

Cerium Oxide Nanoparticles Improve Cotton Salt Tolerance by Enabling Better Ability to Maintain Cytosolic K^+/Na^+ Ratio

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Research

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Abstract

Background

Salinity is a worldwide factor limiting the agricultural production. Cotton is an important cash crop; however, its yield and product quality are negatively affected by salinity. Using nanomaterials such as cerium oxide nanoparticles (nanoceria) to improve plant tolerance to stresses, e.g. salinity, is an emerged approach in agricultural production. Nevertheless, to date, our knowledge about the role of nanoceria in cotton salt response and the behind mechanisms is still rare.

Results

We found that PNC (poly acrylic acid coated nanoceria) helped to improve cotton plant tolerance to salinity, showing the better phenotypic performance, the higher chlorophyll content and biomass, and the better photosynthetic performance in PNC treated cotton plants than the control group. Under salinity stress, in consistent to the results of the enhanced antioxidant enzyme activities, PNC treated cotton plants showed significant lower MDA content and ROS level than the control group, both in the first and second true leaf. Further experiments showed that under salinity stress, PNC treated cotton plants had significant higher cytosolic K^+ and lower cytosolic Na^+ fluorescent intensity in both the first and second true leaf than the control group. This is further confirmed by the leaf ion content analysis, showed that PNC treated cotton plants maintained significant higher leaf K^+ and lower leaf Na^+ content, and thus the higher K^+/Na^+ ratio than the control plants under salinity. Whereas no significant increase of vacuolar Na^+ intensity was observed in PNC treated plants than the control under salinity, suggesting that PNC enhanced leaf K^+ retention and leaf Na^+ exclusion, but not leaf vacuolar Na^+ sequestration are the main mechanisms behind the PNC improved cotton salt tolerance. qPCR results showed that under salinity stress, the modulation of *HKT1* but not *SOS1* refers more to the PNC improved cotton leaf Na^+ exclusion than the control.

Conclusions

Nanoceria enhanced leaf K^+ retention and Na^+ exclusion, but not vacuolar Na^+ sequestration are the main mechanisms behind the nanoceria improved cotton salt tolerance. Our results add more knowledge for better understanding the complexity of plant-nanoceria interaction in terms of nano-enabled plant stress tolerance.

Background

Salinity is an environmental stress limiting agricultural production world-widely and causes billions of dollars' loss annually [1]. Unlike drought and temperature stresses which are either in short period or can be addressed quickly in agriculture practice, salinity is a long-lasting stress in the field. Salinity reduces crop yield, and decreases the quality of agricultural products e.g. seeds, fruits, and fibre [2-4]. Giving the fact that breeding salt tolerant crop species requires long time and the practical approach such as

saturating saline soil with fresh water is not affordable in many areas, especially the semi-arid area, techniques which can help to improve crop salt tolerance in the production period is a feasible option for farmer to choose. Plant nanobiotechnology approach is an emerged technique to tune plant stress response. Many nanomaterials have been reported to improve plant salt tolerance. For example, nanoparticles, i.e. CeO₂, SeNP, TiO₂, and AgNP improved salinity stress tolerance in mature plants such as barley [5], canola [6-8], potato [9], tomato [10,11], broccoli [12] and *Arabidopsis* [13]. Thus, improving crop salt tolerance with plant nanobiotechnology techniques could be an alternative approach to enable the sustainable agriculture.

Cotton (*Gossypium hirsutum* L.) is an important cash and oil crop, and is also one of the most important fibre/textile crops. For example, in marketing year 2019, nearly 20 million bales of cotton which corresponds to ~ \$7 billion were produced in USA (<https://www.ers.usda.gov/topics/crops/cotton-wool/cotton-sector-at-a-glance/>) [14]. Although cotton is regarded as a moderate salt tolerant crop, it still suffers from the salinity stress, especially in the semi-arid area. Salinity not only reduces cotton yield, but also impairs the quality of cotton fibre [15-17]. For example, cotton plants raised at high saline soil showed reduced fibre maturity such as linear density and maturity percent and ratio [18]. Compared with long term process of breeding salt tolerant species, improving cotton salt tolerance in its production period by plant nanobiotechnology approach could be a feasible way. Testing the plant nanobiotechnology approach to improve cotton plant salt tolerance is the first aim to be addressed in this work.

Giving the fact that cotton is an important cash crop across the globe and salinity is a limiting factor affect cotton yield and fibre quality, to our surprise, to date, only one paper tried to investigate the effect of nanoparticles on cotton salt tolerance and was tested with nanoceria seed treatment. An et al. found that nanoceria primed cotton seeds showed higher root length and biomass under salinity and associated it with the modulation of ROS and Ca²⁺ signaling pathways [19]. However, the experiments are conducted under paper roll condition. Besides germination, seedling stage is also critical for cotton plants to survive under stress. Nanoceria (cerium oxide nanoparticles) are potent ROS scavenger and are widely used in industry, medical research and plant research [20-24]. Nanoceria have been reported to improve plant resistance/tolerance to various stress conditions, e.g. salinity [6,7,13], drought [25], light stress (such as highlight and UV [21]), and temperature stress (such as heat and chilling, [21]) etc. Whether nanoceria can help to improve salt tolerance in cotton plants and its associated mechanisms are worthy to be explored.

Under salinity stress, excess Na⁺ entered into the cell and caused massive K⁺ loss from the cell. Thus, K⁺/Na⁺ ratio is a well-known hallmark for plant salt tolerance [26-28]. Also, the ability to maintain K⁺/Na⁺ ratio in plants under salinity is tightly associated with its salt tolerance. However, to date, previous work mainly focused on how nanoceria modulate either K⁺ retention [13] or Na⁺ detoxification [7]. Our previous work showed that nanoceria could improve plant salt tolerance via scavenging of over-accumulated ROS and modulating NSCC and KOR activities to enable better mesophyll K⁺ retention ability [13]. Other

researchers showed that nanoceria improved canola salt tolerance by enhancing plant photosynthesis performance and reducing the root apoplastic barrier to allow more Na^+ being transferred into the shoot [7]. Nonetheless, it should be noticed that possible over-accumulated Na^+ in the shoot also could be an issue to plants since leaf is the main site for plant photosynthesis. Sequestering Na^+ from the cytosol to vacuole and using it for cheap osmoticum is an effective way to avoid the over-accumulation of Na^+ in cell cytosol of plants under salinity stress [29, 30]. Another way to avoid shoot overaccumulation of cytosolic Na^+ is to exclude it to the roots [31, 32]. Investigating the possible contribution of vacuolar Na^+ sequestration and shoot Na^+ exclusion in maintaining cytosolic K^+/Na^+ ratio and thus its role in nanoceria improved cotton salt tolerance is the second aim of this work.

In this work, we investigated the biological responses such as phenotype, chlorophyll content, photosynthetic performance, antioxidant enzyme activities, and leaf Na^+ and K^+ content in the first and second true leaves of cotton plants (two leaf stage). Then, using the confocal microscopy, the distribution of PNC, ROS level, subcellular Na^+ and K^+ level in leaf mesophyll cells in the first and second true leaves of cotton plants were studied. Also, qPCR was performed to investigate the expression level of genes related to Na^+ and K^+ transport.

Materials And Methods

Plant growth

Two leaf stage cotton plants (Xinluzao 74, XLZ 74) were grown in Hoagland solution. Seeds were sown in pots (10×10 cm) filled with standard soil mix (Xingyuxing, Wuhan, China). After cotyledons unfolded, uniformed plants were transplanted into a tray filled with Hoagland solution. Plants were grown in growth room at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation (PAR), $28 \pm 1 \text{ }^\circ\text{C}$ and $25 \pm 1 \text{ }^\circ\text{C}$ at day- and night-time, respectively. Relative humidity was maintained at 70%, and day/night regime was 14 h/10 h. Hoagland solution was refreshed once every five days. After cotton plants reached the two true leaf stage, six uniform plants were selected and transplanted in a tray with 5 L Hoagland solution before treatment. For the salinity stress, NaCl salt was added into the culture solution of the tray to make the salt level to reach 200 mM.

Synthesis and characterization of PNC

The synthesis and characterization of poly (acrylic acid) coated cerium oxide nanoparticles (PNC) were followed the method described in our previous publications [33, 34]. Briefly, 4.5 g poly (acrylic acid) and 1.08 g cerium(III) nitrate were respectively dissolved in 5.0 mL and 2.5 mL deionized water, and the two solutions were mixed thoroughly at 2, 500 rpm for 15 min using a vortex mixer. The mixture was then added dropwise to 15 mL ammonium hydroxide solution (30 %) in a 50 mL beaker and kept stirring at 500 rpm for 24 h at $25 \text{ }^\circ\text{C}$. Then, the solution was centrifuged at 4, 000 rpm for 1 h to remove any debris and large agglomerates. With the centrifugation at 4, 500 rpm for six cycles, 10 K Amicon cells were used to collect the supernatant which is purified from free polymers and other reagents. The final PNC solution

was stored in a refrigerator (4 °C) for two weeks. The absorbance of final PNC solution at 271 nm was measured by the UV-VIS spectrophotometer, and the concentration was calculated using Beer-Lambert's law. All chemicals are from Sigma Aldrich, unless otherwise specified.

The hydrodynamic diameter (DLS size) and zeta potential were determined by 90 Plus PALS (Brookhaven Instruments Corporation, USA). 20 µL of PNC (0.45 mM) was mounted on a holey carbon-coated copper grid, and the PNC TEM imaging was done by a FEI Talos microscope operating at 300 kV.

Dil labeling of PNC

The labelling of PNC with Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) was followed the method described in our previous publications [33, 34]. Briefly, in a 20 mL glass vial, 4 mL, 0.5 mM PNC and 200 µL, 0.3 mg/mL Dil (in DMSO) was mixed at 1000 rpm for 1 min. The resulting mixture was purified using 10 kDa filter (4, 500 rpm for 5 min at least five times) to remove the free chemicals. The final solution was labelled as Dil-PNC and was stored in a refrigerator at 4 °C for further use.

Foliar delivery of PNC and Dil-PNC to cottonplant

Foliar delivery of PNC and Dil-PNC to cotton leaves was followed the method of our previous publication [35] with minor modifications. Briefly, PNC and Dil-PNC formulation were complexed with the surfactant Silwet L-77 (0.05%, Yuanye, Shanghai, China). 0.1 mL, 0.9 mM PNC and Dil-PNC were foliar delivered to each leaf by using a 1000 µL pipette. The 1000 µL pipette tips was cut about 0.3 cm from the top to remove the sharp tip to avoid the possible physical damage during the foliar spraying. The excess solution on the sprayed leaf were removed immediately. After the spraying, cotton leaves were incubated in the room light for 3h to allow the incubation and plant adaption.

Laser confocal microscopy imaging

To quantify ROS *in vivo*, leaf discs (diameter, 5 mm) from the first and second true leaves of the stressed plants (200 mM NaCl, 5 days) were incubated with 25 µM 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA, staining of H₂O₂) or 10 µM dihydroethidium (DHE, staining of [•]O₂⁻) dyes in 1.5 mL tubes for 30 min under darkness. Confocal imaging was performed as described in our previous publication [33] with modifications. After the above-mentioned 0.5 h incubation, the leaf discs were mounted on microscope slides with mounting medium and sealed with a coverslip. Leaf discs were imaged by using Leica SP8 spectral confocal laser scanning microscope. The confocal microscope was manually focused on a region of leaf mesophyll cells. Three individuals (2 leaf discs for each plant) in total were used. The imaging settings were as follows: 488 nm laser excitation; PMT1, 500-600 nm, for DCF and DHE fluorescence; PMT2, 700-785 nm, for chloroplast fluorescence. ROS imaging with DCF and DHE was analysed with Fiji.

The visualization of Dil-PNC in cotton leaves was also performed by using Leica SP8 spectral confocal laser scanning microscope. Briefly, after 3h incubation of the foliar delivered Dil-PNC with the leaves of

the cotton plant, leaf discs (diameter, 5 mm) from the first and second true leaf were made and mounted on the glass slides. PFD (perfluorodecalin) were used to enable better imaging quality. After sealing the slides with a coverslip, the samples are ready for confocal imaging. The imaging settings for DiI-PNC visualization in cotton leaves were as follows: 514 nm laser excitation; PMT1, 550-615 nm, for DiI-PNC fluorescence; PMT2, 700-750 nm, for chloroplast fluorescence. Colocalization between DiI-PNC and chloroplasts was analysed with LAS AF Lite software follow the method described in our previous publications [33]. Three lines of sections were drawn across the ROI (region of interest) with 40 μm interval on the confocal images. The colocalization rate between PNC and chloroplasts was recorded by calculating the proportion of DiI-PNC fluorescence which are overlapped with chloroplast fluorescence emission peaks out of all chloroplast peaks.

Cotton plant performance under salinity stress

The chlorophyll content index (CCI) of the first and second true leaves was daily monitored in salt stressed cotton plant (200 mM NaCl, 5 days). CCI measurements were performed using a chlorophyll meter (SPAD-502 PLUS, Konica Minolta, Japan) with each leaf being measured at three different points (each data point was composed of at least three CCI readout). Plant height was also daily measured for 5 days after the onset of salinity stress. Plant height is defined as the distance between the growth point of the top leaf and the ground [36]. Biomass was determined after 5 days salinity stress. Plants were drying firstly at 105 °C for 30 min, and then at 85 °C for 72 h until reaching the constant weight. The phenotype images of the salt stressed cotton plants with and without PNC were taken by a Nikon D810 camera.

Determination of hydrogen peroxide (H_2O_2), superoxide anion ($\text{O}_2^{\cdot-}$) and malondialdehyde (MDA) content

Measurement of H_2O_2 content in leaf sample was done following a widely accepted method [37] with minor modifications. Approximately 200 mg of fresh samples were placed in liquid nitrogen and then ground with 2 mL of cold acetone. The mixture was then centrifuged (3,000 rpm) for 15 min. 1 mL of $\text{Ti}(\text{SO}_4)_2$ (5%/W/V in concentrated HCl) was added to the supernatant. After shaking, the samples were then centrifuged (3,000 rpm) and the precipitates were solubilized in 1 mL H_2SO_4 . The absorbance of the final solutions was measured at 415 nm vis UV-Vis spectrophotometer.

Estimation of superoxide anion radicals ($\text{O}_2^{\cdot-}$) was done following a widely accepted method [38] with minor modifications. NH_2OH was used as a probe for $\text{O}_2^{\cdot-}$, being oxidized to NO_2^- . NO_2^- can react with α -naphthylamine and sulfamic acid to turn the mixture to red colour. Then, the absorbance of the mixture can be measured at 530 nm by UV-Vis spectrophotometer. The method described in previous publication [39] was followed for the assessment of MDA content. Briefly, 200 mg of samples were homogenized with 5 mL TCA having 0.25% 2-thiobarbituric acid (TBA). After incubating at 90 °C for 30 min, the mixture was immediately cooled down, and then was centrifugate at 8000 \times g for 15 min. The absorbance of the solution was measured at 450 nm, 532 nm and 600 nm.

Measurement of antioxidant enzymes activities

To determine the activity of SOD, CAT and POD, the first and second leaves of the salt stressed cotton plants (200 mM NaCl, 5 days) were separately collected. For the determination of SOD activity, the freshly collected leaf samples were ground with PBS buffer (pH 7.8). The supernatant was collected following the centrifugation at 12,000 g for 20 minutes. The supernatant was mixed with EDTA-Riboflavin, NBT and methionine. The mixture was incubated under $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ lux light for 20 min. The absorbance of the final mixture was measured at 560 nm by a UV-Vis spectrophotometer (UV 1800PC, AOE, Shanghai, China). SOD enzyme activity was calculated by using the measured absorbance value following the equation 1:

$$\text{SOD activity} = (A_b - A_s) \times V_T / (A_b \times 0.5 \times W_s \times V_s).$$

Where A_b and A_s are the blank control and samples' absorbance value, respectively. V_T is the volume of crude leaf extraction mixture. W_s is fresh weight and V_s is the used volume during the sample measurement.

For the determination of POD activity, the collected samples were ground with PBS buffer (pH 5.5). The supernatant was collected following the centrifugation at 3000 rpm for 10 minutes. For POD activity measurement, the solution was prepared with mixing the supernatant of the crude leaf extraction mixture, 20 % TCA, 50mM guaiacol solution, PBS buffer (pH 5.5, 50mM), and 2 % H_2O_2 . The POD activity was measured by calculating the averaged decrease of the recorded absorbance value at 470 nm (1 record/1 min, 3 min) by a UV-Vis spectrophotometer (UV 1800PC, AOE, Shanghai, China). The calculated average decrease value was used to calculate the POD enzyme activity following the equation 2:

$$\text{POD activity} = A_{470} \times V_T / (W \times V_s \times t \times 0.01).$$

Where A_{470} is the averaged decrease of the recorded absorbance value at 470 nm. V_T is the volume of the crude leaf extraction mixture. W is fresh weight. V_s is the used volume during the sample measurement. t is the reaction time and 0.01 was defined as the unit enzyme activity.

For CAT activity measurement, the collected samples were ground with PBS buffer (pH 7.8). The supernatant was collected following the centrifugation at 4,000 rpm for 15 minutes. The supernatant of the crude leaf extraction mixture was vortexed with PBS buffer (pH 7.8) and 10 mM H_2O_2 . The CAT enzyme activity was calculated based on the averaged decrease of the recorded absorbance value at 240 nm (1 record/1 min, 4 min) by a UV-Vis spectrophotometer (UV 1800PC, AOE, Shanghai, China). Then, the measured absorbance value was used to calculate the CAT enzyme activity following the equation 3:

$$\text{CAT activity} = A_{240} \times V_T / (W \times V_s \times t \times 0.1).$$

Where A_{240} is the averaged decrease of the recorded absorbance value at 240 nm. V_T is the volume of the crude leaf extraction mixture. W is fresh weight. V_s is the used volume during the sample measurement. t is reaction time and 0.1 was defined as the unit enzyme activity.

Measurement of chlorophyll content and photosynthetic parameters

Leaf samples (the separated first and second leaves) was mixed with a solution containing acetone and ethanol (1:1) for 24 h at dark condition on a shaker (50 rpm). Following centrifugation at 2000 rpm, 10 min, the supernatant was collected. By using a spectrophotometer, the absorbance of the supernatant was measured at 644 nm and 662 nm for the determination of the content of chlorophyll a and chlorophyll b. The chlorophyll a and b content were calculated using the following equations:

$$\text{Equation 4: Chlorophyll a content} = 9.784 \times A_{662} - 0.99 \times A_{644}.$$

$$\text{Equation 5: Chlorophyll b content} = 21.426 \times A_{644} - 4.65 \times A_{662}.$$

Where A_{662} and A_{644} are the absorbance value measured at 662 nm and 644 nm, respectively.

Photosynthesis rate, intercellular CO₂ concentration, stomatal conductivity and transpiration rate of the first and second leaves were measured by using a portable photosynthetic apparatus Li-6400 XT at D0 (200 mM NaCl, day 0) and D5 (200 mM NaCl, day 5). The measurement settings were set as: 1500 μmol m⁻² s⁻¹ photosynthetic photon flux density, 400 μmol mol⁻¹ CO₂ concentration, and 25 °C leaf temperature.

Estimation of K⁺ and Na⁺ content

For the estimation of leaf K⁺ and Na⁺ content, the first and second leaf samples were milled with a grinder and filtered through a 0.5 mm sieve to collect the grounded samples. 0.2 g of grounded samples were digested for 1.5 h in concentrated H₂SO₄ (18.4 M). After cooling down, 30% H₂O₂ was added into the digested samples to get the transparent mixture solution. The mixture was digested for another 1 h to make sure the H₂O₂ decompose completely. Flame photometer (FP6431, Jiangke, Shanghai, China) was used to determine the content of K⁺ and Na⁺ in the samples. The setup of standard curve can be found in the literatures elsewhere.

qPCR

Total RNA was isolated using the RNeasy Pure Plant Kit (DP441, Tiangen, Beijing, China). 2 μg of total RNA was reverse transcribed into cDNA using the TRUEScript first Strand cDNA Synthesis Kit (PC5402, Aidlab, Beijing, China). The amplification of qRT-PCR products was performed in a reaction mixture of 12.5 μL SYBR Green qPCR Mix (PC3302, Aidlab, Beijing, China) according to the manufacturer's instructions. The qRT-PCR analysis was performed on the Bio-Rad CFX Connect Real-Time PCR System (Bio-Rad, California, USA). Three biological replicates and three technical replicates was used for each investigated gene. The relative gene expression was calculated using the 2^{-ΔΔCt} method. The primers used for qRT-PCR are shown in the Table S1 [40-42].

Statistical analysis

All data were represented as mean \pm SE and analysed using SPSS 23.0. Comparisons were performed by either one-way ANOVA based on Duncan's multiple range test (two tailed) or independent samples t-test (two tailed). * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$. Different lowercase letters mean the significance at $P < 0.05$.

Results

PNC characterization and its distribution in cotton leaves

Hydrodynamic diameter measurements showed the size of PNC is 8.04 ± 1.87 nm (Figure 1a). TEM imaging results showed that the core of the synthesized PNC is spherical with an average diameter of 6.05 ± 0.49 nm (Figure 1b). The zeta potential measurement showed that the surface charge of the synthesized PNC is -15.30 ± 0.11 mV (Figure 1c). The synthesized PNC have a peak absorbance at 271 nm (Figure S1a). Besides the peak absorbance at 271 nm, DiI-PNC have another two peaks at 557 and 517 nm, indicating the successful conjugation of DiI to PNC (Figure S1a). The red colour of DiI-PNC further confirmed the successful synthesis of DiI-PNC (Figure S1b). Confocal imaging results showed that the colocalization rate between DiI-PNC and chloroplasts is 53.34 ± 1.90 % and 52.32 ± 1.51 % in the first and second cotton leaf, respectively (Figure 1d,e). No DiI fluorescence signal was detected in cotton leaves treated with Silwet-L77 (Figure S2).

PNC improved the performance of cotton under salinity stress

Compared to cotton plants without PNC treatment, under 200 mM NaCl, the plants with foliar delivered PNC showed obvious better phenotypic performance (Figure 2a, Figure S3a-c) and significant higher biomass such as leaf fresh weight (Figure 2b), dry weight (Figure S3d) and whole plant weight (Figure S3e), either in the first or the second true leaf. Under 200 mM NaCl, compared with the 0.60 ± 0.09 g/plant and 0.60 ± 0.01 g/plant in the control group, the fresh weight of the first and the second true leaf in PNC treated plants is 0.83 ± 0.07 and 0.72 ± 0.04 g/plant, respectively. Also, under 200 mM NaCl, PNC treated cotton plants showed significant higher chlorophyll content (indicated by CCI, chlorophyll content index) in both the first and the second true leaf than the surfactant Silwet L-77 treated plants (Figure 2c,d). The results of chlorophyll a (0.60 ± 0.03 and 0.91 ± 0.04 mg/g in the first true leaf of plants with and without PNC, 0.70 ± 0.03 and 1.17 ± 0.05 mg/g in the second true leaf of plants with and without PNC) and chlorophyll b (0.21 ± 0.01 and 0.32 ± 0.02 mg/g in the first true leaf of plants with and without PNC, 0.25 ± 0.01 and 0.42 ± 0.02 mg/g in the second true leaf of plants with and without PNC) content (Figure S4a,b) are also consistent with the CCI results. No significant difference of the phenotypic performance and chlorophyll content (indicated by chlorophyll content index) were observed on cotton plants treated with and without PNC under no saline conditions (Figure S5a-c).

PNC improved the photosynthesis performance of cotton under salinity stress

After five days salinity stress (D5, 200 mM NaCl), PNC treated cotton plants showed significant higher carbon assimilation rate in the first true leaf (4.10 ± 0.69 vs 8.84 ± 0.82 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and the second

true leaf (3.62 ± 0.48 vs $8.83 \pm 0.32 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) then the control group (Figure 3a). Similarly, compared with the control group under 200 mM NaCl, 5 days, PNC treated cotton plants showed significant higher stomatal conductance in the first true leaf (0.16 ± 0.05 vs $0.40 \pm 0.01 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and the second true leaf (0.13 ± 0.01 vs $0.46 \pm 0.06 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) (Figure 3b). Figure 3c showed that after five days salt stress, the PNC treated plants have significant higher intercellular CO_2 than the control group, in both the first (255.0 ± 27.68 vs $358.3 \pm 19.20 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and the second (259.67 ± 27.97 vs $340.67 \pm 40.56 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) true leaf. The similar results were also found in the transpiration rate, showing the transpiration rate of the PNC treated plants is significantly higher than the control group, in both the first (0.63 ± 0.43 vs $0.17 \pm 0.07 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and the second (0.36 ± 0.02 vs $0.77 \pm 0.10 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) true leaf (Figure 3d). At day 0 (D0) of salinity stress, no significant difference of carbon assimilation rate, stomatal conductance, intercellular CO_2 and transpiration rate was found between PNC treated cotton plants and the control group, either in the first or the second true leaf (Figure 3a-d).

PNC improved the ROS scavenging ability of cotton under salinity stress

After 5 days' salt stress (200 mM NaCl), compared with the control group, PNC treated cotton plants showed significant lower content of malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and superoxide anion ($\text{}^{\cdot}\text{O}_2^-$) in either the first (3.49 vs $2.32 \mu\text{mol/g}$ for MDA, 20.30 ± 0.12 vs $4.34 \pm 0.14 \mu\text{mol/g}$ for H_2O_2 , 2.60 ± 0.07 vs $1.34 \pm 0.04 \mu\text{mol/g}$ for $\text{}^{\cdot}\text{O}_2^-$) or the second (2.85 ± 0.17 vs $1.61 \pm 0.14 \mu\text{mol/g}$ for MDA, 19.18 ± 0.17 vs $6.47 \pm 0.23 \mu\text{mol/g}$ for H_2O_2 , 1.93 ± 0.01 vs $1.53 \pm 0.02 \mu\text{mol/g}$ for $\text{}^{\cdot}\text{O}_2^-$) true leaf (Figure 4a-c). Confocal imaging results further confirmed the results. Using DCFDA (indicating H_2O_2) and DHE (indicating $\text{}^{\cdot}\text{O}_2^-$) fluorescent dye, Figure 5 showed that compared with the control group, PNC helped to scavenge more ROS in both the first and the second true leaf of salt stressed cotton plants. It shows that PNC treated cotton plants have significant lower DCF and DHE fluorescent dye intensity in either the first (10.61 ± 1.05 vs 3.11 ± 0.68 and 2.12 ± 0.30 vs 0.67 ± 0.38 for H_2O_2 and $\text{}^{\cdot}\text{O}_2^-$, respectively) or the second (6.82 ± 0.69 vs 2.77 ± 0.59 and 1.86 ± 0.15 vs 0.96 ± 0.05 for H_2O_2 and $\text{}^{\cdot}\text{O}_2^-$, respectively) true leaf than the control group after the treatment of 200 mM NaCl, 5 days (Figure 5a-c). In contrast to leaf MDA and ROS content, after 5 days' salt stress (200 mM NaCl), the activities of SOD (superoxide dismutase), CAT (catalase) and POD (peroxidase) in the first (227.38 ± 9.89 vs $343.81 \pm 16.95 \text{ U/g}$ for SOD, 6.61 ± 0.69 vs $13.18 \pm 0.81 \text{ U/g}$ for CAT, 55.26 ± 3.31 vs $102.71 \pm 2.26 \text{ U/g}$ for POD) and the second (252.34 ± 7.08 vs $386.16 \pm 3.15 \text{ U/g}$ for SOD, 8.48 ± 0.91 vs $20.42 \pm 2.52 \text{ U/g}$ for CAT, 81.04 ± 3.04 vs $105.36 \pm 2.38 \text{ U/g}$ for POD) true leaf are significantly higher in PNC treated cotton plants than the control group (Figure S6a-c).

PNC helped to maintain K^+/Na^+ ratio in cotton under salinity stress

After 200 mM NaCl treatment for five days, PNC treated cotton plants showed significant higher APG-2 (K^+ fluorescent dye) intensity in the cytosol (17.98 ± 0.77 vs 111.11 ± 8.92 and 24.61 ± 1.10 vs $58.57 \pm$

2.41 for the first and the second true leaf, respectively) and the vacuole (16.20 ± 0.53 vs 88.20 ± 0.95 and 17.50 ± 0.78 vs 52.66 ± 1.65 for the first and the second true leaf, respectively) of the mesophyll cells than the control group (Figure 6a,b, 6e,f). This is in accordance with the results of total leaf K^+ content, showing that under salinity stress (200 mM NaCl, five days), PNC treated cotton plants have significant higher leaf K^+ content in both the first (28.22 ± 1.18 vs 51.99 ± 1.19 mg/g) and the second (33.14 ± 0.87 vs 60.85 ± 4.58 mg/g) true leaf than the control group (Figure 7a). Whereas, after five days' salt stress, CoroNa Green (Na^+ fluorescent dye) intensity in the cytosol ($33.89 \pm$ vs 7.93 ± 1.10 and 108.45 ± 2.48 vs 35.54 ± 2.11 for the first and the second true leaf, respectively) and the vacuole (13.24 ± 1.28 vs 4.62 ± 0.85 and 17.43 ± 0.73 vs 14.23 ± 1.32 for the first true leaf) of the mesophyll cells is lower in the PNC treated cotton plants than the control group (Figure 6c,d, 6g,h). This is in consistent with the results of total leaf Na^+ content, showing that under salinity stress (200 mM NaCl, five days), leaf Na^+ content in both the first (14.95 ± 0.91 vs 5.71 ± 0.22 mg/g) and the second (11.70 ± 0.42 vs 4.38 ± 0.32 mg/g) true leaf is significant lower in PNC treated cotton plants than the control group (Figure 7b). No significant difference of the vacuolar CoroNa Green intensity was observed in the second true leaf between the PNC treated cotton plants and the control group under salinity stress (200 mM NaCl, five days) (Figure 6h). Moreover, under salinity stress (200 mM NaCl, five days), PNC treated cotton plants have significant higher leaf K^+/Na^+ ratio in both the first (1.91 ± 0.19 vs 9.16 ± 0.57) and the second (2.84 ± 0.17 vs 14.13 ± 1.79) true leaf than the control group (Figure 7c).

PNC modulated the relative expression of genes related to Na^+ and K^+ transport

Under salinity stress (200 mM NaCl), no significant difference of the relative gene expression level of *SOS1* (salt overly sensitive 1, Na^+/H^+ antiporter for Na^+ exclusion) and *NHX1* (Na^+/H^+ exchanger for vacuolar Na^+ sequestration) was observed in cotton plant treated with and without PNC, in both the first and second true leaf (Figure 7d). Whereas, PNC treated cotton plants showed significantly upregulated relative expression level of *HKT1* (high affinity K^+ transporter for Na^+ exclusion) than the control plants under salinity, either in the first or the second true leaf (Figure 7d). In contrast to the upregulated *HKT1*, the relative expression level of *KOR* (K^+ outward rectifying channel for K^+ efflux) was significantly downregulated in PNC treated cotton plants compared with the control plants under salinity, either in the first or the second true leaf (Figure 7d).

Discussion

Nanoceria improved ROS scavenging ability confers the enhanced cotton salt resistance

ROS plays dual role in plants. ROS accumulation is known as a secondary stress in plants under salinity. Over-accumulated ROS could damage protein, DNA, membrane, and other big molecules in plants [43]. ROSs in salt stressed plants mainly refer to hydrogen peroxide (H_2O_2), superoxide anion ($\cdot O_2^-$), hydroxyl radicals ($OH\cdot$) and singlet oxygen (1O_2) [44]. The latter two cannot be scavenged by any known antioxidant enzymes. Among these ROSs, hydroxyl radicals are the most destructive one [45].

Maintaining ROS homeostasis is important for plant salt stress tolerance. Plants evolved enzymatic and nonenzymatic antioxidant system to maintain ROS homeostasis [46, 47]. However, once the production over the scavenging, ROSs start over accumulation which in turn impose negative effect on plant performance under stress. Thus, nanoparticles or nanomaterials with ROS scavenging ability, especially for hydroxyl radicals, may have the potential to maintain ROS homeostasis in plants under stress and thus to improve plant resistance to stress.

Here, we found that besides significant lower leaf MDA content, PNC treated plants showed increased antioxidant enzyme activities and decreased ROS content than the control under salinity stress (Figure 4, 5 and S6). These results are in accordance with previous studies [6, 7, 13, 21], showing that through scavenging of ROS, nanoceria are able to help plants to resist stress conditions, e.g. salinity. Our results further reinforced the conception of nano-enable agriculture, demonstrating the potential use of nanoceria to enhance salinity stress tolerance in cotton. This plant nanobiotechnology approach provides an alternative option of addressing current salinity issue in field and exploring the semi-arid area for cropping to the policy makers and farmers, especially cotton growers.

Better maintained leaf K^+ / Na^+ ratio is important for PNC improved cotton salt tolerance

Plant's ability to maintain both cytosolic K^+ and Na^+ homeostasis is a trait indicating its salinity stress tolerance [27]. Massive over-accumulation of Na^+ in the cytosol not only leads to Na^+ toxicity, but also causes K^+ efflux from the cytosol to the apoplast. K^+ is a co-enzyme which is required for activation of more than 50 enzymes [48]. It also plays important roles in cytosolic pH homeostasis, protein synthesis and cell activities e.g. stomatal opening and closure [49-51]. Under normal condition, the optimal K^+ concentration in plant cell cytosol is about 100 mM [52, 53]. Plants can loss more than 50% K^+ under salinity stress. Our previous work showed that nanoceria modulated the activity of ROS-activated NSCC channels to enable better mesophyll K^+ retention and thus plant salt tolerance [13].

To date, the reported mechanisms for nanomaterials improved plant salt tolerance includes 1) maintaining ROS homeostasis by scavenging over-accumulated ROS or stimulating the activities of antioxidant enzymes [54], 2) shortening the root apoplast barrier to allow more Na^+ been translocated from root to shoot [55, 56], 3) modulating the activities of ion channels (nonselective cation channels, NSCC and K^+ outward rectifying channels, KOR) to enable better mesophyll K^+ retention [13], 4) promoting the production of gas signaling molecules [45, 57]. As mentioned above, leaf cytosolic K^+ / Na^+ ratio is a hallmark for plant salt tolerance [27]. However, the ability of maintaining cytosolic K^+ / Na^+ ratio in cerium oxide nanoparticles improved plant salt tolerance, especially the cash crop cotton, is less addressed. To maintain leaf cytosolic K^+ / Na^+ ratio, the ability to coordinate the level of K^+ and Na^+ in cytosol is important. Here, we found that PNC treated plants showed better ability to maintain cytosolic K^+ and total leaf K^+ than the control plants under salinity, either in the first or second true leaf (Figure 6a-c, Figure 7a). It worth to be noted that *KOR* expression is significantly downregulated in PNC treated plants than the control under salinity stress (Figure 7d), further confirming that PNC helped plants to maintain

higher mesophyll K^+ retention ability. This is in accordance with our previous study [13]. Also, the cytosolic Na^+ level and total leaf Na^+ in both the first and second true leaves of PNC treated plants is significantly lower than in control plants under salinity stress (Figure 6d-f and 7b). These results confirmed that PNC help cotton plants to maintain better leaf cytosolic K^+/Na^+ homeostasis and thus better performance under salt stress. Overall, this work suggests that the ability to maintain mesophyll cytosolic K^+/Na^+ ratio is a component of the mechanisms behind PNC enabled better plant salt tolerance. It adds more knowledge to our understanding of the mechanisms behind nanoceria-plant interactions regarding nano-enabled plant stress tolerance.

PNC enabled lower mesophyll cytosolic Na^+ is not associated with vacuolar Na^+ sequestration

Plants have different measures to modulate cytosolic Na^+ level, either by sequestration more Na^+ into the vacuole [59] or excluding more Na^+ into the roots [32]. Here, our results showed that no increase, even decrease in the first true leaf, of vacuolar Na^+ level in PNC treated plants than the control under salinity stress (Figure 6d-f). It suggests that at least in cotton, PNC enabled lower cytosolic Na^+ is likely not enabled by possible high efficiency of vacuolar Na^+ sequestration which is not observed in this study. Also, qPCR results showed that no difference of *NHX1* expression was found between PNC treated cotton plants and the control under salinity (Figure 7d). In contrast to the *NHX1*, an upregulation of *HKT1* was found in PNC treated cotton plants than the control under salinity, suggesting that PNC enabled better leaf Na^+ exclusion ability. This is accordance with the results showing significant lower leaf Na^+ in PNC treated cotton plants than the control under salt stress (Figure 7b). Overall, our results showed that in cotton plants, PNC enhanced leaf Na^+ exclusion but not the ability of vacuolar Na^+ sequestration. This is different with the mechanisms of PNC enabled salt tolerance in canola plants, by which shortening the apoplastic barrier to allow more Na^+ to be transported into the shoot [7]. It suggests the complexity of the mechanisms behind PNC improved plant salt tolerance. It further calls the research attention about the different employed mechanisms of nanoparticle-plant interactions in different plant species.

The different biological responses of leaves to PNC: more to be addressed

One largely overlooked in nanoparticle-plant interactions is the possible different biological responses of leaves to the nanoparticles. In this work, we investigated the biological responses to PNC of both the first and second true leaves of cotton plants under salinity stress. Our results showed that the biological responses of the first and second true leaves to PNC are different on some the measured parameters. For example, under salinity stress, the second true leaves have higher SOD and CAT activities than the first true leaves treated with PNC (Figure S6a,b). Also, PNC treated 2nd true leaf showed significant higher leaf K^+/Na^+ ratio than the 1st true leaf under salinity stress (Figure 7c). Compared with the first true leaf, the amount of reduced cytosolic Na^+ in leaves with and without PNC is 2.8 times higher in the second true leaf under salt stress (Figure 6g,h). Overall, it suggests that PNC may be able to protect more the younger tissues, for example the second true leaf in this study. These different biological responses between PNC

treated 1st and 2nd true leaves under salt stress calls for more attention on the need to study the nanoparticle-plant interactions at the level of tissue or organ level.

Conclusions

Our previous study tried nanoceria priming on cotton seeds and showed the increased root length and biomass but not the germination rate in cotton under salinity stress [19]. However, the experiments are conducted under paper roll condition and thus could not allow the build-up of normal cotton seedling plants. Also, in some semi-arid area, during the practice, good irrigation was operated at seed sowing but not last for seedling stage. Besides germination, seedling stage is also critical for cotton plants to survive under stress. The present work adds new knowledge about mechanisms behind nanoceria improved cotton salt tolerance at seedling stage. In the present study, our results showed that compared with the control group, PNC could scavenge ROS and enhance antioxidant enzyme activity in the first and second true leaf to maintain better ROS homeostasis in cotton plants under salinity stress. Besides maintaining ROS homeostasis, PNC helped salt stressed cotton plants to retain leaf K⁺ and to exclude over-accumulated leaf Na⁺, thus allowing the higher leaf K⁺/Na⁺ ratio in PNC treated plants than the control plants under salinity stress. Combining the results from confocal imaging and qPCR experiments, our results suggest that not leaf Na⁺ sequestration, but the PNC enhanced leaf K⁺ retention and leaf Na⁺ exclusion are the main mechanisms behind the PNC improved cotton salt tolerance. It adds more knowledge about the mechanisms behind plant-nanoceria interaction.

Declarations

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Author contributions

HW and ZL planned and designed the research. JL and HW performed the physiology experiments of PNC improve cotton salt tolerance. JL, GL and HW performed the confocal imaging experiments. LC performed the qPCR experiments. JG and JL performed the TEM imaging experiments. HW, ZL and JL wrote the manuscript. All authors read the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interests.

References

1. Panta S, Flowers T, Lane P, Doyle R, Haros G, Shabala S. Halophyte agriculture: Success stories. *Environ Exp Bot.* 2014; 107: 71-83.
2. Abdul R, Arfan A, Bin Safdar L, Mubashar Zafar M, Yang R, Amir S, Abbad S, Muhammad A, Wankui G, Youlu Y. Salt stress induces physiochemical alterations in rice grain composition and quality. *J Food Sci.* 2020; 85: 14-20.
3. Lima REM, Farias LFdL, Ferreira JFS, Suarez DL, Bezerra MA. Translocation of photoassimilates in melon vines and fruits under salinity using C-13 isotope. *Sci Hortic.* 2020; 274: 109659.
4. Wang D, Lu X, Chen X, Wang S, Wang J, Guo L, Yin Z, Chen Q, Ye W. Temporal salt stress-induced transcriptome alterations and regulatory mechanisms revealed by PacBio long-reads RNA sequencing in *Gossypium hirsutum*. *BMC Genom.* 2020; 21: 838.
5. Karami A, Sepehri A. Beneficial role of MWCNTs and SNP on growth, physiological and photosynthesis performance of barley under NaCl stress. *J. Soil Sci Plant Nutr.* 2018; 18: 752-771.
6. Rossi L, Zhang WL, Lombardini L, Ma XM. The impact of cerium oxide nanoparticles on the salt stress responses of *Brassica napus* *Environ Pollut.* 2016; 219: 28-36.
7. Rossi L, Zhang WL, Ma XM. Cerium oxide nanoparticles alter the salt stress tolerance of *Brassica napus* by modifying the formation of root apoplastic barriers. *Environ Pollut.* 2017; 229: 132-138.
8. Zhao L, Lu L, Wang A, Zhang H, Huang M, Wu H, Xing B, Wang Z, Ji R. Nano-biotechnology in agriculture: use of nanomaterials to promote plant growth and stress tolerance. *J Agric Food Chem.* 2020; 68: 1935-1947.
9. Mahmoud AWM, Abdeldaym EA, Abdelaziz SM, El-Sawy MBI, Mottaleb SA. Synergetic effects of zinc, boron, silicon, and zeolite nanoparticles on confer tolerance in potato plants subjected to salinity. *Agronomy-Basel.* 2020; 10: 19.
10. Almutairi ZM. Influence of silver nano-particles on the salt resistance of tomato (*Solanum lycopersicum*) during germination. *Int J Agric Biol.* 2016; 18: 449-457.
11. Almutairi ZM. Effect of nano-silicon application on the expression of salt tolerance genes in germinating tomato (*Solanum lycopersicum*) seedlings under salt stress. *Plant Omics.* 2016; 9: 106-114.
12. Martinez-Ballesta MC, Zapata L, Chalbi N, Carvajal M. Multiwalled carbon nanotubes enter broccoli cells enhancing growth and water uptake of plants exposed to salinity. *J Nanobiotechnology.* 2016; 14: 42.

13. Wu H, Shabala L, Shabala S, Giraldo JP. Hydroxyl radical scavenging by cerium oxide nanoparticles improves Arabidopsis salinity tolerance by enhancing leaf mesophyll potassium retention. *Environ Sci Nano*. 2018; 5: 1567-1583.
14. United States Department of Agriculture ERS. Cotton Sector at a Glance. 2020.
15. Li XW, Jin MG, Zhou NQ, Huang JO, Jiang SM, Telesphore H. Evaluation of evapotranspiration and deep percolation under mulched drip irrigation in an oasis of Tarim basin, China. *J Hydrol*. 2016; 538: 677-688.
16. Zhang DM, Li WJ, Xin CS, Tang W, Eneji AE, Dong HZ. Lint yield and nitrogen use efficiency of field-grown cotton vary with soil salinity and nitrogen application rate. *Field Crops Res*. 2012; 138: 63-70.
17. Peng J, Zhang L, Liu JR, Luo JY, Zhao XH, Dong HL, Ma Y, Sui N, Zhou ZG, Meng YL. Effects of soil salinity on sucrose metabolism in cotton fiber. *PLoS ONE*. 2016; 11: e0156398.
18. Razzouk S, Whittington WJ. Effects of salinity on cotton yield and quality. *Field Crops Res*. 1991; 26: 305-314.
19. An J, Hu P, Li F, Wu H, Shen Y, White JC, Tian X, Li Z, Giraldo JP. Emerging investigator series. molecular mechanisms of plant salinity stress tolerance improvement by seed priming with cerium oxide nanoparticles. *Environ Sci Nano*. 2020; 7: 2214-2228.
20. Wu XW, Zhang Y, Lu YC, Pang S, Yang K, Tian ZM, Pei YX, Qu YQ, Wang F, Pei ZC. Synergistic and targeted drug delivery based on nano-CeO₂ capped with galactose functionalized pillar [5]arene *via* host-guest interactions. *J Mater Chem B*. 2017; 5: 3483-3487.
21. Wu HH, Tito N, Giraldo JP. Anionic cerium oxide nanoparticles protect plant photosynthesis from abiotic stress by scavenging reactive oxygen species. *ACS Nano*. 2017; 11: 11283-11297.
22. Mitra RN, Gao RJ, Zheng M, Wu MJ, Voinov MA, Smirnov AI, Smirnova TI, Wang K, Chavala S, Han ZC. Glycol chitosan engineered autoregenerative antioxidant significantly attenuates pathological damages in models of age-related macular degeneration. *ACS Nano*. 2017; 11: 4669-4685.
23. Asati A, Santra S, Kaittanis C, Nath S, Perez JM. Oxidase-like activity of polymer-coated cerium oxide nanoparticles. *Angew Chem Int Ed Engl*. 2009; 48: 2308-2312.
24. Asati A, Santra S, Kaittanis C, Perez JM. Surface-charge-dependent cell localization and cytotoxicity of cerium oxide nanoparticles. *ACS Nano*. 2010; 4: 5321-5331.
25. Djanaguiraman M, Nair R, Giraldo JP, Prasad PVV. Cerium oxide nanoparticles decrease drought-induced oxidative damage in sorghum leading to higher photosynthesis and grain yield. *ACS Omega*. 2018; 3: 14406-14416.
26. Taha R, Mills D, Heimer Y, Tal M. The relation between low K⁺/Na⁺ ratio and salt-tolerance in the wild tomato species *Lycopersicon pennellii*. *J Plant Physiol*. 2000; 157: 59-64.
27. Almeida DM, Oliveira MM, Saibo NJM. Regulation of Na⁺ and K⁺ homeostasis in plants. towards improved salt stress tolerance in crop plants. *Genet Mol Biol*. 2017; 40: 326-345.
28. Assaha DVM, Ueda A, Saneoka H, Al-Yahyai R, Yaish MW. The role of Na⁺ and K⁺ transporters in salt stress adaptation in glycophytes. *Front Physiol*. 2017; 8: 19.

29. Wu HH, Shabala L, Zhou MX, Su NN, Wu Q, Ul-Haq T, Zhu J, Mancuso S, Azzarello E, Shabala S. Root vacuolar Na⁺ sequestration but not exclusion from uptake correlates with barley salt tolerance. *Plant J.* 2019; 100: 55-67.
30. Maathuis FJM. Sodium in plants: perception, signaling, and regulation of sodium fluxes. *J Exp Bot.* 2014; 65: 849-858.
31. Wu HH. Plant salt tolerance and Na⁺ sensing and transport. *Crop J.* 2018; 6: 215-225.
32. Munns R, Tester M. Mechanisms of salinity tolerance. *Annu Rev Plant Biol.* 2008; 59: 651-681.
33. Newkirk GM, Wu H, Santana I, Giraldo JP. Catalytic scavenging of plant reactive oxygen species in vivo by anionic cerium oxide nanoparticles. *J Vis Exp.* 2018; 138: e58373.
34. Hu P, An J, Faulkner MM, Wu H, Li Z, Tian X, Giraldo JP. Nanoparticle charge and size control foliar delivery efficiency to plant cells and organelles. *ACS Nano.* 2020; 14: 7970-7986.
35. Wu H, Santana I, Danise J, Giraldo JP. In vivo delivery of nanoparticles into plant leaves. *Curr Protoc Chem Biol.* 2017; 9: 269-284.
36. Perez-Harguindeguy N, Diaz S, Garnier E, Lavorel S, Poorter H, Jaureguiberry P, Bret-Harte MS, Cornwell WK, Craine JM, Gurvich DE, et al. New handbook for standardised measurement of plant functional traits worldwide. *Aust J Bot.* 2013; 61: 167-234.
37. Ferguson IB, Watkins CB, Harman JE. Inhibition by calcium of senescence of detached cucumber cotyledons - effect on ethylene and hydroperoxide production. *Plant Physiol.* 1983; 71: 182-186.
38. Yang J, Cao Y, Zhang N. Spectrophotometric method for superoxide anion radical detection in a visible light (400-780 nm) system. *Spectrochim Acta A Mol Biomol Spectrosc.* 2020; 239: 118556.
39. Kashyap SP, Kumari N, Mishra P, Moharana DP, Aamir M, Singh B, Prasanna HC. Transcriptional regulation-mediating ROS homeostasis and physio-biochemical changes in wild tomato (*Solanum chilense*) and cultivated tomato (*Solanum lycopersicum*) under high salinity. *Saudi J Biol Sci.* 2020; 27: 1999-2009.
40. Peng Z, He SP, Sun JL, Pan Z, Gong WF, Lu YL, Du XM. Na⁺ compartmentalization related to salinity stress tolerance in upland cotton (*Gossypium hirsutum*) seedlings. *Sci Rep.* 2016; 6: 34548.
41. Qin T, Liu SM, Zhang ZN, Sun LQ, He X, Lindsey K, Zhu LF, Zhang XL. GhCyP3 improves the resistance of cotton to *Verticillium dahliae* by inhibiting the E₃ ubiquitin ligase activity of GhPUB17. *Plant Mol Biol.* 2019; 99: 379-393.
42. Chen XG, Lu XK, Shu N, Wang DL, Wang S, Wang JJ, Guo LX, Guo XN, Fan WL, Lin ZX, Ye WW. *GhSOS1*, a plasma membrane Na⁺/H⁺ antiporter gene from upland cotton, enhances salt tolerance in transgenic *Arabidopsis thaliana*. *PLoS ONE.* 2017; 12: 13.
43. Zhu JK. Abiotic stress signaling and responses in plants. *Cell.* 2016, 167: 313-324.
44. Mittler R. ROS are good. *Trends Plant Sci.* 2017; 22: 11-19.
45. Bianco CL, Toscano JP, Fukuto JM. An Integrated View of the Chemical Biology of NO, CO, H₂S, and O₂⁻. 2017; 9-21.

46. Anjum SA, Tanveer M, Hussain S, Bao M, Wang LC, Khan I, Ullah E, Tung SA, Samad RA, Shahzad B. Cadmium toxicity in Maize (*Zea mays*): consequences on antioxidative systems, reactive oxygen species and cadmium accumulation. *Environ Sci Pollut Res*. 2015; 22: 17022-17030.
47. Mostofa MG, Hossain MA, Fujita M. Trehalose pretreatment induces salt tolerance in rice (*Oryza sativa*) seedlings. oxidative damage and co-induction of antioxidant defense and glyoxalase systems. *Protoplasma*. 2015; 252: 461-475.
48. Wu HH, Zhang XC, Giraldo JP, Shabala S. It is not all about sodium: revealing tissue specificity and signaling roles of potassium in plant responses to salt stress. *Plant Soil*. 2018; 431:1-17.
49. Gierth M, Maser P. Potassium transporters in plants - Involvement in K⁺ acquisition, redistribution and homeostasis. *FEBS Lett*. 2007; 581: 2348-2356.
50. Wang Y, Wu WH. Plant sensing and signaling in response to K⁺-deficiency. *Mol Plant*. 2010; 3: 280-287.
51. Dreyer I, Uozumi N. Potassium channels in plant cells. *FEBS J*. 2011; 278: 4293-4303.
52. Leigh RA, Jones RGW. A hypothesis relating critical potassium concentrations for growth to the distribution and functions of this ion in the plant-cell. *New Phytol*. 1984; 97: 1-13.
53. Britto DT, Kronzucker HJ. Cellular mechanisms of potassium transport in plants. *Physiol Plant*. 2008; 133: 637-650.
54. Das K, Roychoudhury A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Front Environ Sci*. 2014; 2: 53.
55. Plett DC, Moller IS. Na⁺ transport in glycophytic plants: what we know and would like to know. *Plant Cell Environ*. 2010; 33: 612-626.
56. Tester M, Davenport R. Na⁺ tolerance and Na⁺ transport in higher plants. *Ann Bot*. 2003; 91: 503-527.
57. Wang M, Liao W. Carbon monoxide as a signaling molecule in plants. *Front Plant Sci*. 2016; 7: 527.

Figures

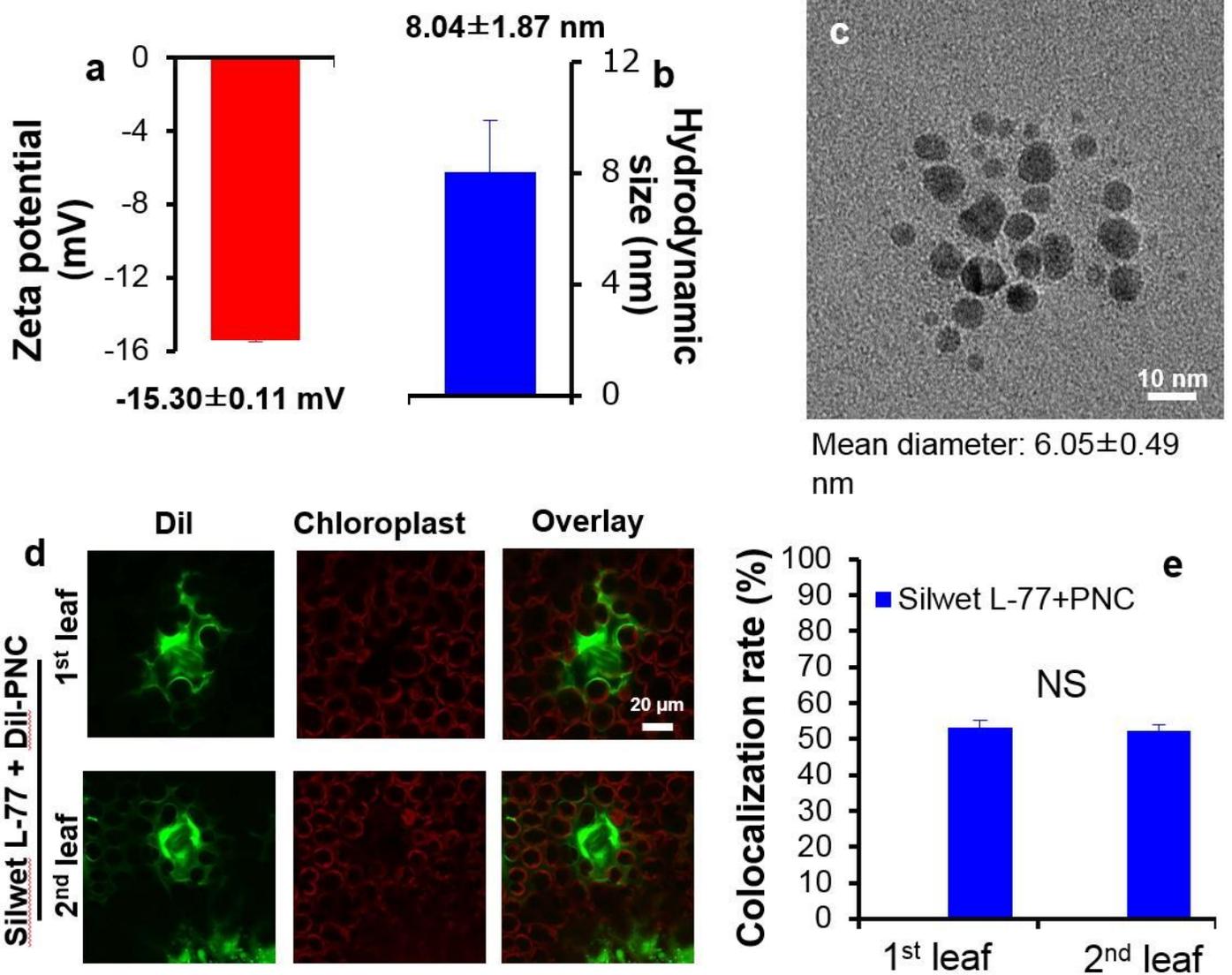


Figure 1

PNC characterization and its colocalization with chloroplasts. a and b, the size and charge of the synthesized PNC. Mean \pm SE (n = 3). c, the TEM imaging of the synthesized PNC. d, confocal imaging results showing the distribution of PNC in the first and second true leaves of the cotton plants. e, the calculated colocalization rate between PNC and chloroplasts in the first and second true leaves of the cotton plants. Mean \pm SE (n = 3). Different lower-case letters indicate the significance level at 0.05. NS, no significant difference.

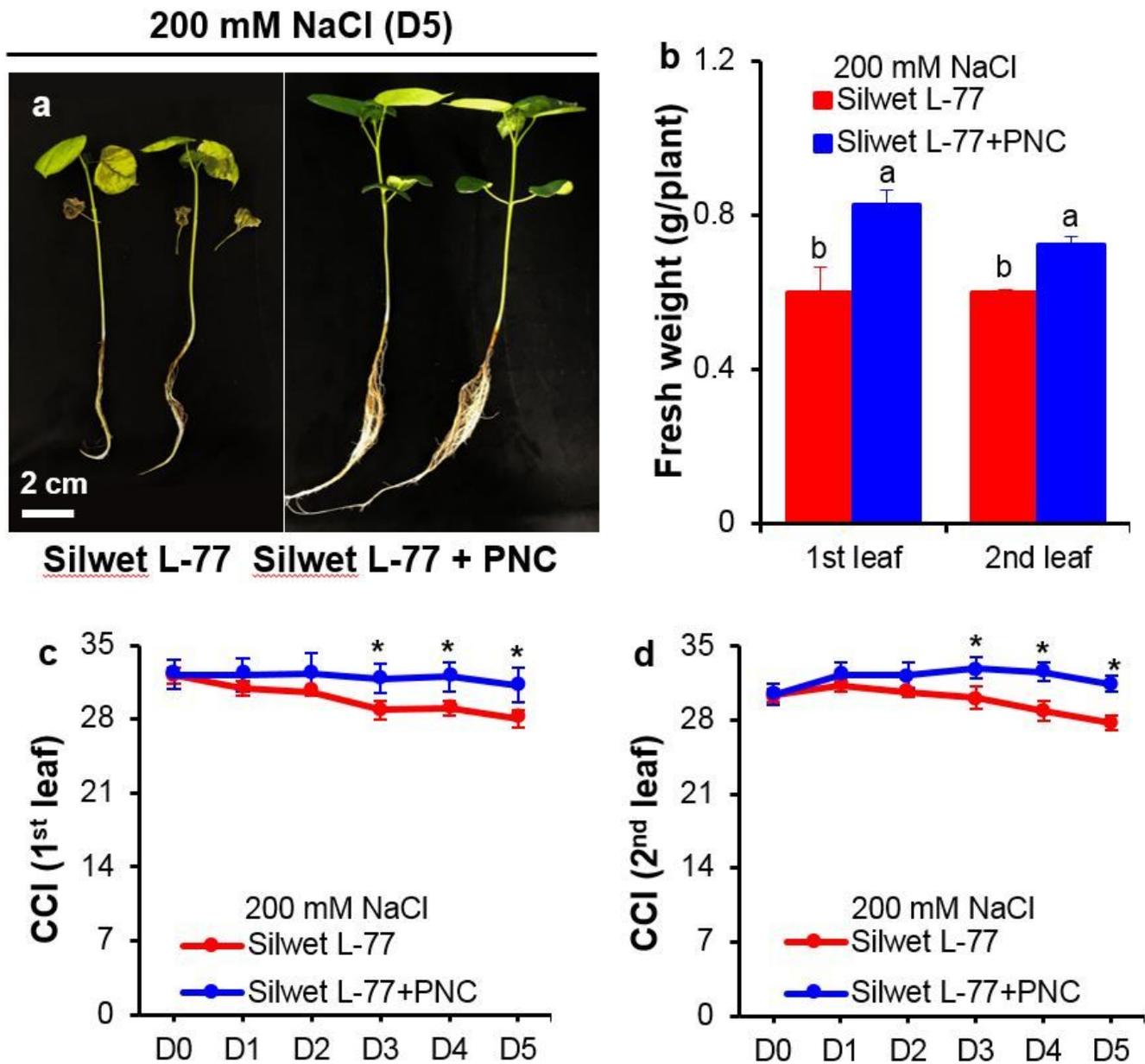


Figure 2

The phenotypic performance and chlorophyll content of salt stressed (200 mM NaCl, 5 days) cotton plants with and without PNC treatment. a, phenotypic performance of salt stressed cotton plants with and without PNC treatment. b, the fresh weight of salt stressed cotton plants with and without PNC treatment. c and d, CCI (chlorophyll content index) readout in the first (c) and second (d) true leaf of salt stressed cotton plants with and without PNC treatment. Mean \pm SE (n = 6). Different lower-case letters indicate the significance level at 0.05. * means P < 0.05.

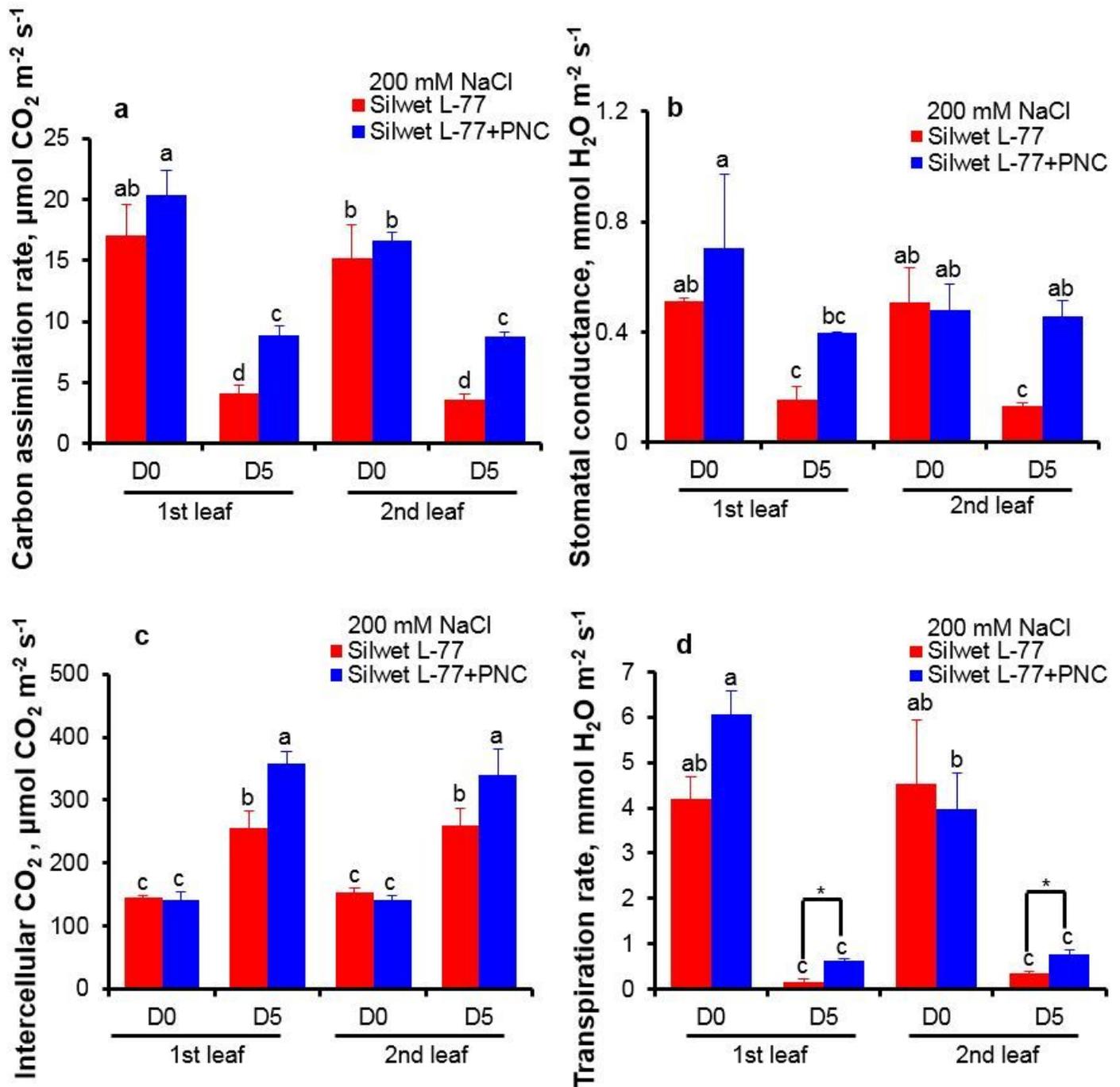


Figure 3

The photosynthetic performance of cotton plants with and without PNC treatment, before and after salt stress (200 mM NaCl, 5 days). a-d, carbon assimilation rate (a), stomatal conductance (b), intercellular CO₂ (c), and transpiration rate (d) of the first and second true leaf of cotton plants with and without PNC treatment before and after salt stress. Mean ± SE (n = 8). Different lower-case letters indicate the significance level at 0.05.

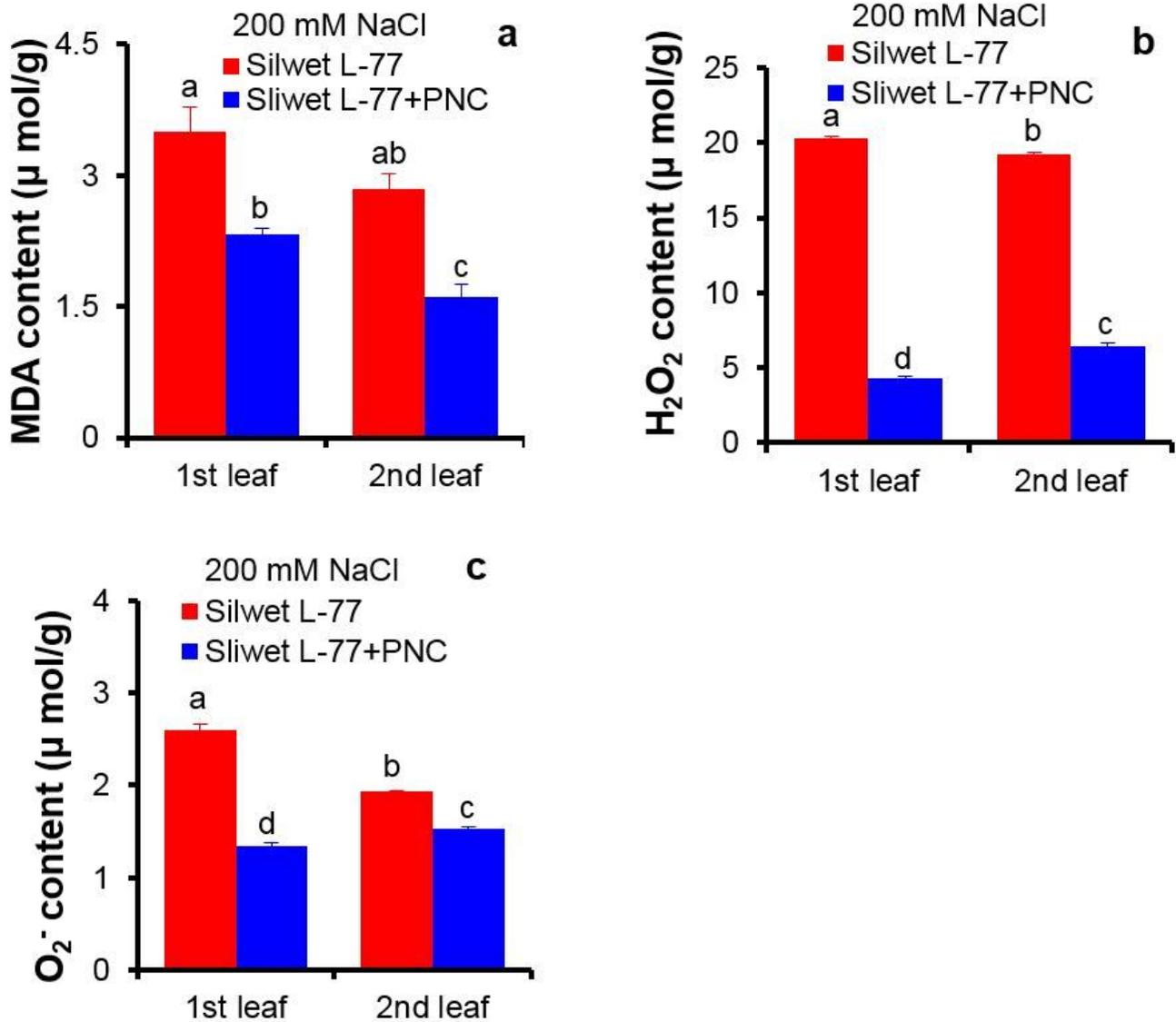


Figure 4

The MDA and ROS content in salt stressed (200 mM NaCl, 5 days) cotton plants with and without PNC treatment. a-c, malondialdehyde (MDA) content (a), H₂O₂ content (b), and O₂⁻ content (c) of the first and second true leaf of salt stressed cotton plants with and without PNC treatment. Mean ± SE (n = 12). Different lower-case letters indicate the significance level at 0.05.

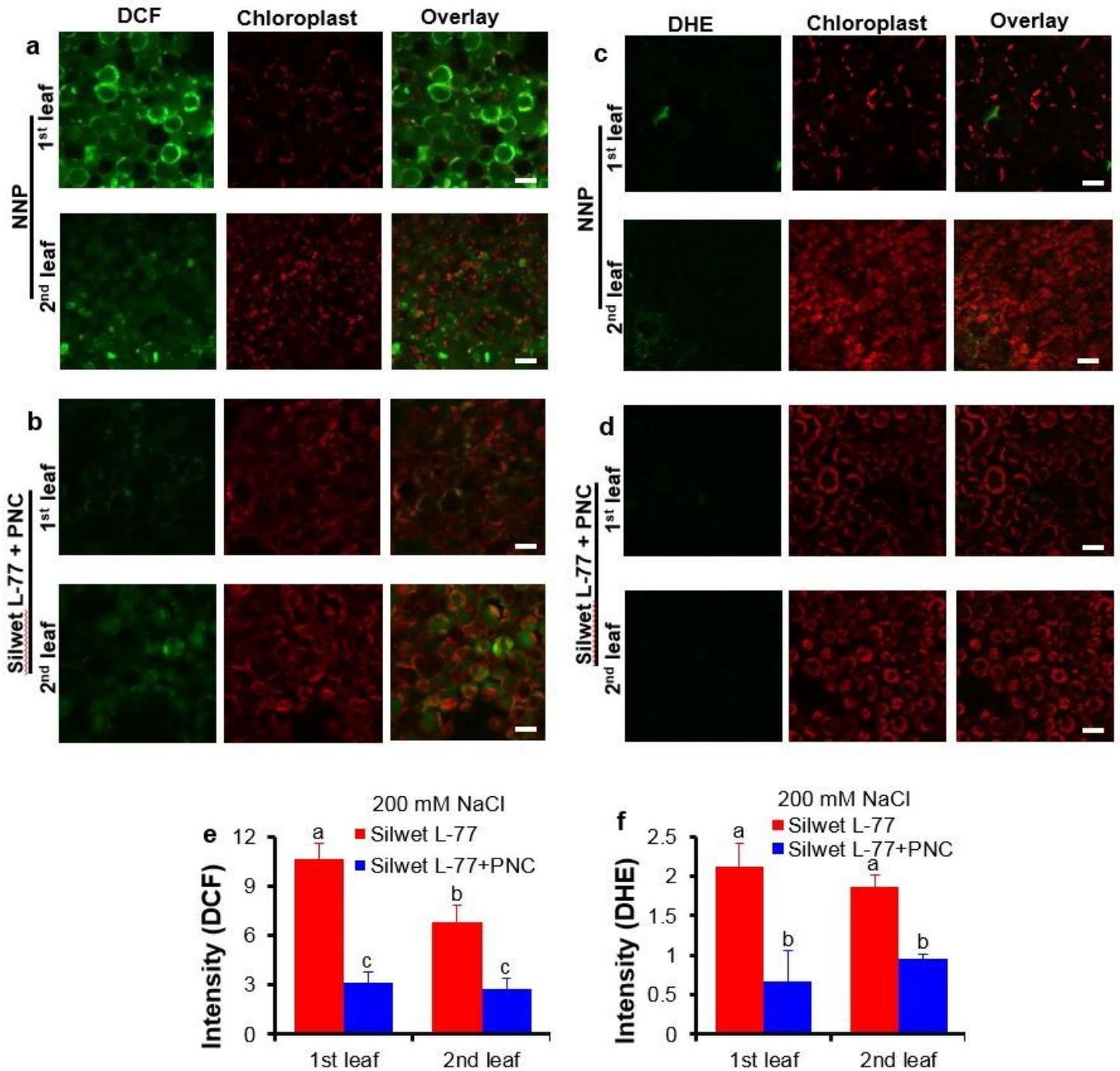


Figure 5

Confocal imaging of ROS fluorescent dye intensity in salt stressed (200 mM NaCl, 5 days) cotton plants with and without PNC treatment. a and b, DCF (largely indicating H₂O₂) fluorescent dye intensity of the first and second true leaf of salt stressed cotton plants without (a) and with (b) PNC treatment. c and d, DHE (indicating •O₂⁻) fluorescent dye intensity of the first and second true leaf of salt stressed cotton plants without (c) and with (d) PNC treatment. e and f, the quantified DCF (e) and DHE (f) fluorescence intensity of the first and second true leaf of salt stressed cotton plants with and without PNC treatment. Mean ± SE (n = 3). Different lower-case letters indicate the significance level at 0.05. Scale bar is 20 μm.

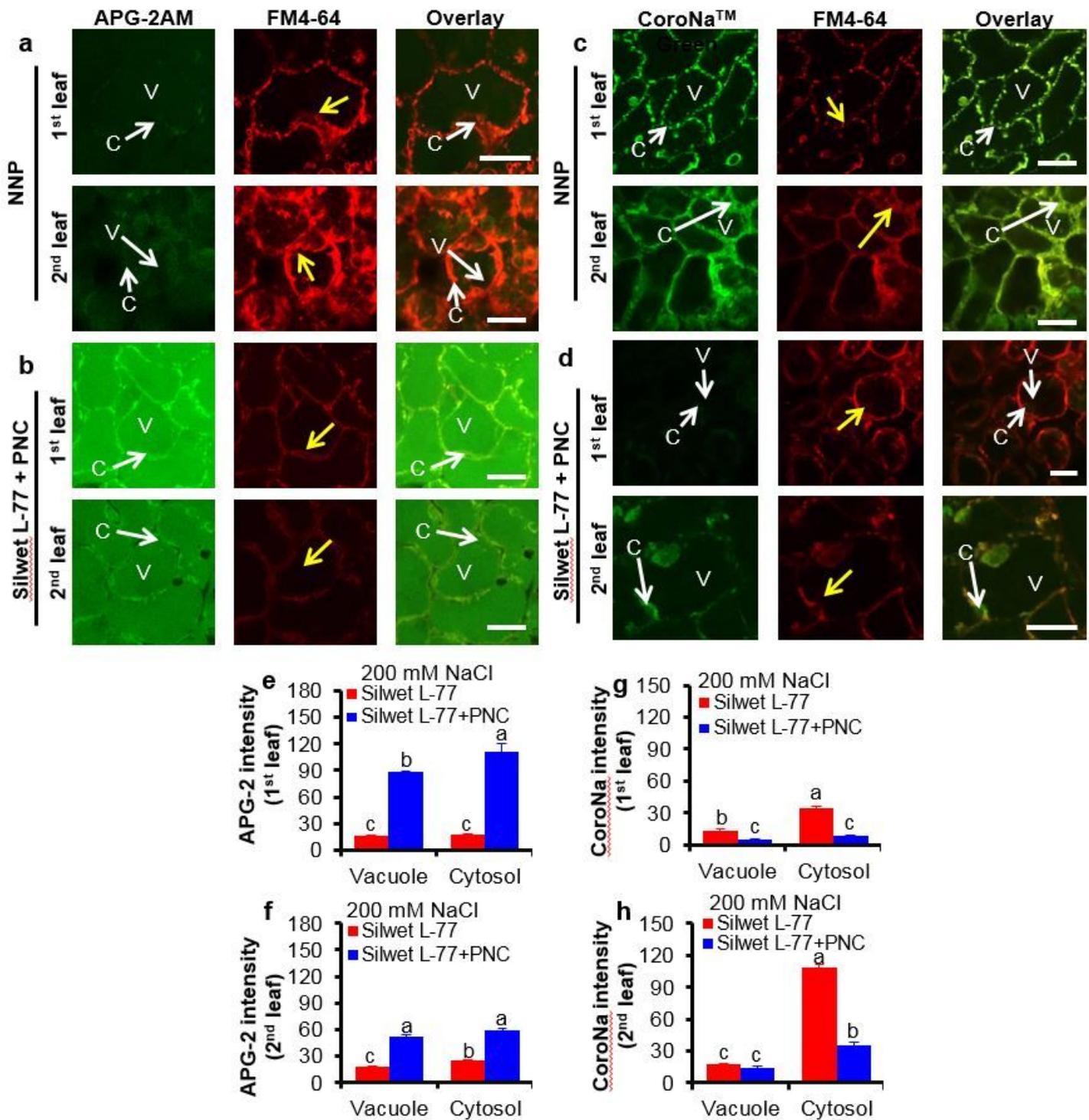


Figure 6

Confocal imaging of K⁺ and Na⁺ fluorescent dye intensity in salt stressed (200 mM NaCl, 5 days) cotton plants with and without PNC treatment. a and b, APG-2AM (indicating K⁺) fluorescent dye intensity of the first and second true leaf of salt stressed cotton plants without (a) and with (b) PNC treatment. c and d, CoroNaTM (indicating Na⁺) fluorescent dye intensity of the first and second true leaf of salt stressed cotton plants without (c) and with (d) PNC treatment. e and f, the quantified vacuolar and cytosolic APG-2AM fluorescent dye of the first (e) and second (f) true leaf of salt stressed cotton plants with and

without PNC treatment. g and h, the quantified vacuolar and cytosolic CoroNa™ fluorescent dye of the first (g) and second (h) true leaf of salt stressed cotton plants with and without PNC treatment. Mean \pm SE (n = 3). Different lower-case letters indicate the significance level at 0.05. C means the cytosol, M means vacuole membrane, and V means the vacuole. The yellow arrows point to the tonoplast membrane. Scale bar is 20 μ m.

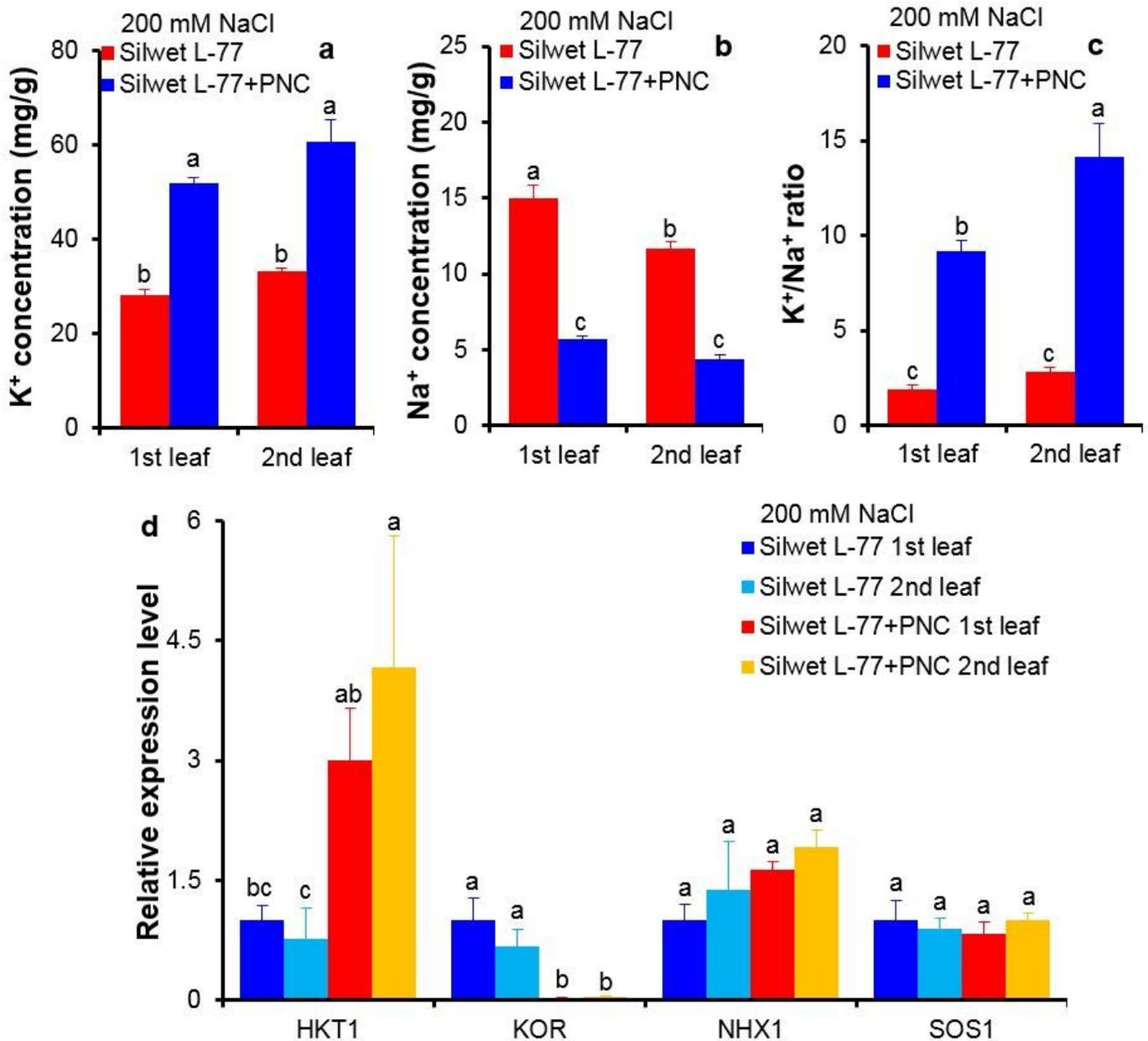


Figure 7

Leaf K⁺ and Na⁺ content, K⁺/Na⁺ ratio and the relative expression of ion channel/transporter genes related to K⁺ and Na⁺ transport in salt stressed (200 mM NaCl, 5 days) cotton plants with and without PNC treatment. a-c, K⁺ content (a), Na⁺ (b) content and K⁺/Na⁺ ratio (c) in the first and second true leaf of salt stressed cotton plants with and without PNC treatment. Mean \pm SE (n = 12). d, the relative expression of ion channel/transporter genes related to K⁺ and Na⁺ transport in the first and second true

leaf of cotton plants with and without PNC treatment, before and after salt stress. Mean \pm SE (n = 3). Different lower-case letters indicate the significance level at 0.05.

Supplementary Files

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