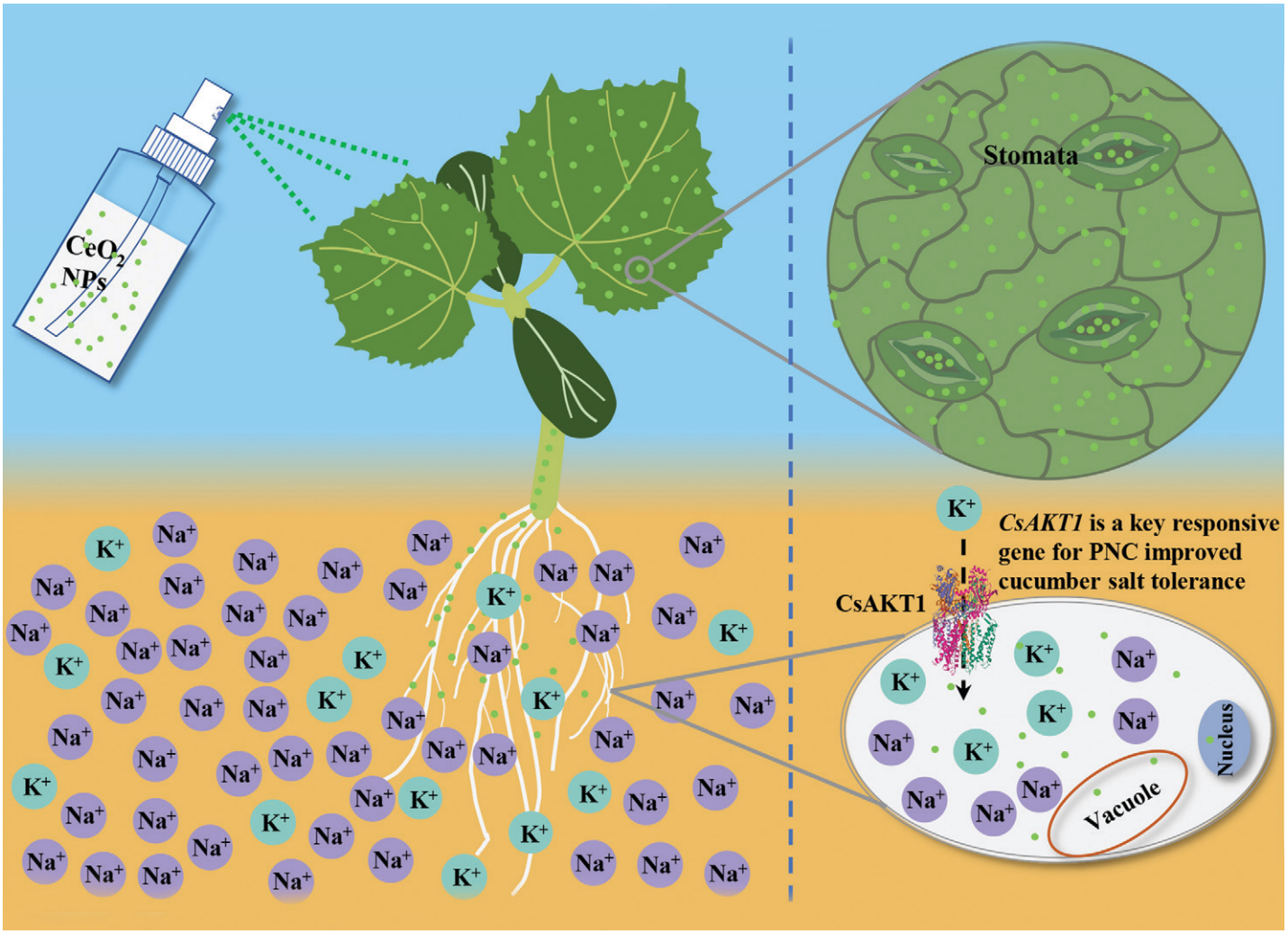

Featuring work from Prof. Zhilong Bie at Key Laboratory of Horticultural Plant Biology, Ministry of Education/ College of Horticulture and Forestry Sciences, Huazhong Agricultural University, China, and Prof. Honghong Wu at MOA Key Laboratory of Crop Ecophysiology and Farming System in the Middle Reaches of the Yangtze River, Huazhong Agricultural University, China.

CsAKT1 is a key gene for the CeO_2 nanoparticle's improved cucumber salt tolerance: a validation from CRISPR-Cas9 lines

PNC (poly acrylic acid coated nanoceria) foliar delivery enabled stronger cucumber salt tolerance than its root application via better maintaining K^+/Na^+ ratio. RNA seq data showed that under salinity, compared with other K^+ transport related genes, *CsAKT1* is the mostly upregulated gene in PNC treated cucumber plants. CRISPR-Cas9 lines further validate that *CsAKT1* is a key responsive potassium uptake gene for PNC improved cucumber salt tolerance.

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CsAKT1 is a key gene for the CeO_2 nanoparticle's improved cucumber salt tolerance: a validation from CRISPR-Cas9 lines†

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Salinity is one of the main factors limiting the crop growth and yield. The application of nanomaterials such as cerium oxide nanoparticles (nanoceria) improves salt tolerance in many plant species. The known mechanisms of the nano-improved crop salt tolerance mostly occur at the physiological and hormonal levels but not at the gene level. The lack of proper methods to precisely investigate the key genes involved in the nano-improved crop salt tolerance is one of the main reasons for this. We found that both the leaf and root applications of PNC (polyacrylic acid-coated nanoceria) improved the cucumber salt tolerance, showing higher shoot and root dry weight and better photosynthetic performance than the control plants. Under salinity stress, PNC-treated cucumber plants showed a significant lower malondialdehyde (MDA) content of leaf (46.7% and 13.3% for leaf and root application of PNC, respectively) and root (31.4% and 19.9% for leaf and root application of PNC, respectively) and reactive oxygen species (ROS) levels such as the H_2O_2 content of leaf (46.6% and 22.0% for leaf and root application of PNC, respectively) and root (50.0% and 29.9% for leaf and root application of PNC, respectively). Further experiments showed that under the salinity stress, compared with control plants, PNC-treated cucumber plants had significantly higher leaf (216.1% and 59.5% for leaf and root application of PNC, respectively) and root (148.2% and 71.8% for leaf and root application of PNC, respectively) K^+ content. The RNA seq showed that under the salinity stress, modulation on *AKT1* is more responsible for the PNC improved K^+ maintenance in cucumber. Further, the *CsAKT1* depletion experiment (CRISPR-Cas9 lines) confirmed that under salinity stress, *CsAKT1* plays an important role in the potassium uptake in cucumber plants treated with PNC. Our results showed that foliar PNC delivery enabled stronger cucumber salt tolerance than the root application associated with a better maintained K^+/Na^+ ratio. CRISPR-Cas9 lines further confirmed that *CsAKT1* is a key gene involved in the PNC-improved cucumber salt tolerance. Our work not only adds more knowledge to the mechanisms underlying nano-improved plant salt tolerance but also suggests a CRISPR-Cas9 approach to study the key genes involved in it.

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Environmental significance

Salinity is a major environmental stress limiting the crop growth and yield. The use of nanomaterials to improve the plant salt tolerance is an emerging approach to facilitate the sustainable development of agriculture. The cerium oxide nanoparticle (nanoceria)-improved plant salt tolerance was widely reported in many species, such as rice, rapeseed, cotton, and *Arabidopsis*. The omics technique helped to identify the up- or down-regulated genes in nanoceria treated plants under salinity. However, our knowledge about the key genes (identified and then validated) involved in nanoceria-improved plant salt tolerance is insufficient. Herein, we created CRISPR/Cas9 lines and validated that *CsAKT1* is a key gene responsible for nanoceria-improved cucumber salt tolerance. Our work not only showed that *CsAKT1* increased the leaf K^+ content and thus helped to maintain the K^+/Na^+ ratio in nanoceria treated plants than control under salinity but also suggests that CRISPR-Cas9 is an efficient approach to study key genes involved in the nanomaterial-improved plant salt tolerance.

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1. Introduction

The global population is expected to rise to 9.3 billion by 2050, which is a big challenge for the food supply.[1] Due to climate change, the abuse of fertilizers and pesticides and irrigation, land salinization has intensified. More than 1.0 × 10^7 km^2 of land around the world is affected by salt, resulting in abundant crop yield.[2–4] Therefore, improving plant salt tolerance is important for securing the food supply. To address food security issues in the background of increased soil salinization, nano-improved salt stress tolerance for plants could be an approach.[5,6]

Nano-improved salt tolerance has been reported in many crop species, including rice,[7] wheat,[8] barley,[9] potatoes,[10,11] rapeseed,[12–14] cotton,[15,16] and cucumber.[17–20] For example, cerium oxide nanoparticles (nanoceria) are potent ROS (reactive oxygen species) scavengers. The formed oxygen deficit due to the co-existence of Ce^{3+} and Ce^{4+} on the surface of nanoceria can scavenge H_2O_2 (hydrogen peroxide), $O_2^{\bullet-}$ (superoxide anion), and $OH^{\bullet}$ (hydroxyl radical) as non-radical counterparts.[21–23] The known mechanisms behind nanoceria-improved plant salt tolerance are mainly associated with maintaining ROS homeostasis to reduce oxidative damage, an improvement in K^+ retention and Na^+ exclusion to maintain the K^+/Na^+ ratio, an increase in gas signaling molecules such as nitric oxide, and modulation at the plant hormone level and α-amylase activities.[7,12,13,16,24,25] However, most of the above-mentioned studies are conducted at physiological, metabolic, and hormonal levels. Our knowledge about the insightful understanding of nano-improved plant salt tolerance at the transcription level is insufficient. The events that happened in nano-improved plant salt tolerance at the transcriptional level might be much earlier than those at physiological, metabolic, and hormonal levels. The RNA seq and qPCR (quantitative real-time PCR) results can point out the modulation of nanomaterials on gene expression levels in plants under salinity stress.[7,13,15,16,20,26,27] While, our knowledge about the validated key genes which are responsible for nanoceria-improved plant salt tolerance is still limited.

Under salinity stress, over-accumulated Na^+ not only inhibits K^+ absorption in plants but also competes with K^+ for the binding sites of enzymes, which specifically requires K^+ as a co-factor.[28,29] Therefore, the ability to maintain a high K^+/Na^+ ratio in the cytoplasm under salt stress is a common index for plant salt tolerance.[16,30] K^+ is an essential nutrient for the plant growth. It involves many physiological processes such as photosynthesis, protein synthesis, osmotic regulation, and the control of cell membrane polarization.[31] Thus, maintaining K^+ homeostasis is important for plant salt tolerance. To maintain K^+ homeostasis in the cell cytosol, mainly two routes are involved in increasing the K^+ influx from the growth medium to cytosol and reducing the K^+ efflux from the cytosol to the apoplast. The known genes for controlling K^+ influx are *AKT* (K^+ transporter) and *HAK* (high-affinity K^+ transporter) families.[32–34] *GORK* (guard cell outwardly rectifying K^+ channel) and NSCC (non-selective cation channels) channels are known to play an important role in the process of K^+ efflux under salinity stress.[32–34] Further, our knowledge about how nanoparticles affect these genes and which gene might be the most responsive for nano-improved plant salt tolerance is still insufficient.

In this study, poly(acrylic) acid-coated nanoceria (PNC) were synthesized and used. Its role in maintaining K^+/Na^+ homeostasis to improve cucumber salt tolerance was investigated. Furthermore, unlike the RNA-seq data obtained at 0 h salt stress in our previous study,[20] in this study, we performed RNA-seq to investigate the changes in the expressions level of K^+ transport-related genes in nanoceria-treated cucumber plants with 7 days' salinity stress. CRISPR/Cas9 lines were created to verify the role of the K^+ transport-related gene with the most significant change of the expression level in nanoceria-improved cucumber salt tolerance. Overall, we found that *CsAKT1* is a key gene responsible for nanoceria-improved cucumber salt tolerance.

2. Materials and methods

2.1. Plant materials

This study used the salt-sensitive cucumber (*Cucumis sativus* L.) variety "Jin Chun No. 4" from Tianjin Kerun Agricultural Science and Technology Co., LTD. Cucumber seeds were sterilized according to earlier investigations by soaking in clean water at 25 °C for three hours followed by at 55 °C for 15 minutes.[35] Then, seeds were added to Petri dishes (10 cm in diameter) that had three layers of filter papers that had been moistened with water. For seed germination, Petri dishes containing seeds were placed in an incubator set at 28 °C. The seeds were sown in the sponge and grown in containers with adequate aeration in 1/2 Hoagland nutrient solution. The seedlings were then transferred to a hydroponic cup with the following dimensions: mouth diameter: 90 mm, a bottom diameter: 57 mm, height: 135 mm, and 400 mL 1/2 modified Hoagland nutritional solution. The mixture contains 1 mM $MgSO_4{\cdot}7H_2O$, 4 mM $CaCl_2$, 10 mM KNO_3, 0.5 mM $Ca(H_2PO4)_2{\cdot}H_2O$, and 74.93 mg L^{-1} coolaber trace element solids (DZPM0059-500G) (pH 6.8 adjusted with 1 mM KOH). The growing environment was configured to have a temperature range of 28 ± 1 °C during the day and 18 ± 1 °C at night, a light intensity of 250 μmol m^{-2} s^{-1}, a day/night cycle of 14/10 hours, and a humidity range of 70–75%. Every experiment employed three replicates, each with two plants unless otherwise stated.

2.2. Synthesis and characterization of PNC

Following the procedure outlined by Wu *et al.*,[21] poly (acrylic) acid-coated nanoceria (PNC) was synthesized and characterised. In a 50 mL conical tube, 1.08 g cerium(III) nitrate (Sigma Aldrich, 99%) and 4.5 g poly (acrylic) acid (1800 MW) were quickly dissolved in 2.5 mL and 5.0 mL of deionized water, respectively. A vortex mixer was used to completely combine the solutions at 2000 rpm for 15

minutes. 15 mL of ammonium hydroxide solution (30%, Sigma Aldrich) was added dropwise to the combined solution in a 50 mL beaker. For 24 hours at room temperature, the mixture was stirred at 500 rpm. To eliminate any big agglomerates and other debris, the mixture was centrifuged at 4000 rpm for 1 h. To purify the supernatant, 10 K Amicon cells (MWCO 10 K, Millipore Inc.) were spun at 4500 rpm for 45 minutes, six times. A UV-vis spectrophotometer (UV-1800, AOE) was used for measurements and using Beer–Lambert's law the concentration of PNC was determined by measuring its peak absorbance at 271 nm. A Brookhaven Zeta-sizer (NanoBrook 173 Plus) instrument was used to measure zeta potential and the hydrodynamic diameter of 0.1 mM PNC suspended in dd H_2O. Then, for later processing, the acquired PNC product was kept in a freezer at 4 °C. 20 μL PNC was mounted on a copper grid with holes covered in carbon for TEM (transmission electron microscope) imaging, then an FEI Talos microscope was set to 300 kV to capture images of the samples. The samples were then kept at 4 °C for future use.

2.3. Synthesis and confocal imaging of DiI-PNC

DiI-PNC was synthesized according to our previous publication,[20] and the final product was stored at 4 °C for further use. Confocal imaging of DiI-PNC was performed 3 hours after DiI-PNC application to cucumber leaves and roots. The confocal imaging was conducted by following the procedures, which are described in our previous publication.[20]

2.4. Incubating cucumber with foliar-delivered or root-applied nanoceria and the salt stress treatment

PNC was administered to cucumber seedlings either foliarly or through the roots (at the period of the growth of the second true leaf). 0.1 mM PNC was utilized, which was determined from a PNC concentration screening experiment.[20] The true leaves of the cucumber were sprayed with 1 mL of 0.1 mM PNC (a total of 0.1 mol PNC in the solution) for foliar delivery.[20] A pipette with clipped tips was used to spray the solution. To aid in the incubation of PNC in leaves, silwet L-77 (0.05% v/w) was utilized. Kimwipes were employed to prevent any solution from dripping onto untreated areas of the plant. For the root application, the seedling roots were placed for 30 minutes in a 50 mL centrifuge tube with 25 mL of 4 μM PNC (containing a total of 0.1 mol PNC in the suspension). As a control, 1 mL of water was equally sprayed on the second true leaves. A 50 mL centrifuge tube with 25 mL of 4 μM PNC (a total of 0.1 mol PNC in the solution) was used for the root treatment, and 1 mL of water was uniformly sprayed on the second true leaves as a control. For applying salt stress, 75 mM NaCl was added to the culture fluid after 3 hours of adaptation under room light.

2.5. Assessing phenotypic and photosynthetic performance of cucumber plants with PNC under salt stress

For measuring, the dry weight of the shoots and roots, the first true leaf's SPAD levels, maximal photochemical efficiency (F_v/F_m), net photosynthetic rate (P_n), stomatal conductance (G_s), and intercellular CO_2 concentration (C_i) of salt-stressed (75 mM NaCl, 7 days) cucumber were carried out according to the protocols described in previous publications.[20,36]

2.6. Determination of cerium, K^+, and Na^+ content

After NaCl treatment for 7 days, cerium, K^+, and Na^+ content samples of the leaves, roots, and whole plant were measured as described in our previous study.[20] Briefly, 0.1 g of the samples were digested with sulfuric acid. Then, the volume of the collected digestion solution was set to 10 mL by using ultrapure water. ICP-MS (Inductively Coupled Plasma-Mass Spectrometry) (Wuhan Qijunchicheng Technology Co., LTD.) technique was used to measure the cerium content. K^+ and Na^+ contents were measured using an atomic absorption spectrophotometer (6300C, Shimadzu).[37]

2.7. Determination of ROS content, MDA, and REC

After 7 days of salt stress (75 mM NaCl), H_2O_2, $O_2^{\bullet-}$, and MDA (malondialdehyde) content and REC (relative electrical conductivity) in leaf and root samples were measured as described in our previous study.[20,39–42] Briefly, H_2O_2 content was determined using a hydrogen peroxide kit from the Nanjing Jiancheng Institute of Biological Engineering.[38] The hydroxylamine hydrochloride oxidation technique was used to measure the concentration of $O_2^{\bullet-}$.[39] The content of $O_2^{\bullet-}$ was then determined using the standard curve for $O_2^{\bullet-}$ standard after measuring the solution's absorbance value at 530 nm. DAB (diaminobenzidine) and NBT (nitrotetrazolium blue chloride) stainings were performed according to the protocols reported in the previous publication.[40] The MDA content was measured following the method described in aprevious publication.[41] The amount of MDA in samples was calculated using the formula: C (nmol g^{-1}) = (6.45 (A532 − A600) − 0.56 × A450) × 5/0.1. The relative electrical conductivity was determined by following the previous study.

2.8. Transcriptome assay

After 7 days of salt stress (75 mM NaCl), the leaves and roots were sampled. The samples were frozen in liquid nitrogen and pulverized into powder before being transferred to a business that specializes in transcriptome sequencing (Megi Bio, Shanghai). Differential expression analysis was performed using the cucumber (Chinese Long) genome V3 (**https://cucurbitgenomics.org/**). The statistical table of the genome alignment results is shown in Table S2.† The software DESeq2 was used to compute and identify differential genes.[43] TPM (Transcription Per Million) was utilized to determine gene expression, and the criteria for

differential gene screening were set as Log 2 (foldchange value) > 1, *p*-value 0.05.[44] The RNA seq data was uploaded to NCBI (**https://www.ncbi.nlm.nih.gov/bioproject/PRJNA874600**), and the accession number is PRJNA874600.

2.9. Quantitative real-time PCR analysis

After 7 days of salt stress (75 mM NaCl), the leaves and roots were sampled for real-time fluorescent quantitative PCR (qRT-PCR). RNA was extracted using the TransZol kit from TransGen in Beijing, and mRNA was reverse transcribed using Hiscript Reverse Transcriptase from Vazyme in China. Reference genes were applied after reverse transcription to assess the reverse transcription's quality. For the next experiment, cDNA and primers that could amplify the reference gene bands were used. ABI6500 (ABI, USA), 10 μL system with 1 μL diluted cDNA, 5 μL 2× TransStartTM Top Green qRT-PCR SuperMix (TransGen, Beijing), and 1 μL forward and reverse primers each were used to perform qRT-PCR. It took 40 cycles of 94 °C pre-denaturation for 30 s, 94 °C denaturation for 5 s, 56 °C annealing for 30 s, and 72 °C extensions for 10 s to complete the PCR. The expression analysis was calculated using $2^{-\Delta\Delta ct}$. Primers were designed using NCBI Primer-BLAST (**https://www.ncbi.nlm.nih.gov/tools/primer-blast/**). For the absolute quantification of *CsGORK*, *CsAKT1*, and *CsHAK5;3*, we prepared their respective standard substances. Using cucumber cDNA as a template, full-length amplification of *CsGORK*, *CsAKT1*, and *CsHAK5;3* was performed using high-fidelity Taq enzyme (I-5™ 2× High-fidelity Master Mix, Engine, China). The PCR amplification procedure was as follows: 95 °C for 3 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min 40 s, and 40 cycles. The obtained PCR reaction products were detected using 1% agarose gel electrophoresis. After confirming the correct band size, the gel was cut and the target band was recovered using the AxyPrepTM DNA gel recovery kit (AxyGEN, USA). Then, the recycled glue samples were sent to the company for sequencing. After the sequencing was correct, the samples were diluted to 100 ng μL^{-1} standard. Then, the standard was diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} of the previous concentration with dd H_2O. These samples were used for qRT-PCR quantification and standard curves were made (Fig. S1a–c†). The absolute quantification of *CsGORK*, *CsAKT1*, and *CsHAK5;3* was calculated using the standard curve of their standard. The used primers are shown in Table S1.†

2.10. *CsAKT1* sequence analysis and subcellular localization

Using TMHMM (**https://services.healthtech.dtu.dk/service.php?TMHMM-2.0**) for analysis of the amino acid sequence of the transmembrane area of *CsAKT1* (CsaV3_1G029650). DNAMAN 9.0 was used for sequence alignment of *CsAKT1*. NCBI (**https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi**) was used to predict *CsAKT1* protein structure. Subcellular localization methods refer to previous studies.[45,46] 1305.4 vectors were used for subcellular localization. Full-length amplification primers were designed based on the Bgl II restriction site sequence and *CsAKT1* CDS sequence on the vector. The recombinant plasmid of *CsAKT1* was transformed into *Escherichia coli* 5α. The method of recombination and transformation was performed according to the operation manual of the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing). *Escherichia coli* was cultured overnight, and several plaques were randomly selected for colony PCR. The positive plaques identified were sent to Optimus for sequencing. Enough recombinant plasmids were obtained by expanding the culture and extracting plasmids from the correctly sequenced *E. coli*. The recombinant plasmid and control plasmid (Empty vector) were transformed into Agrobacterium GV3101 by the freeze–thaw method. The transient expression test was carried out using *Agrobacterium tumefaciens* containing recombinant plasmid, control plasmid, plasmid PM-RK CD3-1007 with cell membrane marker mCherry, and auxiliary plasmid P19. The injected tobacco was placed in an incubator for 48–72 h, followed by observations using a confocal laser microscope (Leica TCS-SP8, Germany).

2.11. *CsAKT1* yeast heterologous expression

According to our previous method,[47] the yeast heterologous expression vector was pYES2-NTA. The CDS (coding sequence) sequence of *CsAKT1* was amplified and inserted into the Hind III restriction site of the pYES2-NTA vector by homologous recombination using cucumber cDNA as a template. The salt-sensitive yeast strain G19 with recombinant pYES2-NTA–*CsAKT1* and empty pYES2-NTA (experimental control) were cultured in the SD-ura liquid medium overnight to OD600 = 0.7, and then the yeasts were diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} of previous concentration with ddH_2O. 5 μL of the solution was pipetted and placed dropwise on the plate containing 0, 100 mM NaCl AP solid medium, after dripping, the plate was placed on an ultra-clean workbench and air was blown. After the plate was blown dry, it was placed upside down in a 30 °C incubator to grow for 4 days and the growth of yeast was observed. The recombinant pYES2-NTA–*Cs*AKT1 and empty pYES2-NTA vectors were transferred into K^+ uptake-deficient yeast strain $W_{\Delta 6}$, cultured in the SD-ura liquid medium overnight to OD600 = 0.7, and then diluted with dd H_2O to 10^1, 10^2, 10^3, 10^4 times, 5 μL liquid was drawn and placed dropwise on a plate of the AP solid medium containing 0, 100 mM KCl. After dripping, the plate was placed on the ultra-clean workbench to blow. After the plate was blown dry, it was placed upside down in a 30 °C incubator to grow for 4 days, and the growth of yeast was observed. A test buffer was prepared as follows, 10 mM MES, 2% galactose, and $Ca(OH)_2$ to adjust the pH to 6.0. 200 μL of yeast $W_{\Delta 6}$ was then added with *Cs*AKT1 to 50 mL of the AP liquid medium containing 50 mM KCl, which was cultured overnight at 28 °C, 220 r min^{-1}, to OD600 = 1.0, then centrifuged at 10 000 r min^{-1} for 1 min; the supernatant was discarded and 50 mL of dd H_2O was added to resuspend the yeast, followed by centrifugation at 10 000 r min^{-1} for 1 min and discarding the supernatant,

and then adding 50 mL of the AP liquid medium without K^+ and incubation for 4 h for K^+ starvation treatment. After the starvation treatment was completed, the solution was centrifuged at 10 000 r min^{-1} for 1 min, the supernatant was discarded, 50 mL of the test buffer was added, and the yeast was resuspended. Three treatments were set up, *i.e.*, inoculating the yeast with the liquid AP medium containing 200 μM NaCl, 200 μM KCl, and 200 μM NaCl + 200 μM KCl. Then, the liquids were placed on a 30 °C shaker, 220 r min^{-1}, and 4 mL of bacterial solution was drawn every 5 min, centrifuged at 12 000 r min^{-1} for the 30 s, and the supernatant was collected for the determination of Na^+ and K^+ contents.

2.12. Creation of CRISPR/Cas9-induced *CsAKT1* deletion plants

CsAKT1 (CsaV3_1G029650) genome sequence was downloaded and constructed from the Cucurbit Genomics Database (**https://cucurbitgenomics.org/**), and then submitted for sequencing to the Geneious software to obtain a collection of sgRNA (small guide RNA) sequences. The target was preferentially selected in the first external display sgRNA sequences with a low off-target rate. According to the sequence design, sgRNA primers were obtained and sent to a primer synthesis company (Tsingke, Beijing) for synthesis. After the synthetic primers were obtained, annealing and ligation of the vector were performed. After the reaction was completed, the ligation products were transformed into *Escherichia coli* 5α. The pKSE402 empty plasmids and pKSE402-containing *CsAKT1* sgRNA were used to transform Agrobacterium K599 (Weidi, Shanghai) referring to the K599 instruction manual. Then, the cucumber was infected. After the infection was successful, the non-GFP roots were removed every week, and the GFP roots were kept. The GFP roots were sampled, DNA was extracted, and PCR amplification was performed. The PCR products were subjected to Hi-TOM sequencing to detect gene editing efficiency.[37] The transformed cucumber seedlings were obtained, cultivated to 2 leaves, and subjected to 75 mM NaCl treatment. The morphological and physiological indices of cucumber were measured at 75 mM NaCl treatment for 7 d.

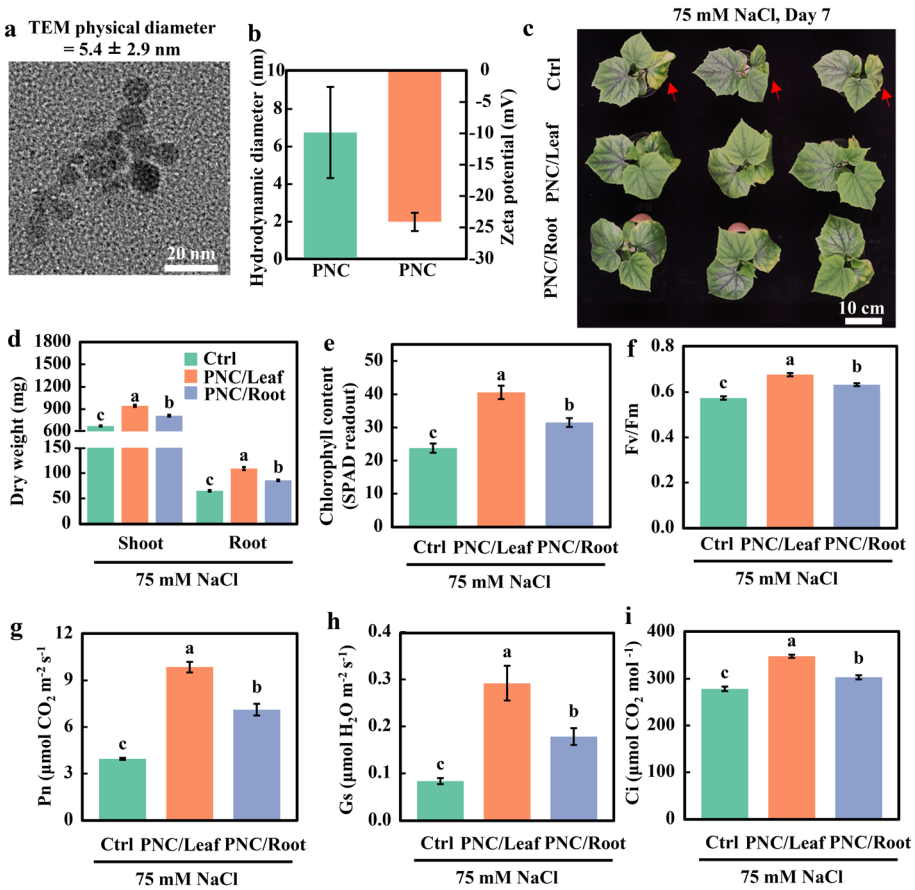

Fig. 1 PNC synthesis and characterization, and its role in improving cucumber salt tolerance (75 mM NaCl, 7 days). (a), TEM image of PNC. (b), the hydrodynamic size and zeta potential of PNC. Mean ± SE (n = 3). Foliar sprayed or root-applied PNC improved cucumber salt tolerance. Phenotypic performance (c), shoot and root dry weight (d), chlorophyll content (SPAD readout, e), F_v/F_m (f), P_n (g), G_s (h), and C_i (i). Mean ± SE (n = 3). Different lowercase letters indicate significant differences among different treatments at the $P < 0.05$ level. Ctrl, control cucumber plants under 75 mM NaCl (7 days); PNC/leaf, cucumber plants with leaf PNC application under 75 mM NaCl (7 days); PNC/root, cucumber plants with leaf PNC application under 75 mM NaCl (7 days).

2.13. Data analysis and image processing

The statistical analysis and sketching of the images were performed using Rstudio 4.03 software. For a significant comparison, the Duncan-type new complex range method was employed. The images were typeset using Photoshop 6.0 software.

3. Results

3.1. PNC characterization and its improvement on the performance of cucumber under salinity stress

In this study, we synthesized poly (acrylic) acid-coated nanoceria (PNC) with a size below 10 nm. Characterization data showed that the TEM size, hydrodynamic diameter (measured in ddH_2O), and zeta potential (measured in ddH_2O) of PNC were 5.4 ± 2.9 nm (Fig. 1a), 6.6 ± 2.4 nm (Fig. 1b), and −24.0 ± 1.4 mV (Fig. 1b), respectively. Confocal imaging results showed that compared with no signals in the control group, clear DiI signals were detected in the leaves and roots of cucumber plants treated with DiI-PNC (Fig. S2a and b†).

Our results showed that compared with control plants under salinity (75 mM NaCl, 7 days), foliar spray or root application of PNC improved cucumber salt tolerance, showing better phenotypic performance (Fig. 1c), increased shoot (40.5 and 20.7% for leaf and root application of PNC, respectively) and root (67.8 and 31.8% for leaf and root application of PNC, respectively) dry weight (Fig. 1d), and higher chlorophyll content (70.8 and 32.5% for leaf and root application of PNC, respectively) (Fig. 1e). Also, cucumber plants with PNC application showed higher total areas of leaf (85.3% and 39.8% for leaf and root application of PNC, respectively) (Fig. S3a†), bigger total lengths of root (41.3% and 13.0% for leaf and root application of PNC, respectively) (Fig. S3b†) and total surface areas of root (33.4% and 15.3% for leaf and root application of PNC, respectively) (Fig. S3c†), and increased root volume (83.3% and 43.0% for leaf and root application of PNC, respectively) (Fig. S3d†) than control plants under salinity stress. Gas exchange experiments showed that compared with control plants under salinity (75 mM NaCl, 7 days), foliar spray or root application of PNC improved the photosynthetic performance of cucumber under salt stress, showing the increase of F_v/F_m (17.8% and 10.3% for leaf and root application of PNC, respectively) (Fig. 1f and S4a†), P_n (149.3% and 80.3% for leaf and root application of PNC, respectively) (Fig. 1g), G_s (248.6% and 113.1% for leaf and root application of PNC, respectively) (Fig. 1h), C_i (24.8% and 8.9% for leaf and root application of PNC, respectively) (Fig. 1i) and T_r (89.5% and 51.6% for leaf and root application of PNC, respectively) (Fig. S4b†). Together, these results suggest that both leaf and root application of PNC could improve the growth and photosynthetic performance of cucumber under salt stress. More importantly, PNC leaf application showed better improvement in cucumber salt tolerance compared to PNC root application. ICP-MS results showed that after 7 days of salinity stress (75 mM NaCl), cerium content in the whole cucumber (20.3 *vs.* 6.3 μg g^{-1}, 3.2 folds), leaf (23.4 *vs.* 4.0 μg g^{-1}, 5.8 folds) and root (8.4 *vs.* 13.4 μg g^{-1}, 62.3%) are significantly higher in the foliar-delivered PNC than the root applied one (Fig. S5a and b†), further confirming that foliar application of PNC is more optimal than the root application, at least for improving cucumber salt tolerance.

3.2. PNC application decreased ROS content in cucumber under salinity stress

Compared with control plants under salinity stress (75 mM NaCl, 7 days), PNC-treated cucumber plants showed a decrease in H_2O_2 content in leaf (46.6% and 22.0% for leaf and root application of PNC, respectively) and root (50.0% and 29.9% for leaf and root application of PNC, respectively) (Fig. S6a†), and significantly lower $O_2^{\bullet-}$ content in leaf (58.7% and 26.4% for leaf and root application of PNC, respectively) and root (53.2% and 33.0% for leaf and root application of PNC, respectively) (Fig. S6b†). Also, significantly lower MDA content in leaf (46.7% and 13.3% for leaf and root application of PNC, respectively) and root (31.4% and 19.9% for leaf and root application of PNC, respectively) (Fig. S6c†) was found in cucumber plants treated with PNC than the control under salinity stress. This is also observed in REC (relative electric conductivity), showing that the PNC-treated cucumber plants have significantly lower REC in leaf (50.6% and 32.8% for leaf and root application of PNC, respectively) and root (33.6% and 13.6% for leaf and root application of PNC, respectively) than the control plants under salinity stress (Fig. S6d†). DAB and NBT staining experiments further confirmed that cucumber plants with leaf or root application of PNC have decreased H_2O_2 and $O_2^{\bullet-}$ content compared to control plants under salinity stress (Fig. S6e and f†).

3.3. PNC-induced *CsAKT1* – mediated absorption of K^+ in cucumber under salinity stress

No doubt, compared with a blank control (0 mM NaCl, 7 days), cucumber plants showed increased Na^+ content in leaf (8.9 folds) and root (2.8 folds), decreased K^+ content of leaf (78.1%) and root (70.5%), and decreased K^+/Na^+ ratio content of leaf (97.8%) and root (92.3%) after salinity stress (75 mM NaCl, 7 days) (Fig. 2a–c). Compared with salt control plants (75 mM NaCl, 7 days), the PNC-treated cucumber showed a decrease in Na^+ content in leaf (21.8% and 8.7% after leaf and root application of PNC, respectively) and root (21.5% and 11.8% after leaf and root application of PNC, respectively) (Fig. 2a). While, the PNC-treated cucumber showed an increase in K^+ content in leaf (216.1% and 59.5% for leaf and root application of PNC, respectively) and root (148.2% and 71.8% for leaf and root application of PNC, respectively) compared to control plants under the salinity stress (Fig. 2b). In terms of the K^+/Na^+ ratio, under salinity stress, the PNC-treated cucumber plants showed increased K^+/Na^+ ratio in leaf (304.5% and 74.7% for leaf and root application of PNC, respectively) and root (216.1% and 95.0% for leaf and root application of PNC, respectively) compared

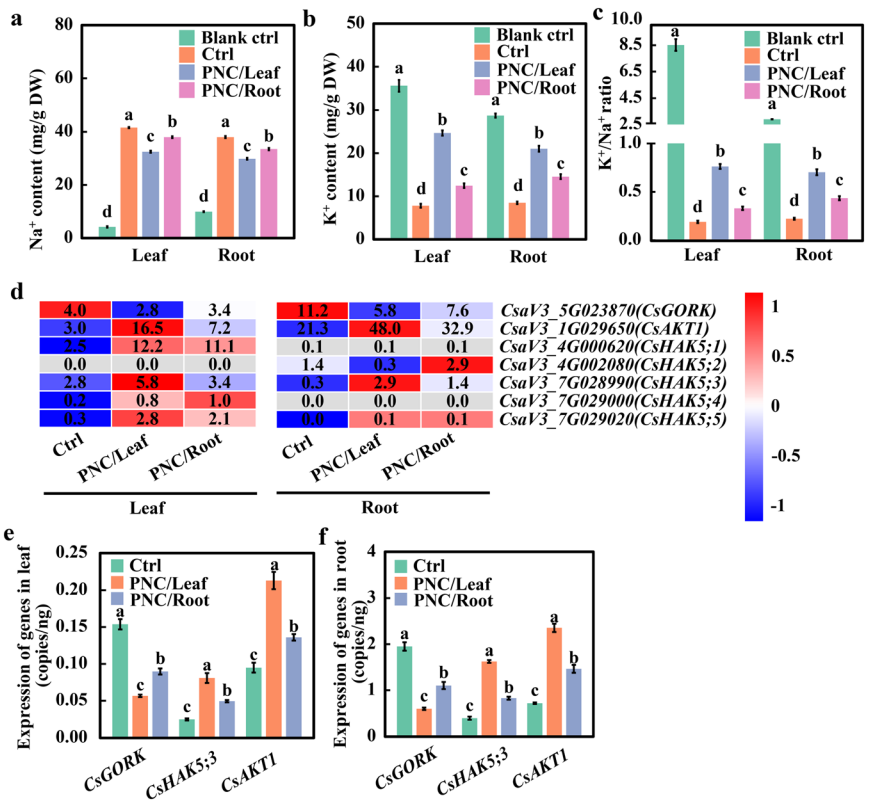

Fig. 2 Effects of PNC on K^+/Na^+ ratio and expression level of K^+ transport genes in cucumber. Na^+ (a) and K^+ (b) content, and K^+/Na^+ ratio (c) in cucumber leaves and roots of cucumber plants with foliar sprayed or root-applied PNC. Heat map (d) and q-PCR analysis of the expression level of *CsGORK*, *CsHAK5;3* and *CsAKT1* in cucumber leaves (e) and roots (f) with foliar sprayed or root applied PNC. Mean ± SE (n = 3). Different lowercase letters indicate significant differences among different treatments at the $P < 0.05$ level. Blank ctrl, control cucumber plants under non-saline conditions; ctrl, control cucumber plants under 75 mM NaCl (7 days); PNC/leaf, cucumber plants with leaf PNC application under 75 mM NaCl (7 days); PNC/root, cucumber plants with leaf PNC application under 75 mM NaCl (7 days).

with the control plants (Fig. 2c). Overall, these results suggest that to maintain K^+/Na^+ ratios under salinity stress, PNC application enabled K^+ retention, contributing to more than its improved Na^+ extrusion in cucumber plants.

RNA seq and qRT-PCR experiments were further conducted to investigate the changes in the expression level of K^+ transporters in cucumber plants with PNC application under salinity stress. Before RNAseq and qRT-PCR, we tested the quality of RNA and found that the integrity of RNA was good without obviously observed degradation (Fig. S7a and b†). Then, PCA analysis (Fig. S8a and b†) was performed on leaf and root transcriptomes of salt-stressed (75 mM NaCl, 7 days) cucumber plants treated with PNC. It showed that the samples with three replicates were all located in 95% confidence ellipses, indicating good repeatability of the samples. qRT-PCR analysis (Fig. S8c†) and correlation analysis (Fig. S8d†) showed that the results of qRT-PCR and RNAseq (Fig. S9†) were consistent. Also, the correlation coefficient R^2 was 0.93 (Fig. S8c†), indicating that the transcriptome results were accurate and reliable. Further data analysis showed that the number of up-regulated genes in leaves and roots was significantly higher than down-regulated genes, regardless of salt-stressed cucumber plants with PNC foliar spray or root application (Fig. S10a and b†). GO enrichment analysis ($P < 0.05$) showed that DEGs in leaves and roots of cucumber with either foliar sprayed or root-applied PNC could be enriched into groups such as cation binding and transporter activity (Fig. S9c–f†).

The phylogenetic tree analysis of cucumber and *Arabidopsis Thaliana* revealed that cucumber *GORK* and *AKT1* had 1 member and *HAK5* had 5 members (Fig. S11†). The heat map analysis showed that compared with H_2O control, potassium ion channel protein genes, such as *GORK*, *HAK5*, and *AKT1* were mostly up-regulated in leaves and roots of cucumber with either foliar delivered or root applied PNC (Fig. 2d). Furthermore, compared with the root application, foliar sprayed PNC downregulated *CsGORK*, upregulated *CsHAK5;3* and *CsAKT1*. qRT-PCR results showed that PNC did not induce changes in the gene expression levels of *CsGORK*, *CsHAK5;3*, and *CsAKT1* in cucumber under non-saline conditions (Fig. S12†). While compared with the control plants under salinity (75 mM NaCl, 7 days), PNC-treated cucumber plants showed a decreased expression level of *CsGORK* (36.8% decrease in leaf and 45.8% decrease in the root of plants with PNC leaf spray over its root application), increased expression level of *CsHAK5;3* (36.2% increase in leaf and 60.8% increase in the root of plants with PNC leaf

spray over its root application), and increased expression level of *CsAKT1* (63.8% increase in leaf and 96.4% increase in the root of plants with PNC leaf spray over its root application) (Fig. 2e and f). Together, these results not only further confirm that foliar-delivered PNC is more optimal than the root application in improving cucumber salt tolerance, but also suggest that *CsAKT1* is more responsible for PNC improved cucumber salt tolerance.

3.4. Expression analysis and functional analysis of *CsAKT1* in yeasts

TMHMM (prediction of transmembrane helices in proteins) was used to predict the transmembrane structure of *CsAKT1*. Our results showed that *CsAKT1* contained five transmembrane domains with a long C-terminal (Fig. S13a and b†), which is consistent with the case in *Arabidopsis*. Further analysis showed that CNBD (cyclic nucleotide-binding domain) of *CsAKT1* was located between 399–485 amino acid sites, anchored repeat domain was located between 532–688 amino acid sites, and KHA (rich in hydrophobic and acidic amino acids) domain is located between the 804 and 866 amino acid sites (Fig. S13c†). Subcellular localization experiments showed that *CsAKT1* was localized to the plasma membrane (Fig. 3a). Yeast G19 (salt-sensitive strain) and $W_{\Delta 6}$ (K^+ deficient strain) strains were used to further verify whether *CsAKT1* could transport K^+. Our results showed that under 100 mM of NaCl treatment, no significant difference in the growth status was found between G19 transformed with *CsAKT1* and the G19 empty (Fig. 3b), indicating that *CsAKT1* could not transport Na^+. Under 100 mM and 0.1 mM KCl treatment, $W_{\Delta 6}$ yeast transformed with *CsAKT1* grew significantly better compared to $W_{\Delta 6}$ empty (Fig. 3c), indicating that *CsAKT1* could transport K^+. Furthermore, the ion depletion experiment showed that compared with no change in Na^+ content in 200 μM NaCl buffer, K^+ content decreased significantly in *CsAKT1* transformed $W_{\Delta 6}$ yeast inoculated into the liquid AP medium

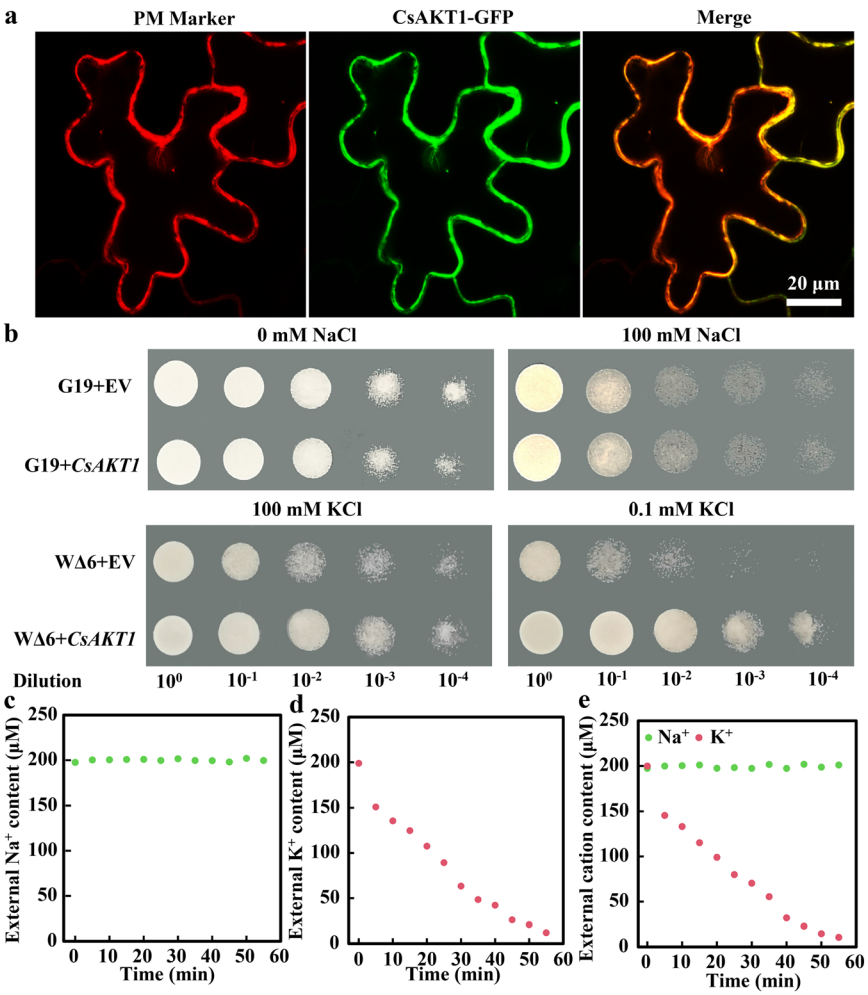

Fig. 3 Subcellular localization and functional analysis of *CsAKT1*. (a), subcellular localization analysis of *CsAKT1* expressed in tobacco plants. (b), growth analysis of yeast mutant G19 under NaCl treatment and *CsAKT1* complements the growth of yeast mutant $W_{\Delta 6}$ under K deficiency medium. The *CsAKT1* expressing $W_{\Delta 6}$ yeast cells were inoculated into liquid AP medium supplemented with 200 μM NaCl, 200 μM KCl and 200 μM NaCl + 200 μM KCl (c–e). EV: Empty vector.

supplemented with either 200 μM KCl or 200 μM NaCl + 200 μM KCl (Fig. 3c–e). This further confirmed the role of *CsAKT1* in K^+ transport.

3.5. Knockout *CsAKT1* impaired PNC enabled improvement of salt tolerance on cucumber

By using the root transformation system, *CsAKT1* was knocked out in the cucumber rootstock. Hi-TOM sequencing showed that the editing efficiency was 60.82% (Fig. 4a and b). Protein translation analysis showed that knock-out lines could induce mismatch and early termination of protein translation (Fig. S14a†). Together, it suggests that Cas9 successfully edited *CsAKT1* of cucumber with high editing efficiency. Compared with EV (Empty vector) plants under salinity (75 mM NaCl, 7 days), *CsAKT1* knockout plants showed worse phenotypic performance (Fig. 4c), significantly lower dry weight of shoot (21.9%, 14.1%, and 19.2% for control, leaf and root application of PNC, respectively, Fig. 4d) and root (27.8%, 42.4%, and 29.5% for control, leaf and root application of PNC, respectively, Fig. 4e), and significantly smaller total area of the leaf (31.7%, 19.6%, and 22.5% for control, leaf and root application of PNC, respectively) (Fig. S15a†). It also showed a significantly decreased total length of root (22.8%, 22.2%, and 24.9% for control, leaf, and root application of PNC, respectively, Fig. S15b†), the total surface area of root (27.2%, 27.0%, and 22.7% for control, leaf and root application of PNC, respectively, Fig. S15c†), and root volume (35.8%, 32.1%, and 28.1% for control, leaf and root application of PNC, respectively, Fig. S15d†) in *CsAKT1* knockout plants than the EV plants under salinity.

K^+ and Na^+ content data showed that compared with EV plants under salinity (75 mM NaCl, 7 days), *CsAKT1* knockout cucumber plants showed significantly increased Na^+ content regardless of the treatment with the control (33.7 ± 0.3 *vs.* 39.3 ± 0.3 mg g^{-1} in leaf and 40.6 ± 0.2 *vs.* 45.0 ± 0.1 mg g^{-1} in root), or foliar PNC delivery (23.6 ± 0.7 *vs.* 29.9 ± 0.5 mg g^{-1} in leaf and 22.3 ± 0.7 *vs.* 36.4 ± 0.5 mg g^{-1} in root), or root PNC application (29.6 ± 1.0 *vs.* 34.2 ± 0.7 mg g^{-1} in leaf and 33.0 ± 0.3 *vs.* 41.2 ± 0.3 mg g^{-1} in root) (Fig. 5a and b). While, under salinity stress, significant lower K^+ content was found in *CsAKT1* knockout cucumber plants than the EV plants with control (8.7 ± 0.1 *vs.* 3.2 ± 0.2 mg g^{-1} in leaf and 10.7 ± 0.5 *vs.* 4.1 ± 0.1 mg g^{-1} in root), foliar PNC application (17.0 ± 0.1 *vs.* 7.9 ± 0.5 mg g^{-1} in leaf and 30.4 ± 0.5 *vs.* 10.9 ± 0.4 mg g^{-1} in root), or root application of PNC (11.3 ± 0.4 *vs.* 5.0 ± 0.2 mg g^{-1} in leaf and 17.8 ± 0.4 *vs.* 7.6 ± 0.2 mg g^{-1} in root) (Fig. 5c and d). Together, not surprisingly, compared with EV plants under salinity (75 mM NaCl, 7 days), *CsAKT1* knockout cucumber plants showed decreased K^+/Na^+ ratios in leaf (69.9%, 63.2%, and 61.7% for control, leaf and root application of PNC, respectively, Fig. 5e) and root (65.7%, 78.0%, and 65.6% for control, leaf and root application of PNC, respectively, Fig. 5f).

Similarly, we found that *CsAKT1* knockout cucumber plants showed significantly higher H_2O_2 content in leaf (57.9%, 34.0%, and 60.5% for control, leaf and root application of PNC, respectively, Fig. 6a) and root (91.5.9%, 154.5%, and 87.5% for control, leaf and root application of

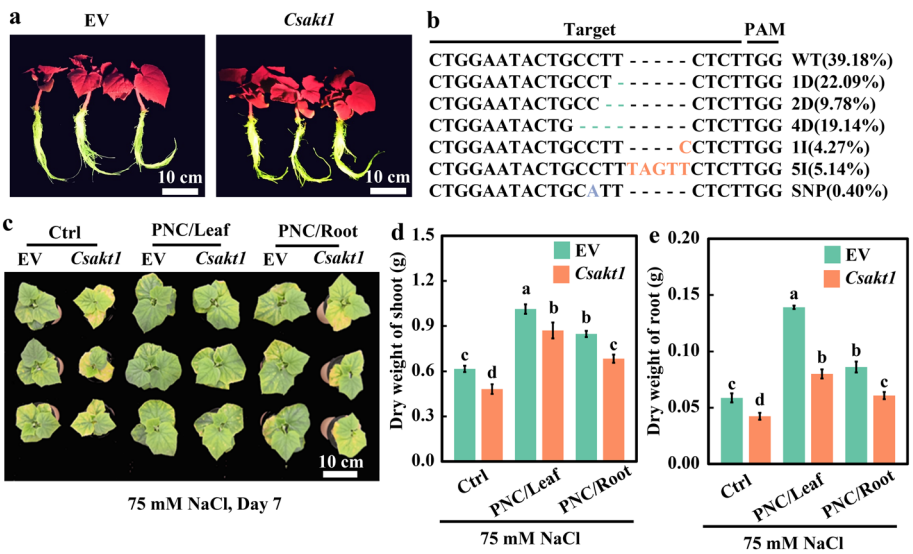

Fig. 4 Creation of *CsAKT1* knockout cucumber plants (CRISPR-Cas 9 lines) and its performance under salinity stress. (a), creation of *CsAKT1* knockout cucumber plants (CRISPR-Cas 9 lines). (b), editing efficiency of *CsAKT1* by Hi-TOM sequencing. Phenotype (c), shoot (d) and root (e) dry weight of *CsAKT1* knockout cucumber seedlings with leaf application and root application of PNC under salt stress. Mean ± SE (n = 3). Different lowercase letters indicate significant differences among different treatments at the $P < 0.05$ level. Ctrl, control cucumber plants under 75 mM NaCl (7 days); PNC/leaf, cucumber plants with leaf PNC application under 75 mM NaCl (7 days); PNC/root, cucumber plants with leaf PNC application under 75 mM NaCl (7 days).

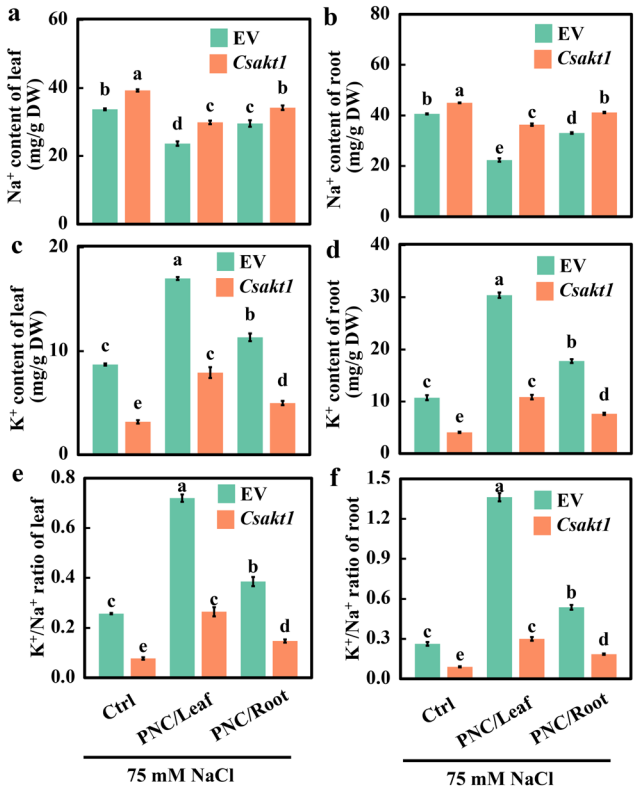

Fig. 5 Ion content and K^+/Na^+ ratio in *CsAKT1* knockout cucumber plants under salinity stress. Leaf (a) and root (b) Na^+ content in *CsAKT1* knockout cucumber seedlings with leaf application and root application of PNC under salt stress. Leaf (c) and root (d) K^+ content in *CsAKT1* knockout cucumber seedlings with leaf application and root application of PNC under salt stress. Leaf (e) and root (f) K^+/Na^+ ratio content in *CsAKT1* knockout cucumber seedlings with leaf application and root application of PNC under salt stress. Mean ± SE (n = 3). Different lowercase letters indicate significant differences among different treatments at the $P < 0.05$ level. Ctrl, control cucumber plants under 75 mM NaCl (7 days); PNC/leaf, cucumber plants with leaf PNC application under 75 mM NaCl (7 days); PNC/root, cucumber plants with leaf PNC application under 75 mM NaCl (7 days).

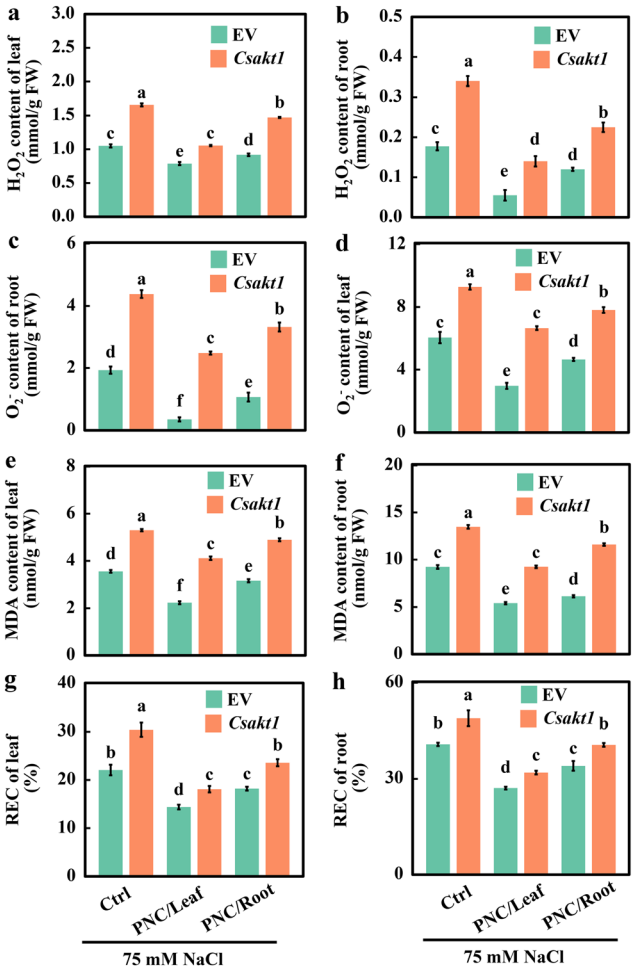

Fig. 6 ROS level, MDA content and REC in *CsAKT1* knockout cucumber plants under salinity stress. Leaf (a) and root (b) H_2O_2 content in *CsAKT1* knockout cucumber seedlings with leaf application and root application of PNC under salt stress. Leaf (c) and root (d) $O_2^{\bullet-}$ content in *CsAKT1* knockout cucumber seedlings with leaf application and root application of PNC under salt stress. Leaf (e) and root (f) MDA content in *CsAKT1* knockout cucumber seedlings with leaf application and root application of PNC under salt stress. Leaf (g) and root (h) REC in *CsAKT1* knockout cucumber seedlings with leaf application and root application of PNC under salt stress. Mean ± SE (n = 3). Different lowercase letters indicate significant differences among different treatments at the $P < 0.05$ level. Ctrl, control cucumber plants under 75 mM NaCl (7 days); PNC/leaf, cucumber plants with leaf PNC application under 75 mM NaCl (7 days); PNC/root, cucumber plants with leaf PNC application under 75 mM NaCl (7 days).

PNC, respectively, Fig. 6b), the $O_2^{\bullet-}$ content in leaf (53.5%, 124.7%, and 67.9% for control, leaf and root application of PNC, respectively) (Fig. 6c) and root (127.1%, 603.5%, and 212.5% for control, leaf and root application of PNC, respectively, Fig. 6d), and MDA content in leaf (48.7%, 83.9%, and 54.3% for control, leaf and root application of PNC, respectively, Fig. 6e) and root (45.9%, 70.8%, and 88.9% for control, leaf and root application of PNC, respectively, Fig. 6f) than the EV plants under salinity (75 mM NaCl, 7 days). This is the same case in the REC results, showing that compared with the EV plants under salinity, *CsAKT1* knockout cucumber plants have significantly increased REC in leaf (37.8%, 25.7%, and 29.5% for control, leaf and root application of PNC, respectively, Fig. 6g) and root (19.8%, 17.9%, and 19.3% for control, leaf and root application of PNC, respectively, Fig. 6h).

4. Discussion

4.1. Leaf but not root nanoceria application resulted in higher improvement in cucumber salt tolerance is associated with a better maintained K^+/Na^+ ratio

As mentioned previously, the K^+/Na^+ ratio is a hallmark of plant salt tolerance.[49,50] Similarly, in nano-improved plant salt tolerance, a better maintained K^+/Na^+ ratio was observed in the nanomaterials treated group than in the control. Cotton plants treated with nanoceria showed a significantly higher K^+/Na^+ ratio than the control under salt stress.[16]

Rapeseed primed with nanoceria also showed a significantly higher shoot and root K^+/Na^+ ratio than the control under salt stress.[12,51,52] This is also found in this work. Cucumber plants with either foliar or root applied nanoceria showed a significantly higher K^+/Na^+ ratio than the control under salt stress (Fig. 2b). Together, it suggests that maintaining the K^+/Na^+ ratio could be a commonly employed mechanism in nano-improved plant salt tolerance.

Many studies have shown that both leaf and root application can improve the salt tolerance of plants such as Arabidopsis,[24] cotton,[16] and rapeseed.[13,25,53] In this study, we found that foliar application of nanoceria resulted in a better improvement of cucumber salt tolerance compared to the root application (Fig. 1c). In our previous study, we found that PNC-induced early stimulation of the antioxidant system (before the onset of salt stress) could be a reason for better salt resistance in cucumber plants with foliar but not root application of PNC.[20] Here, we found that after 7 days of salt treatment, cucumber with foliar PNC application showed a significantly higher K^+/Na^+ ratio than the one with root PNC application (Fig. 2b). It suggests that after salt stress, the ability to maintain the K^+/Na^+ ratio could be a reason for the better salt resistance in cucumber plants with foliar PNC application than the root application. The variations of the K^+/Na^+ ratio were also shown in different organs of nanoparticles treated plants under salt stress. For example, under salt stress, the second true leaf showed a significantly higher K^+/Na^+ ratio than the first true leaf in cotton plants with foliar nanoceria application.[16] Overall, it suggests that the variation of ability to maintain the K^+/Na^+ ratio could be a reason behind the better-improved salt resistance in cucumber plants with foliar PNC application than the root application.

4.2. *CsAKT1* could be a target of nano-improved plant salt tolerance

AKT1, as a class of inwardly rectifying K^+ channels, is widely existing in plants and is important for maintaining K^+ homeostasis.[54,55] It is a typical member of the Shaker family and plays an important role in plant response to salinity stress. Both the N-terminus and C-terminus of Shaker channel proteins are located in the cytoplasm. The N-terminus is a very short domain composed of about 60 amino acids. The C-terminal, at the end of the sixth transmembrane segment, contains a C-junction consisting of about 80 amino acid residues, a cyclic nucleotide-binding domain (CNBD), and an anchor protein domain (Ankyrin-related domain; ANKY) and a KHA domain rich in hydrophobic acid residues.[56] Members of the Shaker family have six transmembrane chains (S1–S6), and the four transmembrane chains S1–S4 are closely related to the ability of potassium ion channels to sense and respond to changes in the membrane potential.[57,58] There is a positively charged arginine or lysine repeat (Arg/Lys–Xaa–Xaa–Arg/Lys) at every two amino acid residues in the S4 transmembrane chain. The presence of this structure enables S4 to move across the membrane, resulting in a conformational change of the membrane channel, which controls the opening and closing of the channel.[59] There is a pore domain between S5 and S6, which consists of a polypeptide fragment embedded in the cell membrane to form a channel. Generally, a single potassium ion channel is a homotetramer structure, consisting of four subunits symmetrically, and can only allow one potassium ion to pass through.[60,61] Shaker-type potassium ion channels can form heterotetramer structures, which is also an important characteristic of the Shaker channel. This structure allows plants to regulate the intracellular potassium transport activity in each organ or tissue relatively independently.[62] In different species, the *AKT1* has the function of the potassium ion transport, *e.g.* tomato,[63] rice,[64] maize,[65] soybean,[54] and Arabidopsis.[66] This further confirms that *AKT1* plays an important role in the absorption of K^+, maintenance of K^+ content in plants, and enhancement of plant salt tolerance.

Previous studies have shown that *GORK*, *AKT1*, and *HAK5* are important potassium ion transporters in response to salt stress. Among them, *GORK* can mediate potassium efflux,[67] while *AKT1* and *HAK5* can mediate active absorption of potassium.[66,68,69] In this study, we found that both leaf and root application of PNC could induce downregulation of *GORK*, which is different from no significant change in *CsGORK* gene expression level in PNC-treated Arabidopsis under salinity stress.[24] Similar results were shown in the PNC-treated rapeseed under salt stress, showing no significant change in the relative expression level of *BnaGORK* between PNC-treated rapeseed and control plants under salt stress, either in the root or in the leaf.[70] While, PNC treatment significantly upregulated *BnaHAK5* in the shoots but not in the root, compared with control plants under salt stress.[70] A similar case was shown in this study. Both leaf and root application of PNC could induce up-regulation of *CsAKT1* and *CsHAK5*, while the absolute expression of *CsAKT1* was significantly higher than that of *CsHAK5*, indicating that *CsAKT1* is the most responsive gene for nanoceria-improved cucumber salt tolerance. Moreover, to date, our knowledge about how nanoparticles induce gene expression in plants under stress is still insufficient. The direct or indirect (*via* ROS or Ca^{2+} signals) modulation effect of nanoparticles on transcription factors might be a reason. This is worthy to be further investigated in future studies.

Domain analysis of *CsAKT1* showed that it has CNBD, anchored repeat domain, and KHA domain, which are consistent with the basic characteristics of Shaker-type channel protein.[62] Unlike the common six transmembrane chains in Shaker-type channel protein,[71,72] the predicted structure analysis showed that *CsAKT1* contains only five transmembrane structures, which may be due to the shorter third transmembrane region. Consistent with the function of *AKT1* reported in other species,[63,65,66] here, we found that *AKT1* is localized in the plasma membrane of cucumber, and executes the transportation of potassium ions. With the

creation of CRISPR/Cas9 lines, we confirmed that *CsAKT1* is a key gene to nanoceria-improved cucumber salt tolerance. Our work suggests that *AKT1* could be a target for nano-improved plant salt tolerance.

4.3. Genome editing is a powerful tool for studying the key genes involved in nano-improved plant stress tolerance

Genome editing is a powerful technique for functional gene analysis. The Cas9-mediated gene editing system is one of the important tools.[48,73,74] Applications of Cas9-mediated gene editing in crops include 1) improving the yield and quality of crops,[75] 2) improving the disease resistance[76] and insect resistance[77] of crops, 3) improving herbicide resistance of crops,[78] 4) improving the abiotic stress resistance of crops,[79] such as drought resistance and salt tolerance.[80] To our surprise, such techniques have not been properly utilized in investigating and verifying the key genes in nano-improved plant salt tolerance.

Previously, we developed a highly efficient root transformation system for Cucurbitaceae that can be used to verify the function of cucumber genes.[37] In this study, in order to verify whether *CsAKT1* is a key gene for nanoceria-improved cucumber salt tolerance, we used Cas9 gene editing technology to obtain cucumber seedlings with root deletion of *CsAKT1*. Our results showed that root deletion of *CsAKT1* significantly reduced the enhancement of nanoceria-improved cucumber salt tolerance (no matter with leaf or root application mode). To the best of our knowledge, this is the first study using the CRISPR/Cas9 technique to verify the key genes in nano-improved plant salt tolerance. This shows that besides making mutants, genome editing such as CRISPR/Cas9 technique could be another powerful tool in verifying the key genes in nano-improved plant stress tolerance. This new approach should be encouraged to be adopted in future studies.

5. Conclusions

This work adds new knowledge about the mechanisms behind nanoceria-improved cucumber salt tolerance. Our results showed that the leaf application of PNC was more effective than the root application of PNC in improving cucumber salt tolerance, which is associated with its better ability to maintain ROS homeostasis and K^+/Na^+ ratio. More interestingly, we found that *CsAKT1* is a key gene for PNC improved salt tolerance in cucumber. Our results not only add more knowledge about the mechanisms behind plant-nanoceria interaction but also provide an approach to investigating the key genes related to nano-improved plant stress tolerance.

Author contributions

Conceptualization, H. W., and B. Z.; writing-original draft preparation, H. W., Y. P., and B. Z.; conducting experiments, Y. P., L. C., L. Z., and L. C.; data analysis, H. W., B. Z., Y. P., and L. Y.; and all authors have read and agreed to the manuscript.

Conflicts of interest

There are no conflicts to declare.

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