

# Photosynthetic Responses of a Wheat Mutant (*Rht-B1c*) with Altered DELLA Proteins to Salt Stress

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**Abstract** Salinity increases in the world's land area and significantly affects the rate of photosynthesis and corresponding plant growth. In this study, the impact of salt stress (200 mM NaCl equivalent to an electrical conductivity of 18.6 mS cm<sup>-1</sup>) on the photosynthetic apparatus and some growth parameters were investigated in wheat DELLA mutant (*Rht-B1c*) and wild-type (*Rht-B1a*) seedlings grown on a half-strength Hoagland solution. Results revealed that salt toxicity was alleviated in the *Rht-B1c* mutant compared to the *Rht-B1a* wild type, as manifested by less-reduced leaf pigment content, relative water content, and photochemical activity of photosystem II (PSII) and photosystem I (PSI) after a 9-day salt exposure of plants. Compared to the wild-type wheat, a higher capacity for PSI-dependent cyclic electron flow, preventing the photosynthetic apparatus from oxidative damage, was observed in the mutant plants before and after salt treatment. In addition, an increase of Psab proteins

was detected in the mutant plants after long-term salt stress unlike the wild type. The observed higher oxidation level of P700 (P700<sup>+</sup>) in the mutant was consistent with higher abundance of PSI-related protein complexes. The data demonstrated that alterations in thylakoid membrane proteins and/or their structural reorganization in wheat DELLA mutant (*Rht-B1c*) significantly contribute to the alleviation of salt-induced damage of the photosynthetic apparatus. Molecular mechanisms involved in the photosynthetic responses of wheat DELLA mutants to salt stress are discussed.

**Keywords** Chlorophyll fluorescence · Cyclic electron flow · DELLA proteins · Photochemical activity · Pigments · *Rht-B1c* mutant · Salt stress tolerance

## Introduction

Salinity is one of the most severe environmental factors limiting productivity of crop plants (Parida and Das 2005; Munns and Tester 2008). High concentrations of salts (especially the accumulation of toxic ions, such as Na<sup>+</sup> and Cl<sup>-</sup>) have various effects on plants, including ionic imbalance, dehydration, and inactivation of enzymes, that influence the metabolic processes, photosynthetic efficiency, plant growth, and thus plant yields (Tester and Davenport 2003; Sudhir and Murthy 2004). Depending on the degree of salinity, its duration, and the plant species employed, the increased amount of salts may also cause destruction of the chloroplast envelope and disintegration of the granal thylakoid systems (Shu and others 2012), accompanied by a decrease in the amount of photosynthetic pigments in leaves. Some reports have suggested that salt-induced phytotoxicity can be prompted by the formation of reactive oxygen species (ROS)

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and subsequent oxidative damage (Sairam and others 2002; Munns and Tester 2008).

Photosynthesis is the primary physiological process to be affected by salinity in all its phases (Sudhir and Murthy 2004; Ashraf and Harris 2013). The effects of salt stress on the photosynthetic apparatus are either direct or secondary as a consequence of the oxidative stress (Chaves and others 2009). Because photosynthesis is a very complex process, including photosynthetic pigments, reaction-center complexes, and the electron transport system, damage at any level caused by salt stress may reduce the overall photosynthetic capacity of a green plant (Sudhir and Murthy 2004; Parida and Das 2005; Chaves and others 2009). Salinity stress reduces the photosynthetic rate by inhibiting PSII at both the acceptor (quinone  $Q_A$ ) and donor sides (oxygen-evolving complex) and by destruction of chlorophylls (see Athar and others 2015).

Achieving stable crop production in saline environments depends largely on the ability of plants to maintain their functions under stress conditions. One of the mechanisms for adaptive modulation of plant growth in response to adverse environmental signals, including salinity stress, is mediated by plant hormones. A probable cross-talk between cytokinin, jasmonic acid (JA), gibberellic acid (GA), and abscisic acid (ABA) signaling in the salinity stress response has been suggested (for review see Nongpiur and others 2016). This cross-talk shows the positive roles of JA and ABA in the salinity stress response, whereas cytokinins and GA have a negative regulatory role.

DELLA proteins have been considered pivotal regulators that mediate the cross-talk of various phytohormones and integrate plant responses to both hormonal and environmental cues (Hou and others 2010; Davière and Achard 2016). The accumulation of DELLA proteins restrains growth and enhances stress tolerance through reduced GA signaling activity and stimulating the expression of some ROS-detoxification enzymes (Achard and others 2008; Harberd and others 2009). Previous studies in *Arabidopsis* have reported that DELLA proteins act as a positive regulator of the salinity stress response by interacting with XERICO, an inducer of ABA biosynthesis, or by inhibiting JAZ proteins, which are negative regulators of the salinity stress response (Nongpiur and others 2016 and refs therein). Although insights into the regulation of plant growth by DELLA proteins have advanced rapidly in *Arabidopsis*, DELLA-mediated regulation of downstream responses in cereals has received little attention to date (see Van De Velde and others 2017).

In wheat, DELLA proteins are encoded by the wild alleles of the reduced height (*Rht*) genes, well known for their impact in the ‘Green Revolution’ wheat cultivars (Saville and others 2012). The allele *Rht-B1c* (*Rht3*) is a mutation within the N-terminal DELLA domain at the *Rht-B1* locus resulting in altered DELLA proteins (Flintham and Gale

1982; Wen and others 2013). The mutant DELLA proteins are unable to interact with the GA receptor, resulting in reduced GA-responsiveness even at adequate hormone levels. This causes DELLA proteins accumulation and, hence, plant height reduction possibly by interfering with the activity of growth promoting transcription factors (Harberd and others 2009; Pearce and others 2011).

Recently, a possible role for *Rht-B1*-encoded DELLA proteins in protective mechanisms and tolerance of the photosynthetic apparatus in wheat plants exposed to drought or cadmium stress has been suggested (Nenova and others 2014; Dobrikova and others 2017). The elucidation of the possible mechanisms of action of wheat DELLA proteins in the plant responses to salinity stress is an important step for better understanding of plant adaptation and acclimation mechanisms, as well as for improving salt tolerance in wheat. The purpose of this study was to examine the role of the mutant DELLA-encoding gene (*Rht-B1c*) in the photosynthetic tolerance of wheat seedlings to moderate salt stress. The relative sensitivity to 200 mM NaCl of two wheat genotypes: *Rht-B1a* (wild type, encoding DELLA proteins) and *Rht-B1c* (dwarf mutant, encoding modified DELLA proteins) was assessed by studying changes in some plant growth parameters, photosynthetic pigments, and the amount of reaction-center proteins of PSI and PSII. The functional activity of the photosynthetic apparatus was also investigated by chlorophyll fluorescence measurements and oxidation–reduction kinetics of P700 with respect to the time of treatment. Data are discussed in the light of possible mechanisms of DELLA protein-mediated acclimation of the photosynthetic apparatus to salt stress.

## Materials and Methods

### Plant Material and Salt Treatment

Two wheat (*Triticum aestivum* L., cv. April Bearded) lines near-isogenic for the *Rht-B1a* allele (wild type, tall plants) and *Rht-B1c* allele (mutant, dwarf plants) were used in this study. The plant material was provided by the seed genebank of the Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK) Gatersleben, Germany and maintained in the experimental field of the Institute of Plant Physiology and Genetics, Sofia, Bulgaria.

Wheat seeds were germinated on moist filter paper in the dark for 3 days. After germination, seedlings were grown hydroponically in pots filled with a half-strength Hoagland nutrient solution (pH 6.0) in a growth chamber with 75% relative humidity, a light intensity of approximately 250  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and 12-h daylight photoperiod at 24/19 °C light/dark. Saline conditions were simulated by employing stock solution of NaCl in distilled water, which was added

to the ½ Hoagland solution to a final concentration of 0 (for control) and 200 mM with conductivity equal to 1.03 and 18.6 mS cm<sup>-1</sup>, respectively. The stress was imposed on 10-day-old seedlings as the nutrient solution was replaced every 3 days. The analyses in vivo were performed on plants exposed to moderate salinity for 2, 7, and 9 days.

### Determination of Relative Water Content of Leaves

Because the dry weight of plants was not significantly affected until the 9th day of treatment, the water status of the plants was checked only at the 9th day. Ten leaves were cut for each of the treatments. Relative water content (RWC) was calculated using the following equation:  $RWC (\%) = [(FW - DW) / (SW - DW)] \times 100$ , where FW is the fresh weight, SW is the water-saturated weight after soaking the leaves in water for 24 h, and DW is the dry weight estimated after drying in an oven for about 12 h at 80 °C or until a constant weight was achieved (Kocheva and others 2014).

### Pigment Analysis

Samples for pigment analysis were collected from fully expanded fresh leaves, which were homogenized in ice-cold 80% (v/v) acetone. Then the homogenates were centrifuged at 5000×g for 5 min at 4 °C. The chlorophyll (Chl) content (Chl *a* and Chl *b*) and total carotenoids (Car) were measured with a spectrophotometer (SPECORD 210 PLUS, Edition 2010, Analytik-Jena AG, Germany) using the method of Lichtenthaler (1987). This method involves measurement of the light absorbed in the plant extract at 470, 646.8, and 663.2 nm, respectively. The pigment content was calculated by the equations of Lichtenthaler (1987).

### Pulse-Amplitude-Modulated (PAM) Chlorophyll Fluorescence

The in vivo chlorophyll fluorescence at room temperature was measured in dark-adapted leaves using a PAM fluorometer (model 101/103, Walz GmbH, Effeltrich, Germany). The minimum fluorescence level ( $F_0$ ) was detected after at least 20 min of dark adaptation by applying a very weak modulated light (0.02 μmol m<sup>-2</sup> s<sup>-1</sup> PPF). The maximum fluorescence levels in the dark-adapted state ( $F_m$ ), the light-adapted state ( $F_m'$ ), and after dark relaxation following the light period ( $F_m''$ ) were obtained by illuminating the leaf samples with a saturating short flash (2500 μmol m<sup>-2</sup> s<sup>-1</sup> PPF, 0.8 s). Photosynthesis was induced by continuous illumination of leaves with actinic light at 250 μmol m<sup>-2</sup> s<sup>-1</sup> PPF, which corresponds to the growth illumination of plants. The steady-state fluorescence level ( $F'$ ) was estimated 7–8 min after turning on the actinic light. The following parameters were determined: the maximum quantum

efficiency of PSII in the dark-adapted state ( $F_v/F_m = (F_m - F_0)/F_m$ ), the effective quantum yield of PSII photochemistry ( $\Phi_{PSII} = (F_m' - F')/F_m'$ ), photochemical ( $qP = (F_m' - F')/F_v'$ ), and non-photochemical (NPQ,  $qE$ ) quenching coefficients, calculated as  $NPQ = (F_m/F_m') - 1$ ,  $qE = (F_m/F_m') - (F_m/F_m'')$  (see Baker 2008; Zivcak and others 2015).

### P700 Redox State Measurements

Steady-state P700 photooxidation (P700<sup>+</sup>) was measured by illumination of the dark-adapted detached leaves with far-red (FR) light supplied by a photodiode (102-FR, Walz, Effeltrich, Germany). The redox changes in P700 were monitored as the far-red light-induced absorbance changes at approximately 830 nm ( $\Delta A_{830}$ ) measured with the PAM 101/103 fluorometer equipped with an ED-800T emitter detector.

### Isolation of Thylakoid Membranes

Thylakoid membranes were isolated from leaves of 9-day-treated plants as described previously (Harrison and Melis 1992) and then were resuspended in a buffer containing 40 mM Tricine (pH 7.6), 10 mM NaCl, 5 mM KCl, 5 mM MgCl<sub>2</sub>, and 400 mM sucrose to appropriate chlorophyll concentrations for further analysis. Analysis of isolated thylakoid membranes was performed on 9-day-treated plants as on the 9th day of salinity, the difference in salt tolerance of the wild type and mutant was better exhibited.

### Low-Temperature (77 K) Chlorophyll Fluorescence

The low-temperature (77 K) chlorophyll fluorescence emission and excitation spectra were measured using a Jobin Yvon (JY3) spectrofluorimeter equipped with red sensitive photomultiplier (Hamamatsu 928) and a liquid-nitrogen device. The isolated thylakoid membranes at a Chl concentration of 20 mL<sup>-1</sup> were quickly frozen in a cylindrical quartz cuvette by plunging into liquid nitrogen. The low-temperature (77 K) chlorophyll fluorescence emission spectra were recorded from 650 to 780 nm with a slit width of 4 nm. The chlorophyll fluorescence was excited at 436 nm (Chl *a*) and 472 nm (Chl *b*). The 77 K fluorescence excitation spectra were monitored at 743 nm.

### SDS-PAGE Electrophoresis and Western blot

The alterations in reaction-center proteins of PSI and PSII of isolated thylakoid membranes from wild-type (*Rht-B1a*) and mutant (*Rht-B1c*) plants, control and salt-treated plants (200 mM NaCl), were analyzed by a Laemmli (1970) SDS-PAGE gel system, modified by adding 4 M urea in the resolving gel. The polyacrylamide concentrations of stacking and resolving gels were 4 and 12%, respectively.

The samples were incubated with sample buffer (3:1) in the dark for one hour at room temperature. Equal volumes of thylakoid membranes from control and treated samples were loaded in every line (20  $\mu$ l), corresponding to 4.5 mg Chl. The proteins from the SDS-PAGE gel was transferred to a PVDF membrane, blocked, and immunodetected with antibodies against D1 and PsaB (AS05 084-10 and AS10 695, respectively, from Agrisera, Sweden) was performed. The membranes were revealed using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad, Hercules, CA) with a goat anti-rabbit (GAR) secondary antibody. The sensitivity of the applied kit is up to 100  $\mu$ g protein. Densitometry analysis (determination of pixel density on membrane spots) was carried out using the Phoretix image analysis software (Phoretix International, Newcastle upon Tyne, UK) and the results were presented as a percent from signals of wild-type thylakoid membranes. The mean values for D1 and PsaB are calculated from 3 and 4 experiments, respectively. Although the observed differences are not considerable, it is a tendency in all experiments.

### Statistical Analysis

The results were calculated and averaged from 3 to 4 independent experiments. All the assays from each independent treatment were carried out in triplicate. The statistical differences among the mean values ( $\pm$  SE) were determined using ANOVA. Values of  $p < 0.05$  and  $p < 0.01$  were considered as significant differences.

## Results

### Effect of Salt Stress on Plant Growth and Leaf RWC

The exposure of wheat plants to 200 mM NaCl resulted in a considerable decrease in root and leaf length (Table 1, Supplementary Fig. S1). In the wild-type plants, the roots were more salt sensitive than the leaves, as the root length was reduced about two times after the treatment. For wild-type wheat, the growth tolerance index, defined as the ratio

between root or leaf length under stress and the corresponding ones in controls, was 42.7 and 65.3%, respectively. In mutant plants, the growth tolerance index to salt stress was higher in comparison to wild-type plants for both root (81.6%) and leaf (73.9%) length (Table 1).

After 9 days of NaCl exposure, RWC (%) declined significantly in both wheat genotypes when compared to the untreated ones (Table 1). During the treatment, higher RWC was observed in the mutant (78.4%) than in the wild type (71.6%).

### Pigment Analysis

Among untreated wheat genotypes, the pigment content was higher in the mutant than in the wild type during all days of growth (Table 2). In the wild type, the content of Chl *a* and *b* significantly decreased by 35.6 and 37.7%, respectively, after 2 days of salt treatment. A similar decrease was observed after 7 and 9 days of treatment (by about 24–28%). The reduction of Chl content was more considerable than that of Car content—by about 28 and 22% on the 2nd and 9th days after exposure to salt stress, respectively (Table 2). In mutant plants, the decrease in the content of Chl *a* and *b* was lower than in the wild type (by around 13–16%) observed after 2, 7, and 9 days of NaCl exposure. The salt-induced changes in pigment composition resulted in a significant increase of the Chl *a/b* ratio only in the wild type (Table 2). The Car content in mutant leaves was slightly reduced (by about 14%) after all days of applied salt stress (Table 2). Therefore, the decrease in the amount of all pigments after 200 mM NaCl exposure was more pronounced in the wild type than in the mutant genotype.

### PAM Chlorophyll Fluorescence Measurements

The chlorophyll fluorescence parameters were measured *in vivo* with a pulse-modulated (PAM) fluorometer to assess the salt-induced changes in the PSII photochemistry for both wheat genotypes. The parameters  $F_v/F_m$ ,  $\Phi_{PSII}$ ,  $qP$ , and NPQ are usually used as sensitive indicators of plant photosynthetic performance (Baker 2008). Our results demonstrated

**Table 1** Effect of 9-day-long salt stress on plant growth parameters and relative water content (RWC) of leaves from both wheat seedlings: *Rht-B1a* (wild type) and *Rht-B1c* (mutant)

Genotype	NaCl (mM)	Leaf length (cm)	% tolerance	Root length (cm)	% tolerance	RWC (%)
<i>Rht-B1a</i>	0	23.9 $\pm$ 1.4 <sup>a</sup>	100	16.4 $\pm$ 2.4 <sup>a</sup>	100	80.3 $\pm$ 2.3 <sup>b</sup>
	200	15.6 $\pm$ 1.9 <sup>b</sup>	65.3	7.0 $\pm$ 0.7 <sup>c</sup>	42.7	71.6 $\pm$ 1.3 <sup>c</sup>
<i>Rht-B1c</i>	0	11.9 $\pm$ 0.8 <sup>c</sup>	100	10.9 $\pm$ 1.3 <sup>b</sup>	100	86.4 $\pm$ 2.5 <sup>a</sup>
	200	8.8 $\pm$ 0.4 <sup>d</sup>	73.9	8.9 $\pm$ 0.7 <sup>bc</sup>	81.6	78.4 $\pm$ 1.9 <sup>b</sup>

The results were averaged from 4 independent experiments with 6–10 replications each. Different letters indicate significant differences between values in the same column ( $p < 0.05$ )

**Table 2** Effect of 200 mM NaCl treatment on leaf pigment content of both wheat seedlings: *Rht-B1a* (wild type) and *Rht-B1c* (mutant)

Time (days)	NaCl (mM)	Chl <i>a</i> ( $\mu\text{g g}^{-1}\text{FW}$ )	Chl <i>b</i> ( $\mu\text{g g}^{-1}\text{FW}$ )	Car ( $\mu\text{g g}^{-1}\text{FW}$ )	Chl <i>a/b</i>
<i>Rht-B1a</i>					
2 days	0	2202 ± 51 <sup>c</sup>	552 ± 18 <sup>b</sup>	614 ± 13 <sup>b</sup>	3.99 ± 0.04 <sup>b</sup>
	200	1417 ± 79 <sup>e</sup>	344 ± 23 <sup>d</sup>	441 ± 21 <sup>d</sup>	4.12 ± 0.04 <sup>a</sup>
7 days	0	2395 ± 62 <sup>b</sup>	606 ± 37 <sup>ab</sup>	631 ± 35 <sup>ab</sup>	3.95 ± 0.13 <sup>b</sup>
	200	1828 ± 41 <sup>d</sup>	445 ± 35 <sup>c</sup>	516 ± 21 <sup>c</sup>	4.12 ± 0.18 <sup>ab</sup>
9 days	0	2128 ± 73 <sup>c</sup>	531 ± 21 <sup>b</sup>	576 ± 43 <sup>b</sup>	4.01 ± 0.03 <sup>b</sup>
	200	1590 ± 37 <sup>e</sup>	380 ± 11 <sup>d</sup>	451 ± 21 <sup>d</sup>	4.20 ± 0.04 <sup>a</sup>
<i>Rht-B1c</i>					
2 days	0	2520 ± 86 <sup>b</sup>	612 ± 41 <sup>ab</sup>	720 ± 48 <sup>a</sup>	4.12 ± 0.15 <sup>ab</sup>
	200	2182 ± 51 <sup>c</sup>	532 ± 28 <sup>b</sup>	618 ± 32 <sup>b</sup>	4.10 ± 0.12 <sup>ab</sup>
7 days	0	2719 ± 39 <sup>a</sup>	684 ± 34 <sup>a</sup>	692 ± 41 <sup>a</sup>	3.97 ± 0.12 <sup>b</sup>
	200	2283 ± 41 <sup>c</sup>	571 ± 31 <sup>b</sup>	592 ± 28 <sup>b</sup>	3.99 ± 0.13 <sup>b</sup>
9 days	0	2571 ± 63 <sup>b</sup>	635 ± 34 <sup>a</sup>	660 ± 33 <sup>a</sup>	4.05 ± 0.11 <sup>ab</sup>
	200	2160 ± 33 <sup>c</sup>	537 ± 33 <sup>b</sup>	568 ± 32 <sup>b</sup>	4.02 ± 0.18 <sup>ab</sup>

Mean values (±SE) were calculated from 4 independent experiments with 3 replications each

Different letters indicate significant differences between values in the same column ( $p < 0.05$ ,  $p < 0.01$ )

that salt stress did not significantly affect the maximum quantum yield of PSII photochemistry during all days of NaCl exposure of both wheat genotypes—the ratio Fv/Fm did not change significantly (data not shown). Changes in PSII activity in response to salt stress were studied by determining the effective quantum yield of PSII photochemistry ( $\Phi_{\text{PSII}}$ ), photochemical quenching ( $qP$ ), non-photochemical quenching (NPQ), and energy-dependent quenching or pH-dependent energy dissipation ( $qE$ ) in both wild-type and mutant wheat plants after 2nd, 7th, and 9th days of salt treatment (Fig. 1). The effective quantum yield of PSII photochemistry (Fig. 1A) and the photochemical quenching (Fig. 1B) were reduced gradually with increasing time of salt treatment of wild-type wheat, as these parameters decreased to 74 and 79%, respectively, after 9 days of treatment. In contrast, the efficiency of PSII photochemistry in mutant plants after the 2nd day of salt stress significantly decreased (more strongly than wild type) by approximately 21% compared to that of the untreated mutant plants ( $p < 0.01$ ). With increasing the days of treatment, these values increased slightly, but the differences were insignificant (Fig. 1A). On the 9th day of salt treatment, the PSII photochemistry (parameter  $\Phi_{\text{PSII}}$ ) in the mutant was higher than that of the wild type (Fig. 1A). Similar alterations were also observed for photochemical quenching (Fig. 1B), as after 7 and 9 days of salt treatment, there was again a slight increase in this parameter in the mutant compared to the wild type. Therefore, after long-term salt treatment for 9 days, the PSII photochemical parameters:  $\Phi_{\text{PSII}}$  and  $qP$ , were reduced more pronouncedly in the wild type than in mutant wheat plants.

In wild-type plants, increased NPQ values were detected for all days of NaCl exposure. The values of NPQ significantly increased by 20 and 35.5% ( $p < 0.01$ ) after 7 and

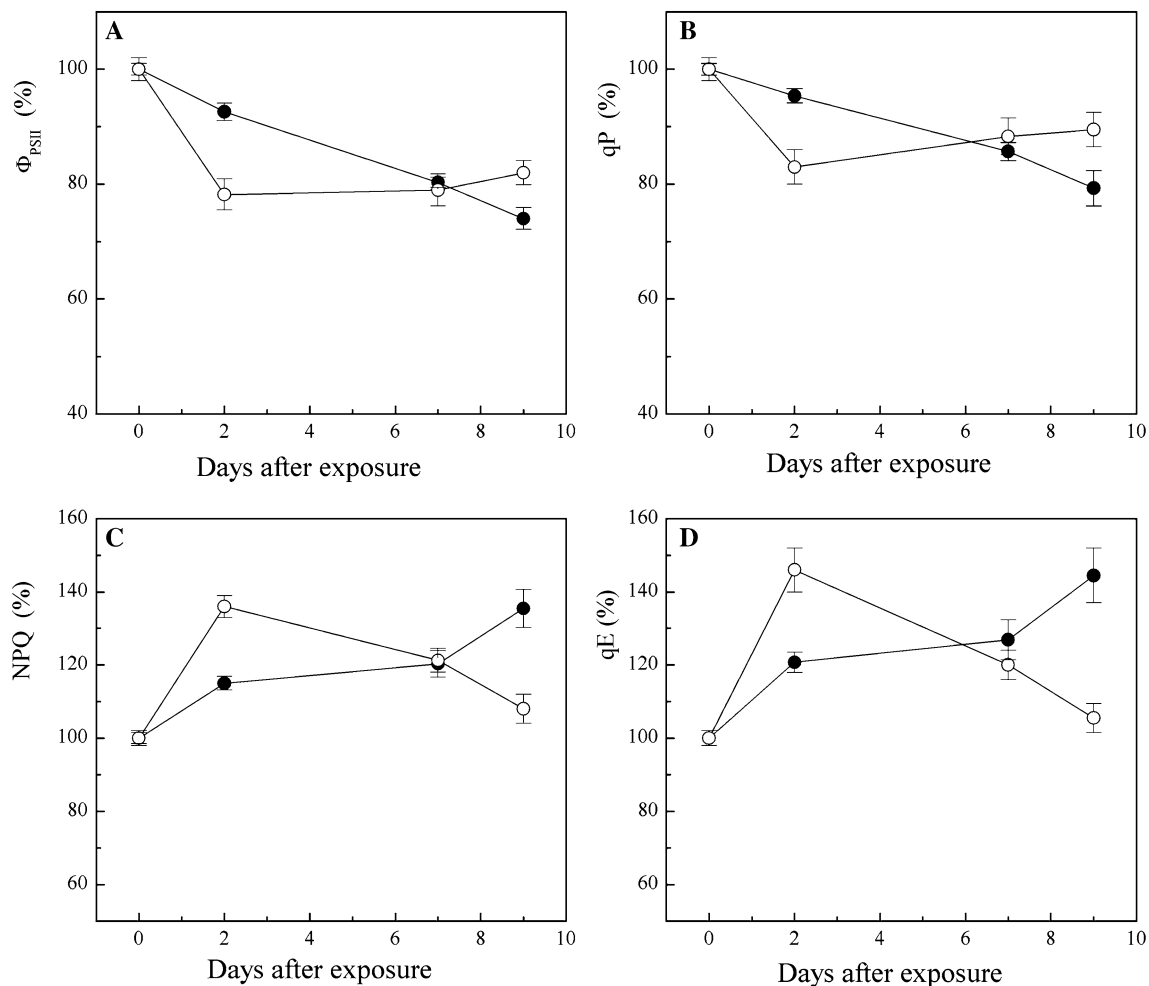
9 days of the treatment, respectively (Fig. 1C). The NPQ values in mutant plants strongly increased by about 36% ( $p < 0.01$ ) after the 2nd day of treatment in comparison to untreated plants, but this value started to decrease after 7 days of salt exposure (with increasing the time of treatment), and reached the control values (100%) on the 9th day of treatment (Fig. 1C).

The values of energy-dependent quenching ( $qE$ ) were also gradually increased with increasing the time of treatment for wild-type plants, a considerable increase in  $qE$  value was observed after the 7th and 9th days of salt treatment by about 27 and 45%, respectively (Fig. 1D). On the contrary, in mutant plants a strong increase in  $qE$  value by 46% ( $p < 0.01$ ) was observed on the 2nd day of the treatment and the  $qE$  values decreased after the 7th day reaching the control values at the 9th day.

Therefore, after long-term salt treatment, the non-photochemical quenching coefficients (NPQ,  $qE$ ) were strongly increased in wild-type wheat and less altered in mutant wheat leaves.

### Oxidation–Reduction Kinetics of P700

To characterize the effect of salt stress on PSI photochemistry of both wheat genotypes, we measured steady-state P700 photooxidation ( $\text{P700}^+$ ) by FR light-induced absorbance changes at 830 nm ( $\Delta A_{830}$ ). The  $\text{P700}^+$  reduction in the dark after turning off the FR light was characterized by half-times of the exponential decay ( $t_{1/2}$ ) (see in Dobrikova and others 2017). The dependence of the relative amplitudes of  $\text{P700}^+$  absorbance changes ( $\Delta A_{830}$ ) and half-times of  $\text{P700}^+$  dark relaxation decay ( $t_{1/2}$ ) on the time of exposure to 200 mM NaCl for both



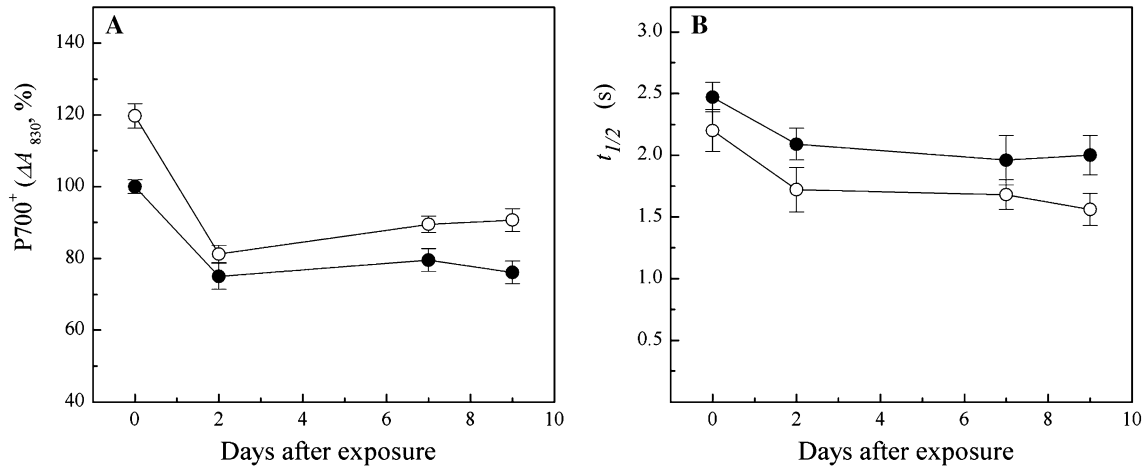
**Fig. 1** Effects of 200 mM NaCl treatment on chlorophyll fluorescence parameters in leaves from both wheat genotypes: *Rht-B1a* (filled circle) and *Rht-B1c* (open circle) after 2, 7, and 9 days of treatment. **A** Effective quantum yield of PSII photochemistry ( $\Phi_{PSII}$ ); **B**

photochemical quenching coefficient ( $qP$ ); **C** non-photochemical quenching (NPQ); and **D**  $\Delta pH$ -dependent energy dissipation ( $qE$ ). Mean values ( $\pm SE$ ) were calculated from 4 independent experiments with 3 replications each

wheat seedlings are presented in Fig. 2. As can be seen, the PSI photochemistry (measured as  $\Delta A_{830}$ ) was higher for mutant leaves before and after all treatment periods than that for the wild type. Results also showed that P700 photooxidation decreased after exposure of both wheat seedlings to 200 mM NaCl; however, this decrease was smaller for the mutant in comparison to the wild-type wheat (Fig. 2A). In addition, the subsequent dark reduction kinetics of P700<sup>+</sup> ( $t_{1/2}$ ) in untreated and NaCl-treated mutant plants was faster than those of wild-type plants during the same measuring period (Fig. 2B). This means that the mutant plants have a higher capacity for PSI-dependent cyclic electron flow (CEF) in response to moderate salt concentrations (see Lu and others 2008).

### Low-Temperature (77 K) Chlorophyll Fluorescence

To investigate the impact of salt stress on the energy transfer between the main pigment-protein complexes in wild-type wheat photosynthetic membranes and mutant ones, the 77 K chlorophyll fluorescence of thylakoid membranes was studied. The chlorophyll fluorescence emission spectra of thylakoid membranes from untreated and salt-treated (200 mM NaCl) plants exhibited three bands at 685, 695, and 743 nm (data not shown). The 685 nm (F685) and 695 nm (F695) bands are related to fluorescence emitted from the PSII complex, whereas the band at 743 nm (F743) originates from the PSI complex (Krause and Weis 1991). The fluorescence ratio F743/F685 was



**Fig. 2** Dependence of P700 photooxidation (P700<sup>+</sup>) measured by  $\Delta A_{830}$  (A) and half-times of P700<sup>+</sup> dark relaxation decay (B) on the time of exposure to 200 mM NaCl for both wheat genotypes: *Rht-B1a*

(filled circle) and *Rht-B1c* (open circle). Mean values ( $\pm$ SE) were calculated from 4 independent experiments with 3 replications each

**Table 3** Effect of 9-day-long salt stress on the chlorophyll fluorescence emission ratio F743/F685 and the ratio E650/E680 calculated from fluorescence excitation spectra at 743 nm of thylakoid membranes from *Rht-B1a* (wild type) and *Rht-B1c* (mutant) leaves

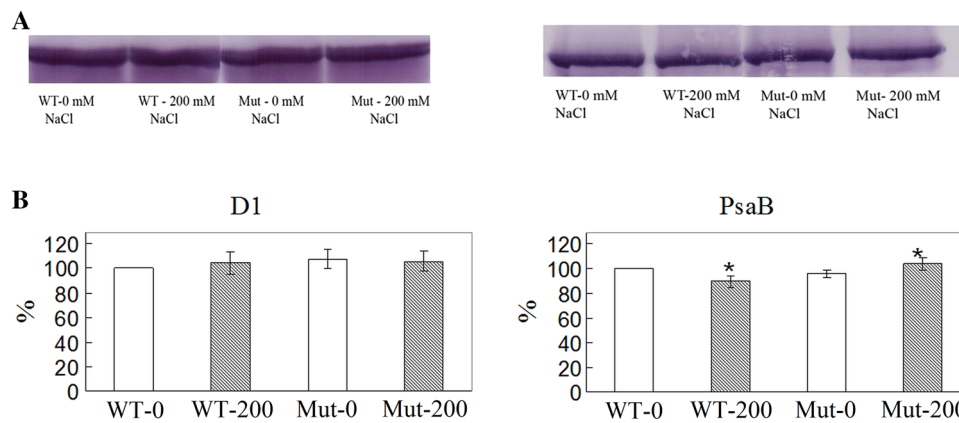
Genotype	NaCl (mM)	F743/F685		E650/E680
		exc. 436 nm	exc. 472 nm	em. 743 nm
<i>Rht-B1a</i>	0	1.88 $\pm$ 0.07 <sup>c</sup>	1.62 $\pm$ 0.02 <sup>c</sup>	0.59 $\pm$ 0.04 <sup>b</sup>
	200	2.70 $\pm$ 0.01 <sup>a</sup>	1.93 $\pm$ 0.06 <sup>a</sup>	0.76 $\pm$ 0.05 <sup>a</sup>
<i>Rht-B1c</i>	0	2.07 $\pm$ 0.06 <sup>b</sup>	1.83 $\pm$ 0.04 <sup>b</sup>	0.72 $\pm$ 0.02 <sup>a</sup>
	200	2.13 $\pm$ 0.07 <sup>b</sup>	1.87 $\pm$ 0.07 <sup>b</sup>	0.73 $\pm$ 0.03 <sup>a</sup>

Mean values ( $\pm$ SE) were calculated from 3 to 4 independent experiments. Different letters indicate significant differences between values in the same column ( $p < 0.05$ )

used as an indicator for the energy redistribution between the two photosystems. Our data revealed that in the wild-type seedlings, the ratios of F743/F685 upon excitation of both Chl *a* (436 nm) and Chl *b* (472 nm) increased after long-term salt exposure (Table 3) suggesting an increase in energy delivery to PSI and an increase of energy transfer from PSII to PSI under moderate salt stress. In addition, this ratio increased more strongly when Chl *a* was excited in comparison to Chl *b*. On the other hand, in mutant thylakoid membranes, this ratio remained almost unchanged after salt treatment, indicating that after long-term salt treatment no changes in the energy distribution between PSII and PSI occurred. The fluorescence excitation spectra at 743 nm for wild-type thylakoid membranes showed that the E650/E680 ratio increased after treatment with NaCl, whereas in mutant thylakoid membranes, this ratio was not changed (Table 3).

### Changes in PSI and PSII Reaction-Center Proteins under Salt Stress

Using Western blot experiments we aimed to find the salt-induced changes, if any, of proteins of the photosynthetic apparatus, especially of populations of PSI and PSII, as a result of salt treatment. Western blot was applied on isolated thylakoid membranes and allowed us to identify reaction-center proteins with high sensitivity (the sensitivity of the applied kit is about 100 pg protein). The proteins of thylakoid membranes from wild-type and mutant plants exposed to 200 mM NaCl treatment were separated by SDS-PAGE and immunoblot analysis was performed using D1 (D1 core protein of PSII) and PsaB (PSI-B core subunit of PSI) antibodies. Both antibodies are specific for the studied proteins—the scans of the full image of colored membranes are presented in Supplementary Fig. S2. It is evident that we observed only one band for each protein that appeared at the right position depending on their molecular weight. The representative scans of typical Western blot membranes and densitometric analyses are presented in Fig. 3. Using the software for evaluation of density of bands, the quantification of immunoblot data for D1 and PsaB proteins was done relative to the amount of corresponding protein in untreated samples from the wild type (Fig. 3B). The results indicated that after long-term treatment with 200 mM NaCl, the content of D1 protein did not change significantly in either wild-type and mutant plants with respect to the untreated ones. The intensity of the PsaB band decreased in wild-type plants and increased in mutant plants after salt treatment, respectively, when compared to untreated plants (Fig. 3). It should be noted that the amount of PsaB proteins in untreated mutant plants was insignificantly decreased (up to 95.7%)



**Fig. 3** **A** Western blot analysis of reaction-center proteins D1 and PsaB of thylakoid membranes from untreated (WT-0 and Mut-0) and treated with 200 mM NaCl (WT-200 and Mut-200) wheat seedlings: *Rht-B1a* (WT) and *Rht-B1c* (Mut). Equal amounts of thylakoid

membranes corresponding to 4,5 µg Chl were loaded to each well. **B** Quantification of immunoblot data for D1 and PsaB proteins, presented as a percentage from the band density of thylakoid membranes from untreated wild-type plants (100%)

compared to that in the untreated wild-type plants. After long-term salt treatment, the amount of PsaB increased in the mutant to about 116.0% in comparison to the treated wild type (Fig. 3B). This indicates that the DELLA mutant (*Rht-B1c*) responds to salt stress by a slight increase of PSI population, but possible changes in mutual organization and arrangements of core complexes of both photosystems and their antenna complexes cannot be excluded. Although this change is not considerable, such changes are observed under other abiotic stresses with the aim to balance the photochemical activities of both photosystems (Schöttler and others 2014; Jia and others 2016).

## Discussion

Salt stress is predicted to have negative effects on plants mainly by affecting the rate of photosynthesis and protein synthesis leading to less production of growth hormones and hence plant growth inhibition (Qados 2011). This study complements data from previous investigations showing the role of DELLA proteins in plant tolerance to salt stress. Our results showed that the wheat DELLA mutant (*Rht-B1c*) exhibits long-term acclimation (over 7–9 days) to salt stress in contrast to the wild-type (*Rht-B1a*) wheat. Plant growth inhibition is the primary injury that could lead to other symptoms such as reduction in Chl content and the overall photosynthetic capacity of green plants (Munns and Tester 2008; Ashraf and Harris 2013). Under moderate salt treatment (200 mM NaCl), a reduction in the leaf and root length and a decrease of the leaf number was observed (Table 1, Supplementary Fig. S1). The applied moderate NaCl stress led to significant changes in the growth parameters and leaf RWC (Table 1). These results confirm earlier reports on the

reducing effect of NaCl on plant height (Memon and others 2010; Kalhor and others 2016). The lengths of both leaves and roots of the DELLA mutant (*Rht-B1c*) plants were significantly less decreased in contrast to the stronger reductions in the growth parameters of the wild-type plants, in particular roots (Table 1). Our data revealed that in the wild-type plants, RWC declined significantly after prolonged salt stress exposure in accordance with previous studies on wheat and barley under moderate salt stress (Sairam and others 2002; Szopko and others 2016). Therefore, the wheat DELLA mutant plants exhibited better tolerance with regard to salt-related leaf water content.

In this study, we explored the protective effect of this mutation on pigment content (Chl and Car) and function of the photosynthetic apparatus under salt stress. The data obtained showed that the salt-induced reduction in leaf elongation was also accompanied by a decrease in Chl *a*, Chl *b*, and total Car leaf content (Table 2). Reduction in photosynthetic pigments has been reported for different plants exposed to higher salt concentrations (Khavari-Nejad and Mostofi 1998; Sairam and others 2002; Ali and others 2004; Yang and others 2011; Akcin and Yalcin 2016). The salt-induced decrease in leaf Chl content could be due to impaired/lowered biosynthesis of the pigments, as reported in previous investigations (see in Ashraf and Harris 2013). Our results demonstrated that the pigment decrease was stronger in the wild-type leaves than in the mutant leaves under salt stress (Table 2). Recently, it has been shown that the dwarfing DELLA mutant genes and wheat participate in the regulation of chlorophyll biosynthesis (Wen and others 2013), which could be related to the less Chl reduction in mutant plants (Table 2). Data presented on the pigment content of wild-type leaves showed that 9 days of salt treatment with 200 mM NaCl caused a more considerable decrease



of Chl *b* than Chl *a*, which resulted in a slight increase of the Chl *a/b* ratio compared to untreated wild-type leaves and mutants (Table 2). The reasons for this increase might be different. Firstly, during the process of Chl degradation, Chl *b* could be converted into Chl *a*, thus resulting in the increased content of Chl *a* (reviewed in Ashraf and Harris 2013). On the other hand, having in mind that the Chl *a/b* ratio correlates to the degree of thylakoid stacking and light-harvesting PSII antenna size (Anderson and Aro 1994), then the slightly increased Chl *a/b* ratio after the treatment could support our assumption that the salt stress most probably causes structural reorganizations in the thylakoid membranes. In line with this hypothesis, electron micrographs of chloroplasts from cucumber and potato plants subjected to salt stress were demonstrated previously (Shu and others 2012; Gao and others 2014).

Because the accumulation of Chl has been proposed as one of the potential biochemical indicators of salt tolerance in different crops (Ashraf and Harris 2013), it could be suggested that the impact of the *Rht-B1c* mutation in Chl and Car biosynthesis might contribute to the alleviated salt-induced pigment reduction and hence higher tolerance of the DELLA mutant plants. Moreover, the higher Car content in the mutant compared with the wild-type wheat before salt treatment is likely also responsible for its higher tolerance, as carotenoids are necessary for photoprotection of photosynthesis and they play an important role as a precursor in signaling during plant development under abiotic stress (for review see Ashraf and Harris 2013). In addition, carotenoids are efficient antioxidants responsible for the quenching of ROS by scavenging singlet molecular oxygen and peroxy radicals (Stahl and Sies 2003). Ziaf and others (2009) have suggested that Car contents and RWC could be used as reliable selection criteria for salt tolerance in pepper plants.

The salt-induced decrease in photosynthetic pigments could also result in a reduced light-absorbing efficiency of both photosystems (PSI and PSII) and hence, an inhibition of the photochemical activity of the photosynthetic apparatus. Chlorophyll fluorescence *in vivo* could be used as criterion for salt sensitivity in wheat plants (Abdeshahian and others 2010). Analysis of the PAM chlorophyll fluorescence parameters in this study showed that the photochemical parameters of PSII ( $\Phi_{\text{PSII}}$  and  $qP$ ) changed to a different degree in both wheat genotypes depending on the time of treatment (Fig. 1A, B). The results demonstrated that moderate NaCl concentrations (200 mM) induced a gradual decrease in the above parameters with increasing the time of treatment up to 9 days for the wild-type plants. Salt-induced inhibition of the effective quantum yield of PSII photochemistry ( $\Phi_{\text{PSII}}$ ) indicated that electron transport processes were down-regulated in the wild-type wheat genotype. In the mutant, the photochemical parameters of PSII were more strongly reduced on the 2nd day of salt treatment and then increased slightly after

7–9 days of treatment, as values of these parameters were higher than that in the wild-type wheat (Fig. 1A, B). This finding indicates that after long-term (9 days) salt treatment, the fraction of the primary quinone electron acceptor of PSII ( $Q_A$ ) in the oxidized state was greater in the mutant (as  $qP$  was greater), as well as the number of open PSII centers (measured by  $\Phi_{\text{PSII}}$ ) was also less negatively affected in the mutant in comparison to the wild type. Therefore, it could be proposed that the mutant DELLA-encoding gene (*Rht-B1c*) is involved in long-term responses of plants during acclimation to salt stress. A recent study has also showed different inhibitory effects of salinity on PSII activity in some wheat cultivars (Abdeshahian and others 2010). In addition, Mehta and others (2010) have reported that salt stress damages the donor side of the PSII to a greater extent than the acceptor side in wheat plants, as well as that the salt-induced damage to PSII is reversible.

Non-photochemical quenching (NPQ) is also an important mechanism to diminish the production of ROS in photosynthetic membranes and plays a key role in the protection of PSII from photodamage. Results obtained here demonstrated that in the wild-type wheat, the values of NPQ increased gradually after the 2nd, 7th, and 9th days of the treatment (Fig. 1C). In the mutant plants, salt stress induced a strong increase in NPQ on the 2nd day followed by a decrease on the 7th day and return to the control values on the 9th day of salt exposure. The increase in NPQ may represent the decreased demand for electron transport which results in heat dissipation of excess excitation energy (Abdeshahian and others 2010), that is, the negative impact of salinity on the photosynthetic rate results in an increase in NPQ. Therefore, the mutant plants showed better photosynthetic tolerance to moderate salt stress than the wild type after long-term exposure because the NPQ is near control values after 9 days of salt exposure (Fig. 1C).

The values of  $qE$ , which is an important component of non-photochemical quenching, follow the same changes as those observed for NPQ in both wheat genotypes (Fig. 1D). The coefficient of  $\Delta\text{pH}$ -dependent non-photochemical quenching ( $qE$ ) is associated with the ATP-generating proton gradient over the thylakoid membrane and the acidification of the thylakoid lumen, which results in activation of violaxanthin de-epoxidase (see Baker 2008; Zivcak and others 2015). In turn, our results showed that both wheat genotypes undergo different salt-induced alterations in the trans-membrane proton gradient ( $\Delta\text{pH}$ ) across thylakoid membranes, as  $qE$  was strongly increased in the mutant genotype after 2 days and recovered after 7–9 days of salt treatment (Fig. 1D). Because zeaxanthin was proposed to be an efficient quencher of excitation energy in the PSII antenna and plays a role in photoprotection of plants, xanthophyll cycle-dependent  $qE$  has been extensively accepted as an important mechanism for energy

dissipation as a defense mechanism (Baker 2008). Therefore,  $\Delta\text{pH}$ -dependent energy dissipation, which is related to the reduction of ROS, is also involved in higher tolerance of mutant wheat plants.

The analysis of the P700 photooxidation ( $\Delta A_{830}$ ) revealed that the PSI photochemistry was inhibited more strongly in the wild type than in the mutant leaves during all treatment time periods (Fig. 2A). In addition, the data also showed that the dark relaxation half-times were more accelerated in the mutant than in the wild type (Fig. 2B). This further indicated that DELLA mutant plants have a higher capacity for PSI-dependent CEF under salt stress as it has also been shown previously upon heavy metal stress (Dobrikova and others 2017). Furthermore, CEF around PSI has been suggested to play a significant role in preventing photooxidative damage of the photosynthetic apparatus to stress factors as it prevents the over-reduction of the electron transport chain and subsequent oxidative damage (Takahashi and others 2009; Szopkó and others 2016; Huang and others 2017). It is known that CEF generates  $\Delta\text{pH}$  across the thylakoid membranes, leading to the formation of ATP, but not NADPH, thus preventing the over-reduction of the acceptor side of PSI. The CEF is also thought to support the regulation of light-harvesting processes via the enhancement of non-photochemical quenching (NPQ,  $qE$ ), thereby contributing to the protection of PSII. Previous studies have shown that CEF is accelerated in salt-tolerant species, which stimulated the higher  $qE$  (Lu and others 2008; Szopkó and others 2016). To protect PSI and PSII against photodamage, CEF-dependent generation of  $\Delta\text{pH}$  primarily enhances lumen acidification, which then activates NPQ, regulates the P700 redox state, and controls the transfer of electrons via the Cyt  $b_6/f$  complex (see Huang and others 2017). Therefore, the enhanced capacity for CEF observed in the mutant leaves could be a possible regulatory mechanism by which these plants alleviate oxidative damage under salt stress.

After prolonged (9 days) salt treatment of wild-type plants, an increase in the F743/F685 ratio in thylakoid membranes was observed (Table 3). This increase could be due to redistribution of excitation energy in favor of PSI as a result of proposed salt-induced destacking of thylakoid membranes and/or alterations of PSI antenna size (Khavari-Nejad and Mostofi 1998; Shu and others 2012; Gao and others 2014). This suggestion was confirmed by the increase of the excitation ratio E650/E680 monitored at 743 nm after treatment with 200 mM NaCl. In contrast, in the mutant thylakoid membrane, the ratio F743/F685 is higher than in the wild type and it was not influenced by salinity. The observed higher amount of rapidly operated PSI centers (by enhanced CEF) together with the increased energy transfer to the PSI (F743/F685 ratio) in untreated mutant thylakoid membranes than in wild-type ones suggests structural reorganizations in the main pigment-protein complexes in

the mutant photosynthetic membranes, which in turn could affect their tolerance to salt stress.

Generally, PSI and PSII photosynthetic activities are more salt-resistant in the *Rht-B1c* mutant than wild-type wheat. The long-term responses of mutant plants to moderate salt treatment include an increase of the PSI-related reaction-center protein (PsaB) and no significant changes in PSII reaction-center protein (D1). Recently, it has been shown that a decrease of D1 in wheat plants after short-time exposure (1 and 3 h) to high light (Chen and others 2017) is related to the intensive generation of ROS under high-light stress. In wheat, it has been shown that long-term salinity induces increased  $\text{H}_2\text{O}_2$  levels and enhanced activity of antioxidant enzymes included in detoxification of ROS (Sairam and others 2002). Moreover, it has been suggested that the modified DELLA proteins also modulate the antioxidant defense system and thus reduce the levels of ROS (Achard and others 2008; Kocheva and others 2014). Data about the content of PsaB proteins showed that the response of mutant plants to 9-day salt treatment (200 mM NaCl) includes an increase of the PSI reaction-center proteins (Fig. 3). These data correspond with higher levels of  $\text{P700}^+$  (higher  $\Delta A_{830}$  values) observed in the mutant plants after 9 days of salt treatment in comparison to the wild-type plants (Fig. 2A). It should be mentioned that before treatment, the amount of PsaB in wild-type and mutant plants does not differ significantly.

Plants are equipped with several protective mechanisms against stress. These include the antioxidant defense system, as well as additional protection mechanisms such as the xanthophyll cycle, non-photochemical quenching (NPQ,  $qE$ ), and PSI-dependent CEF to dissipate the excess excitation energy or scavenge ROS in chloroplasts (Lu and others 2008; Zivcak and others 2015; Huang and others 2017). We suppose that the wheat DELLA mutant (*Rht-B1c*) might stimulate these protection mechanisms to resist the negative effects of salt stress on the photosynthetic apparatus.

## Conclusion

This study demonstrates that the wheat *Rht-B1c*-encoded GA-insensitive DELLA proteins increase seedling tolerance to salt stress in terms of less reduced plant growth and leaf pigment content, as well as less inhibited photosynthetic activity. The mutant tolerance becomes more evident after prolonged salt exposure of plants. Taken together, our results provide new insights into the higher tolerance mechanisms of wheat DELLA mutants (*Rht-B1c*) with respect to photosynthetic performance under moderate salt stress.

It is likely that DELLA proteins participate in long-term responses to salt stress by altering the structural organization of the main pigment-protein complexes in photosynthetic

membranes, which in turn could affect their tolerance to stress. Data demonstrated also that DELLA proteins increase the resistance of photosynthetic apparatus by mitigation of salt-induced damage to chlorophylls and carotenoids, and increased capacity for CEF around PSI as a possible regulatory mechanism by which these mutant plants alleviate oxidative damage under salt stress. To understand the role of DELLA proteins in the regulation of activities of photosynthetic enzymes and hormone homeostasis, more detailed investigations are needed.

The current findings on the possible involvement of the wheat *Rht-B1c*-encoded DELLA proteins in the plant long-term acclimation to salt stress make this allele a suitable candidate to be used in breeding programs for increasing salt tolerance in wheat cultivars tailored for salinity affected areas.

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