**Potassium alleviates over-reduction of the photosynthetic electron transport chain and helps to maintain integrity of the photosynthetic apparatus under salt-stress**

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**Abstract**

Potassium ions enhances photosynthetic tolerance to salt stress. We hypothesized that potassium ions, by minimizing the trans-thylakoid proton diffusion potential difference, can alleviate an over-reduced photosynthetic electron transport chain and maintain the integrity of the photosynthetic apparatus. This study investigated the effects of exogenous potassium on the transcription level and activity of proteins related to the photosynthetic electron-transport chain of tobacco seedlings under salt stress. Salt stress retarded the growth of seedlings, and caused potassium ion outflow from the chloroplast. It also lowered *qP* (indicator of the oxidation state of QA), *Y*PSII (average photochemical yield of PSII) while increasing *Y*NO+NF (non-regulatory energy dissipation), accompanied by reduced expression of most light-harvesting, energy-conversion, and electron-transport genes. Interestingly, Lincomycin (a D1 protein-synthesis inhibitor) significantly diminished the alleviation effect of exogenous potassium on salt stress. We attribute the comprehensive NaCl-induced down-regulation of transcription and photosynthetic activities to ROS-induced retrograde signalling. There probably exists at least two types of ROS-induced retrograde signalling, distinguished by their sensitivity to Lincomycin. Exogenous potassium appears to exert its primary effect by ameliorating the trans-thylakoid proton diffusion potential difference caused by salt stress, thereby alleviating over-reduction of the photosynthetic electron transport chain, and maintaining the integrity of photosynthetic proteins.

**Introduction**

Salt-induced osmotic and ionic stress alters water potential and ionic homeostasis, mainly by inhibiting the efficiency of potassium (K+) uptake by plants (Arif, Singh, Siddiqui, Bajguz, & Hayat, 2020). Additionally, salt stress negatively impacts key physiological attributes of plants, such as photosynthesis, stomatal activity, leaf chlorophyll content, and seed germination rate (Abdul Qados, 2011; Bistgani, et al., 2019; Rahneshan, Nasibi, Lakehal, & Bellini, 2018). Further, excessive accumulation of sodium (Na+) ions impedes soil water and nutrient uptake processes, causing osmotic or water deficit stress and ultimately inhibiting plant growth (Hanin, Ebel, Ngom, Laplaze, & Masmoudi, 2016). Regarding the ion homeostasis in cells, when the concentration of Na+ ions in the cytoplasm is too high, K+ is displaced by Na+, leading to the outflow of potassium ions (Che et al., 2022a), which leads to chlorophyll degradation and disruption of the function of photosynthetic proteins (Suo, Zhao, David, Chen, & Dai, 2017). The accumulation of Na+ and Cl− ions negatively impacts the ultrastructure of the chloroplast photosystem II (PSII), gas exchange, and the transpiration rate, leading to the obstruction of photosynthesis (Khan, Asgher, & Khan, 2014). Salt stress also prevents the transfer of PSII electrons from the primary quinone receptor QA to the secondary quinone receptor QB, increasing the probability of charge recombination and generating high levels of reactive oxygen species (ROS) (Acosta-Motos et al., 2017). When the formation of ROS exceeds the antioxidant capacity of plants, changes can occur in the activity of various ion channels involved in plant ion homeostasis (Demidchik, 2018),while the PSII reaction center and other photosynthetic proteins are also damaged (Zhang et al., 2020b). It is hypothesized that salt stress aggravates the level of excess absorbed light energy, accumulates high levels of ROS, and impairs the PSII repair process (Takahashi & Murata, 2008).

In the process of plant evolution, plants have evolved various strategies by which to adapt to salt stress: 1) increase in the water absorption capacity of plants by increasing osmotic regulatory substances such as soluble sugar and proline (Wang et al., 2021); 2) removal of excessive reactive oxygen species by activating the antioxidant enzyme system to reduce oxidative damage in cells (Zhang et al., 2020a); and 3) combining exogenous salt stress signals with endogenous developmental cues (such as plant hormone signals) in vivo, so as to optimize the balance between growth and stress response (Yu et al., 2020). The photosynthetic system of plants also forms three lines of defense against excess light energy :(1) attenuation of interception and absorption of light energy; (2) Protection of the photosystems by avoiding oxidative damage caused by ROS and promoting the dissipation of excess light energy; and (3) Repair of PSII injury (PSII turnover) (Dong, Jin, & Wang, 2016). Therefore, plant salt tolerance is a complex quantitative trait controlled by multiple genes, which involves salt sensing, signal transduction, osmoregulation, ion transport, hormone synthesis, photosynthesis and metabolism (Yu et al., 2020).

The addition of exogenous potassium ions has been demonstrated to alleviate the damage caused by salt stress to the photosynthetic apparatus, improve the chlorophyll content of leaves under salt stress and improve the photosynthetic function of leaves (Song, Long, Liu & Liu, 2011). Potassium can also improve osmotic and water potential, reduce stomatal resistance, and increase the number of chloroplast grana, electron transfer and photophosphorylation activity, Rubisco and Rubisco activase activity and content, and net photosynthetic rate (Zheng, Cheng, Jiang & Weng, 2002). Overexpression of thylakoid K+/H+ antiporter KEA3 accelerates relaxation of photoprotective energy-dependent quenching after transitioning from high-light to low-light conditions in *Arabidopsis thaliana* and tobacco (Armbruster et al., 2016). Other studies have shown that retention of K+, inhibition of excessive accumulation of Na+, and maintenance of a high K+/Na+ ratio mediated by an H+/K+ antiporter induce tolerance traits in salt-sensitive plants (Wang et al., 2017). Triple mutations of K+ outflow antiporters, such as AtKEA1, AtKEA2, and AtKEA3, substantially changed chloroplast development and proton dynamics on the thylakoid membrane, leading to diminution of carbon assimilation and growth rate (Kunz et al., 2014). Thus, potassium ions play important roles in pigment restoration, energy quenching, carbon assimilation, and ion balance, among other functions. The large amount of ROS produced under salt stress may lead to retrograde signal transduction (Crawford, Lehotai, & Strand, 2018), which would downregulate the transcription level of most genes related to energy harvesting, conversion, and utilization of electron transport chain; however, whether potassium ions are involved in alleviating retrograde signaling and repairing PSII under salt stress is rarely reported.

We hypothesize that (1) salt stress leads to an over-reduced electron transport chain, a phenomenon in which, due to the depletion of stromal K+ under salt stress, K+ is unable to enter the thylakoid lumen to rapidly compensate for the efflux of H+ via the ATP synthase, resulting in insufficient ATP to support rapid carbon assimilation; and (2) an over-reduced electron transport chain enhances the formation of H2O2 and singlet oxygen, both of which could lead to retrograde signalling (Foyer & Shigeoka, 2011; Gollan, Tikkanen, & Aro, 2015; Chan, Phua, Crisp, McQuinn, & Pogson, 2016) that affects the expression of many genes involved in photosynthesis; and (3) supplemental potassium can reverse many of the effects of salt stress. It appears that potassium exerts its effect by primarily by alleviating the trans-thylakoid proton diffusion potential difference via rapid charge compensation as protons exit the thylakoid lumen via the ATP synthase.

**2. Materials and methods**

***2.1 Plant material and growth conditions***

The present experiment was conducted at the Laboratory of Plant Physiology, Northeast Forestry University, Harbin, China. Tobacco seeds were sown into holes in a 5 × 10 cm plate. When tobacco seedlings had grown to a height of 5 cm, they were transplanted into 13-cm diameter, 15-cm tall pots, filled with a 2:1 (*v*/*v*) mixture of peat soil and vermiculite. When each plant had grown five true leaves, the following different treatments were applied: (1) 150 mM NaCl; (2) 150 mM NaCl + 20 mM KCl; and (3) 150 mM NaCl + 20 mM KCl + 1 mM Lincomycin (Linco); each pot was individually watered with the selected solution. The control (CK) utilized distilled water. For all plants, 200 mL of solution was poured into the dish each time, and 200 mL of solution was added each time the dish was dry. After 5 days of treatment, plant indexes were determined and recorded.

***2.2. Determination of parameters and methods***

**Chlorophyll fluorescence kinetics curve:** The chlorophyll fluorescence kinetics curve was measured using a Multi-Function Plant Efficiency Analyser (M-PEA) (Hansatech, King’s Lynn, UK) after dark adaptation for 30 min. To determine the OJIP curve, a pulse of red light (3500 μmol·m−2·s−1) was applied, and the chlorophyll fluorescence signal was recorded for 1 s (Wang, Zhao, Zhang, Lu & Feng, 2022). The time nodes of relative fluorescence intensity *F*O, *F*J, *F*I and *F*P, of points O, J, I, and P are 0.02, 2, 30 and 1000 ms respectively(Che et al., 2020), and the relative fluorescence curve can be described as *V*O-P = (*V*t – *V*O) / (*V*P – *V*O), where the subscript *t* denotes time *t*. The difference between the values of all treatments and the CK value is ΔVO-P. The relative fluorescence curve of points O-K is WOK (L-band) = (*V*t – *V*O) / (*V*K – *V*O), where K is an inflection point at 0.3 ms, and ΔWOK is obtained from the difference between the treatment and CK. Additionally, O-J curve *W*OJ (K-band), Δ*W*OJ, I-P curve *W*IP (G-band), Δ*W*IP, WOI, and Δ*W*OI (Paunov, Koleva, Vassilev, Vangronsveld & Goltsev, 2018) can be similarly obtained. The OJIP curve was analyzed using a JIP-test to obtain the PSII photochemical efficiency (*F*v/*F*m) (Zhang et al., 2020a).

**Chlorophyll fluorescence parameters:** Leaves were first dark adapted for 30 min. The initial fluorescence (*F*o) and maximum fluorescence (*F*m) were measured using an FMS-2 pulse modulation fluorimeter (Hansatech, King’s Lynn, UK) to calculate the dark-adapted maximum quantum efficiency of PSII photochemistry (*F*v/*F*m). Then, under illumination for 3 min at an irradiance of 500 μmol·m−2·s−1, the following were measured: (1) the photochemical yield of PSII averaged over closed and open PSII traps (*Y*II); (2) the fraction of light absorbed by PSII that is non-photochemically dissipated in a light-regulated manner, (*Y*NPQ); (3) the sum of the fractions of light absorbed by PSII that is dissipated in a constitutive, un-regulated manner plus that which is emitted as chlorophyll fluorescence (*Y*NO+NF); (4) the chlorophyll fluorescence parameters *qP* (an indicator of the oxidation state of QA in the puddle model) and (5) the photochemical efficiency of open PSII traps (*F*v′/*F*m′). Calculation and determination of the following parameters were conducted according to the method described by Kornyeyev and Hendrickson (2007) who considered the PSII pool as a whole, without splitting it into functional and non-functional (NF) subpopulations: *Y*II = 1 − *F*s/ *F*m′; *Y*NPQ = *F*s/*F*m′ − *F*s/*F*m; *Y*NO+NF = *F*s/*F*m. Here, *F*m refers to the dark-adapted maximum fluorescence yield when there may or may not be photoinactivated PSII complexes present (= (*F*m)PI as usedby Kornyeyev and Hendrickson 2007). *Y*II + *Y*NPQ + *Y*NO+NF = 1. The parameters *qP* and *F*v′/*F*m′ were calculated as described by Che et al. (2022a).

**Chlorophyll content:** First, 0.1 g of fresh leaf sample was ground to homogenate in a mortar containing 96% ethanol, and then the sample was allowed to stand for 3-5 minutes after fading before proceeding. The extract was filtered through a filter paper funnel into a 25-mL brown volumetric flask, topped up with 96% ethanol to a constant volume. With 96% ethanol as a blank control, the absorbance was measured at wavelengths of 665, 649, and 470 nm. Pigment contents were calculated according to the methods described by Wang et al. (2020).

**Seed germination:** Twenty-five seeds were placed on two pieces of qualitative filter paper in a Petri dish, after which, 3 mL of the various treatment solutions were added to each dish with an eye dropper to soak all the filter paper to ensure that the seeds would absorb the solution. When the germinated seeds had each grown two leaves, images were captured, and root length was measured.

**Potassium ion fluorescence staining:** Treated leaves were cut into 0.5 × 1 cm strips which were then stained for 60 min with Enhanced Potassium Green-4 TMA + Salt (Shanghai Maokang Biotechnology Co., Ltd, Shanghai, China) potassium ion fluorescence probe. The stained leaves were put into 80% ethanol and heated at 80℃ until the colour faded. The discolored leaves were cut into 30-μm-thick slices with a frozen slicer, and the morphology of the leaves was observed under a fluorescence microscope (Nikon 690871, Tokyo, Japan).

**Expression levels of related genes:** The total RNA was extracted from plant tissue samples smaller than 100 mg using the OMEGA plant RNA Kit (Bio-tek, Norcross, GA, USA) kit, and then, single-stranded cDNA template synthesis (Che et al., 2022a) was conducted with the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) and the premixed dye FastStart Universal SYBR Green Master (ROX) (Roche, Basel, Switzerland). The amplification process was conducted using a Mastercycler ®nexus series PCR instrument (Eppendorf, Hamburg, Germany). After amplification, semi-quantitative PCR was conducted using agarose gel electrophoresis. The following gene-specific primers (5′-3′) were used: *psbA* F, ATGCGACCTTGGATTGCTGTTG; *psbA* R, ACCATGAGCGGCTACGATGTTAT; *atpB* F, GTATTTGGCGGAGTGGGTGAAC; *atpB* R, CACAGCGGAAGGCATTCTACC.

**Transcriptome analysis:** Cut tobacco leaves from the three different treatments were wrapped in tin foil and frozen in liquid nitrogen for 1 h. All the samples were then sent to Hua da Gene sequencing Co., Ltd. (Wuhan, China) for transcriptome analysis. RNA-Seq (Quantification) based on next-generation high-throughput sequencing technology was used to study the gene expression pattern and provide accurate digital expression profiles by sequencing and subsequently comparing transcripts. In brief, the experimental procedure included the following steps: mRNA isolation, mRNA fragmentation, cDNA synthesis, end repair, addition of poly-A tails and adaptor ligation, PCR, library quality control, circularization, and sequencing.

The sequence data were filtered with SOAPnuke (v1.5.2) (<https://github.com/BGI-flexlab/SOAPnuke>) and the clean reads were mapped to the reference genome using HISAT2 (v2.0.4) (<http://www.ccb.jhu.edu/software/hisat/index.shtml>). The clean reads were aligned with the reference gene set by Bowtie2 (v2.2.5) (<http://bowtiebio.sourceforge.net/%20Bowtie2%20/index.shtml>), and the gene expression level was calculated by RSEM (v1.2.12) (<https://github.com/deweylab/RSEM>). Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.kegg.jp/>) enrichment analysis of expressed genes was conducted using Phyper. The significance levels of the KEGG terms and pathways were corrected using a Q-value with a strict threshold (Q-value ≤ 0.05).

***2.3. Data processing***

Excel (Office, 2016; Microsoft Corp., Redmond, WA, USA) and SPSS 22.0 (IBM Corp, Armonk, NY, USA) was used for statistical analysis. Origin2019bis (OriginLab, Northampton, MA, USA) was used for drawing figures. Single factor analysis of variance (ANOVA) and least significant difference (LSD) procedures were used to compare differences among treatment groups. Each parameter was determined in triplicate.

**3. Results**

**3.1 Exogenous K+ alleviates the NaCl-induced inhibition of post-germination plant morphology and development in tobacco**

As shown in Figure 1a, although there was no significant difference in the germination rate of tobacco seeds among the four treatments, NaCl affected the vegetative growth of tobacco seedlings. Compared with CK conditions, NaCl for 5 days significantly decreased cotyledon area, reduced radical length and induced cotyledon yellowing (Figure 1a). Compared with the NaCl treatment (Figure 1b), application of K+ significantly increased the cotyledon area, increased radicle length and induced greener cotyledons (Figure 1a). Root length under the NaCl+K+Linco treatment was also significantly greater than that under NaCl; there was no difference in root length between NaCl+K and NaCl+K+Linco treatments, but the cotyledons of NaCl+K+Linco-treated plants were pale green or white (Figure 1a). This indicates that the inhibition of synthesis of D1 and other chloroplast-encoded proteins (despite repair being potentially aided by potassium ions) can seriously affect the synthesis of chlorophyll.

In addition, NaCl had a significant effect on tobacco seedlings that were already in the vegetative growth phase. As shown in Figure 1c, leaves of tobacco plants treated with NaCl for 5 days showed yellowing, while K+ restored the leaf color to that of the CK level. However, after adding Lincomycin, leaf chlorosis was evident in the widespread yellowing (Figure 1c), which means that K+ may promote the reversal of leaf yellowing by promoting the repair of D1 protein in the absence of Lincomycin. We found that under NaCl treatment, potassium ion fluorescence staining was mostly distributed at the edge of the palisade tissue, while after exogenous potassium treatment, the staining spread all over the palisade tissue (Figure 1d). After adding Lincomycin, however, there was no obvious boundary between the interior and edge of the palisade tissue, while the chloroplasts were mainly distributed in the palisade tissue, indicating that the potassium ion outflow from the chloroplast increased under salt stress but that the addition of potassium could supplement the potassium ion level in the chloroplast.

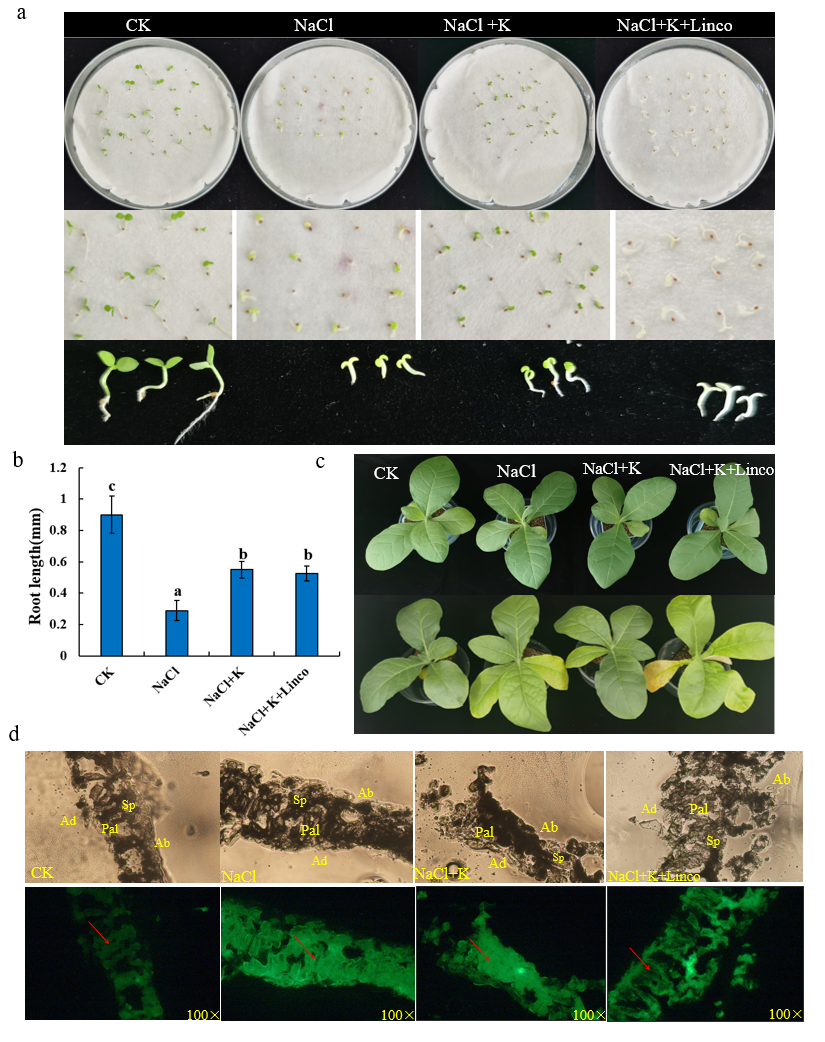


Figure 1. Effects of four treatments on germinated seedling morphology (a), root length (b), leaf morphology (c), and potassium ion fluorescence staining (d) under salt stress. (a) Twenty-five seeds were placed in each Petri dish, and the filter papers were changed every two days. (b) After the seeds had germinated, the root length was measured after seedlings had developed two mature cotyledons. (c) Plants before (above) and after (below) 5 days of treatment. (d) Micrographs of potassium ion distribution in leaf tissue as revealed by fluorescence staining under 100 × magnification. Ad, adaxial; Ab, abaxial; Pal, palisade tissue; Sp, spongy mesophyll. In (d) The top four are the original slides (Che et al., 2022b), and the bottom is the fluorescence of potassium ions. The red arrow points to the palisade tissue, and the distribution of potassium ions at the edge and inside can be observed.

**3.2 Chlorophyll content and expression of related genes**

As shown in Figure 2, compared with the CK, NaCl significantly decreased the chlorophyll *b* content of tobacco leaves, but had no significant effect on the chlorophyll *a* content. Under the NaCl+K treatment, the content of chlorophyll *a* and that of chlorophyll *b* increased compared with the NaCl treatment and reached or exceeded the level of the CK treatment. After adding Lincomycin, the content of chlorophyll (*a*+*b*) was lower than that of the NaCl+K treatment, but slightly higher than that of the NaCl treatment. Relative to the CK, both NaCl and NaCl+K+Linco treatments decreased the content of chlorophyll *a*+*b*, while K+ increased the total chlorophyll content (Figure 2c).

Compared with the CK treatment, the transcription of 21 chlorophyll synthesis genes (*Glu-TR* (LOC107763283, LOC107781666), *ALAD*(LOC107793287, LOC107798564), *PBGD* (LOC107802823, LOC107802603, LOC107830132, LOC107767993), *UROS* (LOC107778553, LOC107797441), *CPOX* (LOC107766888), *PPOX* (LOC107780878, LOC107827378), *POR* (LOC107787316, LOC107820326, LOC107795891, LOC107793976, LOC107825540, LOC107804928), *DVR* (LOC107806278, LOC107773288)) under the NaCl treatment was significantly down-regulated (Figure 2d), and the expression levels of chlorophyll *b* degradation genes (*PPH* (LOC107789349, LOC107787339, LOC107763257, LOC107824158)) were higher than those under the CK treatment as a whole (Figure 2e), consistent with the decrease in chlorophyll *b* content. Compared with the NaCl treatment, 20 chlorophyll synthesis genes were significantly up-regulated after the NaCl+K treatment; among these, all *PBGD*-related genes were up-regulated and chlorophyll *b* degradation genes were down-regulated overall. After the NaCl+K+Linco treatment, 33 chlorophyll synthesis genes were significantly down-regulated and 7 chlorophyll degradation genes were significantly up-regulated compared with the CK treatment (see Figure 2 for details).

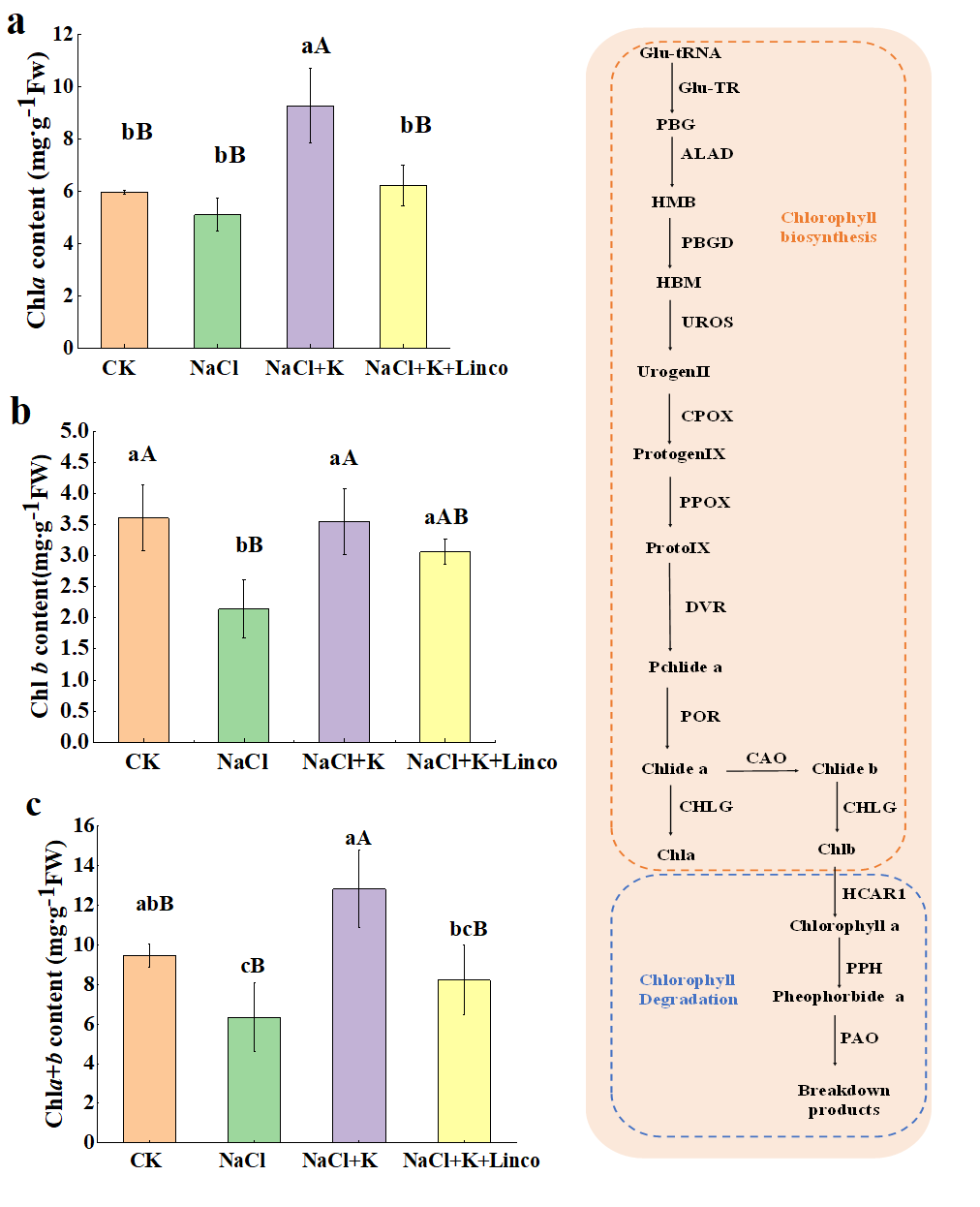






Figure 2. Effects of potassium ions on chlorophyll contents (a, b, c) and its related synthesis (d) and degradation (e) genes in tobacco leaves under salt stress. Glu-TR, glutamyl-tRNA reductase; Glu-tRNA, l-glutamyl-tRNA; PBG, porphobilinogen; ALAD, delta-aminolevulinic acid dehydratase; HMB, hydroxymethylbilane; PBGD, porphobilinogen; deaminase; UROS, uroporphyrinogen III synthase; Urogen II, uroporphyrinogen II; CPOX, coproporphyrinogen-III oxidase; Protogen IX, protoporphyrinogen IX; PPOX, protoporphyrinogen oxidase; Protogen IX, protoporphyrinogen IX; DVR, branched-chain-amino-acid aminotransferase; Pchlide *a*, protochlorophyllide *a*; POR, light-dependent protochlorophyllide oxidoreductase; Chlide *a*, chlorophyllide *a*; CHLG, protoporphyrin IX Mg-chelatase subunit G; Chl *a*, chlorophyll *a*; CAO, chlorophyllide *a* oxygenase; Chlide *b*, chlorophyllide *b*; Chl *b*, Chlorophyll *b*; HCAR, 7-hydroxymethyl chlorophyll a reductase; PPH, pheophytinase; PAO, pheophorbide a oxygenase. The values shown in the heat map become progressively larger from blue to red, and the columns of the heat map correspond to the CK, NaCl, NaCl+K, and NaCl+K+Linco treatments, from left to right. Note: significant differences according to least significant difference (LSD) tests are indicated by different lower-case letters (*P* < 0.05).

**3.3 Carotenoid content and related gene expression**

As shown in Figure 3a, carotenoid content increased under NaCl and NaCl+K+Linco treatments, but not significantly compared with the CK treatment. The NaCl+K treatment, however, significantly increased carotenoid content (Figure 3a). Transcriptome data showed that *PDS* (LOC107816873), and *ZE* (LOC107797654, LOC107763949) genes were significantly up-regulated under salt stress compared with CK conditions, while *GGPS* (LOC107767572, LOC107799556), LBCY (LOC107830918), *VDE* (LOC107763628, LOC107780507), and *LCYE* (LOC107830918, LOC107789691) were significantly down-regulated compared with the CK treatment. After potassium was added to the salt stress treatment, nine genes were significantly upregulated relative to the NaCl treatment, namely *GGPS* (LOC107767572, LOC107799556), *LBCY* (LOC107830918), *VDE* (LOC107763628, LOC107788911, LOC107780507), *LCYE* (LOC107830918, LOC107789691), while 13 genes were significantly down-regulated compared with the NaCl+K treatment. Compared with NaCl treatment alone, only 2 genes were up-regulated, and 11 genes were significantly down-regulated (Figure 3b, c, d), indicating that potassium ions play an important role in promoting carotenoid biosynthesis.



Figure 3. Effect of potassium ions on carotenoid content (a) and the expression of related genes under salt stress (b, c, d). IPP, isopentene pyrophosphate; GGPPS, geranylgeranyl pyrophosphate synthase; GGPP, geranylgeranyl pyrophosphate; PDS, 15-*cis*-phytoene desaturase; ZDS, zeta-carotene desaturase; LCYB, lycopene beta cyclase; LCYE, lycopene epsilon cyclase; LBCY, lycopene epsilon cyclase; BCH, beta-carotene hydroxylase; VDE, violaxanthin de-epoxidase; ZE, zeaxanthin epoxidase. The values shown in the heat map become progressively larger from blue to red, and the columns of the heat map correspond to the CK, NaCl, NaCl+K, and NaCl+K+Linco treatments, from left to right. Note: significant differences according to least significant difference (LSD) tests are indicated by different lowercase letters (*P* < 0.05).

**3.4 Fluorescence parameters related to PSII and PSI**

The OJIP fluorescence intensity curves showed that the latter half of the OJIP curve of the NaCl treatment was lower than that of the CK treatment, while the OJIP curve of the NaCl+K+Linco treatment was higher than that of the CK treatment overall, the increase in Fo under NaCl+K+Linco is also suggestive of inactivation of PSII reaction centers; additionally, the OJIP curve of the NaCl+K treatment had the same shape as that of the CK treatment (Figure 4a). After salt stress treatment, *F*v/*F*m was significantly reduced compared with the CK treatment. After potassium supplementation, *F*v/*F*m was significantly increased compared with the NaCl treatment. After PSII repair was inhibited, *F*v/*F*m reached the lowest value observed (Figure 4b). As can be seen from the O-P curve when normalized to the peak value, the J point under salt stress occurred at about 1 ms, while that under the NaCl+K+Linco treatment appeared earlier than that of the NaCl treatment, at about 0.3 ms. Potassium supplementation under salt stress significantly elevated the J point of these two curves compared with the CK treatment (Figures 4c, 4d).

*W*OK and *W*OJ curves showed that the positive L- and K-bands in the NaCl+K+Linco treatment were significantly higher than those in other treatments, followed by those in the NaCl treatment. The L- and K-bands of the NaCl+K treatment are close to the level of the CK treatment (Figure 4e, f).

Salt stress significantly decreased the yield of PSII (*Y*II); after adding potassium, *Y*II was significantly higher than that under the NaCl treatment (*P* < 0.01). Lincomycin treatment made *Y*II lower than that under the NaCl+K treatment but slightly higher than that under the NaCl treatment (*P* > 0.05), indicating that potassium ions play an important role in restoring PSII function. The *Y*NPQ value under the NaCl+K treatment was higher than that under the NaCl treatment, but after the addition of Lincomycin, *Y*NPQ was significantly lower than that of the NaCl+K treatment (*P* < 0.01). The *Y*NO+NF value under the treatment with NaCl was significantly higher than that of the CK treatment, and *Y*NO+NF under the treatment with NaCl+K was significantly lower than that under the NaCl treatment, being close to that of the CK treatment. The addition of Lincomycin made the level of *Y*NO+NF similar to that under salt stress but significantly higher than those under the CK and NaCl+K treatments. Relative to the CK treatment, the photochemical efficiency of open PSII traps (*F*v′/*F*m′) was significantly lower under salt stress and returned to the level of the CK treatment after adding potassium, though not significantly higher than that of the NaCl treatment (*P* > 0.05). Under Lincomycin treatment, *F*v′/*F*m′ was significantly lower than that of the NaCl+K treatment (*P* < 0.01). Compared with the CK treatment, *qP* decreased significantly under salt stress; *qP* in the treatments with potassium or Lincomycin was the same as that of the CK treatment, but not significantly higher than that of the NaCl treatment (Figure 4g).

The MR820 signal can reflect the activity of the photosystem I (PSI) reaction center (Zhang et al., 2021); from Figures 4h and 4i, it can be seen that the difference between the lowest point and the highest point under the NaCl+K treatment is larger than that under the NaCl treatment, with the NaCl+K+Linco treatment showing the smallest difference.

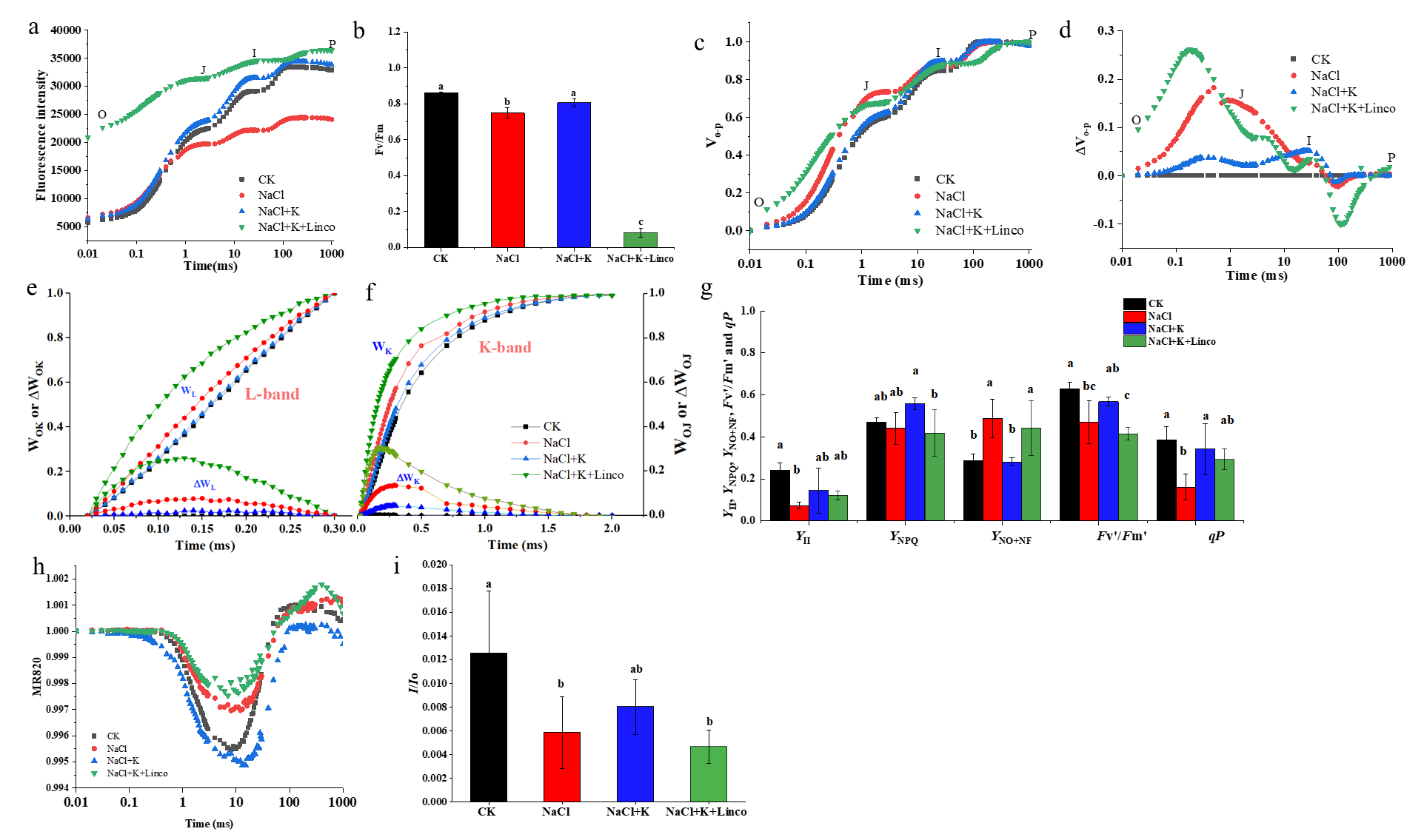


Figure 4. Effects of potassium ions on parameters related to photosystem I and II in tobacco leaves under salt stress. (a) fluorescence curve under different treatments. (b) *F*v/*F*m, maximum fluorescence; (c) fluorescence curve from O point to P point; (d) Δ*V*O-P=*V*O-P(treament)-*V*O-P(CK); (e) WOK is the fluorescence curve of points O-K, *W*OK(L-band) = (*V*t − *V*O) / (*V*K − *V*O), and so on. (g) the fluorescence intensity at *Y*II, *Y*NPQ+NF, *Y*NO, *F*v′/*F*m′, and *qP*. (h) The fluorescence intensity at 820 nm can indicate the activity of PSI. (I) quantify the difference between the lowest point and the highest point of MR820.

**3.5 Quantitative analysis of differentially expressed genes**

The antenna genes of both photosystems decreased under salt stress, *Lhca2*, *Lhca4*, *Lhcb1* (LOC107775016, LOC107773466, LOC107772842), *Lhcb4*, *Lhcb5* (LOC107782430), and *Lhcb6* (LOC107792047) decreasing significantly. After adding potassium in salt stress conditions, transcription levels of antenna genes of the two photosystems were up-regulated as a whole compared with the NaCl treatment, with nine genes significantly up-regulated. Compared with the NaCl treatment, 17 antenna genes were significantly decreased under the NaCl+K+Linco treatment, and the expression levels of 24 genes were significantly lower than those under the NaCl+K treatment (Figures 5a, 5b).

Under NaCl treatment,expression of *PetF* (LOC107803171) was significantly decreased compared with the CK treatment, while it was significantly up-regulated under the NaCl+K treatment compared with the NaCl treatment (Figure 5c). Additionally, *PetE* and *PetF* expression levels under the NaCl+K+Linco treatment were significantly lower than those under the NaCl and NaCl+K treatments.

Expression of ATP synthase genes was significantly down-regulated under the NaCl treatment compared with the CK treatment, but significantly up-regulated compared with salt stress after potassium treatment. After inhibition of PSII repair by Lincomycin, despite the presence of K+ ions, ATP synthase gene expression decreased significantly compared with other treatments.

Under the NaCl treatment, the PSI reaction center genes *PsaD*, *PsaF*, *PsaG* (LOC107794160), *PsaH*, *PsaK* (LOC107768914), and *PsaO* were significantly down-regulated compared with the CK treatment, but after NaCl+K treatment, seven genes were significantly up-regulated compared with the NaCl treatment. After inhibition of PSII repair by Lincomycin, despite the presence of K+ ions, the expression of 14 PSI reaction center genes was significantly lower than that under NaCl treatment, and 15 genes were significantly up-regulated relative to the NaCl+K treatment (Figure 5).

Under salt stress, eight genes (*PsbO* (LOC107820252, LOC107774864, LOC107766588), *PsbP* (LOC107785785), *PsbQ* (LOC107831039, LOC107773657), *Psb27* (LOC107832794, LOC107831545)) related to the PSII reaction center were significantly down-regulated and four genes ( *PsbR* (LOC107816978, LOC107810205), *PsbS* (LOC107805098, LOC107778533) were significantly up-regulated compared with the CK treatment. Under NaCl+K, eight genes were significantly up-regulated compared with salt stress, and the expression of fourteen genes was significantly lower relative to the NaCl treatment after the NaCl+K+Linco treatment, indicating that potassium plays an important role in the repair of the PSII reaction center.





Figure 5. Heat map of differential gene expression in tobacco leaves under salt Stress. Lhca, light-harvesting chlorophyll protein complex I; Pet, photosynthetic electron transport. The values shown in the heat map become progressively larger from blue to red, and the columns of the heat map correspond to the CK, NaCl, NaCl+K, and NaCl+K+Linco treatments, from left to right. Note: significant differences according to least significant difference (LSD) tests are indicated by different lowercase letters (*P* < 0.05).

**4. Discussion**

**A hypothesis on how salt stress exerts its primary effect on photosynthetic processes**

Previous work with mulberry leaves (Che et al., 2022a) led us to propose a hypothesis in which salt stress induces a decrease in the stromal concentration of K+, so that insufficient K+ rapidly enters the thylakoid lumen via the Two Pore K+ channel (TPK3) in exchange for H+ exiting the lumen via the ATP synthase. Consequently, the sluggish counterion compensation makes the electric potential (the so-called proton diffusion potential) of the lumen more negative, thereby retarding the proton efflux. In this hypothesis, the rate of ATP synthesis is limited by the efflux of protons from the lumen (part of a proton circuit around the stroma and lumen) in salt-stress conditions, causing a slow-down of carbon assimilation and, in turn, an accumulation of electrons in the photosynthetic electron transport chain. A highly reduced electron transport chain leads to a deceleration of both linear and cyclic electron transport. For example, a highly reduced plastoquinone (PQ) pool suppresses electron transport by Reduction-Induced Suppression of Electron transport (RISE) (Shaku, Shimakawa, Hashiguchi, & Miyake, 2016), which is a form of photosynthetic control (Shimakawa & Miyake, 2018), in addition to that form in which a high concentration of protons retards the oxidation of PQH2 (West & Wiskich 1968). Indeed, *Y*NPQ, the fraction of excitation energy in PSII dissipated in an energy-dependent, regulatory manner, was not increased (Figure 4g) despite the slower efflux of protons from the lumen expected in the present hypothesis in the NaCl treatment. A highly reduced photosynthetic electron transport chain, in turn, leads to various changes in photosynthetic function that are observed in salt stress conditions. However, addition of potassium alleviates the salinity-induced proton diffusion potential difference in mulberry leaves, thereby reversing many of the salinity-induced effects on chloroplast functional properties (Che et al., 2022a). Below, we further develop this hypothesis based on our observations made in tobacco plants subjected to treatments with NaCl, NaCl+K or NaCl+K+Lincomycin.

**Effects of NaCl treatment on the production of reactive oxygen species (ROS) and on retrograde signalling**

A highly reduced electron transport chain leads to the formation of ROS; the resulting oxidative stress can both induce damage and serve in sending retrograde signals to the nucleus to elicit responses at the level of gene expression (Foyer & Shigeoka, 2011; Gollan, Tiikanen, & Aro, 2015; Chan, Phua, Crisp, McQuinn, & Pogson, 2016; Crawford, Lehotai, & Strand, 2018). In the scheme of Figure 6, for example, superoxide formed after reduction of molecular oxygen by electrons on the acceptor side of PSI is disproportionated by superoxide dismutase (SOD) to form H2O2. Similarly, electrons from plastoquinol can also reduce oxygen at the plastid terminal oxidase (PTOX) to form superoxide, from which H2O2 is obtained. Initially, salt stress will produce a low concentration of H2O2. However, the up-regulation of the genes encoding SOD in tobacco under salt stress (Che et al., 2022b) may increase the SOD content and facilitate the rapid production of H2O2. At the same time, the down-regulation of genes encoding ascorbate peroxidase (APX), glutathione peroxidase and, to a lesser extent, peroxidase (Che et al., 2022b) may slow the scavenging of H2O2. The combined effect could be an increase in the abundance of H2O2, which could then lead to the regulation of the expression of other genes, and/or to the formation of highly-reactive hydroxyl radicals that induce damage. Thus, the smaller MR820 signal under NaCl stress (Figure 4h) could be due to the presence of fewer functional PSI complexes, either through down-regulation of PSI genes (Figure 5e) or damage, or both.

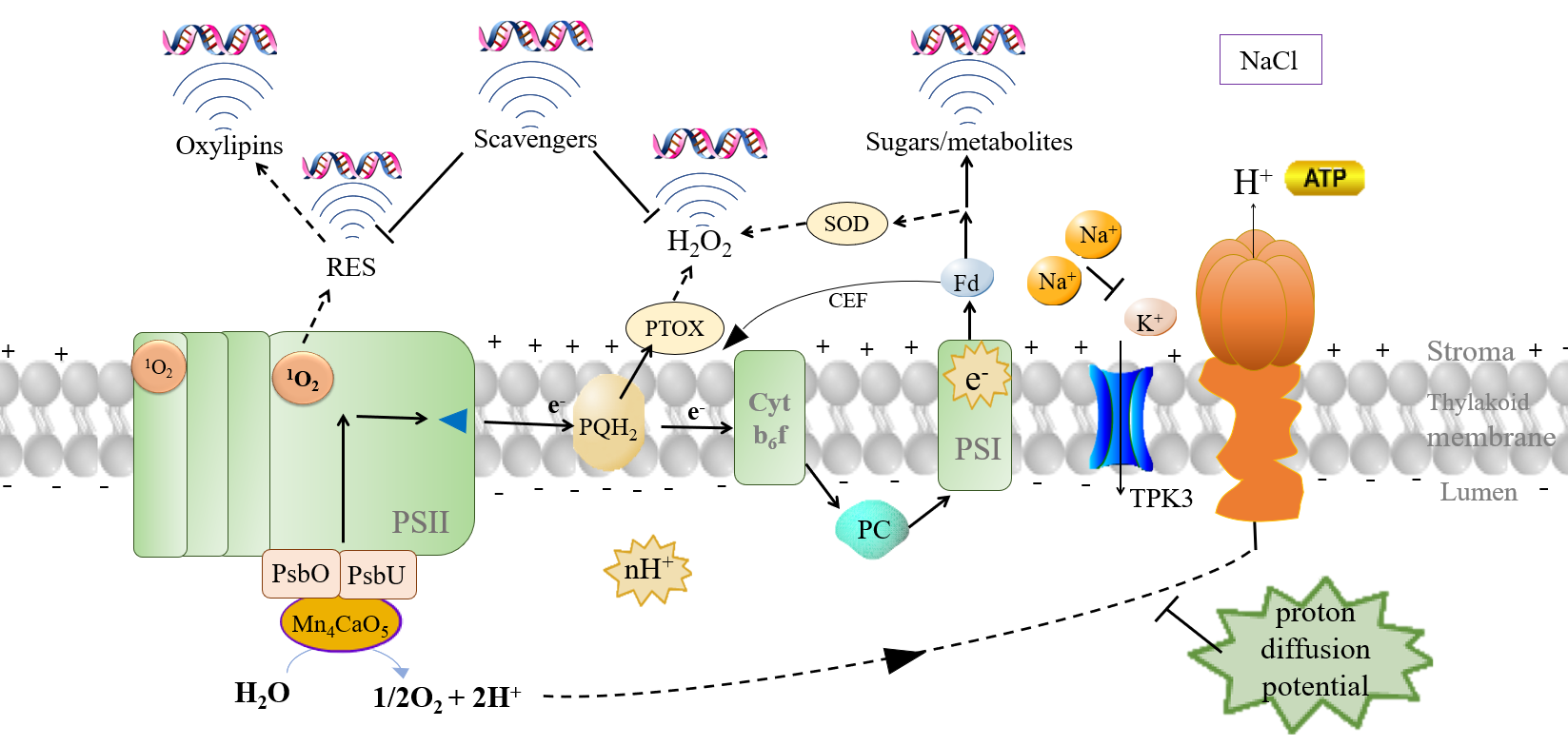


Figure 6. A scheme depicting the effects of NaCl treatment on proton translocation, electron transport and the retrograde signalling that affects gene expression. Salt stress lowers the stromal [K+], impairing the influx of K+ via the Two Pore K+ channel (TPK3) in exchange for H+ exiting the lumen via the ATP synthase. The impaired charge compensation results in a proton diffusion potential (negative in the lumen) that retards the proton efflux, slowing ATP synthesis. Consequently, carbon assimilation is slowed, so that the electron transport chain is in a highly reduced state during illumination. In such conditions, superoxide is formed on the acceptor side of PSI or at the plastid terminal oxidase (PTOX) and converted to H2O2 by superoxide terminal oxidase (SOD). In a highly reduced state of the electron transport chain, there is resistance to the downhill flow of electrons on the acceptor side of PSII (indicated by the blue triangle), resulting in more frequent charge recombination (possibly facilitated by misses of the oxygen evolving complex, Mattila, Mishra, Tyystjärvi, & Tyystjärvi, 2022) that may result in the formation of highly reactive singlet oxygen 1O2. 1O2 Can also be formed in the light-harvesting antenna by triplet chlorophyll after inter-system crossing. Singlet oxygen gives rise to reactive electrophile species (RES), and further products, oxylipins. Scavenging systems can modulate the accumulation of H2O2 and RES. All these redox species/systems can serve in retrograde signalling, as can the levels of sugars/metabolites. Re-drawn according to the scheme proposed by Gollan et al. (2015).

In particular, the backlog of electrons in the electron transport chain in salt stress conditions led to a more reduced state of QA in PSII, as indicated by the lower qP (Figure 4g). The “back pressure” against the downhill flow of electrons on the acceptor side of PSII (indicated by the blue triangle in Figure 6) promotes charge recombination to the ground state or to an excited state of chlorophyll; in either case, energy could be lost in a constitutive, **no**n-regulatory fashion, as indicated by the increase in *Y*NO+NF (Figure 4g) which includes a contribution from any non-functional (NF) PSII complexes present. In the case of charge recombination to an excited state, possibly facilitated by misses of the oxygen evolving complex (Mattila, Mishra, Tyystjärvi, & Tyystjärvi, 2022), there is some chance that triplet chlorophyll (in addition to singlet chlorophyll) is produced; when that occurs, triplet chlorophyll may react with triplet oxygen to form highly reactive singlet oxygen, 1O2 (Figure 6). 1O2 might have damaged PSII, but it appears that repair of PSII was able to mostly counteract the damage, such that the proportion of non-functional PSII was relatively small, as evidenced by only a slight decline in *F*v/*F*m (Figure 4b). Instead, 1O2 might have led to the production of reactive electrophile species (RES) which served in retrograde signalling (Figure 6). Thus, signalling by RES or the resultant oxylipins could have downregulated the expression of various nuclear-encoded genes in the PSII reaction centre (*PsbO*, *PsbP*, *PsbQ*, Figure 5f). PsbR is required to maintain the conformation of the PSII complex and to stabilize the binding of both *PsbP* and *PsbQ* (Allahverdiyeva et al., 2007). Previous work has shown that FUD39 mutants obtained by knockout of *PsbP* and *PsbQ* genes still had *PsbR* in the supercomplex, though the total amount of PSII-LHCII decreased significantly (Mayfield, Rahire, Frank, Zuber, & Rochaix, 1987), which is consistent with the results of the present study (Figure 5), namely, that the expression of *psbR* gene was increased. Additionally, H2O2 could also have participated in the signalling; indeed, there is cross-talk between 1O2- and H2O2-dependent signalling of stress responses in Arabidopsis (Laloi et al., 2007). In any case, down-regulation of gene expression appears to explain the much lower content of PSII per unit leaf area when spinach (Chow, Ball, & Anderson, 1990) or mangrove (Ball, Chow, & Anderson, 1987) plants are subjected to salt stress in the presence of a very low potassium concentration. A lower content of PSII tends to deliver fewer electron to PSI, thereby limiting the production of ROS on the acceptor side of PSI.

Having fewer functional PSII complexes per unit leaf area is not sufficient for photoprotection. Rather, limiting light-harvesting capacity obviously diminishes excitation of the photosystems, resulting in less photodamage. Indeed, the chlorophyll content, particularly that of Chl *b*, was decreased in the NaCl treatment (Figure 2) The decrease was almost certainly induced by the down-regulation of 21 chlorophyll synthesis genes (Figure 2d), accompanied by the up-regulation of genes related to Chl *b* degradation (Figure 2e). Light harvesting is one of the early steps in the synthesis of a photosystem, so the light harvesting antenna must be regulated according to the physiological state and environmental signals of the plant (Wang, Ma, Ma, Qiu, & Wen, 2021). The up-regulation of *Lhcb7* may reflect the temporary enhancement of the light harvesting ability of the plant under salt stress.

Dissipation of harvested light energy is another means of limiting photodamage. Thus, beta-carotene hydrolase (BCH), which enables the synthesis of components of the xanthophyll cycle (zeaxanthin, antheraxanthin and violaxanthin) was up-regulated in the NaCl treatment (Figure 3c), presumably facilitating energy-dependent non-photochemical quenching of excitation energy. Similarly, the gene encoding the PsbS protein that enhances energy-dependent non-photochemical quenching was also up-regulated in the NaCl treatment (Figure 5f). Although the expression of *PsbS* was up-regulated, the expression of VDE was significantly decreased, while that of ZE was increased. We have no explanation for changes in the expression of the VDE and ZE genes, which appears not to be conducive to increasing photoprotection by zeaxanthin. The above adjustments in light harvesting via decreasing antenna pigments and in energy dissipation via PsbS appear to limit the excitation energy reaching the PSII reaction centre, thereby photoprotecting PSII itself. In addition, limiting the flow of electrons from PSII to PSI limits the production of ROS. Such “damage-control” responses would ameliorate the situation in which diminished ATP synthesis leads to slower carbon assimilation.

In the NaCl treatment, genes encoding electron-transport components such as plastocyanin and ferredoxin (Figure 5c), and those encoding the ATP synthase (Figure 5d) were down-regulated. Possibly, the lower electron transport rates in the NaCl treatment did not necessitate the maintenance of an abundance of these protein complexes, hence the lower abundance of their transcripts. Instead, resources could be better deployed in counteracting the effects of salt stress. That is, energy is distributed towards defence and metabolism away from biosynthetic growth and development (Baena-González, 2010).

**Reversal of the effects of salt stress by supplemental potassium**

In the present hypothesis, supplementation with potassium in the NaCl+K treatment increases the stromal [K+] (Che et al., 2022a), so that at a given content of the potassium channel TPK3 (Carraretto et al., 2013), more K+ ions are available to rapidly enter the lumen to alleviate the proton diffusion potential that is set up as protons exit the lumen through the ATP synthase. As illumination continues, it is conceivable that [K+] increases in the lumen; in that situation, a K+ efflux and an H+ influx could occur via the KEA3 antiporter (Armbruster et al., 2016; Wang et al., 2017) to prevent excessive buildup of potassium in the lumen.

As the influx of K+ ions rapidly compensates for the efflux of protons, retardation of the proton efflux by the diffusion potential is ameliorated, so that ATP synthesis and carbon assimilation can speed up, resulting in a less reduced state of the electron transport chain. Thus, under steady illumination qP was much greater while *Y*NO+NF (which tends to increase as QA becomes more reduced) was much smaller in the NaCl+K treatment than in the NaCl treatment (Figure 4g). Additionally, during a light pulse, the pattern of the kinetic rise in the PSII chlorophyll fluorescence yield in the NaCl+K treatment was restored to be similar to that of the control, as was the extent of photo-oxidation of P700 in PSI (Figure 4).

In the presence of a less reduced state of the electron transport chain in the NaCl+K treatment, the transcript levels of PSII reaction centre proteins were generally greater than those of the NaCl treatment, and in many cases similar to those of the control (Figure 5f). The same was true of the transcript levels of light-harvesting chlorophyll-protein complex I (Figure 5a), light-harvesting chlorophyll-protein complex II (Figure 5b), plastocyanin (Figure 5c), PSI reaction centre proteins (Figure 5e), ferredoxin (Figure 5c), and ATP synthase proteins (Figure 5d). These changes in transcript levels are in line with the increases in contents (per unit leaf area) of chlorophyll, PSII assayed as atrazine-binding sites, ATP synthase, and to some extent PSI, as the [K+] increased in a background of 250 mM NaCl (Chow, Ball, & Anderson 1990). Thus, supplemental potassium reversed the effects of salt stress to a large extent.

**Effects of Lincomycin in the NaCl+K treatment**

Photoinactivation of PSII damages not only the D1 protein but also some other proteins of PSII (see, for example, Yi et al., 2022), necessitating repair of PSII by de novo protein synthesis. Lincomycin inhibits the synthesis of chloroplast-encoded proteins The addition of Lincomycin in the NaCl+K treatment led to a drastic decrease in *F*v/*F*m (Figure 4c), suggesting that almost all PSII complexes had been photoinactivated when repair of PSII was prevented. In these conditions qP remained as high as in the NaCl+K treatment (Figure 4g), suggesting that QA reduction was inhibited in photodamaged PSII complexes. At the same time, the K-band (Figure 4f) and L-band (Figure 4e) had obvious positive bands compared with other treatments, indicating that the OEC was seriously damaged and the connectivity or grouping between adjacent PSIIs was weak at the level of the antenna complex (Momchil, Lyubka, Andon, Jaco, & Vasilij 2018).

Interestingly, in the NaCl+K+Lincomycin treatment relative to the NaCl+K treatment, the transcript levels of light-harvesting chlorophyll-protein complex I (Figure 5a), light-harvesting chlorophyll-protein complex II (Figure 5b), PSII reaction centre proteins (Figure 5f), plastocyanin (Figure 5c), PSI reaction centre proteins (Figure 5e), ferredoxin (Figure 5c) and ATP synthase proteins (Figure 5d) were generally decreased. As linear electron transport was drastically diminished, H2O2-induced retrograde signally was unlikely. Instead, 1O2 produced in PSII was most likely to be primary agent leading to retrograde signally that gave rise to wide-spread down-regulation of gene expression (Figure 6). In the NaCl+K+Lincomycin treatment, when linear electron transport was drastically decreased, the much-decreased P700 photo-oxidation signal (Figure 4h) could have been due to a low abundance of PSI complexes as a result of the down regulation of gene expression. It appears that when linear electron transport was severely impaired in the NaCl+K+Lincomycin treatment, there was widespread down-regulation of gene expression, accompanied by up-regulation of genes encoding chlorophyll *b*-degradation enzymes.

**Conclusions**

In the present study, we found that treatment with NaCl suppressed vegetative growth of tobacco seedling, including expansion of cotyledons and growth of roots, while the chlorophyll content of leaves of seedlings already in their vegetative stage was decreased. Application of potassium ions reversed these effects to a large extent. The NaCl treatment also had an impact on the functions of PSII and PSI, as well as affecting the expression of genes associated with light-harvesting, electron transport and ATP synthesis, effects that were also reversed by potassium supplementation. In the context of our previous hypothesis (Che et al., 2022a), supplementary potassium seems to exert its primary effect by ameliorating the high proton diffusion potential difference across the thylakoid membrane caused by salt stress, thereby enhancing ATP synthesis, and consequently accelerating carbon assimilation and avoiding a highly-reduced state of the electron-transport chain.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

YH Che, DY Fan and WS Chow wrote the manuscript；DY Fan and GY Sun designed the research and provided fund support; YH Che, TT Yao and ZH Wang performed the experiments; YH Che, DY Fan and HH Zhang analyzed the resulting data; TT Yao and HB Zhang helped in preparing

the figures.

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