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Root high-affinity K^+ and Cs^+ uptake and plant fertility in tomato plants are dependent on the activity of the high-affinity K^+ transporter SlHAK5

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Abstract

Root K^+ acquisition is a key process for plant growth and development, extensively studied in the model plant *Arabidopsis thaliana*. Because important differences may exist among species, translational research supported by specific studies is needed in crops such as tomato. Here we present a reverse genetics study to demonstrate the role of the SlHAK5 K^+ transporter in tomato K^+ nutrition, Cs^+ accumulation and its fertility. *slhak5* KO lines, generated by CRISPR-Cas edition, were characterized in growth experiments, Rb^+ and Cs^+ uptake tests and root cells K^+-induced plasma membrane depolarizations. Pollen viability and its K^+ accumulation capacity were estimated by using the K^+-sensitive dye Ion Potassium Green 4. SlHAK5 is the major system for high-affinity root K^+ uptake required for plant growth at low K^+, even in the presence of salinity. It also constitutes a pathway for Cs^+ entry in tomato plants with a strong impact on fruit Cs^+ accumulation. SlHAK5 also contributes to pollen K^+ uptake and viability and its absence produces almost seedless fruits. Knowledge gained into SlHAK5 can serve as a model for other crops with fleshy fruits and it can help to generate tools to develop low Cs^+ or seedless fruits crops.

KEYWORDS

caesium, pollen, potassium, salinity, transport

1 | INTRODUCTION

Potassium (K^+) is an essential macroelement for plants which fulfils important functions related to growth and development. It is concentrated in growing tissues, contributing to turgor potential and being crucial for processes that require fast growth or rapid movements such as pollen tube elongation or stomatal movements. Besides, it is involved in many physiological processes such as enzyme activation, neutralization of charges, plasma membrane potential maintenance as well as transcriptional and posttranslational regulation (Amtmann, Hammond, Armengaud, White, & Callow, 2006; Maathuis, 2009; Marschner, 1995). It has a great impact on metabolism (Amtmann & Armengaud, 2009) as well as on the movement of photosynthates and nitrogen compounds to sink tissues (Deeken et al., 2002; Hermans, Hammond, White, & Verbruggen, 2006; Mengel, Kirkby, Kosegarten, & Appel, 2001; White & Karley, 2010), affecting fruit quality and

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productivity of crops (Römheld & Kirkby, 2010). Due to all these functions, K^+ generally has a protective effect against biotic and abiotic stresses and plants with a high K^+ content cope better with environmental stresses (Shabala & Pottosin, 2014).

Due to the essential roles it plays, K^+ is required to maintain production and quality of crops. However, large agricultural areas are deficient in K^+ (Luan et al., 2017; Römheld & Kirkby, 2010) and local areas near the root surface may be depleted of K^+ because of its rapid absorption by the root. In addition, two environmental conditions related to K^+ nutrition, salinity and radiocaesium, challenge current agriculture. Salinity is one of the most important stresses limiting crop production, affecting over 800 million hectares around the world. It is estimated that a 20% of irrigated lands, which produce one-third of world food, suffer from secondary salinization. One of the clear effects that high Na^+ concentrations typical of salinized lands have on plants is the induction of K^+ deficiency (Munns, 2005). In relation to this, increasing K^+ supply to plants reduces Na^+-induced toxicity (Shabala & Pottosin, 2014). A recent analysis suggests that the energy cost of plant K^+ uptake under salt stress can be a limiting factor for plant salt tolerance (Rubio, Nieves-Cordones, Horie, & Shabala, 2020). Radiocaesium released after nuclear accidents has also become an important threat for agriculture due to the large area of land contaminated and its persistence in time (decades) (Yasunari et al., 2011). Radiocaesium, when present in the soil, is rapidly absorbed by crop roots and accumulated in their edible organs (Fujiwara, 2013). Thus, the consumption of radiocaesium-containing food by cattle and humans imposes serious health concerns. It is well-known that K^+ and Cs^+ are taken up by roots in a similar fashion and root K^+ uptake systems may constitute the gate for Cs^+ accumulation into the plant (Collander, 1941; White & Broadley, 2000). Whether for correcting K^+ deficiencies or for reducing the deleterious effects of the mentioned abiotic stresses or others, the fact is that modern agriculture is based on the use of large amounts of K^+ fertilizers. Indeed, 40–60% of crop yields are attributable to fertilization (Stewart, Dibb, Johnston, & Smyth, 2005). This involves important economic and environmental costs and there is a need to generate crop varieties with a higher use-efficiency of K^+ which allow a sustainable agriculture. One of the approaches to meet this goal would be to improve root K^+ acquisition, which requires the characterization of the K^+ uptake systems of crops.

K^+ is taken up from the soil solution by the roots through specific transport systems located at the plasma membrane of epidermal and cortical root cells. Research in the model plant *Arabidopsis thaliana* has allowed the characterization of the main systems involved in root K^+ uptake and their regulatory mechanisms (Aleman, Nieves-Cordones, Martinez, & Rubio, 2011). Thus, the AtHAK5 transporter and the AKT1 channel have been described as the major contributors to root K^+ uptake (Rubio, Nieves-Cordones, Alemán, & Martinez, 2008). AtHAK5 is the only system mediating K^+ uptake at external concentrations lower than 20 μM (Nieves-Cordones, Aleman, Martinez, & Rubio, 2010; Pyo, Gierth, Schroeder, & Cho, 2010; Rubio et al., 2008). At higher concentrations, from 20 to 200 μM, both AtHAK5 and AKT1 contribute to uptake. At concentrations higher than 500 μM K^+, AtHAK5 contribution is very low and AKT1 becomes the predominant pathway for K^+ uptake (Pyo et al., 2010; Rubio, Alemán, Nieves-Cordones, & Martínez, 2010). It is worth to highlight that HAK5-like K^+ transporters have been shown to play an important role in K^+ nutrition under salt stress (Chen et al., 2018; Nieves-Cordones et al., 2010; Shen et al., 2015) and for plant Cs^+ accumulation (Nieves-Cordones et al., 2017; Rai et al., 2017). Thus, this type of transporters is interesting targets for crop biotechnology.

As more information is obtained, it is becoming clear that important differences in the functionality and regulation of HAK5-like transporters exist among species. As an example, it has been observed that AtHAK5 contribution to K^+ uptake is decisive at external concentrations ≤20 μM K^+ in *Arabidopsis* (Rubio et al., 2008) whereas the rice homolog OsHAK1 operates in a much wider range of K^+ concentrations (0.001–1 mM K^+) (Chen et al., 2015; Nieves-Cordones et al., 2017). In addition, K^+ transport systems may have additional functions in crops that are absent in *Arabidopsis*. This is the case of OsHAK1 that contributes to pollen viability, fertility and plant yield (Chen et al., 2018) whereas a similar role for AtHAK5 has not been reported. In conclusion, the *Arabidopsis* model cannot be literally extended to other plant species. Thus, although research in *Arabidopsis* is still fundamental to identify the main actors and regulatory mechanisms for root K^+ uptake, studies in crops are necessary to weight the relative contribution of the different systems and to discover new roles of these actors.

Here we present a study on tomato plants which demonstrates the important role of the high-affinity K^+ transporter SlHAK5 in root K^+ uptake. By using CRISPR-Cas genome edition, KO *slhak5* mutant lines are obtained and characterized. The results show the crucial role of SlHAK5 for root high-affinity K^+ uptake, for maintaining K^+ nutrition and growth under K^+-limiting conditions and especially if they occur under salinity as well as for the accumulation of Cs^+ in the plant, especially in the fruit. In addition, the results show a novel function for a HAK5-type transporter in a dicotyledonous plant with fleshy fruits because SlHAK5 is required for accumulation of K^+ in pollen grains, germination and tube elongation, constituting an important determinant of seed and fruit production.

2 | RESULTS

2.1 | Generation of *slhak5* KO mutants by CRISPR-Cas edition

Previous results by our group pointed to SlHAK5 as a candidate transport system involved in K^+ and Cs^+ accumulation from diluted solutions in tomato plants (Nieves-Cordones, Martinez-Cordero, Martinez, & Rubio, 2007; Nieves-Cordones, Miller, Alemán, Martínez, & Rubio, 2008; Rodenas, Nieves-Cordones, Rivero, Martinez, & Rubio, 2018). In order to demonstrate the role of SlHAK5 in high-affinity K^+ and Cs^+ uptake in tomato roots, the *SlHAK5* locus was edited with the CRISPR-Cas system to produce knock-out mutants. Two single-guide RNA (sgRNAs) were designed to target two genomic

DNA regions corresponding to exon 1 and exon 2 (Figure 1) at positions +147, in the non-coding strand and +1203, in the coding strand, from the ATG, respectively. The coding sequence of the two spacer regions of these sgRNAs was cloned into the same entry vector A59 (multiplex edition). Both sgRNA expression cassettes were subsequently transferred to the A60 plant expression vector and transformed into *Agrobacterium tumefaciens* GV3101 strain (both plasmids A59 and A60 are property of Abiopep Plant Health S.A.). Then, cotyledons of Micro-Tom Wild-type (WT) plants were infected with *A. tumefaciens* and independent plant lines resistant to kanamycin (the antibiotic resistance marker in the transfer DNA) were obtained.

After several transformation attempts, only eight plants were regenerated. After genotyping these plants, four of these lines contained WT alleles in the *SlHAK5* locus. The other four were edited and contained indels that resulted in *SlHAK5* open reading frame truncation and generation of KO mutants (Table 1, Figure S1). Importantly, two of these lines produced no seeds and could not be propagated. The last two lines L1 (*slhak5-1*) and L2 (*slhak5-2*) produced very few seeds and were used for further experiments. It is important to take into account that only the regenerated lines that were not edited (WT allele in the *SlHAK5* locus) produced tomato fruits and seeds to the same levels of a WT plant. By contrast, lines containing KO alleles of *SlHAK5* either produced no or very few seeds (Table 1). This suggested that the low seed production of *slhak5* lines was specific to the edition of the *SlHAK5* gene.

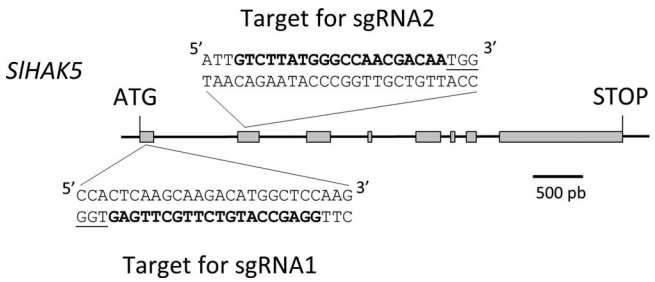

FIGURE 1 Generation of *slhak5* KO mutants with the CRISPR-Cas system. Overview of the *SlHAK5* locus and target sites for the sgRNA-Cas9 complex. Rectangles and lines depict exons and introns in *SlHAK5* gene, respectively. Two spacer sequences were designed in silico to target the sgRNA-Cas9 complex to their corresponding targets sites (sequence provided in bold letters) in exons 1 and 2, respectively. protospacer adjacent motif (PAM) sequences are underlined

2.2 | *slhak5* plants are affected in root high-affinity K^+ uptake

In a first series of experiments we studied the effect of the KO *slhak5* mutation on root K^+ uptake (Figure S2). Rb^+ was used as a tracer for K^+ in uptake experiments at two external concentrations, 1 and 0.02 mM Rb^+ in K^+-sufficient and -starved plants. After growing *slhak5-1* plants under control Hoagland solution containing 0.3 mM K^+ for 14 days, a set of plants were subjected to K^+ starvation by growing them for 7 days in the absence of K^+. Another set remained in the complete solution with 0.3 mM K^+. Then, plants were incubated for 6 hr in nutrient solution with no K^+, supplemented with 1 or 0.02 mM Rb^+. No differences in the dry weights of the different organs were observed between WT and *slhak5-1* plants (Figure 2a). As expected, the content of the K^+-sufficient organs was higher than those of the K^+-starved ones (Figure 2b). The 7-day K^+-starvation treatment reduced organ K^+ content around 50% and no differences were observed in K^+ content between WT and *slhak5-1* organs (Figure 2b). Rb^+ uptake rates were calculated from the Rb^+ accumulation within the plant. It could be observed that WT and *slhak5-1* plants showed similar rates of Rb^+ uptake in K^+-sufficient plants assayed in the presence of 1 mM Rb^+ (Figure 2c). When these K^+-sufficient plants were assayed at 0.02 mM Rb^+, lower rates of Rb^+ uptake than when assayed at 1 mM Rb^+ were observed in both lines. Importantly, the uptake rates of the *slhak5-1* line were of a lower magnitude than those of the WT plants (Figure 2c). K^+ starvation increased the rates of Rb^+ uptake in WT plants from an external concentration of 1 mM Rb^+ and specially from 0.02 mM external Rb^+ (Figure 2d). These results indicated that K^+ starvation induced the high-affinity component of K^+ uptake, in agreement with previous results on both root K^+ uptake and *SlHAK5* expression (Bacha et al., 2015; Nieves-Cordones et al., 2007, 2008; Rodenas et al., 2018). In the

TABLE 1 Allelic sequences of the *SlHAK5* locus at the sgRNAs target sites and number of seeds of T0 tomato plants

		sgRNA1 target (exon 1)		sgRNA2 allele (exon 2)		
Line	Name	Allele 1	Allele 2	Allele 1	Allele 2	Number of seeds per plant
L1	*slhak5-1*	+1	+1	−2	-3	10
L2	*slhak5-2*	+1	+1	-2	−4	6
L3	—	+1	+1	−2	−4	0
L4	—	+1	+1	−2	−3	0
L5	—	WT		WT		152
L6	—	WT		WT		66
L7	—	WT		WT		281
L8	—	WT		WT		214

Note: Positive values and negative values depict the number of bp inserted or deleted, respectively, in the *SlHAK5* locus.

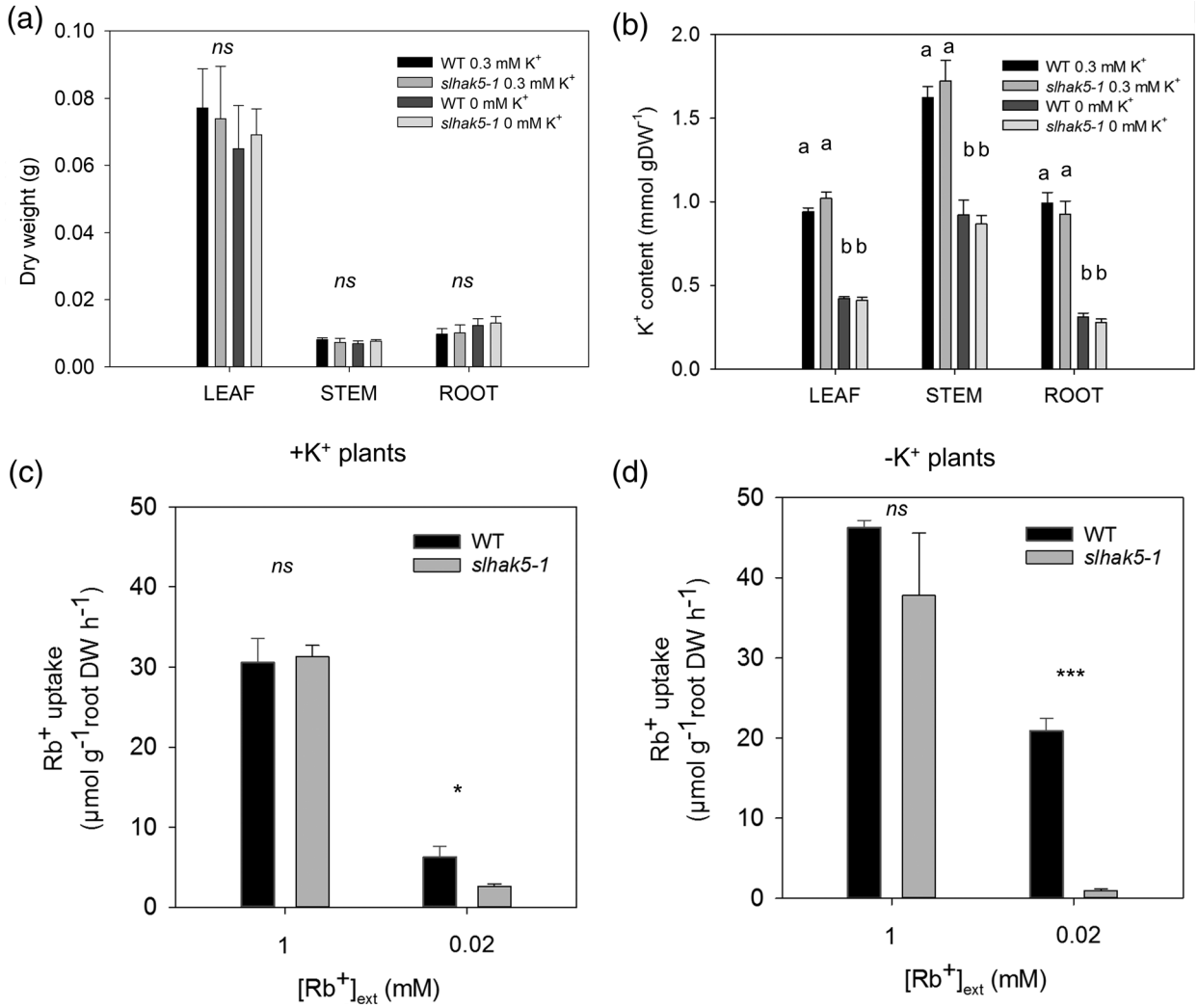

FIGURE 2 Organ dry weight, K^+ content and high-affinity K^+ uptake in K^+-starved WT and *slahk5-1* plants. Plants of the WT and *slhak5-1* lines were grown for 14 days in complete Hoagland solution containing 0.3 mM K^+. Then, a set of plants were grown for 7 days in solution with no K^+ and another set remained in the complete solution. After this growth period, plants were transferred to solution with no added K^+ and 0.02 mM or 1 mM RbCl (as indicated) for 6 hr and then separated in roots, stems and leaves, dried. Four days later their dry weight was determined. After acid digestion, the internal K^+ and Rb^+ content in tissues were determined. (a) Dry weight of leaves, stems and roots. (b) K^+ content of leaves, stems and roots. (c, d) Rb^+ uptake rates of in K^+-sufficient (c) and K^+-starved (d) plants at 1 and 0.02 mM Rb^+. Data are mean values (n = 5) ± *SE*, and bars with different letters are significantly different at $p < .05$ according to Tukey's test. ns denotes not significant. * and *** indicate $p < .05$ and $p < .001$ in Student's t test, respectively

presence of 1 mM Rb^+, K^+-starved *slhak5-1* plants showed a value of Rb^+ uptake rate close to that of WT plants (Figure 2d). However, at 0.02 mM external Rb^+, the *slhak5-1* line did not show the observed increase of Rb^+ uptake displayed by K^+-starved WT plants. Indeed, under these conditions, the *slhak5-1* line showed a 22-fold lower rate of Rb^+ uptake than WT plants (Figure 2d). These results showed that SlHAK5 was required by tomato plants for K^+ uptake in the high-affinity range of concentrations. Rb^+ uptake in the high-affinity range of concentrations was also studied in K^+-starved plants of the *slhak5-2* line, which produced similar results to *slhak5-1* plants (Figure S3).

slhak5-edited plants produced a very low number of seeds (seeds per plant, Table 1). This precluded a comprehensive kinetic characterization of the effect of the mutation on high-affinity K^+ uptake either in short-term Rb^+ uptake or in long-term K^+ accumulation experiments. Therefore, the root cell plasma membrane depolarization produced by increasing external K^+ concentration was used as an alternative approach. As described previously, membrane depolarization induced by increasing external K^+ concentration can be used as a measurement of the K^+ uptake capacity of root cells (Spalding et al., 1999). WT and *slhak5-1* epidermal and cortical root cells of K^+-starved plants were impaled with electrodes and the membrane potential response to changes in external K^+ determined. It was observed that increasing external K^+ produced much higher membrane potential depolarizations in WT roots than in *slhak5-1* roots, except at 0.5 mM K^+, concentration at which similar depolarizations were observed in both lines (Figure 3, Figure S2). The depolarizations

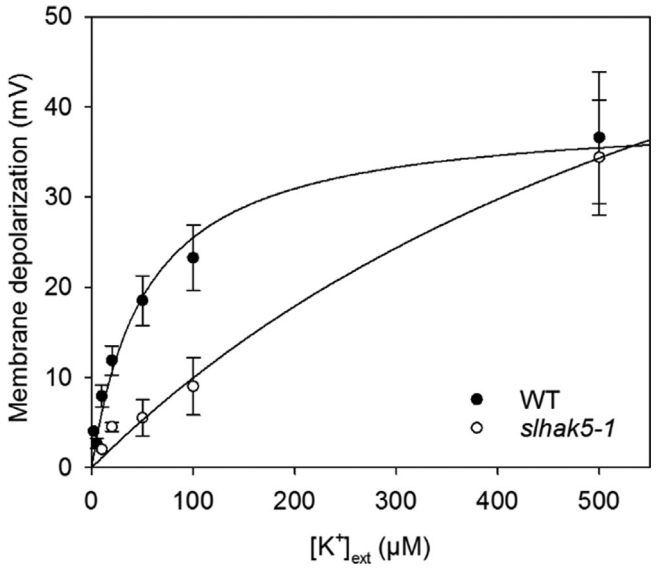

FIGURE 3 Root cell plasma membrane depolarizations induced by K^+ in WT and *slhak5-1* plants. Epidermal and cortical root cells of WT and *slhak5-1* seedlings germinated in water for 10 days were impaled with microelectrodes and their membrane potential registered with an electrometer. Roots were perfused with a solution containing 0.5 mM $CaCl_2$ buffered to pH 6 (10 mM MES/Bis-Tris) and different amounts of KCl to reach the indicated concentrations. Plasma membrane depolarizations induced by external K^+ were plotted versus the external K^+ concentration. Data were fitted to Michaelis–Menten kinetics (R^2 were .96 and .99 for WT and *slhak5-1* data, respectively). Data are mean values of at least three repetitions and error bars denote *SE*

were plotted versus the external K^+ concentrations and the data fitted to Michaelis–Menten equations due to the enzyme kinetics-like behaviour of these measurements (Nieves-Cordones et al., 2017). Kinetic parameters were determined and they showed that in WT plants the depolarizations supported the existence of a K^+ uptake component with an apparent K_m of 26.4 ± 5.2 μM K^+ whereas in the *slhak5-1* line that component showed a K_m of 775 ± 15.9 μM. The higher K_m values in *slhak5-1* roots indicate a lower capacity to take up K^+ at low external K^+ concentrations in comparison to WT roots.

2.3 | SlHAK5 is required for K^+ uptake to sustain plant growth under K^+-limiting conditions in the absence and in the presence of salinity

A long-term growth experiment at limiting external K^+ was performed (Figure S2). Plants of the WT and the *slhak5-1* lines were grown for 7 days in a complete solution with 1.4 mM K^+. Then they were transferred for 14 days to a solution containing 0.01 mM K^+, a concentration where SlHAK5 is expected to have a prominent role in K^+ uptake according to Rb^+ uptake experiments (Figures 2 and 3). In parallel, a set of plants were grown with 50 mM NaCl in the nutrient solution to impair K^+ uptake (external ratio Na^+:K^+ 5,000:1) (Nieves-Cordones et al., 2010).

slhak5-1 plants grown with 0.01 mM K^+ were much smaller than WT plants both in the absence and presence of 50 mM Na^+ (Figure 4a). Importantly, shoots of *slhak5-1* showed chlorosis symptoms characteristic of K^+ deficiency. Root and leaf dry weight of *slhak5-1* plants grown under 0.01 mM K^+ were about one-third the weights of those organs of WT plants, whereas stems dry weight were similar in both plant lines (Figure 4b). It is worth to highlight that the presence of 50 mM Na^+ in WT plants grown under 0.01 mM K^+ increased tissue dry weight. However, this beneficial effect of Na^+ was barely observed in *slhak5-1* plants. Thus, while the presence of Na^+ increased the dry weight of WT plants a 53%, it only increased a 9% that of *slhak5-1* plants. This indicated that SlHAK5 K^+ uptake activity was required to benefit from the additional Na^+ supply.

K^+ content in roots, stems and leaves of these plants were determined. In general, the K^+ contents of *slhak5-1* organs were about 50% lower than those of WT ones (Figure 4c). The presence of 50 mM Na^+ reduced internal K^+ content and this effect was of a similar magnitude in both plant lines. It could be observed that, with the exception of a lower Na^+ content in *slhak5-1* stems of plants exposed to 50 mM Na^+, both plant lines showed similar Na^+ content in their organs (Figure S4a). Thus, the K^+/Na^+ ratios of plants grown in the presence of 50 mM Na^+ were higher in leaves and roots of WT plants than in those of *slhak5-1* plants (Figure S4b).

The plants subjected to the described treatments were assayed for Rb^+ uptake from a 0.02 mM solution. WT plants grown in the absence of Na^+ showed a 23.2 ± 1.5 μmol Rb^+ g^{-1}_{rootDW} hr^{-1} Rb^+ uptake rate, while Rb^+ uptake in *slhak5-1* plants was almost negligible, showing a rate of 0.68 ± 0.07 μmol Rb^+ g^{-1}_{rootDW} hr^{-1} (Figure 4d). These uptake rates of plants grown continuously with 0.01 mM K^+ were in agreement with those shown by K^+ starved plants (Figure 2d). The presence of 50 mM Na^+ reduced about 50% the rate of Rb^+ uptake in WT plants whereas had no effect on *slhak5-1* plants (Figure 4d). This is in conformity with the reported *SlHAK5* expression pattern, induced by K^+ starvation and repressed by the presence of NaCl (Nieves-Cordones et al., 2007, 2008; Rodenas et al., 2018). All together, these results demonstrated the important role of SlHAK5 for K^+ uptake from diluted solutions (Figure 4d) to sustain plant growth (Figure 4a,b). Importantly, the results show that, although the presence of 50 mM NaCl reduced K^+ uptake, SlHAK5 constitutes the only system mediating K^+ uptake at an external concentration of 0.01 mM (Figure 4d).

2.4 | *slhak5* plants show reduced Cs^+ uptake and accumulation

Cs^+ accumulation in plants has been related to the activity of HAK5-like transporters in roots and in a previous report SlHAK5 has been proposed to be the major contributor for Cs^+ accumulation within the plant (Rodenas et al., 2018). In order to clarify the contribution of

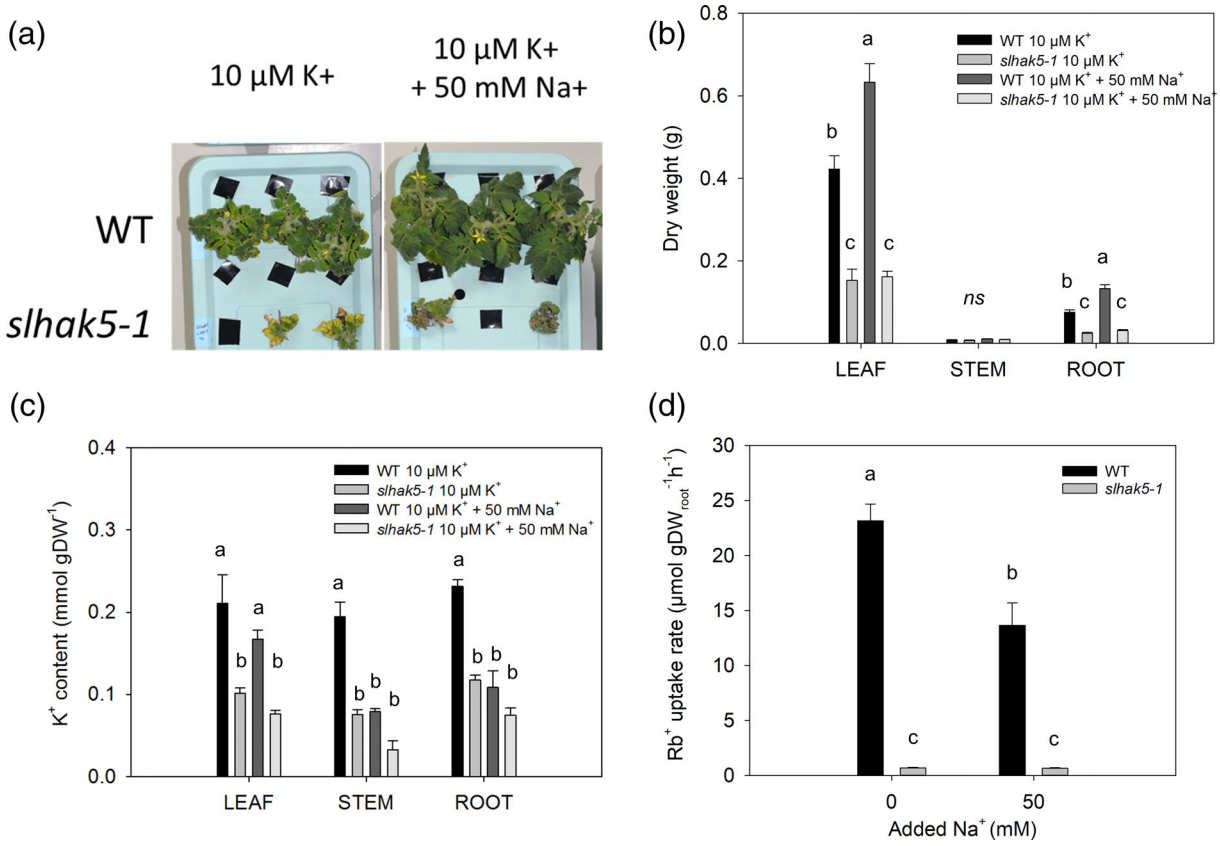

FIGURE 4 Pictures, organ dry weight, K^+ content and high-affinity Rb^+ uptake of WT and *slahk5-1* plants grown with 0.01 mM K^+ in the absence and the presence of 50 mM NaCl. Plants of the WT and *slhak5-1* line were grown for 7 days in nutrient solution containing 1.4 mM K^+ and then transferred to solution containing 0.01 mM K^+ in the absence or the presence of 50 mM NaCl during 14 days. (a) Pictures of plants at the end of the experiment, (b) organ dry weight, (c) internal K^+ content and (d) Rb^+ uptake rates when incubated 6 hr in the presence of 20 µM RbCl. Data are mean values (n = 4–6) ± *SE*, and bars with different letters are significantly different at $p < .05$ according to Tukey's test. ns denotes not significant

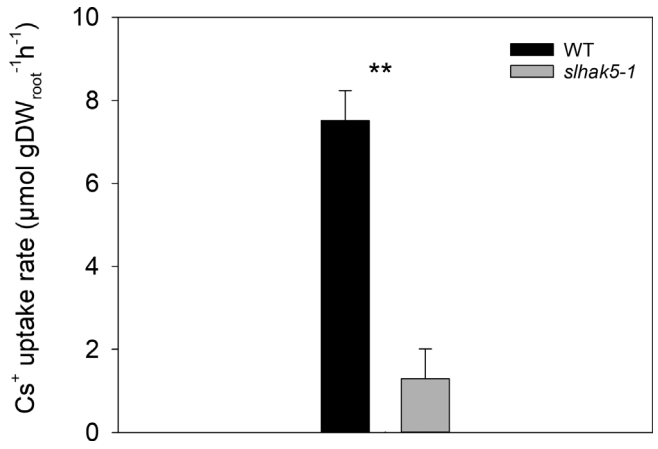

FIGURE 5 Cs^+ uptake rates from 0.02 mM external Cs^+ solution in K^+-starved plants. Plants of the WT and *slhak5-1* lines were grown and starved of K^+ as indicated in Figure 2. Then, plants were transferred to a solution with no added K^+ in the presence of 0.02 mM CsCl for 6 hr. After this, plant material was harvested and processed to determine the rates of Cs^+ uptake. Data are mean values ± *SE* and ** indicates $p < .01$ in Student's t test

SlHAK5 in this process, K^+-starved plants were assayed for Cs^+ uptake from a 0.02 mM solution (Figure S2). The results obtained clearly showed that Cs^+ uptake rates were lower in *slhak5-1* plants in comparison to those of WT plants (1.29 ± 0.72 µmol Cs^+ g^{-1}_{rootDW} hr^{-1} vs. 7.52 ± 0.72 µmol Cs^+ g^{-1}_{rootDW} hr^{-1}, respectively; Figure 5).

Another experiment was designed to study Cs^+ accumulation in different plant organs and importantly in fruits. The latter aspect is of crucial importance in Cs^+-contaminated agricultural lands where fruit commercialization may not be allowed due to a high Cs^+ content. Plants were grown in a solution containing 0.3 mM K^+ and 0.02 mM Cs^+ for 90 days (Figure S2). It was observed that *slhak5-1* plants accumulated much less Cs^+ in their organs than WT plants (Figure 6a). The Cs^+ content was 61-, 52-, 18-, 7- and 22-fold higher in WT roots, stems, young leaves, mature leaves and fruits, respectively, than in *slhak5-1* ones (Figure 6a). The K^+ content of plant organs did not show differences between the two lines, except in fruits (Figure 6b). Therefore, the K^+/Cs^+ ratios were much higher in *slhak5-1* than in WT plants, indicating a higher discrimination between K^+ over Cs^+ in plants lacking SlHAK5 (Figure 6c). The Cs^+ transfer factors (TF_{fruit}) from the external solution to the fruit were calculated and important differences between WT and mutant plants were observed. While

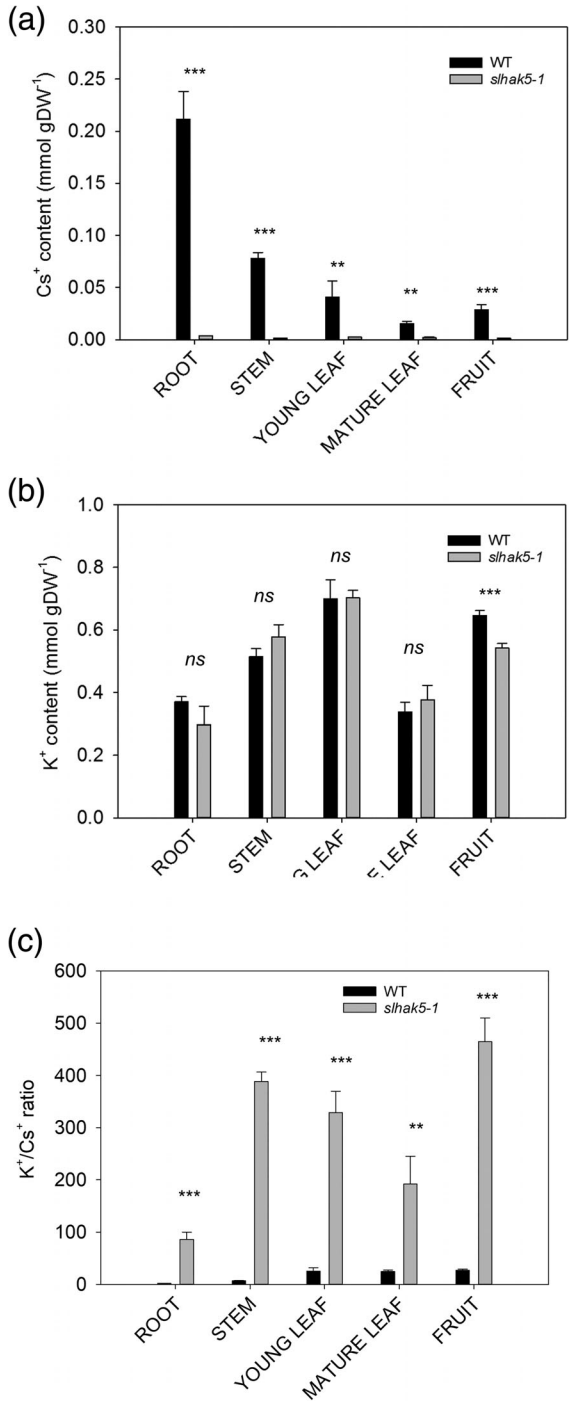

FIGURE 6 Cs^+ and K^+ content and K^+/Cs^+ ratios in organs of plants grown in the presence of 0.3 mM K^+ and 0.02 mM Cs^+. Plants of the WT and *slhak5-1* lines were grown for 90 days in a solution containing 0.3 mM K^+ and 0.02 mM CsCl to allow fructification and fruit harvests. After this, organs were harvested and processed to determine their internal (a) Cs^+ and (b) K^+ content. (c) The internal K^+/Cs^+ ratios were calculated. Data are mean values (n = 3) ± *SE*. ** and *** indicate $p < .01$ and $p < .001$, respectively, in Student's t test. ns denotes not significant

WT plants showed a TF_{fruit} of 151.6 ± 28, *slhak5-1* plants showed a TF_{fruit} of 8.5 ± 0.7. It is worth to mention that the lower TF_{fruit} of *slhak5-1* plants was achieved without affecting plant K^+ content.

2.5 | *slhak5* plants showed reduced fruit and seed production

As indicated above, the CRISPR-Cas edited KO *slhak5* plants produced tomato fruits with very few seeds in comparison to WT plants (Table 1). This effect suggests that SlHAK5 may play a role on fruit physiology, which was further studied. Plants were grown in K^+ sufficient (1.4 mM K^+) nutrient solution until fructification (Figure S2). Tomato fruits were harvested at different time points as they ripen (five harvests in total). Fruit production was delayed in *slhak5-1* in relation to WT plants (Figure 7a). At the date of the first fruit harvest in WT plants, no tomato fruits could be collected in the *slhak5-1* line. In addition, the average weight of *slhak5-1* tomatoes was lower than that of WT plants (Figure 7b). As a consequence, the total fruit yield was lower in the *slhak5-1* line than in the WT (22.6 ± 3.0 g of tomatoes per plant for *slhak5-1* vs. 51.6 ± 9.7 g for WT). Moreover, the number of seeds per tomato fruit was much smaller in *slhak5-1* plants, with 1.5 ± 1.5 seeds per tomato in comparison with the 15.6 ± 4.8 seeds per tomato in WT plants (Figure 7c,d).

2.6 | *SlHAK5* is expressed in stamens and pistils

The reduced fruit and seed production of the *slhak5* lines (Table 1, Figure 7) suggested that SlHAK5 may play a role in a process directly related to ovule fertilization. Therefore, the expression of *SlHAK5* in different plant and flower organs was determined by real-time quantitative polymerase chain reaction (qPCR). Plants were grown under K^+ sufficient supply (1.4 mM K^+) until flowering and samples of roots, stems, leaves, sepals, petals, stamens and pistils were collected to determine *SlHAK5* mRNA levels (Figure S2). qPCR results showed that *SlHAK5* was preferentially expressed in roots followed by stamens and pistils, and that its expression was the lowest in leaves and stems (Figure 8).

2.7 | Pollen germination and pollen tube growth were importantly impaired in *slhak5* plants

Ovule fertilization and seed development directly depend on the germination and tube growth of pollen grains (Wilhelmi & Preuss, 1999). Because the *slhak5* lines were affected in seed production (Table 1) and *SlHAK5* was expressed in stamens (Figure 8), the effect of the *slhak5-1* mutation on pollen physiology was studied (Figure S2). Germination of pollen grains from WT and *slhak5-1* plants was determined on a microscope slide covered with minimal media that contained no added K^+ ($[K^+]_{ext} \approx 100$ μM), or that was supplemented with 1 or 5 mM K^+. Pollen grains were sprinkled on that media and incubated for 4 hr. At first sight, it could be observed that few *slhak5-1* pollen grains germinated and many looked flaccid and wrinkled while most of WT ones had germinated and were rounded and turgid (Figure 9a,b). It could be observed that around 50% of WT pollen grains germinated and that increasing K^+ slightly increased the

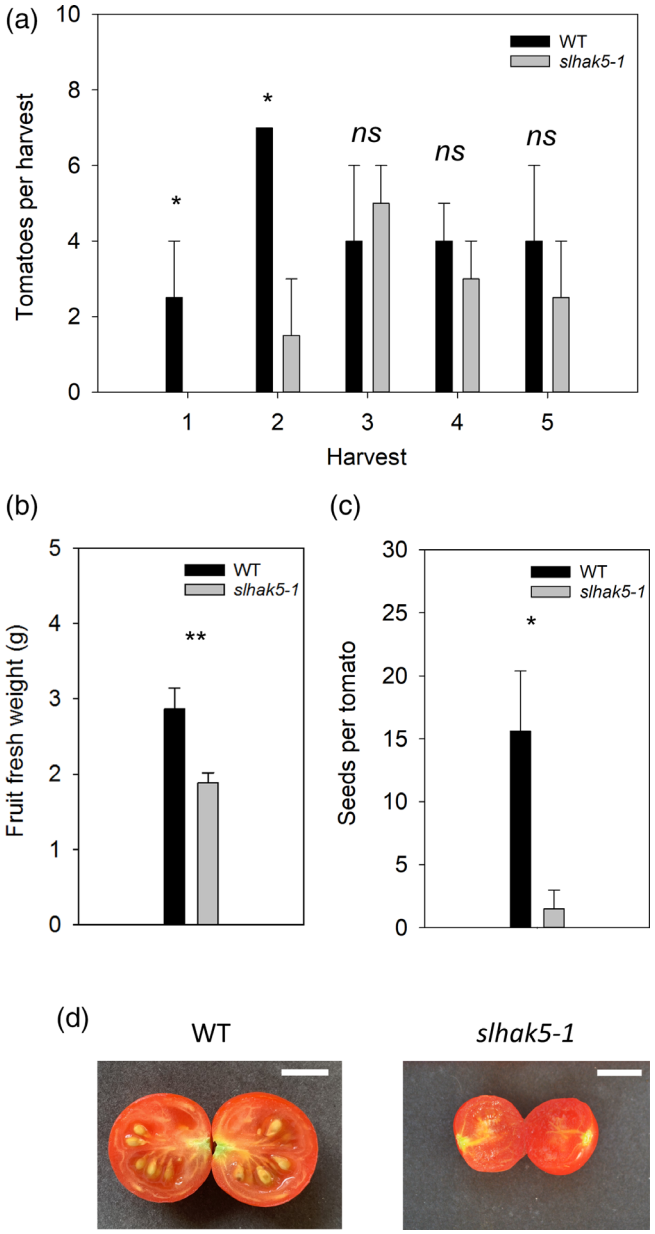

FIGURE 7 Number of tomato fruits harvested, plant yield and seeds per tomato of WT and *slhak5* plants. Plants of the WT and *slhak5-1* lines were grown in the presence of 1.4 mM K^+ until fructification. Tomato fruits were harvested at different time points. (a) Number of tomato fruits per harvest (1, 2, 3, 4 and 5 denote 90, 97, 106, 118 and 129 days after germination) of three plants for each line. (b) Average fruit fresh weight of WT and *slhak5-1* plants. (c) Average number of seeds per tomato in WT and *slhak5-1* plants. Data are mean values ± *SE* and * and ** indicate $p < .05$ and $p < .01$, respectively, in Student's *t* test. ns denotes not significant. $n = 3$ for plant tissues and $n = 15$ for fruits. (d) Photographs of WT and *slhak5-1* tomatoes. Scale bar = 1 cm [Colour figure can be viewed at wileyonlinelibrary.com]

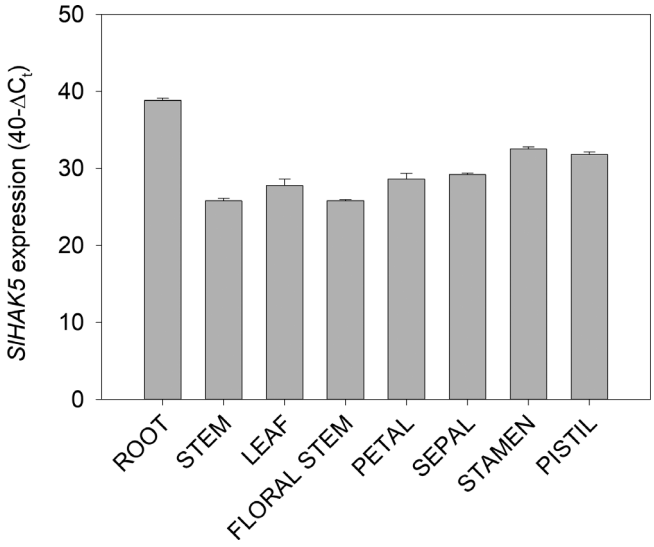

FIGURE 8 Relative expression levels of *SlHAK5* in different organs of WT plants. Plants of the WT line were grown in the presence of 1.4 mM K^+ until fructification. Plants were separated in the indicated plants organs, their RNA isolated and reverse transcribed. Real-time PCR was used to determine relative expression levels of *SlHAK5* with respect to the endogenous gene (*SlEF1α*). Relative expression levels are given as $40 - \Delta Ct$ ($\Delta Ct = Ct_{SlHAK5} - Ct_{SlEF1\alpha}$). Data are means of three biological replicates and error bars denote *SE*

germination rate, although this K^+ effect was not significant (Figure 9c). By contrast, less than a 10% of the *slhak5-1* pollen grains germinated and increasing the K^+ concentration of the germination media did not increase their germination rate but rather it decreased it (Figure 9c). Then, length of pollen tubes 4 hr after germination was determined. In the absence of added K^+, the tube length of WT grains was larger than that of *slhak5-1* grains (Figure 9d). Increasing external K^+ importantly increased the tube length of WT pollen grains. By contrast, K^+ addition had no effect on tube length of *slhak5-1* grains. Germination rates and pollen tube lengths were also determined in pollen grains from the *slhak5-2* line. In addition, pollen from a descendant of the *slhak5-1* line, which lacked the T-DNA cassette, was also characterized (Figure S5). The germination rates and pollen tube lengths of these two lines were comparable to those shown in Figure 9c,d for *slhak5-1* pollen, and gave further support to the idea that the mutation in *SlHAK5* gene was related to poor pollen performance of *slhak5* plants.

The observed phenotypes of pollen could be due to different concentrations of K^+ within pollen grains during germination and tube elongation. Thus, the relative K^+ concentration of pollen was estimated by using the K^+-sensitive fluorescent dye Ion Potassium Green 4 (IPG-4). Pollen grains were germinated in the absence of added K^+ and in the presence of 1 mM K^+ with IPG-4. It was observed that in the absence of added K^+, the fluorescence of WT pollen grains was higher than that of *slhak5-1* ones. When the K^+ of the germination medium was increased to 1 mM, the internal fluorescence of WT pollen grains increased about 1.7-fold whereas that of *slhak5-1* grains was not affected. These results showed that WT pollen grains contained higher K^+ concentrations than *slhak5-1* pollen grains and that the WT pollen grains were able to take up K^+ from the external medium whereas *slhak5-1* ones did not. Similarly, IPG-4 fluorescence

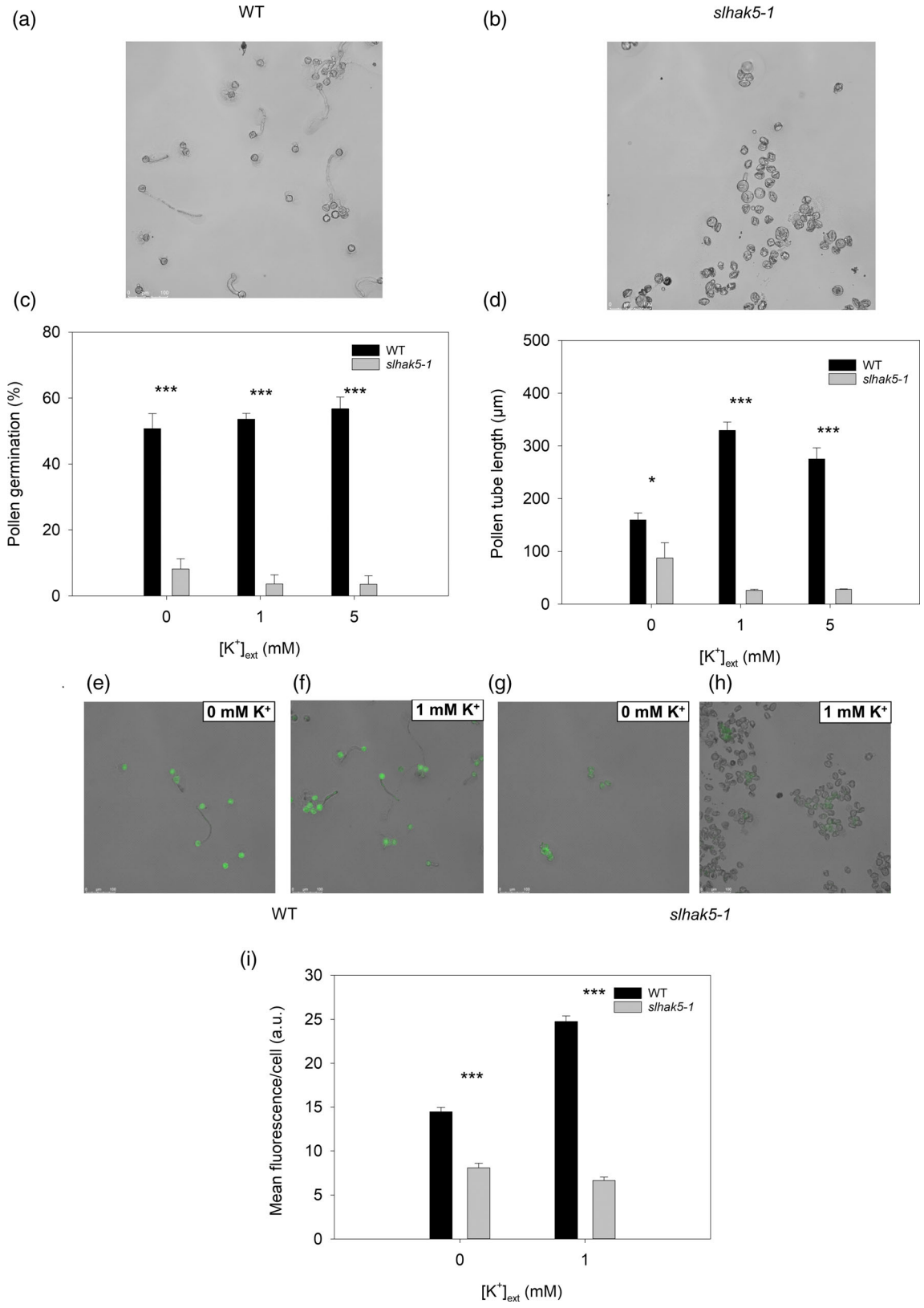

FIGURE 9 Pictures of pollen and germination rates, tube length and relative K^+ concentrations of pollen grains from the WT and *slhak5-1* lines. Pollen of the (a) WT and (b) *slhak5-1* lines were sprinkled on germination media with no K^+ added ($[K^+]_{ext} \approx 100$ µM) or with 1 or 5 mM KCl added and observed 4 hr later under microscope to determine different parameters. (a) and (b) pictures of WT and *slhak5-1* pollen, respectively. (c) Germination rate of pollen grains. (d) Pollen tube length. (e–h) Pictures of (e–f) WT and (g–h) *slhak5-1* pollen germinated on media with (e and g) no K^+ added or (f and h) 1 mM K^+ added, containing the K^+-sensitive fluorescence dye Ion K^+ green 4 (IPG-4) observed under an epifluorescence microscope. (i) Relative IPG-4 fluorescence of WT and *slhak5-1* pollen grains at two levels of external K^+. Data are average ± *SE* of at least 200 pollen grains per each line. * and *** indicate $p < .05$ and $p < .001$, respectively, in Student's *t* test [Colour figure can be viewed at wileyonlinelibrary.com]

was lower in *slhak5-2* and *slhak5-1* (no T-DNA) pollen grains in comparison to the WT ones indicating a lower K^+ concentration in these mutant lines (Figure S5c).

3 | DISCUSSION

Root K^+ uptake in tomato plants has been studied so far by indirect approaches (i.e., pharmacology of K^+ uptake properties) (Bacha et al., 2015; Nieves-Cordones et al., 2007, 2008; Rodenas et al., 2018) and a genetic proof of the individual contribution of candidate K^+ transport systems was pending. Here we showed by using *slhak5* KO mutant lines obtained with CRISPR-Cas that SlHAK5 is the major contributor to root K^+ uptake from diluted solutions. This information was obtained from the phenotypes of *slhak5* plants with three different approaches: Rb^+ uptake assays (Figure 2 and Figure S3), K^+-induced depolarizations of root cell plasma membrane (Figure 3) and plant growth and plant K^+ concentrations at a fixed external K^+ concentration (Figure 4). These approaches have also allowed the establishment of a range of external K^+ concentrations and plant growth conditions where SlHAK5 is the major K^+ uptake system at the root. In K^+ starved plants, SlHAK5 is responsible for most of K^+ uptake observed at external concentrations below 300 μM (Figures 2-4 and Figure S3). In relation to this, it is worth to highlight the negative impact that the *slhak5* mutation had on long-term plant growth at 0.01 mM K^+ (Figure 4a–c). Interestingly, if *slhak5-1* plants were grown under K^+-sufficient conditions and then starved of K^+ for 7 days, the K^+-deficiency phenotype was not observed (Figure 1a,b), probably due to the K^+ reserves that plants had at the end of the K^+ sufficiency period. Thus, the contribution of SlHAK5 to K^+ nutrition in K^+-deficient plants depends on the duration of the low-K^+ period and on plant age, being seedlings more vulnerable to low K^+ stress than adult plants.

When comparing our results with *slhak5* plants with those obtained in *Arabidopsis* and rice with *athak5* and *oshak1* plants, it was evident that remarkable differences in the contribution of HAK5-like transporters to K^+ uptake exist among these species. Here we show (Figures 2d and 3) that the upper limit for the contribution of SlHAK5 to K^+ uptake in tomato plants seems to be located between those for AtHAK5 (<100–200 μM K^+) and OsHAK1 (>1 mM K^+) in *Arabidopsis* and rice, respectively (Chen et al., 2015; Nieves-Cordones et al., 2017; Rubio et al., 2010, 2020). Because HAK5-type transporters are considered K^+–H^+ symporters (Rodríguez-Navarro, 2000), a more general conclusion is that K^+ uptake energization probably differs among land plants. Thus, while some species rely to a higher extent on H^+-coupled K^+ uptake (such as rice and tomato), others preferentially use channel-mediated K^+ uptake (such as *Arabidopsis*). Thus, it remains to be assessed the relevance of these two types of K^+ transport systems in a given species in relation to energy requirements, plant acclimation to changing environments and, in general, for different crops and agriculture.

It is well known that, to some extent, Na^+ may complement K^+ when plants face K^+ deprivation (i.e., as osmoticum in the vacuole) improving plant growth (Horie et al., 2007; Maathuis & Sanders, 1993; Wakeel, Farooq, Qadir, & Schubert, 2011). WT plants grown with 50 mM Na^+ + 0.01 mM K^+ were larger than plants grown without Na^+ (Figure 4a,b) despite those plants had lower tissue K^+ content than WT plants grown without Na^+ (Figure 4c). Interestingly, SlHAK5 activity was required to benefit from this positive effect of Na^+ because growth of *slhak5-1* plants was similar in the absence and in the presence of Na^+ (Figure 4b). The relevance of high K^+/Na^+ ratios for plant salt tolerance has been widely reported (Niu, Bressan, Hasegawa, & Pardo, 1995; Wu, Zhang, Giraldo, & Shabala, 2018). Importantly, although the presence of Na^+ reduced high-affinity K^+ uptake (Figure 4c) the results show that SlHAK5-mediated K^+ uptake was required to maintain plant growth (Figure 4b) and internal K^+ content (Figure 4c). Thus, SlHAK5 is not only crucial for K^+ nutrition at low K^+ but also to benefit from the physiological substitution of K^+ by Na^+. In other words, plant growth requires a minimum K^+ content in plant tissues and, under low K^+ supply, this minimum value is ensured by SlHAK5 function. Our results point to a similar role of SlHAK5 in tomato to that of AtHAK5 in *Arabidopsis*, species in which the transporter was also required to support plant growth under low K^+ plus salinity conditions (Nieves-Cordones et al., 2010).

Production of low-Cs^+ crops in lands affected by nuclear accidents has become a challenge for farmers. It is not a matter of toxicity for crops since radiocaesium concentrations in these scenarios is in the low micromolar range or below (Burger & Lichtscheidl, 2018). On the contrary, it is a problem for people and cattle that eat the radioactive plant material. Radiocaesium can reach crop roots when deposited in the soil or when moved to continental water and thus present in the irrigation water or in hydroponics systems (Nagao et al., 2013; Yasutaka, Miyoshi, & Ito, 2014). At these low concentrations, radiocaesium is rapidly absorbed by plant roots and may be accumulated in edible organs, so Cs^+ uptake is a critical process to reduce its accumulation in the plant. Engineering plants with a low-Cs^+ uptake capacity is a promising approach since the alternative solutions, which are the increase of K^+ fertilizer inputs or removal of Cs^+-containing soil layers, have been shown to be hazardous and expensive (Fujimura et al., 2016; Lepage et al., 2015; Ohmori et al., 2014; Sakai, Gomi, Nunokawa, Wakahara, & Onda, 2014; Wakabayashi et al., 2016). HAK5-like transporters such as AtHAK5 and OsHAK1 have been shown to constitute important pathways for Cs^+ accumulation in *Arabidopsis* and rice, especially in K^+-deprived plants (Genies et al., 2017; Nieves-Cordones et al., 2017; Qi et al., 2008; Rai et al., 2017). A recent pharmacological study suggested that the same might be true for SlHAK5 and tomato plants (Rodenas et al., 2018). Importantly, this low accumulation of Cs^+ also occurred in *slhak5-1* fruits, which are the edible and commercially-relevant part of the plant, that showed a very low Cs^+ transfer factor (TF_{fruit} = 8.5 ± 0.7) in comparison to WT fruits (TF_{fruit} = 151.6 ± 28). It is worth to highlight that the growth conditions used (300 μM K^+ + 0.02 mM Cs^+) gave rise to much lower Cs^+ contents in *slhak5-1* plants (Figure 6a) without remarkable effects in the tissue K^+ content (Figure 6b). Nevertheless, *slhak5-1* plants were prone to have smaller fruits than WT plants (Figure 7b), so it would be desirable to separate the contribution of SlHAK5 to root

Cs^+ uptake from its contribution to fruit development. One approach would be to replace native SlHAK5 with allelic variants with improved K^+/Cs^+ selectivity which would maintain the K^+ transport function, especially in reproductive organs, while reducing root Cs^+ uptake. For example, the F130S mutant of AtHAK5 exhibits a higher K^+/Cs^+ selectivity than the native transporter without penalizing its K^+ transport activity (Aleman et al., 2014).

As stated above, the *slhak5* mutation had deleterious effects on fruit and seed production. These effects were not related to a deficient K^+ nutrition of the plant, because under K^+ sufficient conditions, WT and *slhak5-1* plants showed similar K^+ content (Figure 2b) but a reduced number of fruits and seeds (Figure 7b,c). The reason behind the reduced number of tomato fruits and seeds in the *slhak5* lines (Table 1) may be a defective ovule fertilization. It has been shown that factors that improve pollination increase tomato fruit production and quality (Franceschinelli et al., 2013) and the reduced germination rate (Figure 9c) and tube growth (Figure 9d) of *slhak5-1* pollen grains would result in a reduction of ovule fertilization. Taking into account that the gene encoding *SlHAK5* was expressed in reproductive organs, in particular in stamens (Figure 8), it is very likely that SlHAK5 plays a direct role in pollen physiology. Maintaining K^+ homeostasis is crucial for pollen viability and pollen tube elongation (Fan, Wang, Wang, & Wu, 2001; Mähs et al., 2013). K^+ accumulation in pollen grains is necessary for building up enough turgor pressure which allows the pollen tube to elongate (Weisenseel & Jaffe, 1976). Thus, the lower germination rate (Figure 9c), lower internal K^+ concentrations in pollen grains (Figure 9e–i) and shorter pollen tubes (Figure 9d) in the *slhak5-1* mutant could be due to the absence of SlHAK5-mediated K^+ uptake and, in turn, reduced lower turgor pressure in pollen grains. This is in agreement with the appearance of *slhak5-1* pollen grains and tubes which looked flaccid (Figure 9a). It is worth noting that OsHAK1 has been also related to rice fertility and pollen viability (Chen, Zhang, et al., 2018) and the interaction of this transporter with the receptor-like kinase RUPO may play an important role in this contribution (Liu et al., 2016).

High-affinity HAK K^+ transporters have been suggested to mediate K^+ influx into the cell in co-transport with H^+ (Rodríguez-Navarro,-2000). The question rises why such a mechanism may be required in pollen physiology. Polarized pollen tube growth requires the function of specific transport systems at specific regions of the pollen tube (Michard, Simon, Tavares, Wudick, & Feijó, 2017). It is possible that, at some local points or moments during tube growth, the electrochemical gradient for K^+ makes coupling to H^+ a requirement for K^+ accumulation. In addition, H^+ gradients within the pollen tube have been related to its polarized growth (Weisenseel & Jaffe, 1976), with an acidic tip and an alkaline subapical region, and H^+ may also act in signalling cascades (Michard et al., 2017). Therefore, a K^+/H^+ symport may serve as a mechanism for K^+ accumulation as well as for the movement of H^+ to generate pH gradients.

Importantly, despite the low viability of *slhak5* pollen grains, *slhak5* plants still produce tomato fruits. This is very likely due to the ability of unfertilized tomato flowers to develop fruits (Srivastava & Handa, 2005). The *slhak5-1* fruits were smaller than the WT ones (Figure 7b), probably because the former ones produce less seeds (Figure 7C) and the number of developing seeds influences the final size and weight of a fruit (Varoquaux, Blanvillain, Delseny, & Gallois, 2000). The described observations regarding the production of almost seedless tomato fruits in *slhak5* plants are of great interest for tomato industry (~100 related patents are in the Google patent database) and they may have a future biotechnological application. In particular, this is important for tomato sauce fabrication, since seeds hinder the process of tomato juice extraction (Varoquaux et al., 2000). Moreover, seedless tomatoes are tastier and have longer shelf-life than seeded tomatoes (Varoquaux et al., 2000). There are several natural parthenocarpic tomato cultivars but have disadvantages due to the pleiotropic effects of the genes involved (Varoquaux et al., 2000). In the case of *slhak5* plants, low seed number in tomatoes has no other remarkable defects on plant appearance unless the external K^+ concentration drops below 300 μM for a long time. Thus, the aforementioned features make SlHAK5 an interesting target for breeding tomatoes with few or no seeds.

4 | CONCLUSIONS

Our work provides a molecular mechanism, based on SlHAK5 function, which could help to improve K^+ nutrition and salt tolerance in tomato plants. Plants lacking SlHAK5 may indeed be of interest to produce low-Cs^+ tomatoes in radiocaesium-contaminated environments. However, work is still required to separate the beneficial effect of the *slhak5* mutation at the root, that reduces Cs^+ uptake in the plant, from its detrimental effect at the shoot, which affects pollen viability and reduces fruit yield. Several strategies, from grafting to a root-specific *slhak5* knock-out could be used to circumvent the low-yield phenotype of *slhak5* plants. In contrast, *slhak5* plants can be used to produce tomato fruits with few or no seeds. In addition, the role of SlHAK5 in tomato plants can be regarded as a valuable model for other crops that produce fleshy fruits and to open new avenues for crop biotechnology in the forthcoming years.

5 | MATERIALS AND METHODS

5.1 | Plant material and growth conditions

Tomato plants (*Solanum lycopersicum* L. var Micro-Tom) were used throughout the work. Plants were grown as described previously (Nieves-Cordones et al., 2007). Seeds were germinated in a 0.5 mM aerated $CaSO_4$ solution for 72 hr and then transferred to vermiculite. After 7 days, seedlings were transferred to 8 L containers with aerated nutrient solution that consisted of macronutrients (mM): 1.4 $Ca(NO_3)_2$, 0.2 $MgSO_4$ and 0.1 $Ca(H_2PO4)_2$ and micronutrients (μM): 50 $CaCl_2$, 12.5 H_3BO_3, 1 $MnSO_4$, 1 $ZnSO_4$, 0.5 $CuSO_4$, 0.1 H_2MoO_4, 0.1 $NiSO_4$ and 10 Fe-EDDHA. KCl, NaCl or CsCl were added as indicated in each experiment. Plants were grown in that solution for the indicated time period as indicated in each experiment in a controlled-environment growth chamber with 16/8 hr light/night photoperiod,

at 25°C (day) 20°C (night) temperature, 65% relative humidity and 360 μmol m^{-2} s^{-1} flux density. pH of the nutrient solutions was adjusted daily to 5.5. Fresh solution was supplied once a week.

5.2 | Production of SlHAK5 KO plants with CRISPR-Cas

To produce KO mutants of SlHAK5, two sgRNA sequences (sgRNA1 and sgRNA2), which targeted exons 1 and 2, respectively, of the *SlHAK5* locus (*Solyc12g005670*) (Figure 1) were designed with the web tool Breaking Cas (Oliveros et al., 2016). Target sequences were selected for their high specificity (cut probability in off-target sites <0.4%, Figure S6). Complementary spacer sequences of the sgRNAs were cloned into plasmid A59 and moved to plasmid A60 (property of Abiopep Plant Health S.A.) and the resulting construct transformed into *A. tumefaciens* strain GV3101. The A59 plasmid is an entry vector to clone a sgRNA spacer sequence in frame with a sgRNA scaffold and downstream of an adequate promoter. This vector contains two sgRNAs expression cassettes of this type. Both sgRNA expression cassettes can be transferred into A60 (plant expression vector). The A60 contains the Cas9 expression cassette, a plant marker gene and sgRNAs expression cassettes, all these features located in a T-DNA region (to allow expression of these components in the plant). The presence of two sgRNAs in A60 allows edition of *SlHAK5* at two different sites. Transgenic tomato lines were produced by incubating cotyledons of WT Micro-Tom with suspensions of transformed *Agrobacterium* and subsequent regeneration of whole plants (T0 generation) by in vitro culture (Van Eck, Keen, & Tjahjadi, 2019). Genomic DNA around the target regions of sgRNA was amplified by PCR (Table S3) and sequenced. Then, allele sequences were first deduced using CRISP-Id tool (Dehairs, Talebi, Cherifi, & Swinnen, 2016) and later confirmed by cloning into TOPO PCR2.1 vector and sequencing. Plant lines with interesting alleles were selected for further characterization. Plant genotypes were also determined in T1 and T2 *slhak5* plants (Table S1) and all experiments were carried out with T2 *slhak5* plants. Identification of off-target sites was achieved with the Breaking Cas tool (Figure S6). Off-targets in exon sequences were checked by sequencing in T2 plants and they were found not to be mutated (Table S2). This indicated that *slhak5* plants contained only mutations in *SlHAK5*.

5.3 | Short-term Rb^+ and Cs^+ uptake experiments, ion content and TF_{fruit} determinations

Plants were grown as described in each experiment. For short-term Rb^+ and Cs^+ uptake experiments, plants starved of K^+ for 7 days were transferred to K^+-free solutions supplemented with 0.02 and 1 mM of RbCl or CsCl for 6 hr. Rb^+ or Cs^+ uptake rates were calculated from their accumulation in the plant per unit of time and unit of root dry weight.

In all experiments, plant organs were harvested, their fresh weight determined and dried in a 65°C oven for 4 days to determine their dry weight. Plant material was digested with HNO_3:$HClO_4$ (2:1, v:v) and K^+, Na^+ and Rb^+ concentration was determined by ICP spectrometry analysis (Iris Interpid II, Thermo Electron Corporation, Franklin). Tissue Cs^+ concentration was determined by atomic emission spectrometry in an AAnalist Perking-Elmer 400 spectrometer.

TF_{fruit} was calculated as the ratio between fruit Cs^+ concentration (expressed in mM) and the external Cs^+ concentration (expressed in mM).

5.4 | Root cell plasma membrane potentials determination

Root cell membrane potential was determined as described previously (Nieves-Cordones et al., 2008). Seeds of tomato plants were germinated and grown for 7 days in 0.5 mM $CaSO_4$ solution. Two centimetre root tips were excised and mounted on a perfusion chamber. Root epidermal or cortical cells were impaled with a single-barrelled borosilicate microelectrodes filled with 0.2 M KCl and connected to a duo 773 WPI high-impedance amplifier. Roots were perfused (10 ml/min) with a solution containing 0.5 mM $CaCl_2$ buffered to pH 6 (10 mM MES/Bis-Tris) and different amounts of KCl to reach the indicated concentrations. Membrane depolarizations induced by the presence of K^+ were plotted against the external K^+ concentrations and the data fitted to Michaelis–Menten equations to determine K_m and the maximal depolarization values. Experiments were repeated with different plants and the mean values of at least three repetitions per value of external K^+ used are shown.

5.5 | Real-time qPCR

Expression levels of *SlHAK5* in WT plants were determined by real-time qPCR as described preciously (Rodenas et al., 2018). RNA was isolated with the NucleoSpin, RNA Plant (Macherey-Nagel) kit from the different tissues of plants grown with the complete nutrient solution containing 1.4 mM K^+. After cDNA synthesis with the High capacity cDNA Reverse Transcription Kit (Applied Biosystems), qPCR was performed on a 7500 Real-Time PCR System (Applied Biosystems). Expression levels of *SlHAK5* relative to the expression level of the endogenous gene encoding, the elongation factor SlEF1α are given as $40 - \Delta Ct$ ($\Delta Ct = Ct_{SlHAK5} - Ct_{SlEF1\alpha}$). The primers used are given in Table S3.

5.6 | Pollen germination, tube elongation and estimation of internal K^+

Pollen grains from flowers were germinated as described previously (Maisonneuve & Den Nijs, 1984). Pollen was sprinkled on a microscope slide covered with medium composed of 0.5% agar, 10% sucrose and 50 ppm H_3BO_3. The basal K^+ concentration of this medium was ~100 μM. Slides were incubated on a filter paper soaked

with water inside a petri dish at 25°C for 4 hr. Pollen was observed under a microscope. Germination percentages and pollen tube length were determined by using ImageJ software.

To estimate the internal K^+ concentration of pollen, Ion-K^+ green 4 AM (IPG-4, Iona Biosciences, San Marcos, Texas) dye was added to the germination medium at 20 μM. Slides were observed under an epifluorescence microscope (Leica DM6) after 16 hr. IPG-4 fluorescence was recovered with a 527/30 nm band pass filter after exciting at 480/40 nm. IPG-4 fluorescence was determined by using ImageJ software after subtracting mean fluorescence from pollen grains without dye (pollen autofluorescence).

5.7 | Statistical analysis

Analysis of variance was performed with the Statistix V.8 software for Windows (Analytical Software, Tallahassee, Florida). The differences in means were compared either by Student's *t* test (pair comparisons) or by using a Tukey's multiple range test ($p < .05$) after analysis of variance. Sigma Plot 9.0 was used for data fitting.

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CONFLICT OF INTEREST

The authors have filed a patent application based on the results reported in this study.

AUTHOR CONTRIBUTIONS

M. N. C. and A. L. contributed equally to the manuscript. M. N. C. designed the experiments, obtained the edited mutants, performed experiments, analysed and discussed the results and contributed to writing the paper. A. L. performed the experiments and analysis. M. S. and J. A. contributed to sample collection and analysis. P. R.-S. generated the transgenic lines. R. M. R., V. M. and M. A. B. contributed to design the experiments and results discussion. F. R. contributed to design and perform the experiments, discuss the results and writing the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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