

Pollen tube development and competitive ability are impaired by disruption of a Shaker K⁺ channel in *Arabidopsis*

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Sexual reproduction in plants requires elongation of the pollen tube through the transmitting tissues toward the ovary. Tube growth rate is a major determinant of pollen competitive ability. We report that a K⁺ channel of the Shaker family in *Arabidopsis*, SPIK, plays an important role in pollen tube development. SPIK was found to be specifically expressed in pollen. When SPIK was heterologously expressed in COS cells, its product formed hyperpolarization-activated K⁺ channels. Disruption (T-DNA insertion) of the SPIK coding sequence strongly affected inwardly rectifying K⁺-channel activity in the pollen-grain plasma membrane. Measurements of membrane potential in growing pollen tubes yielded data compatible with a contribution of SPIK to K⁺ influx. In vitro pollen germination assays were performed, revealing that the disruption results in impaired pollen tube growth. Analysis of the transmission rate of the disrupted allele in the progeny of heterozygous plants revealed a decrease in pollen competitive ability, the probability of fertilization by mutant pollen being ~1.6 times lower than that by wild-type pollen. The whole set of data supports the hypothesis that functional expression of SPIK plays a role in K⁺ uptake in the growing pollen tube, and thereby in tube development and pollen competitive ability.

[Key Words: Pollen tube development; Shaker K⁺ channel; *Arabidopsis*; T-DNA knockout mutant]

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In flowering plants, the male gametes reside in the pollen, which is deposited on the receptive surface of the pistil, the female reproductive organ. The pistil is an elongated organ within which three structures can be distinguished: an apical stigma that receives pollen, an ovary that contains the ovules, and a style that connects the stigma to the ovary. On a compatible stigma, the pollen grain germinates and extrudes a tube that elongates through the style to reach the ovary and enter an ovule, where the tube tip bursts, allowing release of the male gametes for fertilization. Rapid elongation of the pollen tube, over a distance of a few hundred micrometers to several centimeters, is therefore an essential process for the sexual reproduction of flowering plants (Wilhelmi and Preuss 1999). Many pollen tubes may grow in the same style toward a few ovules, leading to competition. Tube growth rate is therefore an important determinant of pollen competitive ability, which de-

scribes the reproductive success of a pollen grain (Sari-Gorla and Frova 1997).

Several molecular mechanisms responsible for pollen recognition by the stigma, allowing compatible pollen hydration, germination, and development, have been recently identified (Wolters-Arts et al. 1998; Schopfer et al. 1999; Takasaki et al. 2000; Cabrillac et al. 2001). Progress has also been made in the identification of extracellular cues present in the transmitting tissue that allow tube guidance toward the ovules (Cheung et al. 1995; Franklin-Tong 1999a; Yang 1998; Wilhelmi and Preuss 1999; Lord 2000). A large set of data has been obtained, clearly showing that directed tip growth involves Ca²⁺-based signaling supported by Ca²⁺ influx at the growing tube tip (Holdaway-Clarke et al. 1997; Moutinho et al. 1998; Franklin-Tong 1999b; Li et al. 1999). Control of cytosolic Ca²⁺ concentration at the tube tip also plays a role in the modulation of the rate of pollen tube growth (Pierson et al. 1994; Franklin-Tong 1999b). The other mechanisms specifically controlling growth rate are still poorly documented, despite the importance of this function for pollen competitive ability.

K⁺ uptake is required for tube growth. Here we report the first molecular identification of a pollen ion channel.

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Heterologously expressed in mammalian cells, or in situ on the pollen membrane, it behaves as an inward K^+ channel. Disruption of the gene in *Arabidopsis* results in decreased pollen competitive ability.

Results

Molecular cloning of SPIK

A gene encoding a K^+ channel belonging to the Shaker family (Jan and Jan 1992; Zimmermann and Sentenac 1999) was initially identified in silico (in BAC F3N11, GenBank accession no. AC006053) by The *Arabidopsis* Genome Initiative (2000) and named *AKT5* (Lacombe et al. 2000) or *AKT6* (Mäser et al. 2001) on the sole basis of sequence information. In this work, six cDNAs were isolated by RACE-PCR, identifying the full-length ORF (EMBL AJ309323). The deduced polypeptide is 889 amino acids long, with a predicted molecular mass of 99 kD. Expression studies and functional analyses (see below) led us to name the encoded polypeptide SPIK, for Shaker Pollen Inward K^+ channel.

The plant Shaker channel family contains nine genes in *Arabidopsis*. Members of the Shaker superfamily are thought to share the structural design of the *Drosophila* Shaker gene products (Jan and Jan 1997). Shaker channels consist of four subunits arranged around a central pore (MacKinnon 1991; Jan and Jan 1997). The hydrophobic core region of each subunit shows six transmembrane segments, named S1 to S6. S4 is characterized by the presence of basic amino acids and acts as a voltage sensor. The pore-forming sequence (~20 amino acids) is located between S5 and S6 and harbors a GYGD motif, the hallmark of highly K^+ -selective channels. Analysis of the SPIK deduced sequence (Fig. 1A) revealed the typical hydrophobic core of Shaker-type channels, with the positively charged S4 segment and the GYGD motif in the pore-forming domain (data not shown). Downstream from the channel hydrophobic core, a putative cyclic nucleotide-binding domain is present in SPIK, as in every plant Shaker channel identified up to now. This is followed by an ankyrin domain (Sentenac et al. 1992), as in six out of the nine Shaker channels identified in *Arabidopsis*, and a KHA domain (so-called because of its richness in hydrophobic and acidic amino acids; Ehrhardt et al. 1997), present in every plant Shaker channel (Fig. 1A). The ankyrin domain of plant Shaker channels has been hypothesized to play a role in tethering the channel to regulatory and/or cytoskeleton proteins (Sentenac et al. 1992; Cao et al. 1995). Evidence has been obtained indicating that the KHA domain plays a role in the channel tetramerization process (Daram et al. 1997) and/or in channel clustering in the membrane (Ehrhardt et al. 1997).

Expression of SPIK gene in Arabidopsis

Northern blot experiments revealed that SPIK expression is restricted to flower tissues (Fig. 1B). Localization

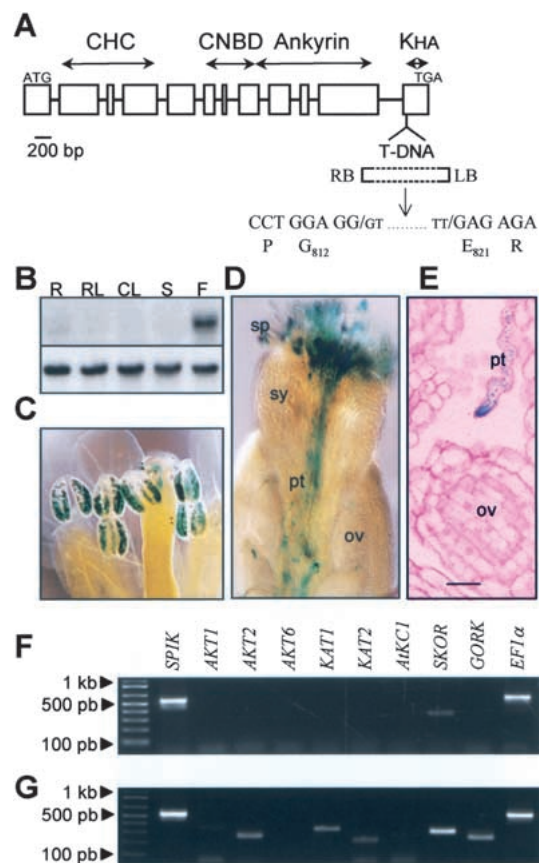


Figure 1. The *Arabidopsis* K^+ channel SPIK gene is specifically expressed in pollen. (A) SPIK gene structure. Boxes indicate exons. (CHC) Sequence encoding the channel hydrophobic core (six transmembrane segments and one pore domain); (CNBD, ankyrin, and KHA) putative cyclic nucleotide-binding domain, ankyrin domain, and domain rich in hydrophobic and acidic residues, respectively. The insertion site of the disrupting T-DNA in the *spik-1* mutant is indicated. (LB and RB) Left and right borders of the T-DNA. (B) Northern blot analysis of SPIK expression. Total RNA was extracted from roots (R), rosette leaves (RL), caulinary leaves (CL), stems (S), or flowers (F). The membrane was first hybridized with SPIK radioactive probe (upper autoradiogram) and thereafter with actin probe (control; lower autoradiogram). (C–E) Histochemical analysis of transgenic *Arabidopsis* expressing a SPIK::GUS reporter construct revealing GUS activity (blue staining) in pollen grains in anthers (C), in pollen germinating on stigma (D), and in pollen tubes (pt) growing through the style tissues toward the ovules (ov) (D, E). (sp) Stigmatic papilla; (sy) style. E shows a longitudinal 0.3- μ m-thick cut of a stigma (bar, 25 μ m). (F, G) RT-PCR experiments performed on total RNA extracted from pollen grains assessing the expression levels of SPIK and of the other members of the *Arabidopsis* Shaker gene family (AKT1, AKT2, AKT6, KAT1, KAT2, AtKC1, and SKOR: Lacombe et al. 2000; GORK: Ache et al. 2000). The *EFl* α gene (Axelos et al. 1989) was used as control. Primers surrounding introns were used to discriminate amplification on genomic DNA. Reactions were stopped after 28, 30, 32, 34, 36, 38, or 45 cycles, and an aliquot of the reaction medium was loaded on a 2% agarose gel. SPIK sequence amplification was almost maximal after 30 cycles. (F) Amplification after 30 cycles. (G) Amplification after 45 cycles. In an independent experiment, another set of primers was used, leading to similar results (data not shown).

of *SPIK* expression was further investigated using transgenic plants carrying the *Escherichia coli* β -glucuronidase gene (*GUS*) under the control of the *SPIK* promoter region (2.5 kb). *GUS* staining was detected in pollen only (Fig. 1C–E; no detectable staining in leaves, stems, roots, and root hairs; data not shown). Staining in pollen was detected both before and after germination: in pollen grains within anthers of flower buds or fully open flowers (Fig. 1C), and in pollen tubes growing toward the ovules, after pollen germination on the stigma (Fig. 1D,E). RT-PCR experiments performed on total RNA extracted from purified pollen grains suggested that *SPIK* is the member from the Shaker K^+ channel gene family displaying the highest expression level in pollen grain. Lower expression levels were suggested for the *SKOR* gene, which encodes an outwardly rectifying channel (Gaymard et al. 1998), and still lower for the other genes (Fig. 1F,G).

Functional characterization of *SPIK* in a heterologous system

In order to elucidate the physiological role of *SPIK* in pollen, the molecular function of the encoded polypeptide was investigated by expression in mammalian COS cells (Fig. 2). Electrophysiological analyses revealed that *SPIK* is a slowly activating, inwardly rectifying channel (Fig. 2A). This channel displayed a high selectivity for K^+ : comparison of whole-cell *SPIK* current (*I*) at -200 mV in external solutions containing either 50 mM KCl or 50 mM XCl, with X^+ being Na^+ , Li^+ , Rb^+ , or NH_4^+ , yielded $I_X/I_K = 0.08 \pm 0.01$ ($n = 7$), 0.06 ± 0.03 ($n = 6$), 0.20 ± 0.04 ($n = 5$), or 0.18 ± 0.04 ($n = 4$) (mean \pm SE), respectively. At an external pH of 6.0, the channel half-maximal activation potential was -205 mV. The channel activation was independent of the external K^+ concentration (Fig. 2B), but highly sensitive to external pH, because acidification shifted the half-activation potential positively (+47 mV shift between pH 7.4 and pH 5.0; Fig. 2B). Increasing the external K^+ concentration from 2 mM to 150 mM revealed a current saturation with an apparent K_M of 14 mM (Fig. 2C). *SPIK* current was not sensitive to external Ca^{2+} in the range 1 to 10 mM (data not shown), as previously reported for the inwardly rectifying K^+ current in *Brassica* or *Arabidopsis* pollen plasma membranes (Fan et al. 1999, 2001). It was blocked by Cs^+ , in an essentially voltage-independent way, with a current inhibition of $82\% \pm 4\%$ ($n = 7$) in 10 mM KCl + 50 mM CsCl (data not shown), and $90\% \pm 2\%$ ($n = 6$) in 10 mM KCl + 90 mM CsCl (Fig. 2D). *SPIK* was identified at the single-channel level (cell-attached configuration; Hamill et al. 1981) on the basis of similarity with characteristics determined in the whole-cell configuration. Indeed, analysis of the most frequently observed channel (Fig. 2E) revealed activation kinetics and voltage dependence (threshold of activation and apparent gating charge Z_g obtained from the Boltzmann fit; Fig. 2F) very similar to those of the whole-cell *SPIK* current (cf. Fig. 2B). The *SPIK* channel conductance was ~ 14 pS in 50 mM external K^+ (Fig. 2G).

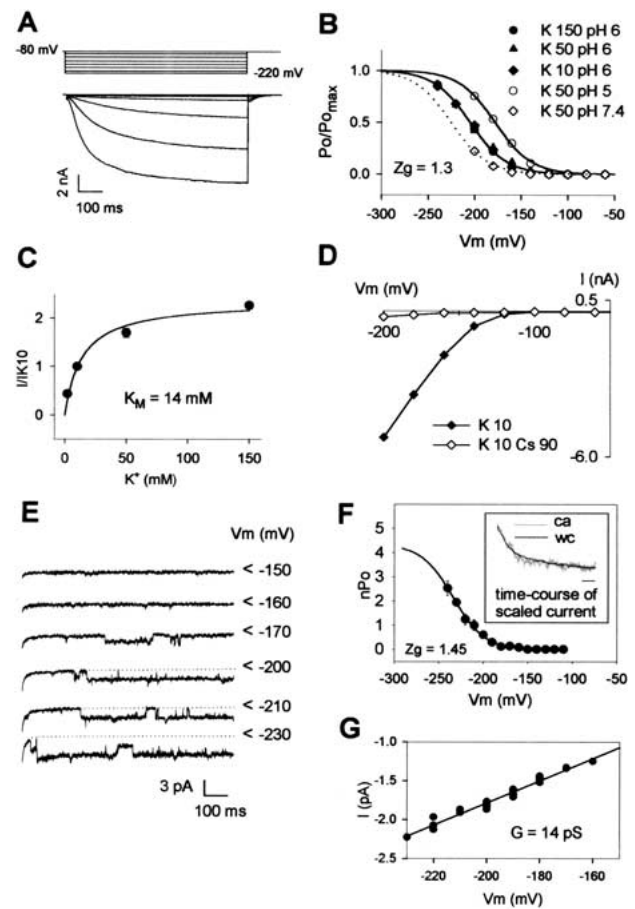


Figure 2. *SPIK*-mediated currents in COS cells. (A) Voltage-clamp protocol and example of whole-cell currents in a COS cell expressing *SPIK*. External K^+ was 10 mM. (B) Analysis of *SPIK* activation at various external K^+ concentrations (10, 50, or 150 mM at pH 6.0) and pH (5, 6, or 7.4 in 50 mM K^+). Lines are Boltzmann fits of the relative open probability ($P_o/P_{o,max}$) versus membrane potential. (Z_g) Equivalent gating charge obtained from the Boltzmann fit. Half activation potentials (from Boltzmann fit) are -178 mV (pH 5.0), -205 mV (pH 6.0), and -226 mV (pH 7.4). (C) Saturation of *SPIK* current with increased external $[K^+]$. Currents were measured at -200 mV. Data are means (normalized to the value obtained in 10 mM K^+) \pm SE ($n = 7$). A Michaelis–Menten fit (solid line) gave an apparent K_M of 14 mM. (D) Block of *SPIK* currents by cesium. External K^+ was 10 mM; added Cs^+ was 90 mM. Data shown are representative of five experiments. (E) Current traces recorded in the cell-attached configuration showing *SPIK* activity at the single-channel level. External K^+ concentration was 50 mM. The patch membrane potential (i.e., the measured cell resting potential, -10 mV in these conditions, minus the imposed pipette potential) is indicated at the right of current traces. The arrows indicate closed channel current levels. (F) *SPIK* single-channel activation features. The mean number of open channels (nPo) was determined from 1-sec recordings ($n = 7$). The solid line is a Boltzmann fit of nPo versus the membrane potential. (Inset) Comparison of *SPIK* activation kinetics in whole-cell (wc) and cell-attached (ca) experiments. The holding and test potentials were -80 mV and -220 mV, respectively. Seven single-channel recordings from the same patch were summed, yielding the ca trace that was scaled (in gray in the inset) to the wc trace (in black) for comparison. Inset, bar, 50 msec. (G) Determination of *SPIK* unitary conductance in 50 mM external K^+ .

Disruption of SPIK coding sequence strongly affects inward K⁺-channel activity in the pollen-grain plasma membrane

A reverse genetic approach based on PCR screening of an insertion mutant library was developed to reveal the functions of the *SPIK* gene in the plant. An *Arabidopsis* mutant line, named *spik-1*, was identified by PCR screening of a collection of T-DNA-transformed plants (Bouchez et al. 1993). The T-DNA insertion (single locus and single copy, as indicated by Southern blot analysis; data not shown) was found to interrupt the last exon of the gene (Fig. 1A), just upstream of the KHA domain. To determine the effect of the *spik-1* mutation on the plant phenotype, homozygous *spik-1* mutant plants and heterozygous *SPIK/spik-1* plants were grown in a growth chamber or in the greenhouse. Comparison with control wild-type plants grown in parallel in the same conditions did not reveal any obvious phenotype. The *spik-1* plants were not found to be impaired in root hair development, excluding the hypothesis that *SPIK* could correspond to the gene identified by the *tip1* mutation affecting both root hair elongation and pollen tube growth (Schiefelbein et al. 1993). Finally, homozygous *spik-1* plants generated as many seeds per silique as wild-type plants (data not shown).

Because *SPIK* is expressed in pollen, we tested the hypothesis that *SPIK* disruption could impair K⁺-channel activity in the pollen-grain membrane. *SPIK* currents were examined in native cells by patch-clamping pollen-grain protoplasts. Whole-cell experiments on wild-type pollen protoplasts revealed both inward and outward currents (Fig. 3A). The inward current was dominated by a slowly activating component, appearing below a threshold potential of ~ -100 mV, strongly reminiscent of that recorded in *SPIK*-expressing COS cells. Indeed, the activation kinetics and the steepness of $P_o/P_{o,max}$ dependence on voltage (i.e., apparent gating charges Z_g , close to 1.3 in both cases) were similar (Fig. 3A). This inwardly rectifying current was highly selective for K⁺ (against Cl⁻ and Ca²⁺; data not shown). Similar experiments were performed on protoplasts prepared from pollen grains obtained from homozygous *spik-1* mutant plants. Remarkably, the inward K⁺ current was strongly reduced, the slowly activating, inwardly rectifying component being no longer observed (Fig. 3B,C), providing evidence that *SPIK* was not expressed in a functional state in the *spik-1* mutant pollen. Furthermore, these experiments suggested that *SPIK* provides the main pathway for K⁺ uptake in these cells at hyperpolarized membrane potentials. Analysis of the remaining current (weakly rectifying) in *spik-1* mutant pollen revealed a reversal potential close to the K⁺ equilibrium potential (Fig. 3C), indicating that this background current was mainly carried by K⁺ ions.

To examine the possibility of *SPIK* involvement in wholesale K⁺ uptake during tube growth, the resting membrane potential of young growing tubes (~ 100 μ m long) was investigated (Fig. 3D). Measurements were carried out in the presence of 1 mM external K⁺. Recordings

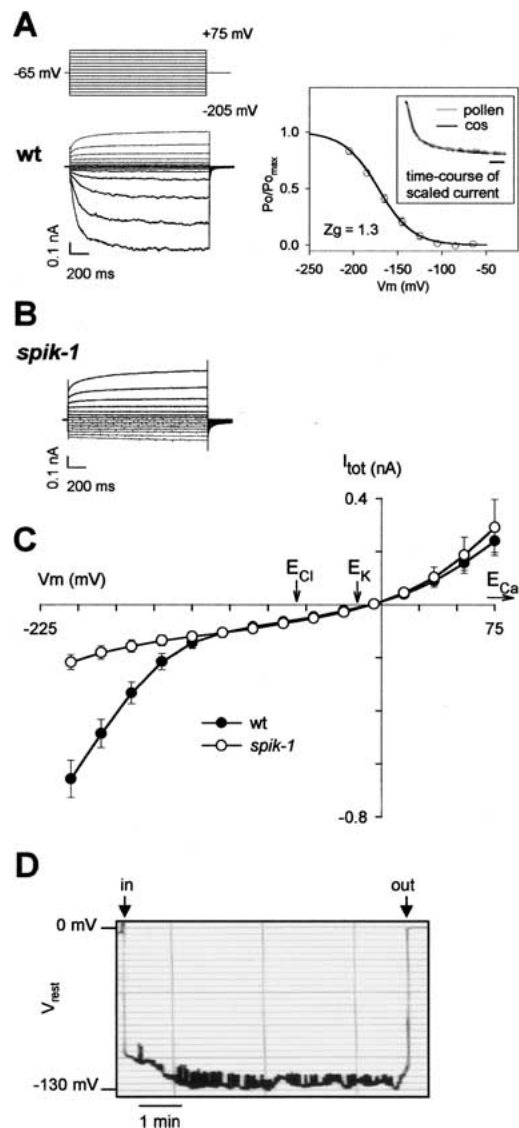


Figure 3. Electrical properties of the wild-type and *spik-1* pollen membrane. (A–C) Patch-clamp measurements on pollen grain protoplasts. External and internal K⁺ concentrations were 50 mM and 100 mM, respectively. (A) Pollen from wild-type (wt) plants. (Left panel) Voltage-clamp protocol and typical example of currents recorded in whole protoplasts. (Right panel) Activation properties of the time-dependent inwardly rectifying conductance. The solid line is a Boltzmann fit of the mean (\pm SE; $n = 6$) relative open probability ($P_o/P_{o,max}$) versus the membrane potential. (Z_g) Equivalent gating charge obtained from the Boltzmann fit. The half-activation potential (from Boltzmann fit) is -172 mV. (Inset) Comparison of activation kinetics of inwardly rectifying current in pollen protoplasts and in *SPIK*-expressing COS cells. Current traces obtained at -205 mV in 50 mM external K⁺ were scaled for easier comparison. Bar: 100 msec. (B) Typical example of currents recorded in pollen protoplasts from homozygous *spik-1* plants. Same protocol as in A. (C) Current/voltage relationship obtained with pollen protoplasts of both genotypes (mean \pm SE; $n = 12$ for wt and 9 for *spik-1*). Arrows point to equilibrium potentials for Cl⁻, K⁺, and Ca²⁺ ions (E_{Cl} , E_K , and E_{Ca}). (D) Recording of resting membrane potential (V_{rest}) in a young growing pollen tube. External [K⁺] was 1 mM. Arrows, (in) start or (out) end of cell impalement.

over 5–10 min generally showed a quite stable hyperpolarized level with values in the -120-mV to -134-mV range (mean, $-127 \pm 2\text{ mV}$, $n = 7$) interspersed with short depolarized (10–20 mV) events (Fig. 3D). Comparison of these values of resting membrane potential with the activation threshold of the slowly activating, inwardly rectifying current recorded in pollen protoplasts ($\sim -100\text{ mV}$; Fig. 3A; Fan et al. 2001) indicates that SPIK was open and at work in these conditions.

The spik-1 mutation strongly affects in vitro pollen germination and tube development

We used the fact that pollen can germinate and grow a tube in vitro to investigate the effects of the *spik-1* mutation on pollen fitness, although in vitro growth does not completely mimic in vivo growth in terms of growth rate or tube length (Taylor and Hepler 1997). Differences in pollen germination and tube growth could be observed from one set of experiments to the other, both with wild-type and *spik-1* pollen. Thus, the effect of the *spik-1* mutation was studied using wild-type and *spik-1* homozygous plants grown in parallel under the same conditions, the wild-type and *spik-1* pollen grains being systematically germinated in parallel on the same slides. The concentration of K^+ in the germination medium was $100\ \mu\text{M}$ or 1 mM . The concentration of K^+ in the apoplast of the style tissues surrounding the growing pollen tube is unknown. However, the $100\ \mu\text{M}$ – 1 mM concentration range can be considered as physiologically relevant.

Six hours after having been deposited on $100\ \mu\text{M}$ K^+ coated slides, wild-type pollen grains (Fig. 4A) displayed longer tubes than *spik-1* grains (Fig. 4B). For both pollen genotypes, detailed examination allowed to distinguish nongerminated grains (no tube) and, among those having grown a tube (germinated), two distinct populations. The first one displayed tubes shorter than $30\ \mu\text{m}$ after 6 h of incubation (as shown in Fig. 4C for *spik-1* pollen) that did not grow further even after 2 d. The second population displayed long tubes, here below called developed tubes, several hundred micrometers long 6 h after pollen deposition. As a consequence, two distinct length ranges were considered in the histogram analysis of tube length in wild-type or *spik-1* pollen grains (Fig. 4D,E). Only the so-called developed tubes were considered for the Gaussian analysis of mean tube length (lines in Fig. 4D,E), which yielded $581 \pm 8\ \mu\text{m}$ and $340 \pm 13\ \mu\text{m}$ (mean \pm SE), respectively, for wild-type and *spik-1* pollen grains grown in 0.1 mM K^+ medium, the difference being significant (t -test, $P < 10^{-10}$). In all subsequent experiments, irrespective of mean values, the mean length of *spik-1* developed tubes was $\sim 40\%$ shorter than that of wild-type ones (see, e.g., Fig. 5C), the difference being always significant ($P < 10^{-10}$).

It is worth noting that in these experiments the mean wild-type tube length, obtained after a 6-h germination, was ~ 5 times greater than that recently reported ($120\ \mu\text{m}$) for *Arabidopsis* pollen after a 6-h germination in 0.1 mM K^+ and otherwise different experimental conditions

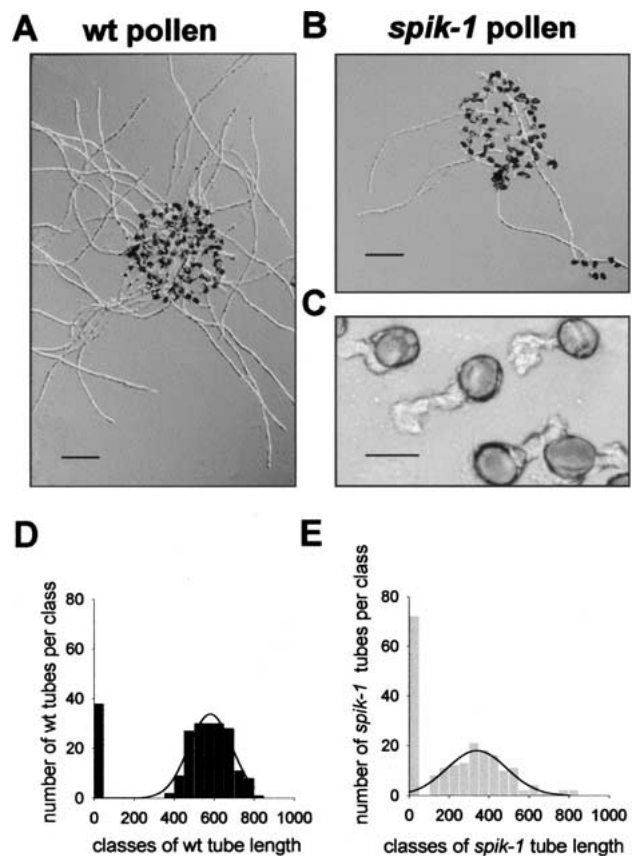


Figure 4. In vitro development of wild-type and *spik-1* pollen. (A) Wild-type (wt) or (B) *spik-1* pollen germinated on 0.1 mM K^+ medium (bar, $100\ \mu\text{m}$). (C) Non-developed (see text) *spik-1* pollen tubes (bar, $15\ \mu\text{m}$). (D,E) Histograms showing the length distribution of (D) wild-type ($n = 214$) and (E) *spik-1* ($n = 202$) germinated grains on the 0.1 mM K^+ medium. The two histograms were fitted according to a Gaussian distribution (lines).

(Fan et al. 2001). In vivo fertilization occurs ~ 2 – 3 h after pollination in *Arabidopsis* (Mansfield and Briarty 1991). The mean distance from stigmatic papillae to ovules being $\sim 1000\ \mu\text{m}$, this suggests a 300 – $500\text{-}\mu\text{m/h}$ tube growth rate in vivo. The germination protocol we used, which clearly favors tube growth when compared with the one used by Fan et al. (2001), is therefore likely to more closely mimic in vivo conditions.

Quantitative analyses of pollen fate (percentage of germinated grains and developed tubes as defined above) are shown for wild-type and *spik-1* pollen grains in 0.1 and 1 mM K^+ media, respectively, in Figure 5, A and B. Of wild-type pollen grains, $\sim 70\%$ – 75% germinated; their germination rate was barely affected by modifying external K^+ from 0.1 mM to 1 mM (Fig. 5A). The *spik-1* mutation poorly affected the overall germination rate, which was 60% – 65% , rather independent of the external concentration of K^+ (0.1 mM or 1 mM ; Fig. 5B). On the other hand, the mutation resulted in a strong decrease (by ~ 3 times in 0.1 mM K^+ and ~ 2 times in 1 mM K^+) in the percentage of developed tubes: the percentage of developed tubes was in the 45% – 50% and 15% – 25% range,

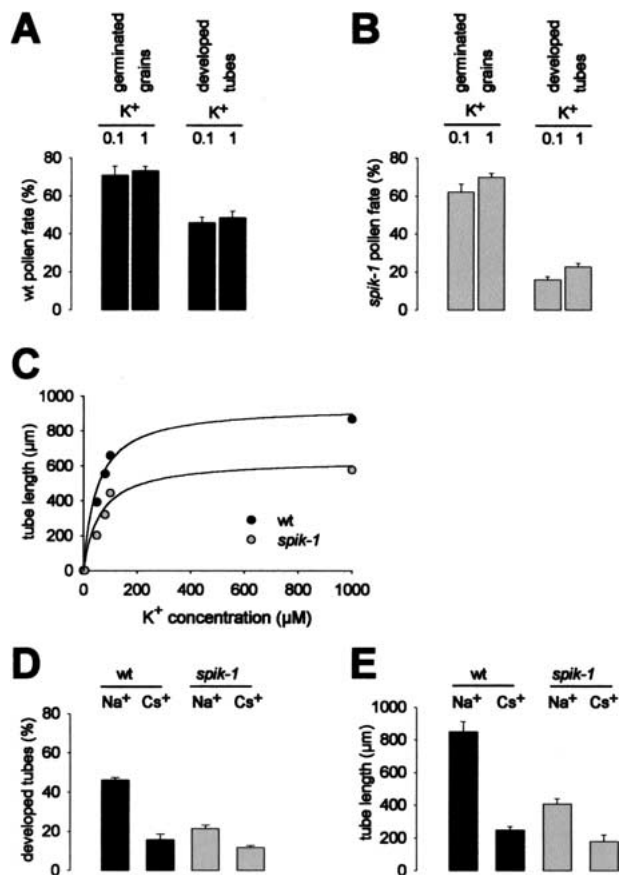


Figure 5. Quantitative analysis of wild-type and *spik-1* pollen fate in vitro. (A,B) Quantification of germinated grains and developed tubes in (A) wild-type (wt) and (B) *spik-1* pollen populations. The germination medium contained either 0.1 or 1 mM K⁺. Means \pm SE of three independent experiments, each one including three replicates (at least 200 pollen grains per replicate). The differences between the percentages of developed tubes in the wild-type and the *spik-1* populations are significant ($P < 10^{-5}$, *t*-test) in both media. (C) Effect of external K⁺ concentration on wild-type (wt) and *spik-1* pollen tube length. K⁺ concentrations below 100 μ M were obtained by supplementing the 100 μ M germination medium with the K⁺ chelator tetraphenylborate (TPB). Addition of 20 μ M, 50 μ M, and 100 μ M TPB was calculated to reduce the free K⁺ concentrations to 80 μ M, 50 μ M, and 5 μ M (lowest concentration shown in the graph), respectively (see Materials and Methods). Mean \pm SE for at least 300 tubes (SE bars smaller than the symbol size). Lines show hyperbolic fits to the data (least squares fits): apparent K_M of 50 and 61 mM for wild-type and *spik-1* pollen grains, respectively. (D,E) Effect of Cs⁺ on percentage of developed tubes (D) and tube length (E) for wild-type (black bars) or *spik-1* (gray bars) pollen grains. Either 2 mM NaCl (control treatment) or 2 mM CsCl was added to 1 mM K⁺ germination medium. Mean \pm SE for at least 800 pollen grains (D) and 50 pollen tubes (E) in both treatments. The depressive effect of Cs⁺ is significant ($P < 10^{-3}$) in each genotype.

respectively, for wild-type and *spik-1* grains (Fig. 5A,B). The effect of K⁺ concentration on the mean tube length was investigated in the 5 μ M to 1 mM range (see Materials and Methods). In both wild-type and *spik-1* pollen,

decreasing K⁺ availability in the germination medium below 0.1 mM was found to strongly affect the mean tube length, that is, the lower the K⁺ concentration the shorter the tubes (Fig. 5C), with a total lack of development in the presence of \sim 5 μ M free K⁺. In the 50 μ M to 1 mM concentration range, the *spik-1* mutation reduced the tube length by an average factor of 40%–50%.

Cs⁺ is known to selectively block inward K⁺ currents through plant K⁺-selective inwardly rectifying Shaker channels while leaving outward K⁺ currents and poorly selective cation channels unaffected (Fig. 2D; White 1997). Adding 2 mM Cs⁺ (CsCl) to the 1 mM K⁺ germination medium reduced both the percentage of developed tubes (Fig. 5D) and the average tube length (Fig. 5E). As expected, the effects of this treatment were less pronounced on *spik-1* pollen than on wild-type pollen.

The spik-1 mutation results in impaired pollen competitive ability

The impairment of pollen development observed in vitro suggested that, in planta, the *spik-1* mutation could result in decreased pollen fitness and competitive ability. Such a decrease would be liable to produce a distorted transmission rate of the *spik-1* and wild-type alleles in the progeny of heterozygous (*SPIK/spik-1*) plants. To analyze this, we took advantage of the fact that the disrupting T-DNA carries a copy of the *nptII* gene, which confers resistance to kanamycin. Thus, F₁ plants free from the transforming T-DNA (i.e., homozygous wild-type plants) could be identified as sensitive to the antibiotic (white plantlets with reduced growth). *Arabidopsis* undergoes efficient self-fertilization with negligible outcrossing even under closely spaced laboratory growth conditions (Snape and Lawrence 1971; Abbott and Games 1989); strict self-fertilization was therefore assumed. In the F₁ progeny of so-called control plants, hemizygous for the *nptII* transgene, the mean percentage of homozygous wild-type plants (sensitive to kanamycin) was found to be close to 25%, as expected for the Mendelian segregation of a single recessive trait (Fig. 6A, white bars). In the F₁ progeny of heterozygous (*SPIK/spik-1*) plants the mean percentage of homozygous wild-type plants was found to be close to 31%. The *spik-1* mutation therefore resulted in a significant decrease in pollen competitive ability. From the frequency of 31%, assuming that the *spik-1* mutation does not affect the ability of the ovules to be fertilized and to develop into seeds, the probability of fertilization by wild-type pollen (P_{wt}) in competition with *spik-1* pollen grain can be estimated to be 0.62 (i.e., 0.31×2). Given this, the probability of fertilization by a *spik-1* pollen grain (P_{*spik-1*}) is 0.38 (i.e., $1 - 0.62$).

The decrease in *spik-1* pollen competitive ability was further confirmed by analyzing the percentage of homozygous wild-type plants in three successive generations obtained by bulk amplification, starting from either *SPIK/spik-1* heterozygous plants or from control plants hemizygous for the *nptII* transgene (Fig. 6B). A persistent distortion in allele segregation was observed from F₁ to

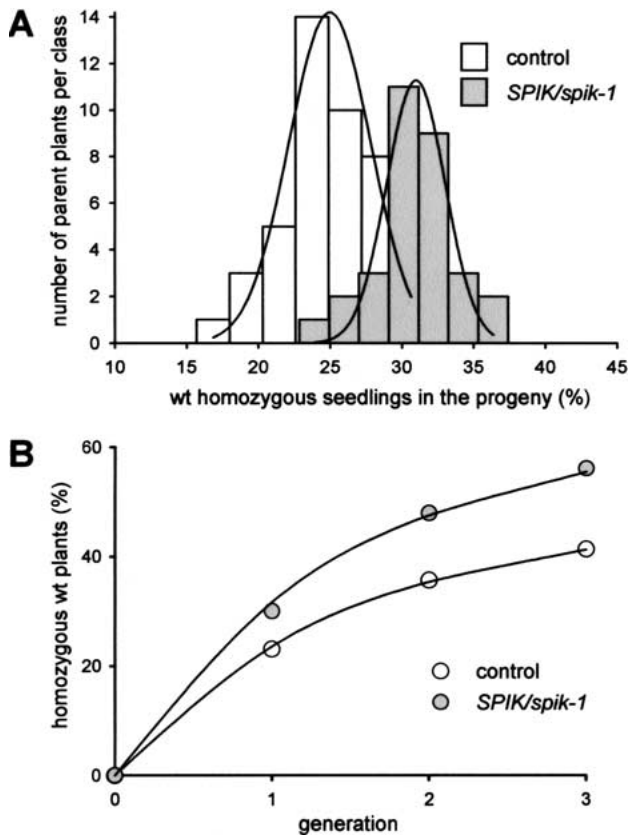


Figure 6. *SPIK* gene disruption results in reduced pollen fitness. (A) Histogram analysis of kanamycin-sensitive seedling percentage in the progeny of 31 *SPIK/spik-1* plants (gray bars) and 44 control plants (white bars). The disrupting T-DNA (Fig. 1A) contains a gene (*nptII*) conferring resistance to kanamycin. Sowing seeds from plants self-fertilized heterozygous (*SPIK/spik-1*) or hemizygous for the transgene *nptII* (control) on kanamycin medium (50 $\mu\text{g}/\text{mL}$) allowed the census (in percent) of homozygous wild-type seedlings (wt, free from disrupting T-DNA and thus kanamycin-sensitive) in the progeny of each plant. At least 300 seedlings were tested for each parental plant. Percentage data were sorted into eight classes for each parental genotype. The data (number of parent plants belonging to each class) were fitted by a Gaussian distribution. (B) Evolution of the census of wild-type homozygous genotype through three subsequent self-fertilizing generations issued from *SPIK/spik-1* plants (gray symbols) or from control plants (white symbols). Thirty-five plants (F_0) of each genotype were grown. Their seeds (F_1) were harvested in bulk, of which at least 1200 seeds were sown on kanamycin medium (homozygous wt genotype census) and at least 100 seeds were sown on compost and grown in the greenhouse for producing the subsequent (F_2) generation. The same protocol was applied to analyze the F_2 seeds, and to produce and analyze the F_3 generation. It was assumed that pure self-fertilization occurred and that the *spik-1* mutation affected only pollen competitive ability. Within this framework, the predicted percentage of homozygous wild-type genotype in generation n is $\%wt_n = 100Pwt \times S_n$, where

$$S_n = \sum_{i=1}^n 1/2^i,$$

and Pwt is the probability of fertilization by wild-type pollen in competition with *spik-1* pollen. The value of the unknown parameter Pwt was adjusted to fit the data using a Marquardt-Levenberg algorithm (least-squares fitting). This yielded $Pwt = 0.47$ for the control genotype and $Pwt = 0.64$ for the *SPIK/spik-1* genotype.

F_3 in the progeny of the *SPIK/spik-1* heterozygous plants. Over the three generations, the probability of fertilization by wild-type pollen in competition with *spik-1* pollen was estimated to be 0.63, and therefore the probability of fertilization by *spik-1* pollen to be 0.37 (i.e., $1 - 0.63$).

In vitro tests (Figs. 4,5) revealed that wild-type pollen grains displayed both a two-to-three-times higher tube development score and a 1.7-times longer tube length than *spik-1* pollen grains. Therefore, assuming a cumulative effect of these advantages, a Pwt/P_{spik-1} ratio of 3.4 to 5.1 would be predicted from these in vitro tests, which is much higher than the 1.7 ratio ($0.63/0.37$) deduced from in vivo tests (Fig. 6B). This indicates that the *spik-1* mutation has more drastic effects on pollen tube development in vitro than in vivo, in agreement with the contention that in vitro media fall short of mimicking the in vivo conditions (Lord 2000).

Discussion

The SPIK Shaker channel mediates inward K^+ current in the pollen plasma membrane

SPIK is the first ion channel active in the pollen membrane to be identified at the molecular level. This channel has been cloned in the model species *Arabidopsis thaliana*. Based on sequence analysis, the two closest relatives of *SPIK* within the *Arabidopsis* Shaker family are *AKT6* (Lacombe et al. 2000; in BAC F8B4, GenBank accession no. AL034567; 76% identity with *SPIK*), also called *AKT5* (Mäser et al. 2001), and *AKT1* (Sentenac et al. 1992; 55% identity with *SPIK*). The *AKT6* channel (Lacombe et al. 2000) has not yet been characterized at the functional level. *AKT1* has been characterized both in planta (Hirsch et al. 1998) and in heterologous contexts (Gaymard et al. 1996; Bertl et al. 1997) as an inwardly rectifying K^+ (Kin) channel. When expressed in COS cells, *SPIK* also behaves as a Kin channel. Its single channel conductance is in the range of that of other Shaker channels characterized in *Arabidopsis*. Like the other *Arabidopsis* Shaker channels characterized so far, *SPIK* is highly selective for K^+ . It activates independently of the external K^+ concentration, as also shown for the *AKT1* (Gaymard et al. 1996) and *KAT1* (Véry et al. 1995) Kin channels from *Arabidopsis*.

The strong sensitivity of *SPIK* to external pH (Fig. 2B) could allow regulation of K^+ influx by the apoplastic pH, itself under the control of H^+ transport activity of the germinating pollen grain and/or of the neighboring cells. The hypothesis that the sensitivity of Shaker channels to pH could play a role in coupling K^+ and H^+ fluxes has been also proposed for the guard cell Kin channels *KAT1* from *Arabidopsis* and *KST1* from potato (Müller-Röber et al. 1995; Hoth et al. 1997; Hoth and Hedrich 1999).

Comparison of Kin currents recorded in *Arabidopsis* pollen protoplasts (this study; Fan et al. 2001) with the functional properties of *SPIK* analyzed in COS cells reveals similarity in the following features: current activation kinetics, apparent gating charge, sensitivity to ex-

ternal pH, and absence of sensitivity to external Ca^{2+} in the 1 mM–10 mM range. The slight difference in activation threshold of \sim –100 mV in pollen protoplasts (Fig. 3A; Fan et al. 2001) versus \sim –125 mV in COS cells (Fig. 2B) can be ascribed to the fact that the activation threshold of Shaker channels expressed in heterologous systems is known to be sensitive to the host context, varying from one expression system to the other (Dreyer et al. 1999), and also to the expression level within the same expression system (Véry et al. 1994). It is also worth noting that, at the single-channel level, the only Kin channel so far described in pollen plasma membrane has a unitary conductance (in Lily) of 19 pS in 100 mM external K^+ (Obermeyer and Kolb 1993; Fig. 4), very close to that of SPIK (14 pS in 50 mM K^+). Finally, comparison of the electrophysiological recordings obtained in wild-type and *spik-1* pollen-grain protoplasts indicates that the *spik-1* mutation prevents expression of any slowly activating Kin channel in the cell membrane. Therefore, the simplest hypothesis is that SPIK is the main channel endowed with these functional features active on the pollen membrane, and that the *spik-1* mutation prevents expression of functional SPIK channels. This hypothesis is further supported by RT-PCR analyses suggesting that, within the Shaker channel gene family, the inward channel displaying the highest expression level in pollen is SPIK (Fig. 1F,G). Alternatively, if other Shaker Kin-channel subunits were expressed in pollen at significant levels (e.g., AKT2, KAT1, or KAT2; see Fig. 1G), allowing formation of heterotetrameric channels endowed with functional properties similar to those of SPIK (in COS cells), the hypothesis would be that *spik-1* behaves as a dominant-negative mutation. Whatever the mechanisms involved, it is clear that functional expression of SPIK is required for the pollen membrane to display significant Kin channel activity, and that the *spik-1* mutation knocks out this activity.

Role of SPIK in pollen

The depressive effect of decreased K^+ availability on pollen tube development, resulting in a total absence of tube growth when the free concentration of K^+ is decreased to \sim 5 μM , indicates that a minimal concentration of K^+ is absolutely required for sustained growth, in agreement with previous observations (Weisenseel and Jaffe 1976). K^+ influx in the growing tube is necessary for maintenance of the cytosolic K^+ concentration despite the increase in cell volume. It is also widely accepted (although not directly shown) that the pollen tube uses K^+ (Weisenseel and Jaffe 1976; Weisenseel and Wenisch 1980; Messerli et al. 1999), together with other osmotics such as sucrose (Stadler et al. 1999), to control its turgor. A minimal cell turgor is, indeed, necessary for rapid tube growth, although turgor alone is not sufficient to explain the variation in tube growth rates seen in vitro (Benkert et al. 1997).

The *spik-1* mutation strongly affects the capacity of the pollen to develop a tube in vitro, in the presence of

physiologically relevant K^+ concentrations. The depressive effect of the Kin-channel blocker Cs^+ (mimicking the effects of the *spik-1* mutation) further confirms that Kin-channel activity mediating K^+ uptake is required for pollen tube development. It can be calculated that a continuous K^+ current of \sim –10 pA is required for maintaining an internal K^+ concentration of \sim 120 mM (Weisenseel and Jaffe 1976; Bashe and Mascarenhas 1984) in a tube of 5- μm diameter growing at 2.5 $\mu\text{m}/\text{min}$. Could SPIK activity significantly contribute to this current? In young *Arabidopsis* pollen tubes in 1 mM external K^+ , we recorded resting-membrane potential values (-127 ± 2 mV) quite similar to those reported in germinated lily pollen grains in the presence of 1 mM K^+ (Weisenseel and Jaffe 1976). They are more negative than the activation potential of SPIK (–100 mV in Fig. 3A), and slightly more negative than the K^+ equilibrium potential (E_{K} , expected to be close to –120 mV; Weisenseel and Jaffe 1976; Bashe and Mascarenhas 1984). In these conditions (1 mM external K^+ and membrane potential of –127 mV), from the current–voltage curves obtained in pollen protoplasts (Fig. 3C) and the curve describing the effect of external K^+ concentration on SPIK current (Fig. 2C; K_{M} of 14 mM), it can be estimated that the slowly activating Kin current (dependent on SPIK expression) entering the pollen grain is close to –4 pA. This value is likely to underestimate the SPIK contribution to K^+ influx into the germinating pollen because measured membrane potentials are likely to have been underestimated. This is widely assumed when membrane potentials are determined by intracellular microelectrodes, as “even in the case of high-quality impalement a significant portion of the membrane potential would be dissipated at the impalement site” (Gassmann and Schroeder 1994). For instance, assuming that the actual membrane potential was \sim 20 mV more negative than the measured values, the SPIK current entering the pollen grain would be close to –9 pA. Therefore, our data support a model in which SPIK-dependent K^+ -channel activity would significantly contribute to wholesale K^+ influx, allowing control of internal K^+ concentration for sustained tube growth.

No estimate of the membrane potential of the growing pollen tube is available in planta. Also, the concentration of K^+ in the apoplast of the cells surrounding the growing pollen tube is unknown. However, the fact that the *spik-1* mutation affects in vitro pollen tube development even when the concentration of K^+ in the external solution is as low as 50 μM (Fig. 5C) suggests that SPIK-channel activity could allow high-affinity K^+ uptake, sustained by very negative membrane potentials. It is worthwhile noting that a similar situation has been described for the *Arabidopsis* AKT1 K^+ channel expressed in root hairs and cortical cells (Hirsch et al. 1998). The possible involvement under low external K^+ conditions of Kin channels in wholesale K^+ uptake during pollen tube germination has, however, been questioned (Messerli et al. 1999), highly negative resting membrane potentials having rarely been reported (Weisenseel and Wenisch 1980; Obermeyer and Blatt 1995; Messerli et al. 1999). Active $\text{K}^+:\text{H}^+$ symport activity has therefore been

assumed to be required for efficient K⁺ uptake, especially at high growth rate (Messerli et al. 1999, 2000).

In planta, the *spik-1* mutation strongly affects pollen competitive ability, the probability of fertilization by the mutant pollen being ~1.6–1.7 times lower than that by the wild-type one. In the absence of SPIK-type channel activity, pollen tube development in planta (and in vitro, in ~20% of the cells; Figs. 4B,5B) could be owing to transport systems responsible for the background inward K⁺ current observed in *spik-1* (and wild-type) pollen (Fig. 3B,C).

Conclusion

Pollen tube growth has been extensively studied, in particular because it provides an attractive model for investigating cell polarity and morphogenesis in tip growing cells (Derksen 1996; Taylor and Hepler 1997; Feijó et al. 2001). The molecular mechanisms underlying rapid and polarized growth in these cells are still poorly understood. The demonstration that the *spik-1* mutation affects pollen tube growth, and thereby pollen fitness, is therefore a highly significant result. Regarding the effect on pollen fitness, it has been emphasized that alleles that are measurably lowered in frequency in a few generations have, at the population level, essentially the same fate as a lethal mutation (Gilliland et al. 1998). The fact that, in the progeny of *SPIK/spik-1* plants, the wild-type genotype rapidly and significantly takes the advantage over the mutant genotype indicates that *SPIK* is essential, and is under a high selection pressure.

Materials and methods

SPIK cDNA cloning

A sequence encoding a K⁺ channel of the plant Shaker family (Zimmermann and Sentenac 1999) was identified in the F3N11 BAC (GenBank accession no. AC006053). A PCR fragment was amplified from this BAC and used as a probe for Northern blot experiments. Expression having been localized in flowers (Fig. 1), flower poly(A)⁺ RNAs were isolated and used for cDNA isolation by 5' and 3' RACE-PCR. Six cDNAs were obtained, sharing the same 2667-bp ORF. This *SPIK* ORF was amplified with *Pfu* polymerase (Promega) by reverse transcription-PCR on flower poly(A)⁺ RNAs with primers containing an *NheI* site upstream of the ATG codon (5'-GCTAGCATGGAGAAGAA GAAGGTATGGTTTTGGGG-3') and an *NotI* site downstream of the Stop codon (5'-GCGGCCGCTCAAGGATCCCTTGATAGAAGGAGAAAATCGCCATCTC-3'). The amplified fragment was cloned into the pCI expression vector (Promega) for heterologous expression in COS cells.

RT-PCR experiments

About 800 inflorescences from *Arabidopsis thaliana* plants grown in the greenhouse were split into four vials filled with 30 mL of the basal germination medium (see below). After vigorous shaking (vortex) to allow the release of pollen grains, the medium was filtered through one layer of Miracloth (CalBiochem) and centrifuged (4000g for 5 min), allowing recovery of a pure

pollen-grain preparation (checked under the microscope). The pellet was resuspended in 200 µL of RNA extraction buffer (Lobréaux and Briat 1992) and ground in a mortar, and the total RNA was purified according to Lobréaux and Briat (1992). The final pellet was resuspended in 20 µL of H₂O, and 5 µL was used for first-strand synthesis by MMLV reverse transcriptase (Promega) with 10 pmole of oligo(dT)₂₀ (final volume 20 µL). The reaction medium was diluted 10-fold in water, and 5 µL was used for each PCR reaction. For each *Shaker* gene, two independent couples of primers (sequences available upon request) surrounding introns were used for the PCR reaction (final volume 50 µL; ExtraPol I, Eurobio). Expression of the *EF1α* gene (Axelos et al. 1989) was monitored as a control.

Expression analysis using the GUS reporter gene

The *SPIK* promoter region was isolated by PCR on the BAC F3N11 DNA with a direct primer containing a *Bam*HI site (5'-GGATCCGTGTAACGAGAATAAAAAGTGGTTTC-3') and a reverse primer introducing an *Nco*I site at the initiation ATG codon (5'-CCATGGTTCAGATCCCTCTCTCTTTCTTTTCC-3'). The amplified fragment (~2.5 kb) was translationally fused to the *GUS* coding sequence in pBI320.X (this plasmid carries a unique *Nco*I site at the first initiation codon of the promoterless *GUS-3'* nopaline synthase reporter gene). This construct was introduced into the *Sac*I site of the pMOG 402 binary vector. The resulting plasmid was introduced into *Agrobacterium tumefaciens* MP90 (Höfgen and Willmitzer 1988). *Arabidopsis thaliana* was transformed with agrobacteria using the infiltration method (Bechtold et al. 1993). GUS histochemical staining was performed as previously described (Lagarde et al. 1996).

Isolation of a T-DNA-tagged plant disrupted in the *SPIK* gene

The *spik-1* mutant was obtained by PCR screening of a collection of ~40,000 *Arabidopsis* T-DNA insertion mutants (Wassilevskija ecotype; library constructed by the Laboratoire de Génétique et Amélioration des Plantes, INRA Versailles, France) with primers corresponding to the *SPIK* gene and to the T-DNA left and right borders. The exact position of the T-DNA insertion was determined by sequencing the T-DNA flanking sequences. Plants homozygous for the disruption and hemizygous plants were selected (by PCR) in the progeny of the positive line.

Expression and characterization of *SPIK* in COS cells

COS-7 cells were cotransfected with 33 ng of the transfection marker pIRES-CD8 plasmid and 2 µg of pCI-SPIK per dish as described (Reyes et al. 1998). Patch-clamp experiments were carried out 2–3 d after transfection. Transfected cells were identified by anti-CD8 antibody-coated beads (Dynabeads M450, Dynal ASA). Classical patch-clamp methods (Hamill et al. 1981) were applied, using an Axopatch 200A amplifier (Axon Instruments Inc.). Data were sampled (2 kHz, filtering 1 kHz) and analyzed with pClamp6 (Axon Instruments) or WinASCD (G. Droogmans, University of Leuven, Belgium) softwares. Voltage drops through series resistance were corrected (75%) during acquisition using the built-in compensation feature of the amplifier. Leak current was digitally subtracted before quantitative analyses of the data (current recordings shown in figures are raw data). At the end of cell-attached patch-clamp experiments, the cell resting potential was measured after rupture of the patch, by clamping the whole-cell current to zero. All patch-clamp solutions were adjusted to 300 mOsm with mannitol. The ex-

ternal basal solution (bath in whole-cell configuration and pipette filling in cell-attached configuration) contained 1 mM CaCl₂, 3 mM MgCl₂, 10 mM Mes/Tris (pH 5.0 or pH 6.0), or 10 mM Hepes/Tris (pH 7.4). Unless otherwise stated, the pH was 6.0 and the concentration of K⁺ (added as Cl⁻ salt) was 50 mM. When the concentration of K⁺ was lower than 50 mM, the solution was supplemented with NaCl for ionic strength adjustment (sum of the K⁺ and Na⁺ concentrations set to 50 mM), assuming no significant block of SPIK by Na⁺ as for several other plant Shakers (e.g., KAT1, Véry et al. 1995; AKT1, Gaymard et al. 1996; AKT2, Lacombe et al. 2000). For Cs⁺ block experiments, the external concentration of KCl was 10 mM, and that of CsCl was 0 (control), 50 mM, or 90 mM (supplemented with 90, 40, and 0 mM NaCl, respectively). In experiments addressing SPIK ionic selectivity, currents measured in 50 mM KCl were compared with those recorded in 50 mM XCl (X⁺ being Na⁺, Li⁺, Rb⁺, or NH₄⁺). The internal medium (whole-cell experiments) was comprised of 150 mM KCl, 3 mM MgCl₂, 5 mM MgATP, 5 mM EGTA, and 10 mM Hepes/NaOH (pH 7.2).

Patch-clamp on pollen protoplasts

Ten flower buds were collected and vortexed in 1 mL of a solution containing 1 mM KNO₃, 0.2 mM KH₂PO₄, 1 mM MgSO₄, 1 μM KI, 0.1 μM CuSO₄, 5 mM CaCl₂, 5 mM Mes, 1.6 mM Tris (pH 5.8), 1 M sorbitol, and 0.5 M glucose. Filtration on 100-μm mesh resulted in recovery of a fairly pure pollen-grain preparation. After centrifugation (10,000g for 1 min), the supernatant was discarded, and 0.5 mL of the digestion solution (same as above, complemented with 1% [w/w] cellulase [RS, Onozuka], 0.5% macerozyme [Onozuka], and 0.2% BSA) was added. Digestion occurred at 30°C for 1 h with gentle shaking. The suspension was then centrifuged for 5 min at 1000g, and the supernatant was replaced by 1 mL of patch-clamp bath solution containing 50 mM K-glutamate, 10 mM CaCl₂, 5 mM Mes (pH 5.7), and 1.35 M sorbitol (for osmolarity adjustment). Patch-clamp experiments were performed in the whole-cell configuration as described above for COS cells. The patch pipette solution was 100 mM K-glutamate, 1 mM MgCl₂, 5 mM EGTA, 2 mM MgATP, 10 mM HEPES, 20 mM Tris (pH 7.2), and 1.25 M sorbitol.

Resting-membrane potential measurements

Pollen grains were allowed to germinate for 1–2 h on a slide coated with a gelled germination medium containing 1 mM KCl (see next section below). Then the slide was covered with liquid medium (2-mm height) of the same composition for resting-membrane potential measurements. Tubes continued to grow in these conditions for at least 0.5 h. Impalement electrodes were pulled from soft glass capillaries (Micro hematocrit, Modulohm) with a one-step vertical puller (Narishige), and filled with 3 M KCl. Their resistance was 10–20 MΩ. The electrometer was an Axoprobe-1A (Axon Instruments). Resting-membrane potentials of young pollen tubes (50–150 μm) were recorded on a chart recorder.

Pollen germination analysis

Plants were grown in the greenhouse until flowering. The pollen was collected from freshly dehiscent anthers and dabbed on glass slides coated with a thin layer (~30 μm) of germination medium. Wild-type and *spik-1* pollen grains were always germinated in parallel on the same slides. Slides were incubated at 25°–28°C for 6 h and then photographed (under microscope), to determine germination rates and tube lengths (AnalySIS Pro 3

software, Soft Imaging System GmbH). The basal germination medium contained 1% low melting point agarose, 200 mM sucrose, 1.6 mM boric acid, and 3 mM calcium nitrate at pH 6.0 (Thorsness et al. 1993). Because of contaminating K⁺ in agarose and sucrose, the nominal concentration of this cation in the medium was ~80–90 μM. Addition of KCl ensured that the final concentration of K⁺ in the medium was 100 μM (controlled by flame spectroscopy assay). KCl or the K⁺ chelator tetraphenylborate (TPB; Bellando 1989) was added in some experiments to increase or decrease the concentration of free K⁺ in the germination medium. The dissociation constant of the TPB–K⁺ complex was estimated to be ~0.3 μM by measuring the changes in K⁺ activity in response to increased amounts of TPB with a K⁺-selective electrode, and assuming a first-order dissociation kinetics for the TPB–K⁺ complex.

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