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Production of low-Cs^+ rice plants by inactivation of the K^+ transporter OsHAK1 with the CRISPR-Cas system

Manuel Nieves-Cordones[1,*,†], Sonia Mohamed[1,2], Keitaro Tanoi[3,4], Natsuko I. Kobayashi[3], Keiko Takagi[3], Aurore Vernet[2], Emmanuel Guiderdoni[2], Christophe Périn[2], Hervé Sentenac[1], Anne-Aliénor Véry[1,*]

[1]*Biochimie et Physiologie Moléculaire des Plantes, Institut de Biologie Intégrative des Plantes, UMR 5004 CNRS/ 386 INRA/Montpellier SupAgro/Université Montpellier, 34060 Montpellier Cedex 2, France;* [2]*CIRAD, UMR AGAP, 34398 Montpellier Cedex 5, France;* [3]*Graduate School of Agricultural and Life Sciences, University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan;* [4]*PRESTO, Japan Science and Technology Agency (JST), 4–1–8 Honcho, Kawaguchi, Saitama 332–0012, Japan.*

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*Correspondence (e-mail: mncordones@gmail.com and very@supagro.inra.fr)

†Present address: Departamento de Nutrición Vegetal, CEBAS-CSIC, Campus de Espinardo, 30100 Murcia, Spain.

SUMMARY

The occurrence of radiocesium in food has raised sharp health concerns after nuclear accidents. Despite being present at low concentrations in contaminated soils (below µM), cesium (Cs^+) can be taken up by crops and transported to their edible parts. This plant capacity to take up Cs^+ from low concentrations has notably affected the production of rice (*Oryza sativa* L.) in Japan after the nuclear accident at Fukushima in 2011. Several strategies have been put into practice to reduce Cs^+ content in this crop species such as contaminated soil removal or adaptation of agricultural practices, including dedicated fertilizer management, with limited impact or pernicious side-effects. Conversely, the development of biotechnological approaches aimed at reducing Cs^+ accumulation in rice remain challenging. Here, we show that inactivation of the Cs^+-permeable K^+ transporter OsHAK1 with the CRISPR-Cas system dramatically reduced Cs^+ uptake by rice plants. Cs^+ uptake in rice roots and in transformed yeast cells that expressed OsHAK1 displayed very similar kinetics parameters. In rice, Cs^+ uptake is dependent on two functional properties of OsHAK1: (i) a

poor capacity of this system to discriminate between Cs^+ and K^+; and (ii) a high capacity to transport Cs^+ from very low external concentrations that is likely to involve an active transport mechanism. In an experiment with a Fukushima soil highly contaminated with $^{137}Cs^+$, plants lacking OsHAK1 function displayed strikingly reduced levels of $^{137}Cs^+$ in roots and shoots. These results open stimulating perspectives to smartly produce safe food in regions contaminated by nuclear accidents.

KEYWORDS: rice, cesium, HAK1, CRISPR-Cas, soil contamination by radioactivity

INTRODUCTION

Crop production has to be increased in the coming decades in order to meet the rise in world population to 9 billion in the 2050s (Bongaarts, 2009), while the total area of lands available for agriculture is likely to decrease as a result of the population growth, climate change and pollution. In this challenging context, agriculture has also to produce safe food.

The contamination of large areas of arable lands resulting from anthropogenic activity can compromise such goals. Nuclear accidents, such as those of the nuclear power plants of Chernobyl in 1986 and Fukushima in 2011, are ecological disasters that can contaminate large areas of soils for very long periods of time. For instance, resulting from the accident in Fukushima, the large emission of radioactive cesium (Cs) isotopes, ^{134}Cs (2-year half-life) and ^{137}Cs (30-year half-life), are expected to have contaminated approximately half of the soils in Japan (Yasunari *et al*., 2011). In polluted areas, radioactive Cs^+ is taken up by plant roots and translocated to the shoots (Fujiwara, 2013). Rice is the major crop in most polluted regions in Japan and it is cultivated for production of both grains for humans and straw for cattle. Therefore, radioactive Cs^+ accumulation in this crop has become a crucial issue for authorities, farmers and researchers.

Cesium (Cs) is a group I alkali metal with chemical properties similar to that of potassium (K). Cs^+ can be toxic for plants when present in the soil at high concentrations, in the mM range, but the aforementioned environmental concerns result from the presence of very low concentrations of radioactive isotopes, below the µM range (White and Broadley, 2000). Cs^+ ions are poorly mobile in the soil, so that contaminating radioactive Cs^+ remains mainly present in the 0–5 cm top soil layer (Lepage *et al*., 2015). To reduce ^{134}Cs and ^{137}Cs accumulation in rice, several strategies have been developed, including top soil layer removal, water management and potassium fertilizer addition (Ohmori *et al*., 2014b; Sakai *et al*., 2014; Lepage *et al*.; 2015, Fujimura *et al*., 2016; Wakabayashi *et al*., 2016). Besides

being expensive, such strategies are hazardous as they can have side-effects such as the production of large amounts of radioactive soil waste (due to soil removal) and fertilizer effluents reaching other ecosystems. So far, biotechnological approaches aimed at directly reducing Cs^+ uptake and accumulation in rice plants have remained relatively unexplored.

A preliminary step to develop such a biotechnological strategy is the identification and characterization of the molecular mechanisms involved in Cs^+ uptake by rice roots. While Cs^+ is not an essential nutrient for plants, many species, including rice, pea, tobacco and maize, poorly discriminate between Cs^+ and K^+ and exhibit similar uptake rates for these two cations (Collander, 1941; Bañuelos *et al*., 2002). Further evidence for the existence of close interactions between Cs^+ and K^+ uptake have been provided by functional analyses showing that several cloned plant K^+ transporters, notably from the HAK/KUP/KT family, are permeable to Cs^+ when heterologously expressed in bacteria, yeast or *Xenopus* oocytes (Rubio *et al*., 2000; Bañuelos *et al*., 2002; Qi *et al*., 2008; Kobayashi *et al*., 2010; Véry *et al*., 2014; Scherzer *et al*., 2015). *In planta*, the main root K^+ uptake pathways have been analysed, most extensively in the model plant Arabidopsis, using knock-out mutant lines. Evidence has thereby been obtained that K^+ transporters from the HAK/KUP/KT family and inwardly rectifying K^+ channels from the Shaker family are the major contributors to root K^+ uptake from the external medium (Hirsch *et al*., 1998; Gierth *et al*., 2005; Rubio *et al*., 2008). Conversely, less information is known about the transport systems that actually contribute to Cs^+ uptake *in planta*. In Arabidopsis, evidence is available that several transport systems, including AtHAK5, and voltage-independent channels that are presently unidentified at the molecular level, contribute to Cs^+ uptake with a large redundancy amongst them (Broadley *et al*., 2001, Qi *et al*., 2008).

The development of new strategies for efficient production of transgene-free improved crops has attracted much attention as an alternative to transgenic crops in plant biotechnology (Schaart *et al*., 2016). The Clustered Regularly Interspaced Short Palindromic Repeats-Cas (CRISPR/Cas) system has turned out to be a powerful tool in this domain, with particular success in rice (Zhang *et al*., 2014; Khatodia *et al*., 2016; Weeks *et al*., 2016). This method relies on engineered sequence-specific small RNAs that target DNA nucleases to genes of interest to create double-stranded breaks (DSBs). Such breaks can result in gene mutations due to non-homologous end-joining (NHEJ) repair, which can lead to loss-of-function alleles, or gene replacement, or correction if homologous recombination-based repair (HR) takes place.

Here we report the production, using the CRISPR-Cas system, of rice plants with

strongly reduced radioactive cesium contents when grown in Fukushima soil highly contaminated with $^{137}Cs^+$. These plants have been obtained by inactivating the Cs^+-permeable OsHAK1 transporter, which is shown to be the major pathway for Cs^+ uptake and translocation in rice.

RESULTS

High-affinity Cs^+ uptake in rice plants

Cs^+ uptake by roots was assumed to be a major determinant of Cs^+ accumulation in rice plants. Thus, to produce low-Cs^+ rice plants, we first aimed at identifying which systems contribute to the uptake of this cation from the soil in rice. Cs^+ concentrations in contaminated fields are low (below the µM range) (White and Broadley, 2000), and thus the uptake of this cation under such conditions was hypothesized to occur through high-affinity transport systems. High-affinity monovalent cation uptake is known to be particularly enhanced under K^+ deprivation (Zhu and Smolders, 2000; Rodríguez-Navarro and Rubio, 2006). As a first approach to characterize Cs^+ accumulation at low external concentrations, we germinated and grew cv. Nipponbare rice plants for 7 days in a solution free from added K^+ (0 K^+ solution) and 7 further days in the absence or presence of 30 µM Cs^+ and/or 30 µM K^+, the corresponding treatments being named 0K-0Cs, 0K-30Cs, 30K-0Cs and 30K-30Cs. The combination of Cs^+/K^+ treatments was expected to bring some insight into competition that could occur between these two cations to be taken up by roots. Unless stated, Cs^+ stands for $^{133}Cs^+$ (a non-radioactive isotope). The aforementioned Cs^+ treatments did not have any significant effect on plant dry weight (biomass) (Figure 1(a)). Regarding Cs^+ tissue content, we found that rice plants were able to accumulate large amounts of this cation, especially in roots and in the absence of K^+ in the external solution (0K-30Cs treatment). In such conditions, the Cs^+ content in roots was even higher than the K^+ content (0.27 vs. 0.17 mmol/g DW, respectively) (Figure 1(b, c)). These results suggest that rice roots display a large capacity to take up Cs^+ upon K^+ starvation. Addition of K^+ to the external solution reduced Cs^+ accumulation in roots by about 35%. However, this reduction was without significant effects on shoot Cs^+ contents under our experimental conditions (Figure 1(b)). The presence of Cs^+ in the medium did not affect the K^+ contents in plants grown in the absence of K^+, while it reduced both root and shoot K^+ contents in plants grown in the presence of this cation (30K-30Cs treatment) (Figure 1(c)). It is also worth noting that the Cs^+ contents were always lower, by about three times, in shoots than in roots (Figure 1(b)), whereas the opposite was observed for the K^+ contents (Figure 1(c)). Such results provide further support

to the hypothesis that Cs^+ translocation to shoots is restricted in rice (Kobayashi *et al*., 2016).

The capacity of rice roots to deplete Cs^+ from 30Cs solution and the capacity to deplete K^+ from the 30K solution were then compared. The two depletion kinetics were strikingly similar (Figure 1(d)). The depletion data were further analysed according to the method described in Bañuelos *et al*. (2002), derived from the classical Michaelis–Menten equation (Epstein *et al*., 1963; Bañuelos *et al*., 2002), allowing the determination of V_{max} and apparent K_M parameters (here related to net transport, possibly integrating a varying efflux component). The V_{max} values obtained for Cs^+ and K^+ were not significantly different, and were 1.7 ± 0.5 nmol min^{-1} (mg^{-1} root dry weight) for the former cation, and of 1.6 ± 0.4 nmol min^{-1} (mg^{-1} root dry weight) for the latter one. The main difference between Cs^+ and K^+ uptake curves stemmed from a higher apparent K_M (i.e. a lower affinity) for Cs^+ than for K^+, by about two times (34 ± 7 µM vs. 16 ± 6 µM).

Using the microelectrode impalement technique, we then compared the effects of Cs^+ and K^+ on the cell membrane potential in rice root periphery cells. Addition of chloride Cs^+ or K^+ salts to the external solution depolarized the membrane in a concentration-dependent manner (Figure 1(e, f, g)). The cell membrane permeability to Cl^- can be expected to be negligible at low external concentrations (Spalding *et al*., 1999), and thus the depolarization induced by the chloride salts can be assumed to essentially result from K^+ or Cs^+ influx. Data were fitted using the Michaelis–Menten equation, allowing the derivation of the D_{max} ('maximal depolarization') parameter and an apparent K_M. In agreement with the results of the depletion experiments, D_{max} values were similar for Cs^+ and K^+ [D_{max} (Cs^+) = 71 ± 2 mV vs D_{max} (K^+) = 74 ± 2 mV] while the apparent K_M was higher, by about 1.5 times, in the case of Cs^+ than in that of K^+ [K_M (Cs^+) = 34 ± 5 µM vs K_M (K^+) = 22 ± 3 µM]. Altogether, these results were consistent with the hypothesis that the transport systems that mediate high-affinity Cs^+ uptake in rice roots display a comparable transport capacity (depicted by V_{max} and D_{max}) for Cs^+ and K^+, with a slightly lower affinity for Cs^+ than for K^+, evidencing little discrimination between both cations.

The strong capacity of rice roots to deplete the external concentrations of Cs^+ and K^+ below 0.1 µM (Figure 1(d)) raised the question of how cation uptake can be achieved from such very low external concentrations. The activity of K^+ in the cytosol is very likely to be in the 10–100 mM range (Szczerba *et al*., 2008). Thus, in these depletion experiments, K^+ was efficiently taken up against a concentration gradient of at least five orders of magnitude. Based on the current knowledge of the energetic coupling of K^+ absorption in plants (Maathuis and Sanders, 1994; Rodríguez-Navarro, 2000; Véry and Sentenac, 2003), K^+

transport against such a large concentration gradient cannot be passively mediated by channels. For example, at 30 µM K^+, Em was -84 ± 9 mV while E_K would be of about −190 mV (if the K^+ activity in the cytosol is 50 mM (Szczerba *et al*., 2008)). The fact that this E_K estimate is strongly more negative than Em, by more than 100 mV in these experimental conditions, provides support to the hypothesis that K^+ transport was actively mediated by co-transporters (Rodríguez-Navarro, 2000).

Further analysis of electrophysiological recordings allowed us to get insight into the energetic coupling of K^+ and Cs^+ uptake from low concentrations. Plotting the membrane potential against the external concentration of Cs^+ or K^+ (in log10 scale) resulted in a linear relationship, with a slope of 29 mV per decade of cation concentration for both cations (Figure 1(h)). Such a slope value is far from the 58 mV per decade of cation concentration expected for channel-mediated (non-coupled) uptake. Altogether, these results suggest that K^+ and Cs^+ are taken up at low external concentrations via a co-transport mechanism.

OsHAK1 mediates high-affinity Cs^+ uptake when heterologously expressed in yeast

When considering candidate transport systems, evidence is available that high-affinity K^+ transporters belonging to the HAK family can mediate active K^+ uptake in plants, and that some of them are permeable to Cs^+, in addition to K^+ (Rodríguez-Navarro and Rubio, 2006; Véry *et al*., 2014). Furthermore, it has been previously shown that a chimeric construct of the K^+ transporter *OsHAK1* harbouring the first 16 bp of *HvHAK1* from barley exhibited high-affinity Cs^+ uptake when expressed in a yeast mutant strain defective for K^+ transport (Bañuelos *et al*., 2002). Such a chimeric transporter was constructed as the *OsHAK1* clone that could then be amplified was incomplete and not functional (Bañuelos *et al*., 2002). A complete *OsHAK1* clone has since become available in the Japanese cDNA database Knowledge-based Oryza Molecular Biological Encyclopedia (KOME) (Rice Full-Length Consortium *et al*., 2003). We investigated the K^+ and Cs^+ transport activity of the encoded transporter by heterologous expression in yeast and analysis of the capacity of the transformed cells to deplete Cs^+ and K^+ from 30 µM solutions (same concentration as in the experiment with plants, Figure 1d). The *S. cerevisiae* strain PLY246 (Bertl *et al*., 2003), impaired in K^+ uptake, was transformed with the pYPGE15 plasmid containing *OsHAK1* coding sequence or no insert (empty vector control, EV). Yeast cells transformed with the plasmid allowing *OsHAK1* expression efficiently took up Cs^+ and K^+ whereas cells transformed with the empty plasmid failed to do so (Figure 2). The Cs^+ and K^+ depletion curves observed with OsHAK1-expressing yeast cells resembled those previously obtained

with rice roots (Figure 1(d)). Further evidence for this similitude was obtained by using the Michaelis–Menten equation to derive kinetic parameters. As for rice roots, the V_{max} values for K^+ and Cs^+ uptake in the transformed yeast cells were similar: $V_{max}(Cs^+)$ = 6.5 ± 0.7 nmol mg^{-1} min^{-1}, and $V_{max}(K^+)$ = 7 ± 0.6 nmol mg^{-1} min^{-1}, respectively. Also, similarly to what was observed in rice roots (Figure 1(d)), the apparent K_M in the transformed yeast cells was slightly higher for Cs^+ than that for K^+, by about twice: $K_M(Cs^+)$ = 32 ± 5 µM vs. $K_M(K^+)$ = 16 ± 2 µM. Altogether, these similarities suggested that OsHAK1 contributed to Cs^+ uptake in rice roots. This hypothesis was checked directly by production of *oshak1* loss-of-function mutant plants with the CRISPR-Cas system.

Inactivation of *OsHAK1* in rice plants using the CRISPR-Cas system

Two sgRNAs, sgRNA1 and sgRNA2, targeting *OsHAK1* exons 1 or 2, respectively, were designed (Figure 3(a)) in order to guide the nuclease Cas9 to the corresponding complementary regions to produce DNA DSBs. To allow co-expression of the corresponding sgRNA with Cas9, their coding sequences were cloned into the T-DNA sequence of the pH-Ubi-Cas9-7 vector (Miao *et al*., 2013). Transformation of rice calli with *Agrobacterium tumefaciens* cells containing either the sgRNA1-pH-Ubi-Cas9-7 or the sgRNA2-pH-Ubi-Cas9-7 vector, allowed insertion of the T-DNA cassette in the rice genomic DNA and gave rise to expression of the sgRNA-Cas9 complex. Forty-one plants (T0 generation) were successfully regenerated. DNA sequence analyses revealed that 34 of these contained mutations in the *OsHAK1* gene at the expected sites (83% mutation rate). Four lines were selected for the next physiological analyses: three lines in which frameshift mutations in both *OsHAK1* alleles were identified [KO1 was transformed with sgRNA1 and KO2 and KO3 were transformed with sgRNA2; Figure 3(c–e)] as knock-out plants, and a ‘wild-type’ line used as the control, named WT-c, issued from the transformation with sgRNA2 but in which the *OsHAK1* gene was not mutated (Figure 3(b)). Frameshift mutations were +1(T)bp, +1(A)bp (at position +1765 bp) in KO1, −2 bp, −4 bp (at positions +2075 bp and +2073 bp, respectively) in KO2, and −5bp, −5 bp (at position +2072 bp) in KO3 (Figure 3(c–e)). All these mutations gave rise to premature stop codons (Table S1). It is worth noting that no mutation was identified in potential off-target loci (Table S2).

***oshak1* plants are unable to take up Cs^+ at low external concentrations**

To characterize Cs^+ uptake in *OsHAK1*-edited mutant plants, we grew T1 plants from the WT-c and the three mutant (KO1 to KO3) lines in solution free from added K^+ (0 K^+

solution) for 14 days. We assessed the segregation of *OsHAK1* alleles in the T1 KO plants and found the same alleles as those identified in T0 plants (Table S3). Thus, the KO plants carried homo- or bi-allelic frameshift mutations, which inactivated the OsHAK1 sequence. When plants were grown in 0 K^+ solution (from seeds which displayed comparable K^+ content among genotypes; Figure S2), the three KO plant lines did not display any significant difference in root biomass (dry weight) when compared with WT-c plants, but had smaller shoots (Figure 4(a)). Depletion assays from 30 µM Cs^+ solution revealed that plants from the three KO lines did not reduce external Cs^+ concentration at all, while WT-c plants did so efficiently (Figure 4(b)) as initially observed with WT plants (Figure 1(d)). Furthermore, the three KO plant lines were also unable to deplete K^+ from 30 µM K^+ solution (Figure 4(c)), in contrast with WT-c (Figure 4(c)) and WT plants (Figure 1(d)). We also observed impaired Cs^+ and K^+ uptake capacity in Dongjin cultivar rice mutant plants (Figure S1) carrying a null *OsHAK1* mutant allele due to T-DNA insertion (Chen *et al*., 2015). Altogether, these data indicated that the three lines KO1 to KO3 behave as loss-of-function *oshak1* mutant plants. Furthermore, these data also indicated that OsHAK1 mediates both Cs^+ and K^+ high-affinity uptake in rice roots, in agreement with the results of the analysis of OsHAK1 transport activity when this system was heterologously expressed in yeast (Figure 2).

We then investigated the consequences of the absence of OsHAK1 transport activity on Cs^+ accumulation in the KO 1 to KO3 mutant lines. In roots from mutant plants used for the Cs^+ depletion experiments, the Cs^+ content was extremely low, about 35 times lower than in the corresponding WT-c plants (Figure 4(d)). The KO plants also displayed a significant reduction in K^+ tissue contents, by about two to three times in both shoots and roots when compared with WT-c plants (Figure 4(e)). Then, we investigated the behaviour of *oshak1* loss-of-function plants upon a longer time of exposure to Cs^+, 7 days in 30 µM Cs^+ (30Cs solution), following 7 days of growth in 0 K^+ solution since sowing. The KO plants displayed markedly lower Cs^+ contents when compared with WT-c plants, both in roots, by about 45 times, and in shoots, by 12 times (Figure 4(f)). Altogether, these experiments provided evidence that OsHAK1 was the major contributor to Cs^+ uptake from low concentrations, and that this activity was by far the major determinant of Cs^+ accumulation in rice plants faced with µM external concentrations of this cation.

The residual K^+ and Cs^+ transport activities by root periphery cells in *oshak1* mutant plants were investigated by membrane potential recordings in the 10–1000 µM concentration range (Figure 4(g–l)). In the absence of Cs^+ and K^+ in the external solution, stable impalements of root cortical cells with microelectrodes provided similar membrane potential

values in WT-c and *oshak1* mutant plants (experiments performed using the KO1 and KO2 lines; Table S4). Increasing the external concentration of Cs^+ or K^+ resulted in a rapid and pronounced depolarization of the cell membrane in WT-c plants. The experimental depolarization vs. external cation concentration curve (Figure 4(k, l)) displayed a hyperbolic shape with an apparent saturation above 300 µM, completely in agreement with the previous results obtained with WT plants (Figure 1(g)). In contrast, the membrane potential of *oshak1* KO mutant plants did not display any sensitivity to changes in external Cs^+ concentrations, from 0 up to 1000 µM (Figure 4(g, h, k)). It was however sensitive to the changes in the external concentration of K^+, but to a lower level than in WT-c (and WT) plants, and with a different kinetics, quasi-linear instead of hyperbolic. Thus, OsHAK1 appears as the single transport system that significantly contributes to Cs^+ uptake from external solutions containing this cation at a concentration of up to 1 mM, while at least another type of electrogenic transport system, besides OsHAK1, can contribute to root K^+ uptake. The impact of this other type of transport system on the cell membrane potential appeared only at K^+ external concentrations higher than 100 µM (Figure 4(i, j, l)). Altogether, these results are consistent with the current view that K^+ uptake by plant roots from low concentrations (in the µM range) involves active high-affinity transporters belonging to the HAK family, while K^+ channels from the Shaker family contribute to passive K^+ uptake from higher concentrations (Maathuis and Sanders, 1994; Nieves-Cordones *et al*., 2016a).

$^{137}Cs^+$ accumulation in *oshak1* plants grown on Fukushima soils

The previous analyses of Cs^+ uptake and of the membrane potential, both carried out in hydroponics conditions, clearly indicated that inactivation of OsHAK1 resulted in strongly reduced Cs^+ uptake and accumulation in plant tissues. To check the effects of this inactivation on radioactive cesium accumulation in conditions resulting from nuclear accidents, KO#1 to KO#3 *oshak1* mutant plants and WT-c plants were grown on $^{137}Cs^+$-contaminated soils from Fukushima Prefecture. Two different soils were used (Figure 5(a)). The first soil contained 7×10^3 Bq of $^{137}Cs^+$ per kg of soil dry weight (DW), and a level of exchangeable K^+ (177 mg/kg DW) close to the recommended range for reducing radiocesium concentration in rice plants to levels allowing grain consumption (Figure 5(a)) (Kato *et al*., 2015; Ishikawa *et al*., 2017). T2 plants grown for 113 days in this batch of soil displayed low $^{137}Cs^+$ in grains (Figure 5(b)), within the permitted range (<100 Bq/kg) permitted by the 2012 Food Sanitation Act (Ohmori *et al*., 2014b). Shoot to grain $^{137}Cs^+$ transfer coefficient was found to be within 0.25 to 0.55 in the different genotypes (Figure 5(b, c)), which

corresponded to the range of values previously reported for rice grown on Fukushima soils (Ohmori *et al*., 2014a; Ohmori *et al*., 2014b; Kato *et al*., 2015). No significant differences between WT-c and mutant plants were found with respect to grain $^{137}Cs^{+}$, leaf $^{137}Cs^{+}$ and K^{+} contents, shoot biomass and grain yield in this experiment (Figure 5(b–e)).

The second soil displayed a much higher $^{137}Cs^{+}$ content (2.1×10^{6} Bq per kg of soil DW) together with lower exchangeable K^{+} (67 mg per kg of soil DW), which could be expected to greatly favour plant $^{137}Cs^{+}$ uptake. After growing plants for 25 days under these conditions (see Figure S3 for plant phenotype), tissue $^{137}Cs^{+}$ contents were assayed. Roots from *oshak1* mutant plants displayed about 30 times lower $^{137}Cs^{+}$ contents than those from WT-c plants (Figure 5(f)). The $^{137}Cs^{+}$ content was lower in shoots than in roots in both WT-c and *oshak1* mutant plants, by about eight times in the WT-c plants but by more than 15 times in the mutant plants (Figure 5(g)). The shoot content in $^{137}Cs^{+}$ was thus remarkably lower, by more than 60 times, in the mutant plants (Figure 5(g)). In this experiment, the three KO plant lines displayed a root biomass (DW) similar to that of the control WT-c plants grown in parallel conditions, and a slightly lower shoot biomass (Figure 5(h), see also Figure S3). Regarding K^{+}, the root and shoot contents in this cation were about two times lower in the *oshak1* mutant than in the WT-c plants (Figure 5(i)). No information on $^{137}Cs^{+}$ in grains was collected from this experiment, which used younger plants (at the early tilling stage; Figure S3), rendering possible the recovery of intact root systems. However, due to the available information on shoot to grain $^{137}Cs^{+}$ transfer, with coefficients for rice in the range of 0.2 to 0.5 (Figure 5(b, c)) (Ohmori *et al*., 2014a; Ohmori *et al*., 2014b; Kato *et al*., 2015), including that for plants of cv. Nipponbare grown on soils displaying low contents in exchangeable K^{+} as in our second soil (Ishikawa *et al*., 2017), radiocesium in grains in our WT-c plants would undoubtedly have been high (>100 times higher than the permitted level). Under such conditions, and taking into account the huge difference in shoot $^{137}Cs^{+}$ between WT-c and *oshak1* mutant plants (Figure 5(g)), it is very likely that the grains of KO plants grown on this soil would have had far lower $^{137}Cs^{+}$ contents than the grains of WT-c plants (by at least a factor of 10).

Overall, the results of the two experiments with contaminated soils indicated that inactivation of OsHAK1 has no beneficial effect on rice $^{137}Cs^{+}$ accumulation when plants are grown under field conditions in which radiocesium uptake by the plant is sufficiently low to enable rice consumption, but is a promising strategy to reduce $^{137}Cs^{+}$ accumulation in plants grown in conditions favouring high radiocesium uptake by the plant.

DISCUSSION

Role of OsHAK1 in rice plants

OsHAK1 belongs to cluster Ia of HAK/KUP/KT K^+ transporters (Nieves-Cordones *et al*., 2016b), whose members are widely associated with root high-affinity K^+ uptake in several plant species beside rice, for example, barley, Arabidopsis, tomato, pepper and *Eutrema salsugineum* (Santa-María *et al*., 1997; Rubio *et al*., 2000; Bañuelos *et al*., 2002; Martínez-Cordero *et al*., 2004; Nieves-Cordones *et al*., 2007; Alemán *et al*., 2009). OsHAK1 localizes to the plasma membrane and is upregulated in roots under low K^+ conditions (Bañuelos *et al*., 2002; Chen *et al*., 2015). Reverse genetics analyses using Arabidopsis or rice mutant plants displaying loss-of-function mutations in genes encoding K^+ transport systems have revealed that the HAK K^+ transporters AtHAK5 and OsHAK1 mediate active K^+ uptake from low K^+ concentrations, and that voltage-gated K^+ channels from the Shaker family, especially AKT1 in Arabidopsis and OsAKT1 in rice, play a major role in passive K^+ uptake from less diluted solutions (Nieves-Cordones *et al*., 2010; Li *et al*., 2014; Chen *et al*., 2015).

We provide here an electrophysiological investigation of the activity of OsHAK1 in rice root periphery cells. First, recordings of cell membrane potential in WT-c and *oshak1* loss-of-function plants reveal that OsHAK1 transport activity is strongly electrogenic and depolarizing (Figure 4(l)). Large variations in the cell membrane electrical polarization were observed in response to changes in the external concentration of K^+ in the 1–1000 µM range in WT (and WT-c) plants (Figures 1(f, g) and 4(l)). This sensitivity of the membrane potential to external K^+ can be described by an apparent K_M value close to 30 µM, which is similar to the K_M value of root K^+ uptake versus the external concentration of K^+ in WT rice plants in the same experimental conditions (Figure 1(d)) as well to the K_M values of high-affinity K^+ uptake in roots of other plant species (Epstein *et al*., 1963). Second, the membrane potential in WT (and WT-c) rice roots in the presence of low external K^+ concentrations did not appear to be sufficiently negative to allow passive K^+ uptake (Figures 1(h) and 4(l) and Table S4). Thus, this uptake should involve active transporters. Finally, as the K^+-induced depolarization was extremely weak for concentrations below 100 µM in *oshak1* root cells, it can be assumed that the depolarization events observed in WT plants in response to the changes in K^+ external concentration essentially reflected changes in OsHAK1 transport activity (Figure 4(i, j, l)). Thus, the calculated slope of 29 mV of depolarization per decade of K^+ concentration (Figure 1(h)) is likely to result essentially from OsHAK1 activity, suggesting that OsHAK1 active K^+ uptake involves a depolarizing co-transport mechanism.

In the absence of OsHAK1 functional expression, i.e. in the *oshak1* mutant plants, the

membrane potentials recorded in the presence of K^+ concentrations higher than ca. 100 µM (e.g. about −130 mV at 300 µM K^+) are consistent with the hypothesis that K^+ uptake was passive in such conditions. As K^+, and not Cs^+, depolarized the cell membrane in these conditions, the depolarizing effect of K^+ in *oshak1* root cells can be assumed to result from the uptake of this cation through voltage-gated K^+ channels belonging to the Shaker family that are known to be permeable to K^+ but not to Cs^+ (Véry *et al*., 2014). Thus, our results provide further evidence that the general organization of K^+ uptake is similar in rice and in Arabidopsis except that, in the latter species, K^+ uptake can be passive and Shaker channel-mediated from very low concentrations, of a few µM, because of the more negative membrane potentials in the presence of µM K^+ concentrations than those observed in rice in the present study (Spalding *et al*., 1999, Nieves-Cordones *et al*., 2016a; Figure 1(h)). In other words, rice depends on OsHAK1 activity for active uptake in a wide range of external concentrations, larger by at least one order of magnitude than the K^+ concentration range within which uptake of this cation has to be active in Arabidopsis.

Towards plants with strongly reduced Cs^+ contents in shoots

The present analysis of the effects of the external concentration of Cs^+ on Cs^+ uptake and the electrical polarization of the cell membrane in rice roots indicates that OsHAK1, which is permeable to Cs^+ when heterologously expressed in yeast (Figure 2), behaves *in planta* as a high-affinity Cs^+ uptake transporter, able to accumulate Cs^+ from low concentrations, with a K_M close to 30 µM. Furthermore, loss-of-function mutations in *OsHAK1* gene results in a strong reduction of the plant Cs^+ contents when growth occurs in the presence of this contaminating cation (Figures 4 and 5). Thus, rice roots accumulate Cs^+ from low external concentrations essentially because of the poor ability of OsHAK1 to discriminate between K^+ and Cs^+ and the high uptake capacity of this transporter. Besides OsHAK1, seven other HAK transporters belonging to cluster Ia are encoded by the rice genome (Nieves-Cordones *et al*., 2016b). The present results indicate that their contribution to Cs^+ uptake from low external concentrations, as well as that of any other transport system, can be considered as very weak and probably negligible in comparison with the contribution of OsHAK1. It is worth noting that this situation is quite different from that reported in the model plant Arabidopsis. Indeed, although AtHAK5 contributes to K^+ uptake in Arabidopsis roots and can efficiently transport Cs^+ when heterologously expressed in yeast (Rubio *et al*., 2000, Qi *et al*., 2008), the uptake of Cs^+ from low concentrations by Arabidopsis roots is not significantly affected in *athak5* null mutants, irrespective of the K^+ concentration present in the growth solution (Qi *et al*.,

2008). In this species, non-selective channels are likely to play an important role in Cs^+ uptake as this transport is inhibited by Ba^{2+} and Ca^{2+} (Broadley *et al*., 2001, Caballero *et al*., 2012). Thus, the absence of contribution of AtHAK5 to Cs^+ uptake in *athak5* KO mutant plants would be fully compensated by non-selective channels or similar transport systems in Arabidopsis.

It has been recently shown that Cs^+ translocation to shoots in rice is slower than K^+ translocation. Thus, control mechanisms seem to reduce Cs^+ entry into the xylem vasculature in this species (Kobayashi *et al*., 2016). In plants grown for 25 days on Fukushima highly contaminated soil, the depressive effect of the *OsHAK1* loss-of-function mutations on radioactive Cs^+ accumulation appears much larger in shoots (Figure 5(g); radioactive Cs^+ only found in one plant out of the nine plants tested, the shoot content in this plant being about 70 times lower than that observed in control WT plants) than in roots (Figure 5(f)) and in the whole plant (roots + shoot contents, computed from the data shown in Figure 5(f, g)). Thus, besides being the major contributor to Cs^+ uptake from the soil solution, OsHAK1 could also play a role in Cs^+ translocation towards the shoots. This hypothesis is consistent with the fact that *OsHAK1* is expressed in root stele tissues (Chen *et al*., 2015), where it can contribute to K^+ transport towards the shoots in conditions of low K^+ availability (Chen *et al*., 2015). Thus, OsHAK1 transport activity in the root stele could significantly contribute to the restricted pathway through which Cs^+, once taken up by root periphery cells, can migrate in the root stele towards the xylem sap (Kobayashi *et al*., 2016).

Accidents in nuclear power plants, such as in Fukushima and Chernobyl, can affect large areas of lands for long periods of time, and then raise crucial issues about the exploitability of the contaminated agricultural areas. The main soil contaminant due to such accidents has been radiocesium. In Fukushima, different strategies are pursued to minimize radioactive Cs^+ accumulation in plants in the contaminated zones, including strong K^+ fertilization (Kato *et al*., 2015). Incidentally, our results provide detailed information about the mechanisms by which K^+ fertilization can reduce the uptake of radioactive cesium in rice and other plants that would behave similarly to this species (White and Broadley, 2000; Ohmori *et al*., 2014b; Wakabayashi *et al*., 2016). High availability of K^+ results in decreased expression of OsHAK1 (Bañuelos *et al*., 2002, Chen *et al*., 2015), which provides the main pathway for Cs^+ entry into the root. Other cations such as NH_4^+ (Bañuelos *et al*., 2002) and Na^+ (which can be present at high concentrations in paddy fields), may affect OsHAK1 activity. In our experiments on contaminated soils from Fukushima, N fertilization using NH_4^+ salts for mimicking paddy conditions were performed. Analyses of *oshak1* mutant performance in the

presence of varying concentrations of NH_4^+ and Na^+ have however to be performed to gain further insights into the influence of these cations on OsHAK1-mediated radiocesium transport.

Based on the current status of knowledge, it seems however very clear that biotechnological programs aiming at breeding plants that do not significantly take up and accumulate cesium would provide the most controlled and safest solutions to reduce the consequences of nuclear accidents on crop contamination and food production. So far, the numerous programs carried out in order to characterize the mechanisms of radioactive Cs^+ uptake and translocation in rice plants (Fujiwara, 2013, Nemoto and Abe, 2013, Ishikawa *et al*., 2017) have not identified biotechnological targets as promising as OsHAK1. The present results indicate that OsHAK1 is a major contributor to Cs^+ accumulation in rice. A similar situation might also exist in various plant species that display a low K^+/Cs^+ discrimination for high-affinity uptake (Collander, 1941, Epstein and Hagen, 1952), unlike Arabidopsis (Sheahan *et al*., 1993). In conclusion, despite OsHAK1 contributes to K^+ uptake in rice plants, inactivation of this transporter had much more pronounced effects on Cs^+ tissue contents (>30 times decrease) than on K^+ tissue contents (about a two-fold decrease) or on shoot biomass (comparable values between WT and *oshak1* plants) when grown on low K^+ Fukushima soil, thus making *oshak1* plants the most valuable genetic background identified so far in order to produce low-Cs^+ rice plants.

EXPERIMENTAL PROCEDURES

Plant seedlings and growth conditions

Rice (*Oryza sativa* L. cv Nipponbare) seeds were germinated in filter paper wetted with tap water for 5 days. Then, seedlings were hydroponically grown for 14 days on 1 mM $CaSO_4$, 2 mM MES-1,3-bis[tris(hydroxymethyl) methylamino]propane, pH 6.0 at 28°C day/25°C night 80% humidity. The nominal concentration of K^+ in this 0 K^+ solution ranged from 0.5 to 3 µM. The solution was renewed after 7 days. It was then added or not to 30 µM CsCl and/or 30 µM KCl, the corresponding treatments being named 0K-0Cs, 0K-30Cs, 30K-0Cs and 30K-30Cs. External Cs^+ and K^+ concentrations were periodically measured by flame spectrophotometry in all growth solutions and such cations were resupplied when necessary.

External Cs^+ and K^+ depletion by rice plants

Intact plants grown over 14 days in 0 K^+ solution were used for external Cs^+ and K^+ depletion experiments. Rice roots (not detached from the plant) were rinsed in cold 0 K^+ solution and

transferred onto plastic containers (one plant per container) containing 10 mL of 0 K^+ solution supplemented with 30 µM CsCl or 30 µM KCl, as stated. Solution samples were taken periodically and their concentrations in Cs^+ and K^+ were determined by flame spectrophotometry.

Cs^+ and K^+ tissue content analysis

At the end of the experiments, plants were separated into shoots and roots. Roots were briefly washed in 0 K^+ solution. Plant organs were dried at 80°C in paper bags over 4 days and dry weight (biomass) was measured. Then, ion content analyses of plant organs were carried out after ion extraction with 0.1 N hydrochloric acid for 2 days. K^+ and Cs^+ contents were determined by flame spectrophotometry. The same protocol was followed to measure seed K^+ content.

Yeast growth conditions and Cs^+ and K^+ uptake in yeast

The K^+ uptake deficient yeast (*Saccharomyces cerevisiae*) strain PLY246 (*trk1*Δ *trk2*Δ *tok1*Δ) (Bertl *et al*., 2003) was used for cation uptake experiments. *OsHAK1* coding sequence was obtained from KOME database (https://dbarchive.biosciencedbc.jp/en/kome/desc.html; clone AK119883) (Rice Full-Length <Author: Please provide missing text>c *et al*., 2003) and cloned in the pYPGE15 vector (Brunelli and Pall, 1993) between *Bam*HI and *Eco*RI sites. Yeast transformants containing either *OsHAK1* in pYPGE15 or the EV (control transformants) were selected after plating on uracil-free SD medium supplemented with 100 mM K^+ (Sherman, 1991). Cation uptake experiments were performed as described in (Rubio *et al*., 2000; Nieves-Cordones *et al*., 2008). Kinetic analysis of cation depletion from the external medium was performed as described in (Bañuelos *et al*., 2002).

Electrophysiological recordings in root cells with microelectrodes

Rice seedlings were grown in 0 K^+ solution for 5–7 days. Intact seedlings were fixed in a plexiglas chamber filled with 0 K^+ solution. Microelectrode preparation, cell impalement and setup details were described in Mian *et al*. (2011). After stabilization of the membrane potential in 0 K^+ solution was observed, membrane potential measurement solutions containing CsCl or KCl at different concentrations were percolated, in the plexiglas chamber, the chamber was washed with 0 K^+ solution between two successive measurements. A Michaelis–Menten equation was fitted to depolarization data obtained with the different CsCl and KCl concentrations.

Production of *OsHAK1*-edited plants with CRISPR/Cas9

Two sgRNA sequences (sgRNA1 and sgRNA2), which targeted exons 1 and 2, respectively, of the *OsHAK1* locus (Os04g32920) (Figure 3) were designed with the web tool CRISPR Plant (Xie *et al*., 2014). The 20-bp target sequence has to be immediately followed by a NGG motif (PAM), which is essential for Cas9 binding to the target DNA. sgRNA cloning into the pOs-sgRNA plasmid and subsequent transfer to pH-Ubi-cas9-7 was performed as described in (Miao *et al*., 2013). Transgenic plants were obtained by co-culture of seed embryo-derived calli (*Oryza sativa* L. cv. Nipponbare) with *A. tumefaciens* strain EHA105 (harbouring the sgRNAs in the pH-Ubi-cas9-7 plasmid) following the procedure of Sallaud *et al.* (2003). To identify mutations in regenerated plants, genomic DNA surrounding the regions targeted by sgRNA1 and sgRNA2 was amplified by PCR (1012-bp amplicon using OsHAK1gDNA1513-F and OsHAK1gDNA2506-R primers). PCR products were cloned into the pGEM-T Easy vector (Promega) and analysed by cycle sequencing. Plant lines with interesting alleles were selected for phenotypic analyses. Experiments were performed with T1 plants, which contained either WT (WT-c line) or mutated alleles (KO1 to KO3 lines). The analysis of edited alleles in T1 plants showed segregation of those identified in the corresponding T0 generation (Table S3).

Off-target-analysis

The potential off-target sites of the target sequence were predicted with the web tool of CRISPR-P (Lei *et al*., 2014) using the 20-bp target sequence plus the PAM sequence for the Blastn algorithm. Top-ranking potential off-target sites containing a NGG motif at 3′ of the 20-bp target sequence were selected for further analysis (Table S2). Genomic DNA fragments (about 700 bp) encompassing the potential off-target sites were amplified from T0 plants using specific primers (Table S5). PCR products were analysed by cycle sequencing.

$^{137}Cs^{+}$ determination and experiments in Fukushima soil

WT-c and KO1 to KO3 plants were grown on soils containing $^{137}Cs^{+}$ derived from the Fukushima Daiichi Nuclear Power Plant accident. Soil 1 was a paddy soil from Iitate (Fukushima Prefecture), which was placed in a container (1/5000a Wagner pot). Soil 2 was a mixture of uncultivated soil from Iitate (250 g) and gravels (400 ml) to which were added 400 mL of a modified half-strength Kimura-B nutrient solution that contained no potassium, which was placed in a 2-L container. Both soils were fertilized by addition of N and P as

$NH_4H_2PO_4$ and $(NH_4)_2SO_4$, the level of fertilization corresponding to 5 kg of N and 10 kg of P per 10 a.<Author: Please define 'a'> After soil conditioning, water only was added twice a week to maintain soil water capacity. For soil exchangeable cation determination, a mixture of dried soil (5 g) and 25 ml of 1 M ammonium acetate (pH 7.0) was shaken for 1 h. The extract was filtered and its potassium and sodium concentrations were determined using inductively coupled plasma-atomic emission spectrometry (Optima 7300 DV, PerkinElmer).

The plants were grown at 30°C with a 8:16 h light:dark photoperiod. In experiments with soil 1, plants were cultivated until grain production. $^{137}Cs^{+}$ content in shoots and grains harvested after 113 days of plant cultivation, was measured using a germanium semiconductor detector (GEM type, SEIKO EG&G CO., LTD., Tokyo, Japan). The detection limit was about 0.01 Bq per sample. In experiment with soil 2, $^{137}Cs^{+}$ was measured in roots and shoots after plant cultivation for 25 days, with a well-type NaI (Tl) scintillation counter (2480 WIZARD, PerkinElmer, Massachusetts, USA). The $^{137}Cs^{+}$ detection limit was 0.25 to 0.5 Bq per sample.

Identification of an *oshak1* homozygous T-DNA insertion mutant

A T-DNA insertion mutant line of rice in the Dongjin genetic background was identified through Rice GE (the Rice Functional Genomic Express Database) (http://signal.salk.edu/cgi-bin/RiceGE) and ordered from POSTECH-RISD (Korea) (Jeon *et al*., 2000, Jeong *et al*., 2006). Identification of homozygous mutant plants was performed by PCR using primers described in Table S5.

Statistical analyses

Statistical analysis was conducted using the Statistix 8.0 free software using analysis of variance (ANOVA) and Tukey's post-hoc test. Sigma Plot 9.0 was used for Michaelis–Menten and logarithmic fits.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information is available online at the publisher's website.

Figure S1. High-affinity Cs^+ uptake is impaired in the T-DNA insertion *oshak1* mutant from Donjin cultivar.

Figure S2. Seed K^+ content is not affected in *oshak1* KO plants.

Figure S3. Rice plant growth on Fukushima soil.

Table S1. Mutated OsHAK1 alleles obtained after CRISPR-Cas9 edition of *OsHAK1* locus.

Table S2. Mutation analysis of putative off-target sites.

Table S3. *OsHAK1* allele segregation in T1 plants.

Table S4. Resting membrane potentials in intact rice root cells of *OsHAK1*-edited and control plants bathed in the 0 K^+ solution.

Table S5. Oligonucleotides used in this study.

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FIGURE LEGENDS

Figure 1. High-affinity Cs^+ uptake in Nipponbare plants.

(a–c) Biomass (a), expressed as mg of dry weight, Cs^+ (b) and K^+ (c) content of Nipponbare root and shoots grown under hydroponic conditions in the presence or absence of 30 μM K^+ and/or 30 μM Cs^+ over 7 days. Bars denote mean values ± standard error (SE) (n = 6). Letters denote different groups obtained from Tukey's test ($P < 0.05$).

(d) Time courses of external Cs^+ or K^+ depletion by Nipponbare roots grown in 0 K^+ solution for 14 days. Roots were rinsed in cold 0 K^+ solution and then transferred into 0 K^+ solution supplemented with 30 μM CsCl or KCl. Kinetic analysis of depletion data allowed the calculation of apparent K_M and V_{max} values of depletion for each cation as described in Bañuelos *et al*. (2002) (see Results section). Data points are mean values ± SE (n = 4). The solid and dashed lines show expected depletion curves using obtained K_M and V_{max} values for Cs^+ and K^+, respectively.

(e, f) Representative recordings displaying membrane depolarizations elicited by different CsCl (e) and KCl (f) concentrations in Nipponbare cortex cells. Black bars denote the exposure period of the root to a given concentration of Cs^+ or K^+, and absence of black bars denotes a period of washing by 0 K^+ solution.

(g) Concentration dependence of membrane depolarizations elicited by CsCl or KCl in root cortical cells. Data points are mean values ± SE (n = 3–6). The solid and dashed lines correspond to Michaelis–Menten fits to depolarization data elicited by CsCl and KCl, respectively.

(h) Membrane potential (E_m) values at different external Cs^+ or K^+ concentrations (logarithmic scale). The linear fits to E_m versus the logarithm of the external concentration of Cs^+ (solid line) or of K^+ (dashed line) display a slope of ca. 29 mV per decade of cation concentration for both cations. The values of the predicted equilibrium potential for K^+ (E_K), assuming a cytosolic K^+ concentration of 50 mM, are provided by the gray line (slope of 58 mV per decade of external K^+ concentration).

Figure 2. OsHAK1 mediates Cs^+ uptake in yeast.

Kinetics of external Cs^+ and K^+ depletion by yeast mutant cells (Δ*trk1* Δ*trk2* Δ*tok1*) expressing OsHAK1 or the EV pYPGE15. Analysis of the depletion data allowed the calculation of apparent K_M and V_{max} values of depletion for each cation as described in Bañuelos *et al*. (2002) (see Results section). The solid and dashed lines show expected depletion by a yeast cell culture expressing OsHAK1, using the calculated K_M and V_{max}

values for Cs^+ and K^+, respectively. Symbols denote mean values ± standard error (SE) (n = 3).

Figure 3. Targeted mutagenesis of *OsHAK1* in rice using the CRISPR-Cas system.

(a) Overview of the *OsHAK1* locus and target sites for the sgRNA-Cas9 complex. Exons and introns in *OsHAK1* gene are depicted by rectangles and lines, respectively. Untranslated 5′ and 3′ sequences are shown in gray. Two single-guide RNAs (sgRNA1 and sgRNA2) were designed *in silico* to target the sgRNA-Cas9 complex to their corresponding targets sites (sequence provided in gray letters) in exons 1 and 2, respectively. PAM sequences are underlined.

(b–e) Allelic sequences at the target sites within the *OsHAK1* locus are identified in the four lines used in this study. Genomic DNA fragments (1012 bp long) covering target sites within the *OsHAK1* locus were amplified by PCR from T0 plants and sequenced after cloning. Sequencing results were aligned to the reference genome sequence. Insertions and deletions are depicted by lower case or black dashes, respectively. The target sequence in the *OsHAK1* gene is shown in gray and PAM is underlined. Among regenerated T0 plants, one line harbouring wild-type alleles (transformed with sgRNA2) was selected to be the wild-type control (sgRNA2–1, WT-c) (b), and three lines harbouring frameshift mutations were selected as *oshak1* mutants: one line transformed with sgRNA1 (sgRNA1-1, KO1) (c) and two lines transformed with sgRNA2 (sgRNA2-2, KO2 and sgRNA2-3, KO3) (d, e). Translated protein sequences from mutant alleles are shown in Table S1.

Figure 4. Frameshift mutations in the *OsHAK1* gene leads to reduced Cs^+ uptake in rice plants.

(a) Root and shoot biomass of WT-c and KO plants grown in 0 K^+ solution. Letters above the bars denote different groups obtained from Tukey's test ($P < 0.05$).

(b, c) Kinetics of Cs^+ (b) or K^+ (c) depletion by WT-c and KO plants. Experimental points: mean values ± standard error (SE) (n = 4).

(d, e) Root Cs^+ contents (d) and root and shoot K^+ contents (e) in plants used in (a–c). Data are mean values ± SE (n = 4).

(f) Cs^+ root and shoot contents in plants grown in 0K-30Cs solution over 7 days. Prior to the Cs^+ treatment, plants were grown in 0 K^+ solution for 7 days. Bars denote mean values ± SE (n = 6).

(g–j) Representative recordings displaying membrane depolarizations elicited by different

CsCl (g, h) and KCl (i, j) concentrations in KO1 (g, i) and KO2 (h, j) cortex cells. Black bars denote the exposure period of the root to a given concentration of Cs^+ or K^+, and the absence of black bars denotes a period of washing by 0 K^+ solution (as in Figure 1(e, f)).

(k, l), Concentration dependence of membrane depolarizations elicited by CsCl (k) and KCl (l) in WT-c and *oshak1* rice cortex cells (mean values ± SE; n = 3–6). Michaelis–Menten equation was used to fit the data. The solid, dashed and dotted lines correspond to the fits obtained for WT-c, KO1 and KO2 lines, respectively. For the WT-c roots, the values of the apparent K_M corresponding to these fitted data were 53 ± 5 µM for Cs^+ and 32 ± 6 µM for K^+, and the maximal depolarization were 81 ±2 mV for Cs^+ and 78 ± 3 mV for K^+, in agreement with the results obtained with WT Nipponbare plants (Figure 1(g)).

Figure 5. $^{137}Cs^+$ accumulation in *oshak1* plants grown on Fukushima contaminated soils.

(a) Average $^{137}Cs^+$ in soils collected in the area of Iitate in Fukushima Prefecture (adapted from Nihei *et al*., 2015). Soil 1, from paddy field, contained 7000 Bq of $^{137}Cs^+$ per kg dry weight (DW) and, as exchangeable cations, 177 mg K^+ and 309 mg Na^+ per kg DW. Soil 2, from non-cultivated land, contained 2.1×10^6 Bq of $^{137}Cs^+$ per kg DW and, as exchangeable cations, 67 mg K^+ and 4 mg Na^+ per kg DW.

(b–e) Data from WT-c and *oshak1* KO plants grown for 113 days in soil 1. (b, c) $^{137}Cs^+$ contents in grains (b) and shoots (c). Measurements were performed on pooled grains or shoot tissues from four plants from each genotype. (d, e), Grain and shoot biomass (d) and shoot K^+ content (e). Bars denote mean values ± SE (n = 4). No significant differences were obtained when performing ANOVA ($P > 0.05$).

(f–i), data from WT-c and KO plants grown for 25 days in soil 2. (f, g) $^{137}Cs^+$ contents in roots (f) and shoots (g). In eight out of nine KO mutant plants, $^{137}Cs^+$ could not be detected in shoots. (h, i) Root and shoot biomass (h) and K^+ tissue content (i).

Bars denote mean values ± SE (n = 4 for WT-c and KO2, n = 2 for KO1, n = 3 for KO3). Letters denote different groups according to Tukey's test ($P < 0.05$). B.D., below detection limit.