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Exogenous calcium-alleviating effect on sodium salt-induced phytotoxicity associated with changes in photosynthetic characteristics of wheat seedlings

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Abstract

To evaluate the Ca-alleviating effect on sodium salt-induced phytotoxicity, wheat (*Triticum aestivum* L.) cultivar Xihan 3 seedlings were treated with 150 mM NaCl, $CaCl_2$ (0.1, 0.5, and 1 mM), Ca^{2+}-channel blocker $LaCl_3$, and/or Ca^{2+} chelator, ethylene glycol tetraacetic acid (EGTA) alone or in combination, to investigate seedling growth and photosynthetic characteristics. NaCl (150 mM) exposure alone suppressed a growth of seedling, weakened photosynthetic efficiency and chlorophyll (Chl) fluorescence parameters, reduced photosynthetic pigments, Ca^{2+} and calmodulin (CaM) contents, and downregulated *TaCaM* expression in wheat leaves. The opposite changes of these parameters were caused by 0.5 or 1 mM $CaCl_2$ treatments alone. Moreover, 0.5 or 1 mM $CaCl_2$ application effectively alleviated sodium salt-induced changes of these parameters, which was blocked by $LaCl_3$ or EGTA. Therefore, exogenous Ca presence effectively promoted the growth of NaCl-stressed wheat seedlings through the enhancement of photosynthesis and Chl synthesis mediated by the Ca–CaM signal.

Keywords: calcium; chlorophyll fluorescence parameters; photosynthetic characteristic; photosynthetic pigment; sodium salt stress; *Triticum aestivum*.

Introduction

Photosynthesis can convert inorganic matter into organic matter (Gu *et al*. 2017), thus providing energy and material sources for plant growth and development. In recent years, soil salinization has become increasingly aggravated due to climate change, environmental deterioration, and unreasonable agricultural irrigation (Mukhopadhyay *et al*. 2021). In general, photosynthesis is the most sensitive physiological process to salinity environment and the main target of salinity toxicity (Hussain *et al*. 2017, Patanè *et al*. 2013). Gas-exchange parameters, net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and transpiration rate (E), are important indicators for evaluating plant photosynthesis (Huang *et al*. 2019). Salinity-induced inhibition of photosynthesis is reflected by the significant reduction of these four parameters in salt-tolerant and salt-sensitive wheat seedlings (Dugasa *et al*. 2019). In addition, chlorophyll (Chl) fluorescence parameters

Highlights

- The sodium salt-inhibitory effect on wheat seedling growth is alleviated by $CaCl_2$
- $CaCl_2$ application promotes photosynthesis in sodium salt-stressed wheat seedlings
- Activated Ca–CaM signal results in enhanced Chl synthesis in NaCl + $CaCl_2$-treated wheat seedlings

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Abbreviations: CaM – calmodulin; Car – carotenoid; Chl – chlorophyll; C_i – intercellular CO_2 concentration; DM – dry mass; E – transpiration rate; EGTA – ethylene glycol tetraacetic acid; ETR – electron transport rate; F_0 – minimal fluorescence yield of the dark-adapted state; F_0' – minimal fluorescence yield of the light-adapted state; FM – fresh mass; F_m – maximum fluorescence yield of the dark-adapted state; F_m' – maximal fluorescence yield of the light-adapted state; F_s – the steady-state Chl fluorescence yield; F_v – variable fluorescence; F_v/F_m – maximal quantum yield of PSII photochemistry; g_s – stomatal conductance; NPQ – nonphotochemical quenching; P_N – net photosynthetic rate; q_P – photochemical quenching; ROS – reactive oxygen species; $Y_{(II)}$ – the actual photochemical efficiency; $Y_{(NPQ)}$ – the electron yield of regulatory energy dissipation.

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can reflect the primary photosynthesis reaction. Among those, the actual photochemical efficiency ($Y_{(II)}$), electron transport rate (ETR), and photochemical quenching (q_P) represent the changes in photochemical efficiency and electron transport of the PSII (Lu *et al*. 2019), and the electron yield of regulatory energy dissipation ($Y_{(NPQ)}$) and nonphotochemical quenching (NPQ) reflect the alteration in energy dissipation of the PSII. NaCl treatment (100–300 mM) negatively affected the maximal quantum yield of PSII photochemistry (F_v/F_m) in wheat (*Triticum aestivum* L.) cultivars (Kafi 2009), while this parameter of two wheat species (*T. durum* and *T. aestivum*) remains unaltered under 50 or 100 mM sodium salt treatment (Ouerghi *et al*. 2000). In contrast to a notable decrease of ETR and q_P, $Y_{(II)}$, $Y_{(NPQ)}$, and NPQ enhance gradually in the leaves of *Haloxylon ammodendron* seedlings with the increase of the salinity concentration (Han *et al*. 2010). Photosynthetic pigments are the material basis of plant photosynthesis, and the effects of sodium salt on Chl are dependent on salt categories and concentrations, salt treatment durations, or various plant species. For example, Chl *a* and Chl *b* contents in seven tetraploid wheat genotypes (*T. durum* subspp. *dicoccum* and *T. turgidum* subspp. *durum*) decrease with increasing NaCl concentration (Tabatabaei *et al*. 2016), and the Chl content in the leaves of wild-type (*Rht-B1c*) wheat seedlings significantly increase for 7 d but decrease for 9 d under sodium salt treatment (Jusovic *et al*. 2018). Salt stress can also alter photosynthesis by affecting carotenoid (Car) or lutein in plants (Sankari *et al*. 2019, Wang *et al*. 2019a).

Calcium (Ca), one of the essential macronutrients for plant growth, participates in regulating various physiological processes in plants (Dayod *et al*. 2010). Plant organelles chloroplasts possess a high concentration of Ca^{2+} (Hochmal *et al*. 2015), and specific Ca^{2+} signals can be observed in the chloroplast under a high salt environment (Nomura *et al*. 2012). Calmodulins (CaMs), a group of well-characterized Ca^{2+} sensors, can interact with the target proteins and affect their biological activity, thereby regulating plant growth and response under environmental stresses (Zielinski 1998, Bouché *et al*. 2005, Zeng *et al*. 2015). The study of Vafadar *et al*. (2020) highlighted that the influx of extracellular Ca^{2+} through plasma membrane Ca^{2+} channels and the formation of the Ca^{2+}–CaM complex are essential in mediating *Dracocephalum kotschyi* tolerance to salt stress. Yang *et al*. (2020) found that the detection of most *TaCaM* implies the wide involvement of these genes in wheat growth and development. The overexpression of *TaCaM2-D* in *Arabidopsis* enhances plant tolerance to drought and salt treatment (Li *et al*. 2022). Ethylene glycol tetraacetate (EGTA) is a Ca^{2+} chelator, and $LaCl_3$ is a blocker of the Ca channel in the plasma membrane. Ma *et al*. (2019) reported that applying $CaCl_2$ significantly induces Ca^{2+} burst and increases the CaM content in barley under salinity stress, but the addition of $LaCl_3$ or EGTA impairs these responses.

Although the research associated with the effects of Ca on plant stress responses has attracted much attention, the mechanism of exogenous Ca regulation of photosynthetic characteristics in salt-stressed plants remains unclear. In addition, few reports have analyzed the relationship between plant growth and photosynthetic characteristics under Ca salt and sodium salt treatment alone or in combination. Our previous investigations showed that different sodium salt concentrations (100–300 mM NaCl) retarded the growth of wheat (*T. aestivum* L.) seedlings including cultivar Xihan 3 (Li *et al*. 2018a, Xu *et al*. 2021). Based on these studies, in the present study, wheat cultivar Xihan 3 seedlings were treated with 150 mM NaCl, different $CaCl_2$ concentrations, EGTA, and/or $LaCl_3$ alone or in combination to evaluate the changes in plant growth, photosynthetic characteristics, and Ca–CaM components, thereby revealing the regulatory mechanism of Ca–CaM signal involved in salinity-tolerant enhancement of wheat seedlings by adding exogenous Ca.

Materials and methods

Cultivation and treatment of plant materials: The seeds of wheat 'Xihan 3' purchased from Gansu Agricultural University were sterilized with 0.1% $HgCl_2$ for 10 min, washed with water for 8–10 h, and germinated in darkness at 25°C for 24 h. Wheat seedlings were cultured in 1/4 Hoagland nutrient solution [945 mg$(Ca(NO_3)_2)$ L^{-1}, 506 mg(KNO_3) L^{-1}, 80 mg(NH_4NO_3) L^{-1}, 136 mg(KH_2PO_4) L^{-1}, 439 mg$(MgSO_4)$ L^{-1}, 2.5 mL of EDTA-FeNa·$3H_2O$, and 2 μL of trace element] with or without NaCl, $CaCl_2$, $LaCl_3$, and/or EGTA, and the nutrient solution or treatment solution was changed every other day. At least three replicates were set for each treatment. The seedlings were cultured at 25 ± 0.5°C and 12-h light/12-h dark under a light irradiance of 300 μmol(photon) m^{-2} s^{-1} in the incubator *LRH-250-A* (*Medical Instruments Factory*, Guangdong, China). The seedlings grown for 6 d were used for detecting physiological indicators.

Growth parameters: After 6 d of growth, twenty five wheat seedlings were randomly selected from each treatment to determine root and stem lengths as well as plant fresh mass (FM). Wheat seedlings were dried at 80°C to constant mass and plant dry mass (DM) was recorded. Twenty-five wheat seedlings were chosen from each treatment to measure the length (A) and width (B) of wheat flag leaves, and the leaf area was calculated using the coefficient method (leaf area = A × B × 0.77, 0.77 in the formula is the corrected value for wheat leaf area) (Li *et al*. 2018b).

Photosynthetic gas-exchange parameters: Gas-exchange parameters including P_N, g_s, C_i, and E were determined in the fully developed young leaves (the first true leaf) using a portable photosynthesis-fluorescence measurement system (*GFS-3000*, *Heinz Walz*, Germany). The measurement was performed at 9:00–11:00 h on the sixth day after treatment. Leaf temperature was maintained at 25°C, together with 60–80% relative humidity, 600 ± 10 μmol mol^{-1} external CO_2 concentration, and 600 μmol(photon) m^{-2} s^{-1} illumination intensity (Zhou *et al*. 2016). Fifteen wheat seedlings were randomly selected from each treatment, with three biological replicates for each treatment.

Chl fluorescence parameters: *PAM-2500* Chl fluorometer (*Walz Heinz GmbH*, Effeltrich, Germany) was used to analyze Chl fluorescence parameters (Han *et al*. 2017, Zhao *et al*. 2017), such as minimal fluorescence yield of the dark-adapted state (F_0), maximum fluorescence yield of the dark-adapted state (F_m), variable fluorescence (F_v), F_v/F_m, $Y_{(II)}$, q_P, ETR, $Y_{(NPQ)}$, and NPQ. Ten seedlings were selected for each treatment, and each plant was tested three times in duplicate. Wheat leaves were first dark-adapted for 30 min to determine the F_0, and a red saturation pulse (Int: 9) was applied for 5 s to determine F_m and calculate $F_v/F_m = (F_m - F_0)/F_m$. Actinic light (red light) was applied to measure the steady-state Chl fluorescence yield (F_s). Maximal fluorescence yield of the light-adapted state (F_m') and minimal fluorescence yield of the light-adapted state (F_0') were measured in the light-adapted state, which was used to calculate $Y_{(II)}$, q_P, NPQ, and $Y_{(NPQ)}$ (Han *et al*. 2017). ETR was automatically calculated using *WinControl* software (*v2.08* and *v2.13*; *Heinz Walz GmbH*, Effeltrich, Germany) under the standard default settings for rapid light curves (Ritchie *et al*. 2021).

Photosynthetic pigment content: Fresh leaves (0.5 g) were thoroughly homogenized with 4 mL of 95% ethanol, and the homogenate was centrifuged at 9,000 × *g* for 10 min to collect the supernatants, and then the precipitate was extracted again with 4 mL of 95% ethanol and centrifuged according to the above operation. The supernatant obtained by two centrifugations was mixed and the volume was set to 25 mL. The absorbances at 470, 485, 646, and 663 nm were recorded by using a UV-Visible spectrophotometer (*UV-Vis*, *Agilent 8453*, Palo Alto, USA). The contents of Chl *a*, Chl *b*, total Chl, Car, and lutein, as well as the ratio of Chl *a* to Chl *b* (Chl *a*/*b*), were calculated according to dos Santos Araújo *et al*. (2018) and Li *et al*. (2018a).

Ca^{2+} content: The amount of Ca^{2+} was detected based on the method described by Sheteiwy *et al*. (2019). A dry sample (0.2 g) was digested with 8 mL of concentrated nitric acid in a microwave digester. The program was set to the first stage at 150°C for 5 min and a power of 1,600 W, followed by 190°C for 10 min and a power of 1,600 W in the second stage. After complete digestion, the solution was transferred and a volume was set to 50 mL with 1 M dilute nitric acid, and the Ca^{2+} content was determined by inductively coupled plasma mass spectrometer (ICP-MS) model *7500a* (*Agilent Technologies*, USA).

CaM content: The amount of CaM in 0.5 g of wheat leaves was detected by enzyme-linked immunosorbent assay (ELISA) with the kit purchased from *Hongyu Biochemical Co.*, *Ltd*.

Real-time quantitative PCR (RT-PCR) of *TaCaM* expression: To investigate the expression levels of *TaCaM*, wheat leaves were collected after culturing for 6 d. Total RNA extraction and first-strand cDNA synthesis were accomplished using *TRIZOL* reagent and a *PrimeScript*™ RT reagent kit (*Takara*, Shiga, Japan). The PCR solution (20 mm^3) consisted of 10 mm^3 of 2 × *TransStart Tip Green* qPCR super mix (*TransGen*, Beijing, China), 0.4 mm^3 of 10 μM each primer, 1.5 mm^3 of cDNA, and distilled water. RT-PCR was completed on the detection system *Bio-Rad iQ5* (CA, USA). The PCR conditions were as follows: 94°C for 30 s, followed by 40 cycles of 94°C for 5 s and 60°C for 30 s. Gene-specific primers used for real-time quantitative PCR were designed using the primer design software *Primer Premier 5* from the wheat *CaM* gene fragment (forward: 5'-GCTCTTCACAAGGCAAACATCA-3'; reverse: 5'-CTACAGAACTCAAACCGAAATCCA-3'). The *T. aestivum glyceraldehyde-3-phosphate dehydrogenase* (*TaGAPDH*) gene (forward: 5'-TTAGACTTGCGAAGCCAGCA-3'; reverse: 5'-AAATGCCCTTGAGGTTTCCC-3') was used as a reference gene. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. RT-PCR experiment was carried out three times with three biological replicates (Xu *et al*. 2021).

The data analysis: Data analysis software *SPSS* 17.0 was used for one-way analysis of variance (*ANOVA*), and the significant differences between treatments were indicated with different lowercase letters ($P \leq 0.05$). Statistical data were expressed as average value ± standard error (SE), and *Origin 2018* software was used for mapping.

Results

In this work, 150 mM NaCl stress significantly restrained the growth of wheat roots and stems with reduced leaf area and a loss in plant FM and DM (Table 1). In addition to reduced stem length, these parameters of 0.1 mM $CaCl_2$ exposure alone remained unaltered, when compared to CK. By contrast, the individual application of 0.5 mM $CaCl_2$ promoted stem growth and increased leaf area with plant FM and DM increment, 1 mM $CaCl_2$ treatment only stimulated stem growth. In contrast to 0.5 mM $CaCl_2$ alone, exposure to 5 mM $CaCl_2$ caused the opposite effects on these parameters (Table 1). Adding 0.5 or 1 mM $CaCl_2$ stimulated the growth of roots and stems and enhanced plant FM and DM in sodium salt-stressed seedlings when compared with 150 mM NaCl stress alone, and the stimulating effect of 0.5 mM $CaCl_2$ was more significant than that of 1 mM $CaCl_2$. However, the stem or root length as well as plant FM and DM under 150 mM NaCl + 0.1 mM $CaCl_2$ treatment did not alter notably. Additionally, the application of all $CaCl_2$ concentrations increased leaf area in sodium salt-stressed seedlings. Therefore, 150 mM NaCl or/and 0.1, 0.5, and 1 mM $CaCl_2$ alone or in combination were selected for subsequent study.

Photosynthetic gas-exchange parameters P_N, g_s, C_i, and E in wheat leaves significantly weakened under 150 mM NaCl stress (Fig. 1). Compared with CK, 0.1 mM $CaCl_2$ treatment alone significantly upregulated P_N, C_i, and E, respectively, but insignificantly altered g_s. By contrast, 0.5 mM $CaCl_2$ treatment alone increased P_N, g_s, C_i, and E to 1.22, 1.14, 1.27, and 1.35 folds of CK, respectively, whereas for 1 mM $CaCl_2$ treatment wheat leaves exhibited about 146 and 127% enhancement of P_N and C_i, respectively, along with decreased g_s and E (Fig. 1).

Table 1. Changes of growth parameters in wheat seedlings under different treatments. Values are expressed as the average value ± SE based on at least three replicates, and *different lowercase letters* indicate significant differences (P<0.05) according to *Duncan*'s test. CK – control, S – 150 mM NaCl, C1 – 0.1 mM $CaCl_2$, C2 – 0.5 mM $CaCl_2$, C3 – 1 mM $CaCl_2$, C4 – 5 mM $CaCl_2$, S + C1 – 150 mM NaCl + 0.1 mM $CaCl_2$, S + C2 – 150 mM NaCl + 0.5 mM $CaCl_2$, S + C3 – 150 mM NaCl + 1 mM $CaCl_2$, S + C4 – 150 mM NaCl + 5 mM $CaCl_2$. FM – fresh mass, DM – dry mass.

Treatment	Root length [cm]	Stem length [cm]	Leaf area [cm^2]	Plant FM [g per treatment]	Plant DM [g per treatment]
CK	8.43 ± 0.36^{e}	16.55 ± 0.17^{g}	4.28 ± 0.03^{g}	4.50 ± 0.07^{e}	0.47 ± 0.01^{ef}
C1	8.18 ± 0.14^{e}	16.73 ± 0.24^{g}	4.13 ± 0.01^{g}	4.21 ± 0.08^{d}	0.44 ± 0.01^{e}
C2	8.83 ± 0.28^{e}	17.63 ± 0.16^{h}	4.50 ± 0.10^{h}	4.81 ± 0.11^{f}	0.51 ± 0.01^{g}
C3	7.13 ± 0.29^{d}	17.57 ± 0.23^{h}	4.31 ± 0.02^{gh}	4.75 ± 0.12^{f}	0.47 ± 0.00^{ef}
C4	6.07 ± 0.28^{c}	14.72 ± 0.25^{f}	3.69 ± 0.08^{f}	3.84 ± 0.10^{c}	0.41 ± 0.01^{d}
S	3.81 ± 0.13^{b}	10.45 ± 0.26^{cd}	1.67 ± 0.08^{a}	2.62 ± 0.04^{a}	0.33 ± 0.01^{a}
S + C1	5.71 ± 0.33^{c}	9.58 ± 0.22^{b}	2.11 ± 0.04^{b}	2.75 ± 0.05^{a}	0.35 ± 0.01^{ab}
S + C2	6.97 ± 0.33^{d}	12.62 ± 0.32^{e}	3.17 ± 0.04^{e}	3.87 ± 0.05^{c}	0.46 ± 0.01^{ef}
S + C3	5.43 ± 0.31^{c}	12.17 ± 0.21^{e}	2.90 ± 0.01^{d}	3.74 ± 0.09^{bc}	0.39 ± 0.00^{cd}
S + C4	4.29 ± 0.17^{b}	10.97 ± 0.36^{d}	2.62 ± 0.07^{c}	3.52 ± 0.01^{b}	0.36 ± 0.00^{bc}

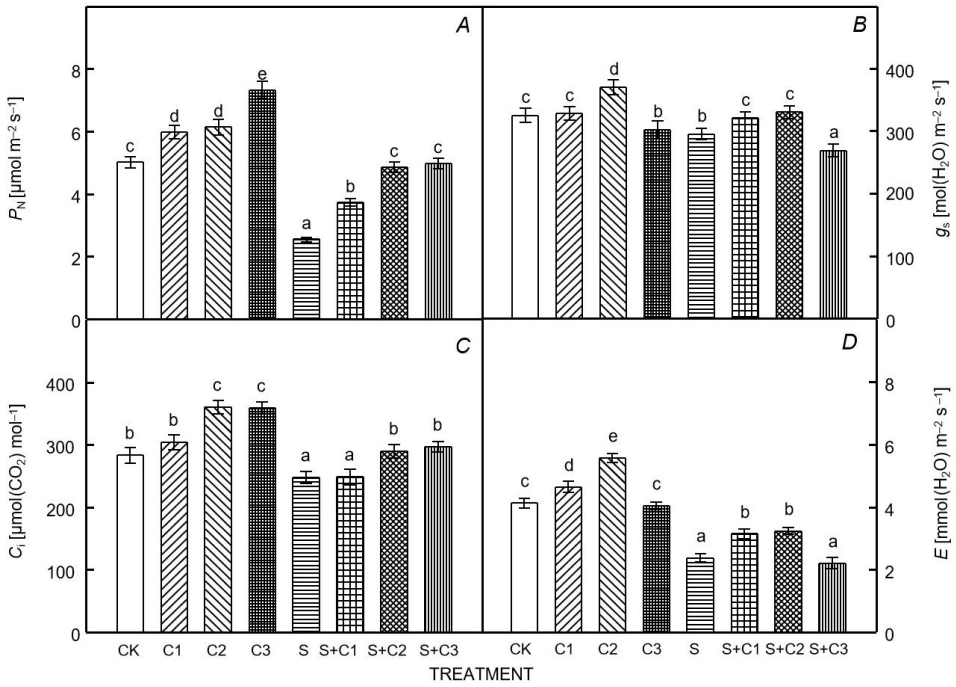

Fig. 1. Changes of (*A*) net photosynthetic rate (P_N) [μmol m^{-2} s^{-1}]; (*B*) stomatal conductance (g_s) [mol(H_2O) m^{-2} s^{-1}]; (*C*) intercellular CO_2 concentration (C_i) [μmol(CO_2) mol^{-1}]; (*D*) transpiration rate (E) [mmol(H_2O) m^{-2} s^{-1}] in wheat leaves under different treatments. CK – control, S – 150 mM NaCl, C1 – 0.1 mM $CaCl_2$, C2 – 0.5 mM $CaCl_2$, C3 – 1 mM $CaCl_2$, S + C1 – 150 mM NaCl + 0.1 mM $CaCl_2$, S + C2 – 150 mM NaCl + 0.5 mM $CaCl_2$, S + C3 – 150 mM NaCl + 1 mM $CaCl_2$. Values are expressed as the average value ± SE based on at least three replicates, and *different lowercase letters* on bars indicate significant differences (P<0.05) according to *Duncan*'s test.

In comparison with NaCl stress alone, the presence of 0.1 mM $CaCl_2$ did not change C_i but elevated P_N, g_s, and E in the leaves of sodium salt-stressed seedlings; 0.5 mM $CaCl_2$ application significantly increased these four parameters of NaCl-treated seedlings by 91, 12, 17, and 36%, respectively. However, adding 1 mM $CaCl_2$ to sodium salt-stressed wheat seedlings upregulated P_N and C_i by about 95 and 20%, respectively, together with further decreased g_s and E (Fig. 1).

Chl fluorescence parameters F_v/F_m, $Y_{(II)}$, q_P, and ETR of 150 mM NaCl stress significantly lowered in wheat leaves to 59, 30, 72, and 74% of CK, respectively (Fig. 2). By contrast, 0.1 mM $CaCl_2$ alone did not affect F_v/F_m, $Y_{(II)}$, q_P with upregulating ETR, whereas these four parameters of 0.5 mM $CaCl_2$ treatment notably elevated by about 13, 35, 17, and 26%, respectively; q_P and ETR of 1 mM $CaCl_2$ rose about 16 and 12%, respectively, when compared with CK. No statistical difference for F_v/F_m, q_P, and ETR

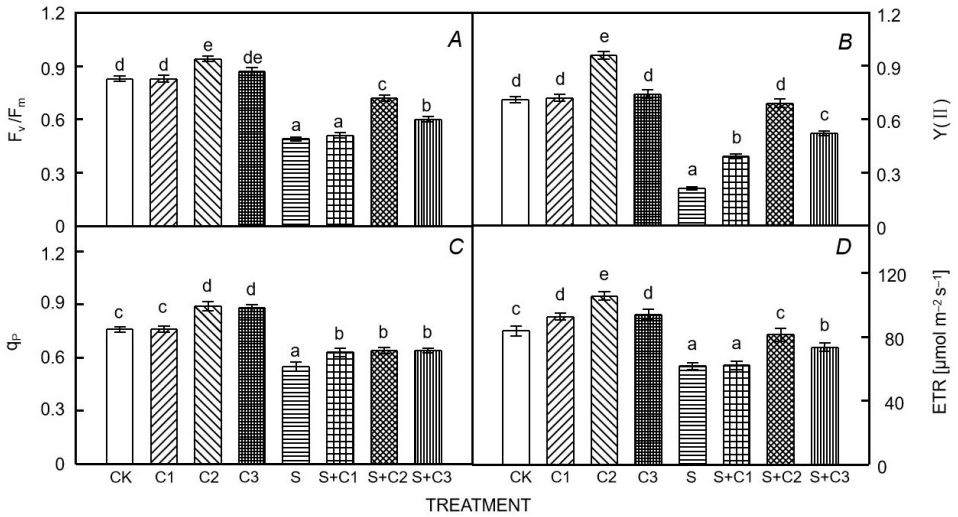

Fig. 2. Changes of (*A*) maximal quantum yield of PSII photochemistry (F_v/F_m), (*B*) the actual photochemical efficiency ($Y_{(II)}$), (*C*) photochemical quenching (q_P), and (*D*) electron transport rate (ETR) in wheat leaves under different treatments. CK – control, S – 150 mM NaCl, C1 – 0.1 mM $CaCl_2$, C2 – 0.5 mM $CaCl_2$, C3 – 1 mM $CaCl_2$, S + C1 – 150 mM NaCl + 0.1 mM $CaCl_2$, S + C2 – 150 mM NaCl + 0.5 mM $CaCl_2$, S + C3 – 150 mM NaCl + 1 mM $CaCl_2$. Values are expressed as the average value ± SE based on at least three replicates, and *different lowercase letters* on bars indicate significant differences (P<0.05) according to *Duncan*'s test.

was observed between NaCl-alone-treated and 150 mM NaCl + 0.1 mM $CaCl_2$-treated seedlings. The presence of 0.5 or 1 mM $CaCl_2$ effectively prevented NaCl-induced decreases of F_v/F_m, $Y_{(II)}$, q_P, and ETR, with about 47, 229, 16, and 32% increases due to 150 mM NaCl + 0.5 mM $CaCl_2$ treatment and 22, 148, 16, and 19% increments to 150 mM NaCl + 1 mM $CaCl_2$ exposure, respectively, when compared with sodium salt alone (Fig. 2).

In contrast, Chl fluorescence parameters $Y_{(NPQ)}$ and NPQ in wheat leaves rose significantly under NaCl stress but markedly lowered under $CaCl_2$ treatment alone, when compared with CK (Table 2). Adding $CaCl_2$ to 150 mM NaCl-stressed seedlings significantly reduced $Y_{(NPQ)}$, whereas NPQ of NaCl-stressed seedlings remained unaltered due to 0.1 or 1 mM $CaCl_2$ presence but notably lowered by about 31% under 0.5 mM $CaCl_2$ application when compared with NaCl stress alone.

Exposure to NaCl alone led to a significant reduction in the photosynthetic pigment content in wheat leaves, with approximately 20, 46, 24, 27, and 17% decreases in the amount of Chl *a*, Chl *b*, total Chl, Car, and lutein, respectively, together with about 48% increase of Chl *a*/*b*, when compared with CK (Fig. 3). By contrast, 0.1 mM $CaCl_2$ treatment alone obviously increased the content of lutein but did not affect other photosynthetic pigments, while the exposure of wheat seedlings to 0.5 or 1 mM $CaCl_2$ alone resulted in notable increases of these five parameters and an obvious decrease of Chl *a*/*b*. No significant difference of Chl *a* and total Chl contents was found between the leaves of sodium salt-alone-treated and NaCl + 0.1 mM $CaCl_2$-treated seedlings, while an obvious increase for the amount of Chl *b*, Car, and lutein was observed, together with a significant decrease in Chl *a*/*b*. Adding 0.5 or 1 mM $CaCl_2$ to sodium salt-stressed seedlings effectively enhanced Chl *a*, Chl *b*, total Chl, Car, and lutein contents to about 133, 177, 137, 119, and 152% of 150 mM NaCl treatment alone, respectively, whereas Chl *a*/*b* significantly decreased, when compared with sodium salt stress alone (Fig. 3).

Table 2. Changes of the electron yield of regulatory energy dissipation ($Y_{(NPQ)}$) and nonphotochemical quenching (NPQ) in wheat leaves under different treatments. Values are expressed as the average value ± SE based on at least three replicates, and *different lowercase letters* indicate significant differences (P<0.05) according to *Duncan*'s test. CK – control, S – 150 mM NaCl, C1 – 0.1 mM $CaCl_2$, C2 – 0.5 mM $CaCl_2$, C3 – 1 mM $CaCl_2$, S + C1 – 150 mM NaCl + 0.1 mM $CaCl_2$, S + C2 – 150 mM NaCl + 0.5 mM $CaCl_2$, S + C3 – 150 mM NaCl + 1 mM $CaCl_2$.

Treatment	$Y_{(NPQ)}$	NPQ
CK	0.39 ± 0.02^b	0.26 ± 0.01^b
C1	0.25 ± 0.02^a	0.19 ± 0.01^a
C2	0.21 ± 0.02^a	0.17 ± 0.01^a
C3	0.20 ± 0.02^a	0.17 ± 0.01^a
S	0.79 ± 0.02^d	0.39 ± 0.01^c
S + C1	0.55 ± 0.01^c	0.35 ± 0.01^c
S + C2	0.52 ± 0.02^c	0.27 ± 0.01^b
S + C3	0.53 ± 0.02^c	0.35 ± 0.02^c

Further studies were conducted to analyze the effect of Ca^{2+} channel blocker $LaCl_3$ or Ca^{2+} chelator EGTA on the Chl content in wheat leaves under the combined treatment of NaCl and $CaCl_2$. The application of 0.5 mM $LaCl_3$ or 0.5 mM EGTA significantly eliminated the $CaCl_2$-alleviating effect on sodium salt-induced destruction of Chl (Table 3). For example, the amount of Chl *a*, Chl *b*, and total Chl in the leaves of sodium

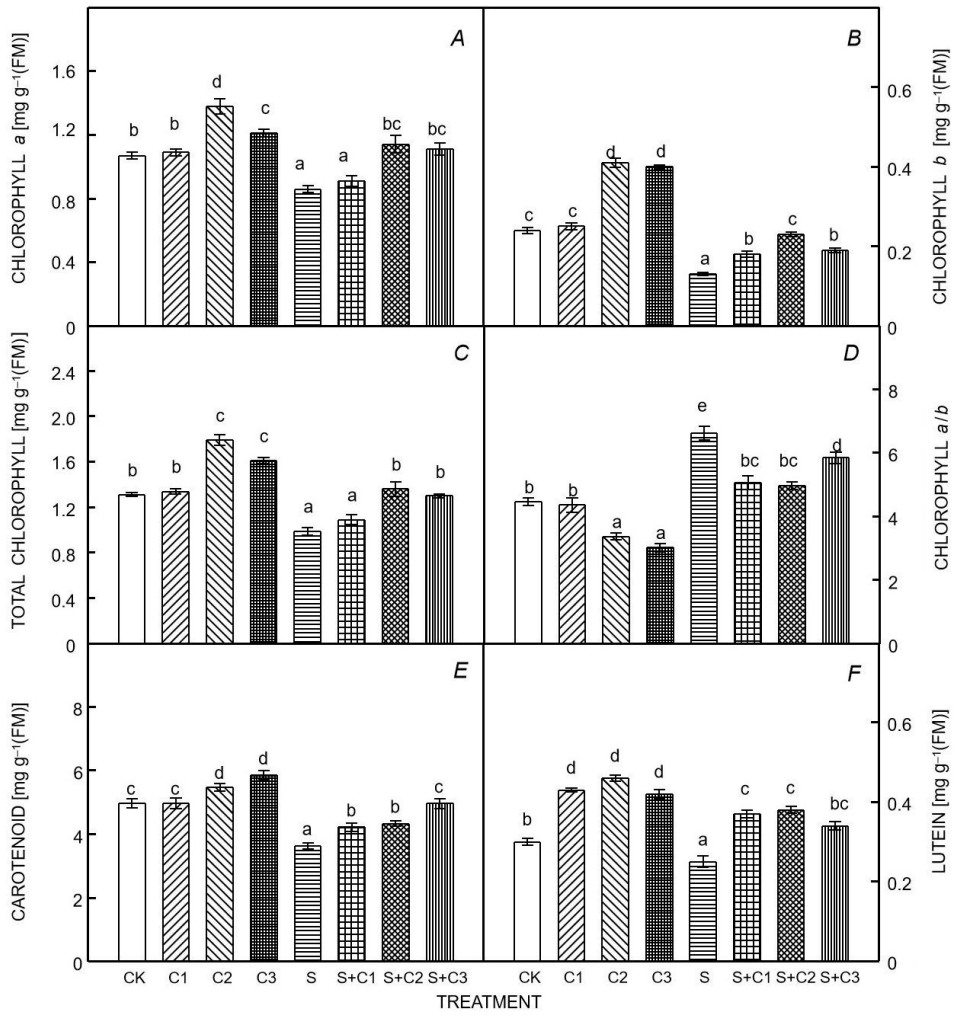

Fig. 3. Contents of chlorophyll (Chl) *a* (*A*), Chl *b* (*B*), total Chl (*C*), Car (*E*), and lutein (*F*) [mg g^{-1}(FM)] and Chl *a*/Chl *b* (*D*) in wheat leaves under different treatments. CK – control, S – 150 mM NaCl, C1 – 0.1 mM $CaCl_2$, C2 – 0.5 mM $CaCl_2$, C3 – 1 mM $CaCl_2$, S + C1 – 150 mM NaCl + 0.1 mM $CaCl_2$, S + C2 – 150 mM NaCl + 0.5 mM $CaCl_2$, S + C3 – 150 mM NaCl + 1 mM $CaCl_2$. Values are expressed as the average value ± SE based on at least three replicates, and *different lowercase letters* on bars indicate significant differences (P<0.05) according to *Duncan*'s test.

salt-stressed seedlings was 1.19 ± 0.027, 0.17 ± 0.008, and 1.36 ± 0.019 [mg g^{-1}(FM)], respectively, and the presence of $CaCl_2$ increased these three indexes to 1.26 ± 0.048, 0.29 ± 0.017, and 1.55 ± 0.024 [mg g^{-1}(FM)] in the leaves of sodium salt-treated seedlings, whereas adding 0.5 mM $LaCl_3$ to NaCl + $CaCl_2$-treated seedlings reduced the amount of Chl *a*, Chl *b*, and total Chl to 1.15 ± 0.019, 0.19 ± 0.011, and 1.34 ± 0.016 [mg g^{-1}(FM)], respectively. Similarly, the addition of 0.5 mM EGTA to NaCl + $CaCl_2$-treated seedlings reduced Chl *a*, Chl *b*, and total Chl to 1.19 ± 0.062, 0.20 ± 0.013, and 1.39 ± 0.020 [mg g^{-1}(FM)], respectively.

When compared with CK, 150 mM NaCl significantly reduced the Ca^{2+} content in wheat leaves by about 25% (Fig. 4*A*). Insignificant changes were found between CK and 0.1 mM $CaCl_2$ treatment, whereas exposure to 0.5 and 1 mM $CaCl_2$ alone notably increased the amount of Ca^{2+} to about 126 and 132% of CK, respectively. No statistical difference for the Ca^{2+} content was observed between the leaves of sodium salt-alone-stressed and NaCl + 0.1 mM $CaCl_2$-treated seedlings, while this parameter significantly increased in the leaves of sodium salt-stressed seedlings in the presence of 0.5 or 1 mM $CaCl_2$ by about 22% when compared with 150 mM NaCl stress alone.

The CaM content in the leaves of both untreated and salinity-treated seedlings notably decreased with the extension of growth time (Fig. 4*B*). In untreated seedlings that grew on days 2 and 6, the amount of CaM in the leaves was 152.26 ± 14.64 and 89.73 ± 9.24 [U g^{-1}(FM)], respectively. Compared with untreated seedlings, 150 mM NaCl-alone-stressed ones exhibited about 47 and 35% reduction of the CaM content on days 2 and 6, respectively. The CaM content of different $CaCl_2$ concentrations was significantly higher than that of CK, and the most prominent increase was due to 0.5 mM $CaCl_2$ exposure alone (Fig. 4*B*). Adding 0.1, 0.5, or 1 mM

Table 3. Changes of Chl content [mg g^{-1}(FM)] in wheat leaves under different treatments. Values are expressed as the average value ± SE based on at least three replicates, and *different lowercase letters* indicate significant differences (P<0.05) according to *Duncan*'s test. CK – control, S – 150 mM NaCl, C2 – 0.5 mM $CaCl_2$, S + C2 – 150 mM NaCl + 0.5 mM $CaCl_2$.

Treatment	Chl *a*	Chl *b*	Total Chl	Chl *a*/*b*
CK	1.51 ± 0.03^{d}	0.34 ± 0.00^{c}	1.85 ± 0.04^{c}	4.44 ± 0.21^{a}
0.5 mM $LaCl_3$	1.31 ± 0.03^{c}	0.26 ± 0.01^{b}	1.57 ± 0.02^{b}	5.04 ± 0.18^{b}
0.5 mM EGTA	1.34 ± 0.02^{c}	0.31 ± 0.01^{c}	1.65 ± 0.03^{b}	4.32 ± 0.22^{a}
S	1.19 ± 0.03^{a}	0.17 ± 0.01^{a}	1.36 ± 0.02^{a}	7.00 ± 0.26^{d}
S + C_2	1.26 ± 0.05^{b}	0.29 ± 0.02^{b}	1.55 ± 0.02^{b}	4.34 ± 0.27^{a}
S + C2 + 0.5 mM $LaCl_3$	1.15 ± 0.05^{b}	0.19 ± 0.01^{a}	1.34 ± 0.02^{a}	6.05 ± 0.28^{c}
S + C2 + 0.5 mM EGTA	1.19 ± 0.06^{a}	0.20 ± 0.01^{a}	1.39 ± 0.02^{a}	5.09 ± 0.33^{b}

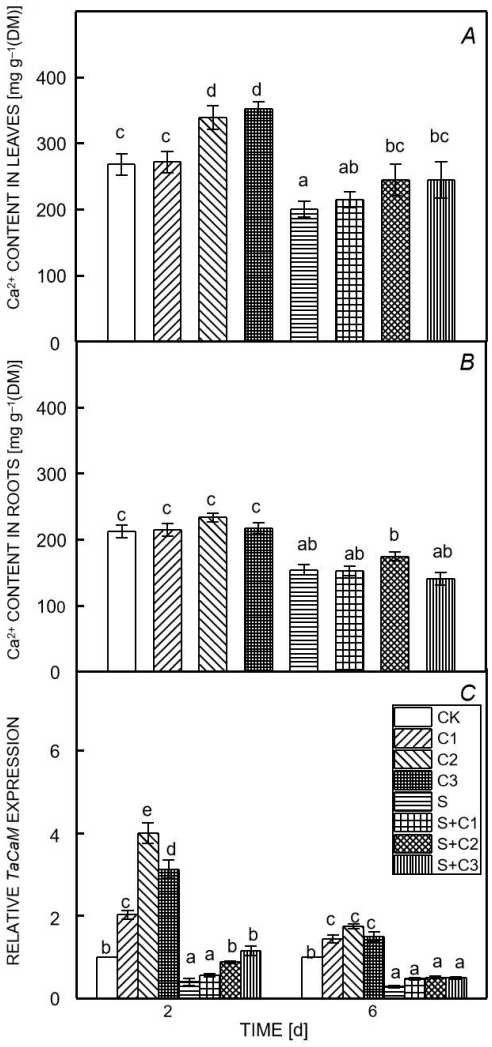

Fig. 4. Changes of Ca^{2+} content [mg g^{-1}(DM)] (*A*) and calmodulin (CaM) content (*B*) [U g^{-1}(FM)] and *TaCaM* expression (*C*) in wheat leaves measured under different treatments after 2 d and 6 d. CK – control, S – 150 mM NaCl, C1 – 0.1 mM $CaCl_2$, C2 – 0.5 mM $CaCl_2$, C3 – 1 mM $CaCl_2$, S + C1 – 150 mM NaCl + 0.1 mM $CaCl_2$, S + C2 – 150 mM NaCl + 0.5 mM $CaCl_2$, S + C3 – 150 mM NaCl + 1 mM $CaCl_2$. Values are expressed as the average value ± SE based on at least three replicates, and *different lowercase letters* on bars indicate significant differences (P<0.05) according to *Duncan*'s test.

$CaCl_2$ to wheat seedlings treated with sodium salt for 2 d increased the amount of CaM to 141, 164, and 148% of sodium salt stress alone, respectively, but did not affect this parameter on day 6. We speculated that the addition of exogenous $CaCl_2$ affected the amount of CaM in seedling leaves at the early stages of 150 mM NaCl stress.

As shown in Fig. 4*C*, stress with 150 mM NaCl on days 2 and 6 downregulated *TaCaM* expression by about 60 and 72%, respectively, in comparison with CK. However, the expression of this gene was significantly enhanced in response to different $CaCl_2$ concentrations, among which the 0.5 mM $CaCl_2$-stimulating effect was the most predominant, and the early effect of all $CaCl_2$ concentrations was notably stronger than the late effect (Fig. 4*C*). No significant difference was found between NaCl-alone-stressed and NaCl + 0.1 mM $CaCl_2$-treated seedlings, whereas the sodium salt-induced downregulation of *TaCaM* expression was effectively blocked by the application of 0.5 or 1 mM $CaCl_2$ for 2 d but remained unaltered for 6 d (Fig. 4*C*).

Discussion

Photosynthesis is the main determinant of plant growth (Chang *et al.* 2020). Photosynthetic gas-exchange parameters and Chl fluorescence parameters can reflect the influence of adverse environments on plant photosynthetic apparatus (Yin *et al.* 2019, Zhang *et al.* 2021). In the present study, photosynthetic gas-exchange parameters P_N, g_s, C_i, and E, as well as Chl fluorescence parameters F_v/F_m, $Y_{(II)}$, q_P, and ETR, notably decreased in wheat leaves under 150 mM NaCl treatment. The changes in these parameters might be due to the excessive reactive oxygen species (ROS) accumulation in wheat leaves caused by sodium chloride stress (Li *et al.* 2018a), because more ROS generation not only causes oxidative damage on plant cells but also disturbs processes within the various photosynthetic apparatus (Wu *et al.* 2023). Therefore, sodium salt-induced restraint of wheat seedling growth (Table 1) was caused by the weakening of photosynthetic capacity (Zhao *et al.* 2007). These present findings suggested that salinity-mediated decrease in photosynthesis might be attributed to stomatal constraints, such as stomatal closure and CO_2 absorption reduction, and nonstomatal factors including the reduction of leaf

area (Table 1) and photosynthetic pigment content (Fig. 4), and the inhibition of photochemical reactions. Several previous observations also demonstrated that decreased photochemical efficiency of the PSII reaction center was responsible for the inhibition of plant growth (Hu *et al.* 2022) and that the inhibition of photosynthesis was attributed to stomatal limitation (Sehar *et al*. 2019, Rasouli *et al*. 2021), when various plants were exposed to sodium salt stress. However, sodium salt-induced weakening of P_N is accompanied by increased C_i and decreased E, F_v/F_m, and q_p in the leaves of rice (*Oryza sativa* L., cv. Ningjing No. 7) seedlings (Yan *et al*. 2021). Also increased P_N of *Puccinellia distans* (Dashtebani *et al*. 2014) and *Typha domingensis* Pers. (Akhter *et al*. 2021) together with markedly decreased g_s and E under sodium salinity stress is indicating that the retardation or enhancement of photosynthesis in these plants is due to nonstomatal factors. It could be concluded from the above that sodium salt treatment mediated different stomatal responses to modify photosynthesis in various plants. Contrary to the variation of the above parameters, the obvious increases of $Y_{(NPQ)}$ and NPQ in wheat leaves under sodium salt stress indicated enhancing thermal dissipation, which was beneficial to attenuate the damage of excessive energy to photosynthetic apparatus.

The calcium-salt environment can also affect plant growth by altering photosynthesis. In the present study, notable improvement of P_N and C_i was observed in wheat leaves under all $CaCl_2$ concentrations (0.1–1 mM), along with obvious increment of E due to 0.1 mM $CaCl_2$, significant increases of g_s and E to 0.5 mM $CaCl_2$ but marked decreases of g_s and E to 1 mM $CaCl_2$. These results suggested that all $CaCl_2$ concentrations (0.1–1 mM) enhanced photosynthesis in wheat leaves because Ca^{2+} is an essential element for photosynthesis in some plants (Jones and Lunt 1967, Schwartz *et al*. 1988) and a certain Ca^{2+} concentration can maintain the stability of chloroplast structure and photosynthetic efficiency (Guo *et al*. 2023). Meanwhile, these present findings together with $CaCl_2$-induced positive effects on F_v/F_m, $Y_{(II)}$, q_p, or/and ETR also manifested that enhanced photosynthetic efficiency depended on both stomatal and nonstomatal factors under $CaCl_2$ treatment alone (Fig. 2). Differently, stomatal limitation is mainly responsible for the suppression of photosynthesis in 80 mM $Ca(NO_3)_2$-treated tomato and cucumber (Yuan *et al*. 2012, Wu *et al*. 2023). More recently, some researchers observed that combined sodium salt and Ca treatments increased P_N but decreased g_s, C_i, and E in *Populus deltoides* female clones (Miao *et al*. 2020). The present findings showed that appropriate Ca application effectively alleviated the NaCl-inhibitory effect on wheat seedling growth (Table 1). More importantly, in addition to decreased g_s and unchanged E in NaCl + 1 mM $CaCl_2$-treated seedlings, the application of 0.1–1 mM $CaCl_2$ concentrations to NaCl-exposed wheat seedlings significantly increased P_N, g_s, C_i, and E, confirming that $CaCl_2$ presence could relieve stomatal restriction to promote photosynthetic efficiency in salinity-stressed wheat seedlings. Additionally, the marked improvement of F_v/F_m, $Y_{(II)}$, q_P, or/and ETR in sodium salt + $CaCl_2$-treated wheat seedlings, along with lowered $Y_{(NPQ)}$ and unaltered or decreased NPQ, suggested that nonstomatal factors also were responsible for the enhanced photosynthesis. According to the above results in this study, we speculated that exogenous $CaCl_2$ application might relieve the damage to photosynthetic apparatus and the restriction of stomatal factors under sodium salt stress, thereby reducing heat dissipation and promoting photosynthesis and seedling growth. This is because Ca can maintain the photosynthetic capacity of plants by increasing stomatal conductivity and maintaining chloroplast membrane structure under abiotic stress (Lionetti *et al*. 2014, Xu *et al.* 2017). Our speculation was also supported by Kang *et al*. (2017), who reported that $CaCl_2$ treatment alone did not alter PSII photochemistry, but that water deficit-caused inhibition of PSII photochemistry could be blocked by adding $CaCl_2$.

Photosynthetic pigments affect the rate of photosynthesis and the formation of photosynthates (Hussain *et al*. 2021). According to the literature, the amount of Chl, Car, and lutein in the leaves of various plants gradually decreases under sodium salt stress (Shafi *et al*. 2009, Zhang *et al*. 2021, Hu *et al*. 2022). Similarly, the amount of Chl, Car, and lutein in wheat leaves significantly lowered under NaCl treatment alone, along with the obvious increase in Chl *a*/*b*, implying stronger destruction of Chl *b* concerning Chl *a* and weakened photosynthesis. Increased Chl *a*/*b* was also found in salt-adapted halophyte *Artimisia anethifolia* (Lu *et al*. 2003) and rice cultivars (Frukh *et al*. 2020) under sodium salt. Reduced Chl content might be caused by the destruction of photosynthetic apparatus and the decomposition of Chl under sodium salt stress (Shu *et al*. 2013, Yang *et al*. 2015), because sodium salt toxicity improves ROS accumulation (Li *et al*. 2018a) and the instability of protein complexes and stimulates Chl-degrading enzymes chlorophyllase and pheophorbide α oxygenase (Reddy and Vora 1986, Zhu *et al*. 2019), and ROS-induced peroxidation of membrane lipids in wheat chloroplasts enhances the direct contact between Chl and Chl-degrading enzymes and thus accelerates Chl degradation (Hörtensteiner and Kräutler 2011). Different from sodium salt treatment alone, 0.1 mM $CaCl_2$ only significantly increased lutein content, while the individual application of medium- or high-concentration $CaCl_2$ markedly improved the amount of Chl *a*, Chl *b*, Car, and lutein with an obvious decrease in Chl *a*/*b* in wheat leaves, proving that appropriate Ca concentration was necessary for the stability or synthesis of photosynthetic pigments; in particular, Chl *b* to Chl *a* was strongly dependent on the Ca content in wheat leaves. This conclusion was further supported by the Ca^{2+}-induced increase of the Chl content in rice (Cha-um *et al*. 2012), $CaCl_2$-preventing Chl degradation in butterhead lettuce (León *et al*. 2009), and the Chl *a* content of foxtail millet seedlings independent of Ca^{2+} concentration (Han *et al*. 2019). Different from these observations, exogenous Ca application causes a greater positive effect on Chl *a* than Chl *b* in honeysuckle (*Lonicera japonica*) (Huang *et al*. 2019), and high-concentration Ca as a stress factor destroyed photosynthetic apparatus and inhibited photosynthetic pigments synthesis (Yuan *et al*. 2012).

We also found that the addition of 0.5 or 1 mM $CaCl_2$ effectively blocked the reduction of photosynthetic pigment contents in wheat leaves under sodium salt treatment, further confirming that appropriate Ca^{2+} presence might be beneficial for enhancing the stability of photosynthetic pigment or/and weakening the decomposition of photosynthetic pigment in wheat leaves under sodium salt stress. Several previous studies also demonstrate that exogenous $CaCl_2$ application can partially eliminate the decrease of the Chl content and the inhibition of plant growth under sodium salt stress in honeysuckle (*Lonicera japonica*) (Huang *et al*. 2019) and foxtail millet (*Setaria italica* L.) (Han *et al*. 2019). Moreover, $CaCl_2$ treatment can successfully prevent the accumulation of $O_2^{\bullet-}$ and H_2O_2 and thus delay Chl degradation (Ganesan and Thomas 2001).

The Ca–CaM complex in the Ca-signal pathway can activate a variety of target proteins to regulate various cellular processes (Villalobo and Berchtold 2020). EGTA or/and $LaCl_3$ can inhibit Ca–CaM-mediated signal transduction (Wang *et al*. 2019b). In this experiment, EGTA or $LaCl_3$ treatment alone or combined with NaCl significantly reduced the Chl content in wheat leaves, implying that Chl metabolism might depend on Ca–CaM signal pathway. Similarly, EGTA or $LaCl_3$ can eliminate the Ca-alleviating effect on Chl destruction induced by sodium salt stress in excised etiolated barley leaves and cucumber cotyledons (Legocka *et al*. 2014). Therefore, we further analyzed Ca and CaM contents in wheat leaves under NaCl or NaCl + $CaCl_2$ treatment. Sodium salt stress alone significantly reduced the Ca and CaM contents, whereas all $CaCl_2$ concentrations markedly enhanced Ca content. On the contrary, the CaM content in tobacco (Che *et al*. 2022) and sweet tomato [*Ipomoea batatas* (L.) Lam.] (Chen *et al*. 2012) rose observably under sodium salt stress. Additionally, the CaM content together with *TaCaM* expression under NaCl + $CaCl_2$ treatment was upregulated for 2 d but remained unaltered for 6 d, further supporting the conclusion that exogenous $CaCl_2$ application enhanced the amounts of photosynthetic pigments in sodium salt-stressed seedlings through activating the Ca^{2+}–CaM messenger pathway. Similarly, exogenous Ca^{2+} addition dramatically increased the CaM content in perennial ryegrass (*Lolium perenne* L.) seedlings under sodium salt stress (Hu *et al*. 2016).

In conclusion, sodium salt treatment alone significantly retarded seedlings' growth and photosynthetic efficiency, which were related to the decreases in photosynthetic electron transport, light energy conversion efficiency, and photosynthetic pigment contents. Moderate-concentration $CaCl_2$ treatment alone or combined with sodium salt promoted the growth of wheat seedlings by enhancing the efficiency of photosynthetic electron transport light energy conversion and the amount of photosynthetic pigments. The positive effect of $CaCl_2$ application on growth and photosynthesis was associated with the adverse improvement of photosynthetic pigment contents, photosynthetic efficiency, and photochemical reactions in NaCl-stressed wheat seedlings. Additionally, exogenous Ca application promoted Chl synthesis by activating the Ca–CaM messenger system in the leaves of sodium salt-treated wheat seedlings.

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