method as described8, with modifications. Pollen tubes after 5 h germination were pipetted into glass bottom dishes. An aliquot (60 μl) of original growth medium was collected for osmolarity measurement denoting (πe). Pollen tubes were observed under CLSM. Liquid pollen germination medium with 10% sucrose and 10% mannitol was added into the growth medium until the plasma membrane retracted from the cell wall of the tip. An aliquot of 60 μl of solution was collected for osmolarity measurement denoting πi. The osmolality was measured by Fiske Micro-Osmometer. Turgor pressure is given by πi − (πe).

Ca2+ imaging of pollen tubes. To express GCaMP6s-expressing pollen tubes, pollen tubes after 5 h germination were pressed with GCaMP6s-expressing Col-0. The resulting F0 progeny was allowed to self-pollinate with limited pollen grains. The genotype of individual F1 plants was determined by PCR. Pollen grains from GCaMP6s-expressing Col-0 or Col were germinated on agarose medium. Imaging of growing pollen tubes was performed using CLSM with a 488-nm argon laser. Emission was measured at 470–580 for ECFP and 515–550 for Venus. Venus/ECFP ratio images were processed by Image J and Image-pro Plus.

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