Protoplasma (2012) 249:1109–1117
DOI 10.1007/s00709-011-0356-4

ORIGINAL ARTICLE

A possible role for C_4 photosynthetic enzymes in tolerance of *Zea mays* to NaCl

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Received: 31 July 2011 / Accepted: 21 November 2011 / Published online: 1 December 2011
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Abstract Treatment of 14-day-old maize cultivars (Hybrid351 and Giza2) with 250 mM NaCl significantly reduced shoot fresh and dry weights and protein content during the subsequent 12 days. The magnitude of reduction was more pronounced in Giza than Hybrid. Both cultivars contained converging levels of protein for the enzymes phospho*enol*pyruvate carboxylase (PEPC), malate dehydrogenase (MDH), pyruvate phosphate dikinase (PPDK) and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) under normal conditions; however, NaCl led to increase these levels in Hybrid and decrease them in Giza. Moreover, NaCl significantly inhibited the activities of PEPC, MDH and PPDK in both cultivars during the first 2 days, thereafter the inhibition nullified only in Hybrid; nonetheless, Rubisco was the least affected enzyme in both cultivars. In addition, NaCl slightly increased V_{max} of PEPC, MDH and PPDK in Hybrid with no change in K_m; nevertheless V_{max} dropped in Giza with an increase in K_m of only PEPC and MDH. Also K_{cat}, K_{cat}/K_m and V_{max}/K_m of all enzymes were lower in treated Giza than in treated Hybrid. The increased V_{max} of all enzymes in only Hybrid by NaCl confirms that they were synthesised more in Hybrid than in Giza. However, the decreased V_{max} in Giza concomitant with the increased K_m points to an interference of salinity with synthesis of enzymes and their structural integrity. This would lead to a noncompetitive inhibition for the enzymes. These findings declare that maize tolerance to NaCl was larger in Hybrid compared to Giza due to a role for C4 enzymes.

Keywords C4 photosynthetic enzymes · Kinetic parameters · Maize · NaCl · Tolerance

Abbreviations

DTT	Dithiothreitol
K_{cat}	Catalytic constant
K_m	Michaelis constant
LSD	Least significant difference
NADP-MDH	NADP-malate dehydrogenase
OAA	Oxaloacetate
PEP	Phospho*enol*pyruvate
PEPC	Phospho*enol*pyruvate carboxylase
PPDK	Pyruvate phosphate dikinase
Rubisco	Ribulose 1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
[*S*]	Substrate concentrations
v	Velocity
V_{max}	Maximum velocity

Introduction

Salinity is one of the major environmental factors affecting plant growth and productivity (Azevedo et al. 2006; Wei et al. 2006; Aghaleh and Niknam 2009). The effects of salinity arise from osmotic and ionic toxicity that would result in molecular damage, growth arrest and even death of plants (Younis et al. 2003; Duan et al. 2007). Three physiological mechanisms are suggested to be involved in reduction of

Handling Editor: Bhumi Nath Tripathi

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plant growth resulted from osmotic stress: (1) turgor pressure reduction, (2) reduction in photosynthetic activity and (3) effects of accumulated salts on the metabolic activities (Neumann 1997; Younis et al. 2003). Salt stress reduces CO_2 availability in the leaves and inhibits carbon fixation with a consequent of growth reduction. Osmotic stress causes stomatal closure and results in a lower intracellular CO_2 concentration. The rate of photosynthesis in salt-treated plants is reduced (El-Shihaby et al. 2003; Munns and Tester 2008). The high efficiency of C4 photosynthetic pathway is attributed to its unique mode of carbon assimilation which involves two different photosynthetic cell types, mesophyll and bundle sheath cells (Westhoff and Gowik 2004).

CO_2 is firstly fixed by phospho*enol*pyruvate carboxylase (PEPC) in the mesophyll cells to oxaloacetate (OAA) which is then reduced to malate by malate dehydrogenase (MDH). Malate is transported to bundle sheath where CO_2 is omitted leaving pyruvate which is then passed back to mesophyll cells for the phosphorylation by pyruvate phosphate dikinase (PPDK) to form phospho*enol*pyruvate (PEP) (Tovar-Mendez and Munoz-Clares 2001; Osamu et al. 2004). In the bundle sheath, the omitted CO_2 is re-fixed by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) to start the Calvin cycle (Nishimura et al. 2008). Rubisco is the key enzyme in this cycle. It catalyses the irreversible carboxylation of ribulose-1,5-bisphosphate (RuBP) to form two 3-phosphoglyceric acid molecules. Its activity is limiting to CO_2 assimilation rate and net photosynthesis (El-Shihaby et al. 2003; Yokota and Shigeoka 2008). It is the most abundant protein in plants. It comprises as much as half of the leaf total soluble proteins and about 30% of total nitrogen compounds of the leaf (Spreitzer 1993; Osamu et al. 2004). Any decrease in the amount of Rubisco means an inhibition of CO_2 assimilation and consequently growth retardation. Osmotic stress reduces the content of chlorophyll and so decreases the efficiency of Rubisco for carbon fixation (Munns and Tester 2008).

These photosynthetic enzymes are influenced by stress due to alterations in enzyme concentration and/or in enzyme–substrate affinity (Engel 1984). Therefore, an accurate determination of the kinetic parameters [maximum velocity (V_{max}), Michaelis constant (K_m) and catalytic constant (K_{cat})], which define the rate behaviour of an enzyme-catalysed reaction, would declare to what extent the stress can interfere with the activities of the enzymes. Such activities vary with plant tolerance to stress elicitors. Therefore, the present work is aimed to examine the effects of salinity stress imposed by 250 mM NaCl on the activities, contents and kinetics of the C_4 enzymes PEPC, NADP-malate dehydrogenase (NADP-MDH) and PPDK in addition to Rubisco in two cultivars of *Zea mays* (Hybrid351 and Giza2) vary in tolerance to salinity in order to evaluate the possible role of enzymes in tolerance to NaCl.

Materials and methods

Plant material and growth conditions

A homologous lot of maize seeds (*Z. mays* L.) of two genotypes, Hybrid351 and Giza2, was obtained from the Agricultural Research Center, Cairo, Egypt. The grains were surface sterilised by immersing in 3% sodium hypochlorite solution for 10 min, thoroughly washed, soaked for 8 h and germinated in loamy/sand soil (1/1, *v*/*v*) in plastic pots (25 cm diameter×20 cm height). The pots were kept in a growth cabinet (growth chamber Conviron, CMP 3244; controlled environments, Winnipeg, Manitoba, Canada) at 14 h photoperiod with 450–500 μmol m^{-2} s^{-1} photosynthetic photon flux density, 75–80% relative humidity and 28/16°C day/night regime and irrigated daily by an adequate quantity of one fourth strength Hoagland solution. When seedlings were 14 days old, the pots containing seedlings with uniform sizes were divided into two groups; one was left to serve as control and the other for treatment with 250 mM NaCl (a concentration approaching salinity in brackish water of many areas in Egypt). Plants were harvested after being left for at least 4 h in illumination. The leaf samples were taken from five different plants in each plot just before treatment with NaCl (to be considered as zero time) and also after 1, 2, 5 and 12 days from treatment. The leaves were thoroughly washed with water, dried by plotting with paper towels, immediately immersed in liquid nitrogen and stored at −80°C until sample preparation for analysis. The fresh weight of shoots (n=6) was recorded and then dried in an oven at 80°C for 2 days to obtain the dry weight.

Determination of protein content

About 200 mg of frozen leaves from five seedlings were homogenised in 80 mM Tris–HCl, pH 7.4 (Laemmlli 1970). After centrifugation at 14,000×g for 5 min, the extracted protein was precipitated over night at 4°C by adding 10% chilled trichloroacetic acid in acetone (*w*/*v*). Protein pellets were separated by centrifugation at 12,000×g for 15 min and reconstituted in the buffer. Protein was determined using Commassie Brilliant Blue G-250 at 595 nm (Bradford 1976).

Extraction of PEPC, NADP-MDH, PPDK and Rubisco

Extraction procedures of PEPC, NADP-MDH and PPDK were performed according to Ashton et al. (1990). About 200 mg of frozen leaves was homogenised with extraction buffer containing 50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES–potassium hydroxide (KOH) (pH 7.5) and 10 mM dithiothreitol (DTT). For

extraction of PEPC only, 5 mg ml^{-1} bovine serum albumin, 5 mM $MgCl_2$ and 2 mM potassium phosphate were added. The mixtures were centrifuged at 10,000×*g* at 4°C for 10 min and the supernatants were applied on Sephadex G-25 (Pharmacia) equilibrated with HEPES–KOH buffer.

Extraction of Rubisco was performed in extraction buffer containing 20 mM Tris–HCl (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM DTT, 0.002% chlorohexidine diacetate (Hibitane) and 1% (*w*/*v*) polyvinyl pyrrolidone (Keys and Parry 1990). The homogenate was centrifuged at 5,000×*g* for 15 min and the supernatant was treated with $(NH_4)_2SO_4$ to give 35% saturation, centrifuged at 20,000×*g* for 15 min and then saturation was raised to 55%. After centrifugation at 20,000×*g* for 15 min, the pellet was dissolved in 20 mM Tris–HCl (pH 8.0) containing 1 mM DTT, 1 mM $MgCl_2$ and 0.002% Hibitane.

SDS-PAGE of PEPC, NADP-MDH, PPDK and Rubisco

According to Laemmlli (1970), an aliquot of the partially purified extract of each enzyme from 200 mg of frozen leaves—as mentioned above—was denatured by heating at 80°C for 3–5 min. The proteins were resolved by SDS-PAGE. The resolving gel was prepared using Bio-Rad Mini Protean 3 cell from mixing the monomer solution with 50 µl ammonium persulfate (APS, 10%) and 5 µl tetramethyl ethylenediamine (TEMED). The monomer solution contained 4.1 ml deionized water, 3.3 ml 30% acrylamide (11% acrylamide/bis), 2.5 ml gel buffer (1.5 M Tris–HCl, pH 8.8) and 0.1 ml SDS (10% *w*/*v*). TEMED was used with APS to catalyse acrylamide polymerization. The stacking gel was prepared from mixing the monomer solution with 50 µl APS and 5 µl TEMED. The monomer solution contained 5.7 ml deionized water, 1.7 ml 30% acrylamide (5% acrylamide/bis), 2.5 ml gel buffer (0.5 M Tris–HCl, pH 6.8) and 0.1 ml SDS (10% *w*/*v*). Protein samples were loaded into lanes. The gels were stained with 0.25% Brilliant Blue R250 in 40% methanol and 10% acetic acid, destained with water for 24 h and dried with Savant gel dryer (BioRad, Hercules, CA, USA). The gels were then scanned and the band volumes were measured by using Lab Image V 2.7.2 software. The protein level of each enzyme, based on the scanned density, was expressed as arbitrary units.

Assay of activity of PEPC, NADP-MDH, PPDK and Rubisco

The activity of PEPC was assayed at 340 nm by following the reduction of OAA by nicotinamide adenine dinucleotide (NADH) in the presence of MDH (Ashton et al. 1990) using a Varian Model Cary 210 double-beam grating spectrophotometer (Melbourne, Australia). Reaction mixture (1 ml) contained 25 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 2 mM DTT, 1 mM $NaHCO_3$, 5 mM glucose-6-phosphate, 5 mM PEP, 0.2 mM NADH and 2 U of MDH. The reaction was initiated by the addition of 0.2 ml of the enzyme extract and incubated at 30°C. The decrease in absorbance due to the oxidation of NADH was measured at 340 nm. The enzyme activities were expressed as kat (the release of 1 mol of NAD s^{-1}) mg^{-1} protein. The activity of MDH was measured in the direction of OAA reduction by following the oxidation of NADPH at 340 nm. Reaction mixture (1 ml) contained 25 mM Tricine–KOH (pH 8.3), 70 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM OAA and 0.2 mM NADPH. The reaction was started by the addition of 0.2 ml of the enzyme extract and the contents were allowed to react at 30°C, during which the decrease in absorbance at 340 nm was measured. The enzyme activities were expressed as kat (the release of 1 mol of NADP s^{-1}) mg^{-1} protein.

The activity assay of PPDK was performed at 340 nm in the forward direction by coupling the production of PEP to NADH via PEPC and MDH. Reaction mixture (1 ml) contained 25 mM HEPES–KOH (pH 8.0), 8 mM $MgSO_4$, 10 mM DTT, 10 mM $NaHCO_3$, 2 mM pyruvate, 5 mM $(NH_4)_2SO_4$, 1 mM glucose-6-phosphate, 1 mM ATP, 2.5 mM KPO_4, 0.2 mM NADH, 0.5 U PEPC and 2 U of MDH. The reaction was started by the addition of 0.2 ml of the enzyme extract and the contents were allowed to react at 30°C and the decrease in absorbance at 340 nm was measured. The enzyme activities were expressed as kat (the release of 1 mol of NAD s^{-1}) mg^{-1} protein.

Rubisco activity was assayed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADPH (Keys and Parry 1990). Reaction mixture (1 ml) contained 50 mM HEPES (pH 7.8), 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 0.66 mM ribulose-1,5-bisphosphate, 0.2 mM NADPH, 5 mM ATP, 5 mM creatine phosphate, 2.0 U creatine phosphokinase, 2.8 U glyceraldehydes-3-phosphate dehydrogenase and 2.0 U phosphoglycerate kinase. The reaction was started by the addition of 0.2 ml of the enzyme extract and the contents were allowed to react at 30°C and the decrease in absorbance at 340 nm was measured. The enzyme activities were expressed as kat (the release of 1 mol of NADP s^{-1}) mg^{-1} protein.

Determination of kinetic parameters

The determination of the kinetic parameters (K_m, V_{max} and K_{cat}) was performed for the enzymes extracted from plants treated with NaCl for 5 days. K_m was determined as millimolar substrate concentrations [*S*], V_{max} as kat mg^{-1} protein and K_{cat} as V_{max}/enzyme concentration. In this experiment, enzyme velocity (*v*) was plotted as a function of varied substrate concentrations ([*S*]). The substrate concentration of

PEPC (PEP), MDH (OAA), PPDK (pyruvate) and Rubisco (RuBP) varied from 1 to 20 mM, 0.2 to 4 mM, 0.4 to 10 mM and 0.2 to 2 mM, respectively. A plot of v against $[S]$ gives the curve of Michaelis–Menten plot (Engel 1984). The values of V_{max} and K_m were accurately calculated from Lineweaver–Burk Eq. 1 and its linear transformation 2:

$$1/v = (1/[S])(K_m/V_{max}) + (1/V_{max}) \quad (1)$$

$$v = (V_{max}[S])/(K_m + [S]). \quad (2)$$

The plot of $1/v$ against $1/[S]$ gives a straight line with an ordinate intercept of $1/V_{max}$, a slope of K_m/V_{max} and an abscissa intercept of $1/K_m$ (Lineweaver–Burk plot). Dividing V_{max} on enzyme concentration gives K_{cat}.

Statistical analysis

Each experiment was repeated twice in triplicate, so that the mean obtained was for six replicates. The full data were statistically analysed using analysis of variance and least significant difference (LSD) test.

Results

It is clear from Fig. 1 that the treatment of 14-day-old maize cultivars (Hybrid351 and Giza2) with 250 mM NaCl resulted in significant reductions in shoot fresh weight, shoot dry weight and protein content of both cultivars during the first 5 days as compared to the respective control. Thereafter, the magnitude of reduction was retracted in Hybrid but augmented in Giza. By the end of the experiment on the 12th day following treatment, the magnitude of reduction in fresh weight retracted to become only about 3% in Hybrid relative to 37% in Giza (Fig. 1a). Meanwhile, shoot dry weight was decreased by 12% and 37% in Hybrid and Giza, respectively (Fig. 1b); however, the decrease of protein content was only 4% in Hybrid relative to 38% in Giza (Fig. 1c).

Figure 2 shows that both cultivars contained similar levels of protein for each of PEPC, MDH, PPDK and Rubisco under normal conditions; there was no great difference in the protein levels of these enzymes among the untreated control of both cultivars. Nonetheless, treatment with NaCl resulted in varied effects; there were fluctuations in the enzyme protein levels in treated samples of both cultivars. In response to the treatment, PEPC, MDH and PPDK were significantly increased in Hybrid but significantly reduced in Giza (Fig. 2a–c). On the contrary, Rubisco was decreased in both cultivars; the magnitude of decrease was more pronounced in Giza than Hybrid

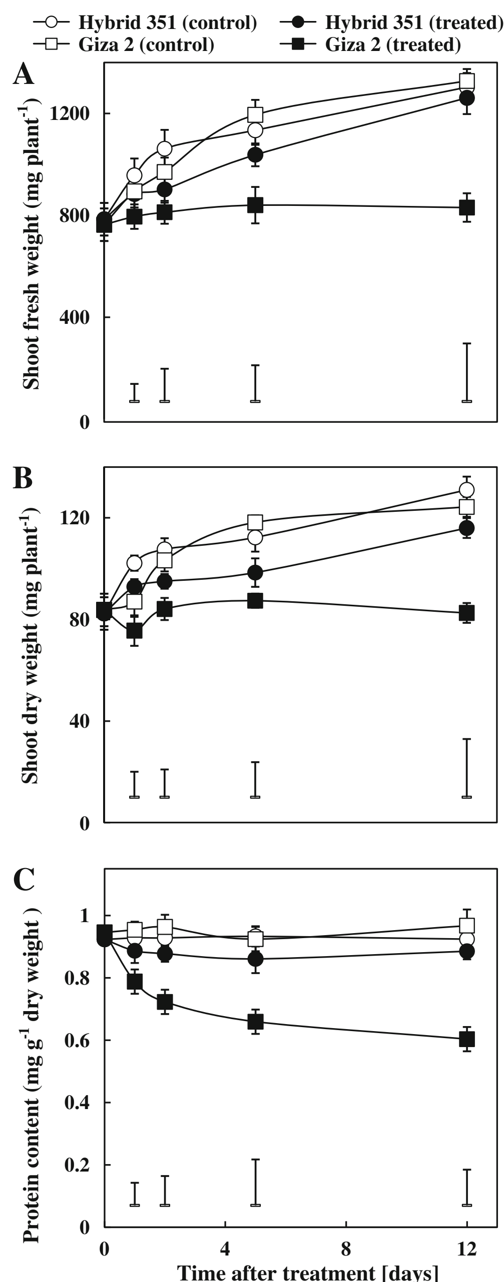

Fig. 1 Effect of treatment with 250 mM NaCl on **a** shoot fresh weight, **b** shoot dry weight and **c** shoot protein content of 14-day-old *Zea mays* (Hybrid351 and Giza2) for the subsequent 12 days. Means ± SE, $n=6$. *Vertical bars* represent LSD at $p<0.05$

(Fig. 2d). At the end of the experiment, protein levels of PEPC, MDH and PPDK increased in treated Hybrid by about 24%, 16% and 10%, respectively, but these levels decreased in treated Giza by about 53%, 51% and 64%, respectively. However, Rubisco protein was decreased following NaCl treatment by 12% and 22% in Hybrid and Giza, respectively.

The activities of PEPC, MDH and PPDK were significantly inhibited in both cultivars by NaCl treatment during

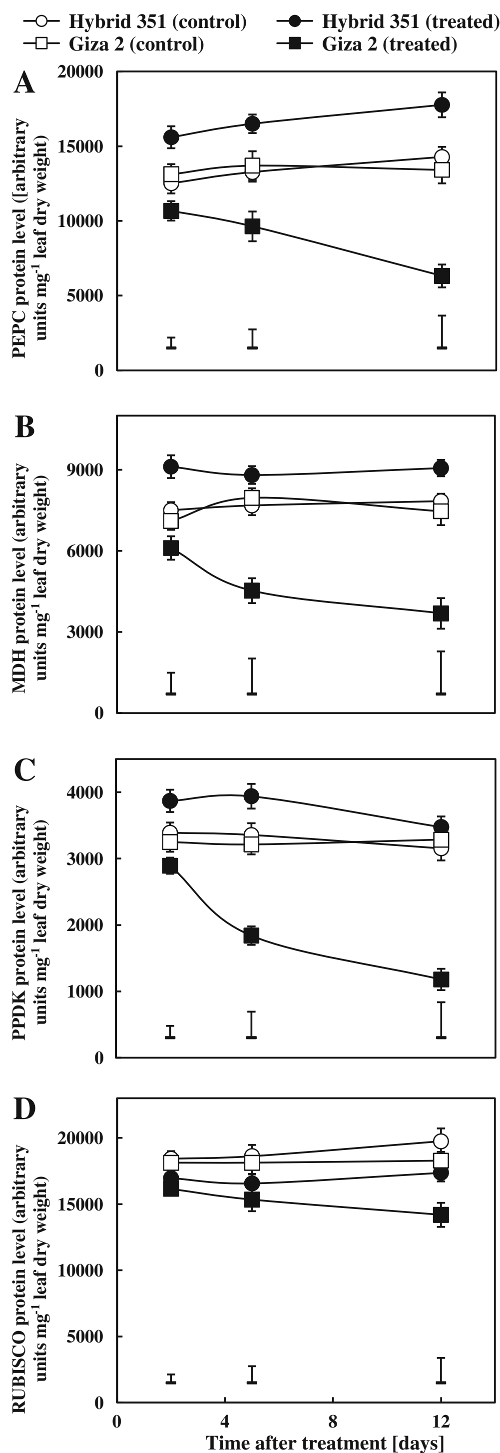

Fig. 2 Effect of treatment with 250 mM NaCl on protein levels of **a** phospho*enol*pyruvate carboxylase (PEP-C), **b** NADP-malate dehydrogenase (NADP-MDH), **c** pyruvate phosphate dikinase (PPDK) and **d** ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) of 14-day-old *Zea mays* (Hybrid351 and Giza2) for the subsequent 12 days. Means ± SE, n=6. *Vertical bars* represent LSD at p<0.05

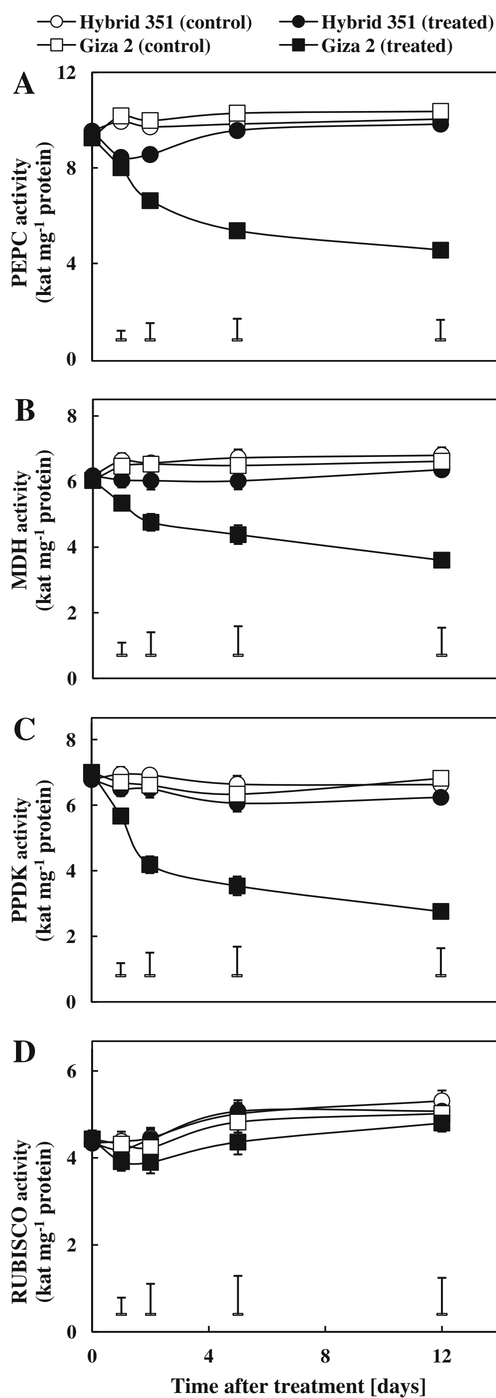

Fig. 3 Effect of treatment with 250 mM NaCl on specific activity of **a** phospho*enol*pyruvate carboxylase (PEP-C), **b** NADP-malate dehydrogenase (NADP-MDH), **c** pyruvate phosphate dikinase (PPDK) and **d** ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) of 14-day-old *Zea mays* (Hybrid351 and Giza2) for the subsequent 12 days. Means ± SE, n=6. *Vertical bars* represent LSD at p<0.05

the first 2 days, thereafter the inhibition was continued and augmented in Giza; however, it was nullified in Hybrid (Fig. 3). The magnitude of inhibition of PEPC activity by NaCl in Hybrid was withdrawn from 15% on the second day from treatment to only 2% on the 12th day, meanwhile this inhibition was magnified in Giza from 21% to 56%

(Fig. 3a). MDH activity was consistently inhibited in Hybrid by about 8% but increased in Giza from 17% on the second day to 46% on the 12th day (Fig. 3b). Similarly, the inhibition of PPDK activity ranged only about 6% in Hybrid but increased in Giza from 15% to 60% (Fig. 3c). The activity of Rubisco was least affected by NaCl; a slight inhibition of only about 4% was detected in both cultivars on the 12th day (Fig. 3d).

Michaelis–Menten plot shows the changes in the velocities of enzymes extracted from plant leaves 5 days following treatment as a function of changing the substrate concentration (Fig. 4a–d). NaCl induced an increase in the velocities of enzymes extracted from Hybrid but it provoked a great drop in those extracted from Giza; however, the velocity of Rubisco was slightly influenced by NaCl. Lineweaver–Burk plot was used for the accurate determination of K_m and V_{max} values. The plot indicates that the ordinate intercept ($1/V_{max}$) was high for the enzymes extracted from treated Giza but low for those extracted from treated Hybrid; such variations were less obvious for Rubisco (Fig. 4e–h). On the contrary, the abscissa intercept ($1/K_m$) was low for PEPC and MDH in treated Giza but unchanged in Hybrid; however, there were not distinct variations among both cultivars for Rubisco or PPDK.

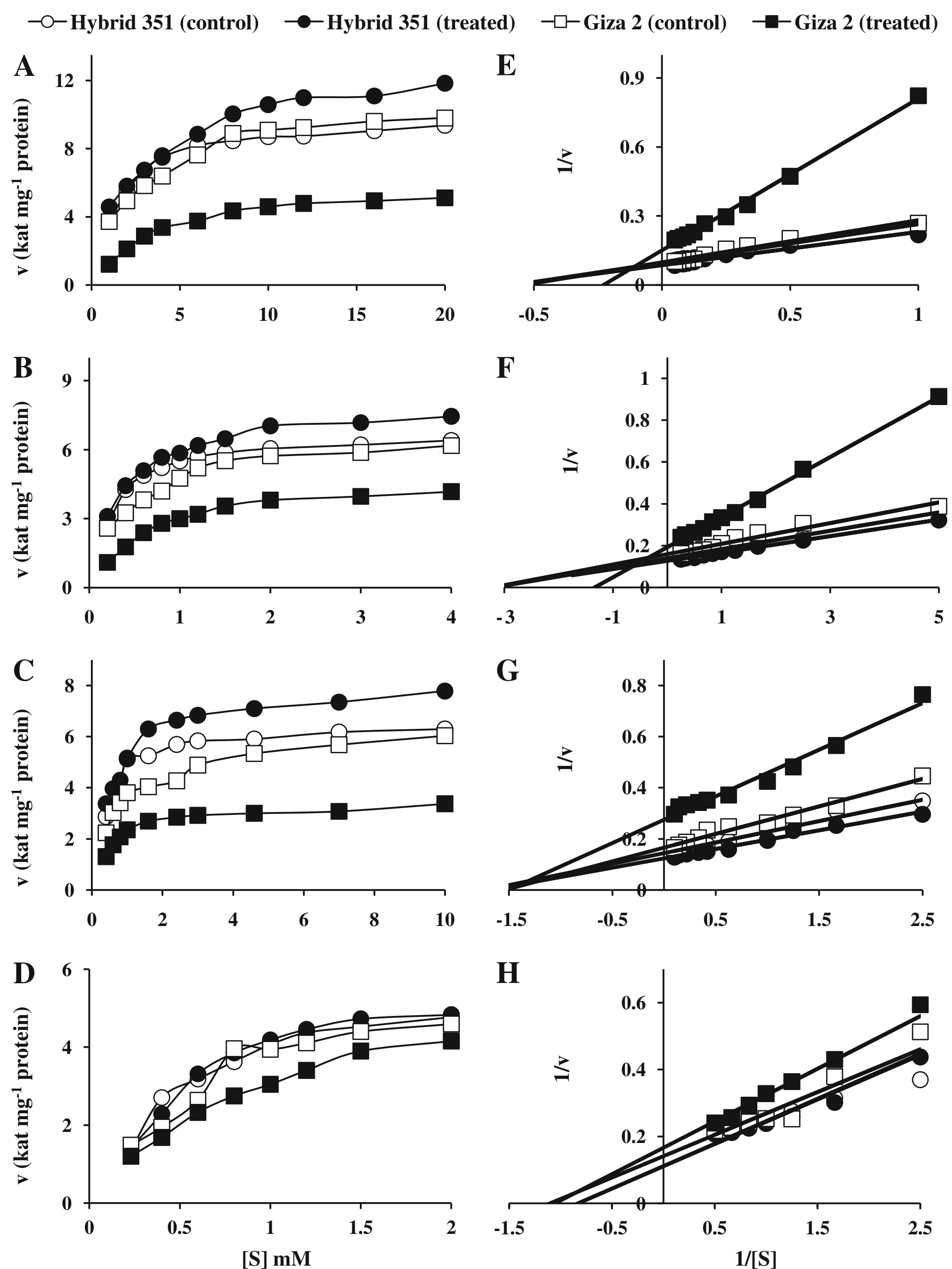

Fig. 4 Effect of treatment with 250 mM NaCl on kinetic parameters of **a** phospho*enol*-pyruvate carboxylase (PEP-C), **b** NADP-malate dehydrogenase (NADP-MDH), **c** pyruvate phosphate dikinase (PPDK) and **d** ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) of *Zea mays* (Hybrid351 and Giza2). **a**–**d** Michaelis–Menten plot for the enzymes extracted 5 days following treatment as a function of concentration [*S*] (phospho*enol*-pyruvate, oxaloacetate, pyruvate and ribulose 1,5-bisphosphate, respectively). **e**–**h** Lineweaver–Burk plot

For easier interpretation of results, the values of kinetic parameters were determined and obtained by data interpolation from equations presented in Fig. 4e–h (Table 1). These data clearly show that V_{max} values of PEPC, MDH and PPDK were significantly higher in treated Hybrid but significantly lower in treated Giza than their respective controls. Meanwhile, NaCl significantly lowered Rubisco V_{max} in Giza but had no significant effects in Hybrid. In contrast, K_m values were slightly affected by NaCl; only increases were detected for PEPC and MDH from treated Giza with no effects for PPDK and Rubisco; the magnitude of increase was most pronounced in PEPC. On the other hand, there were no changes in K_m for all enzymes from treated Hybrid. Moreover, NaCl raised K_{cat} of enzymes from Hybrid; however, these values for the enzymes obtained from Giza were relatively low. The same sequence of trend was detected also for both K_{cat}/K_m and V_{max}/K_m; these ratios were mostly higher in Hybrid and lower in Giza than their respective untreated controls.

Table 1 Changes in kinetic parameter values of phospho*enol*pyruvate carboxylase (PEPC), NADP-malate dehydrogenase (NADP-MDH), pyruvate phosphate dikinase (PPDK) and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) from *Zea mays* (Hybrid351 and Giza2 cultivars) 5 days following treatment with 250 mM NaCl as a function of substrate concentration ([*S*]): phospho*enol*pyruvate, oxaloacetate, pyruvate and ribulose 1,5-bisphosphate, respectively

	PEPC	MDH	PPDK	Rubisco
V_{max} [kat mg^{-1} protein]				
Hybrid351 control	10.31	7.19	6.94	9.09
Hybrid351 treated	11.76*	7.87*	8.13*	9.01
Giza2 control	10.42	6.29	6.06	7.09
Giza2 treated	6.71*	5.21*	3.64*	6.02*
$LSD_{0.05}$	1.59	0.57	0.44	0.32
K_m [mM substrate]				
Hybrid351 control	1.73	0.31	0.58	1.20
Hybrid351 treated	1.70	0.31	0.59	1.20
Giza2 control	1.87	0.31	0.65	0.90
Giza2 treated	4.41*	0.75*	0.66	0.95
$LSD_{0.05}$	0.33	0.08	0.10	0.16
K_{cat} [kat mg^{-1} protein]				
Hybrid351 control	3.03	2.32	2.39	2.67
Hybrid351 treated	3.66*	2.12	2.94*	2.65
Giza2 control	3.80	2.03	1.96	2.29
Giza2 treated	1.10*	0.85*	0.60*	0.99*
$LSD_{0.05}$	0.42	0.39	0.31	0.47
K_{cat}/K_m				
Hybrid351 control	1.75	7.48	4.12	2.23
Hybrid351 treated	2.15	6.84	4.98	2.21
Giza2 control	2.03	6.55	3.02	2.54
Giza2 treated	0.25	1.13	0.91	1.04
V_{max}/K_m				
Hybrid351 control	5.96	23.19	11.97	7.58
Hybrid351 treated	6.92	25.39	13.78	7.51
Giza2 control	5.57	20.29	9.32	7.88
Giza2 treated	1.52	6.95	5.52	6.34

Values were obtained by data interpolation from equations presented in Lineweaver–Burk plot (Fig. 4). Values within the same column followed an asterisk are significantly different from the respective control at 5% level. Significance was not applied for K_{cat}/K_m and V_{max}/K_m. These are ratios obtained from dividing mean values

PEPC phospho*enol*pyruvate carboxylase, *MDH* malate dehydrogenase, *PPDK* pyruvate phosphate dikinase

Discussion

The present results showed that maize growth was significantly reduced by NaCl. Despite these reductions, NaCl was less deleterious to Hybrid. As the time elapsed, the magnitude of reductions retracted in Hybrid and increased in Giza. These results suggest that the two maize cultivars have a differential and relative tolerance to NaCl and this tolerance was larger in Hybrid than Giza. So, Hybrid is considered as more tolerant cultivar to NaCl than Giza. In this account, Qasim et al. (2003) found that some lines of *Brassica napus* were salt tolerant and some others are salt sensitive with regard to shoot dry matter production and seed yield under saline conditions.

In general, salinity reduces plant growth and lowers plant production worldwide (El-Shihaby et al. 2003; Younis et al. 2003; Gao et al. 2008; Aghaleh and Niknam 2009; Yang et al. 2009). Khan et al. (2000) concluded that deleterious effects of salt stress on plants result from low water potentials, ion toxicities, nutrient deficiencies or a combination of all of these factors. Wei et al. (2006) found that 200 mM NaCl significantly decreased leaf chlorophyll, gas exchange, photochemical efficiency, leaf area and soluble sugar contents of *Lycium barbarum*. The physiological mechanisms involved in the reduction of plant growth resulted from salinity are turgor pressure reduction, reduction in photosynthetic activity, effects of accumulated salts on the metabolic activities, molecular responses and oxidative stress (Younis et al. 2003; Hassan and Nemat Alla 2005; Duan et al. 2007; Zushi and Matsuzoe 2009; Yang et al. 2009). The rate of photosynthesis is reduced in salt-treated plants due to inhibition of Rubisco and C4 photosynthetic enzymes (Rivelli et al. 2002). These enzymes have roles in plants to cope with stress conditions. Any inhibition in activities of these enzymes would lead to disturbance in CO_2 assimilation. Abiotic stress such as osmotic stress decreases the efficiency of these enzymes for carbon fixation. Water deficit causes decreases in the activities of Rubisco and PEPC in *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica* (Soares-Cordeiro et al. 2009). Moreover, biotic stress also influences photosynthetic

enzymes. Castillejo et al. (2010) identified 63 protein spots in three *Medicago truncatula* genotypes, in response to *Uromyces striatus*; most of which correspond to enzymes involved in photosynthesis, energy metabolic pathways and stress.

In the present work, the protein of enzymes was most likely equal in the untreated control among both cultivars; however, PEPC, MDH and PPDK were increased in treated Hybrid but decreased in treated Giza with no significant change in Rubisco in both cultivars. Gonzalez et al. (2003) found that PEPC plays an important role in CO_2 fixation in C4 plants; they indicated that abiotic stresses, affecting water status, increased PEPC expression in wheat. In support, treatment of *Mesembryanthemum crystallinum* with high salinity caused an increase in PEPC protein and a concomitant rise in the activity of PEP carboxykinase (Li and Chollet 1994). Moreover, Bouraima et al. (1987) stated that the response of PEPC and MDH in Pearl millet to mild treatment with NaCl depends on the tolerance of seedlings to the salt. They found that PEPC enhanced in the salt-tolerant genotype and decreased in the most sensitive one. They concluded that PEPC and MDH may be effective markers of salt tolerance in the C4 plant. Nemat Alla et al. (2011) reported that NaCl doubled PEPC protein in *Atriplex halimus* while Rubisco was unchanged. They concluded that *A. halimus* tolerates NaCl treatment through decreasing growth and saving the photosynthetic enzymes particularly PEPC. On the contrary, Marques Da Silva and Arrabaça (2004) found that rapid stress increased Rubisco activation in *Setaria sphacelata*; however, PEPC showed a deep decrease of activity.

Therefore, increases in these enzymes by NaCl in Hybrid only suggest that they are playing a possible role in tolerance of maize to salinity. Gonzalez et al. (2003) suggested an important role for PEPC in the adaptation of plants to environmental changes. Contrarily, Debez et al. (2006) reported that Rubisco activity of *Cakile maritime* significantly increased at salinity levels of 100–200 mM NaCl but declined at higher levels while PEPC activity did not show any difference from control. Also, Zhu and Meinzer (1999) found a decrease in Rubisco and PEPC activities in response to salt stress in *Atriplex lentiformis*. In the more tolerant maize cultivar, Rubisco was less inhibited by NaCl while the activities of PEPC, MDH and PPDK were increased indicating the ability of this cultivar to preserve the photosynthetic enzymes under harsh environment. In accordance, Stepien and Klobus (2005) observed a much better capacity of maize to preserve the photosynthetic apparatus against overproduction of reactive oxygen species resulted from salinity stress. Despite the low protein content, NaCl increased the enzymes protein levels in Hybrid. These increases could reveal that this cultivar tends to gather these enzymes to withstand salinity conditions. Nonetheless, the slight changes in Rubisco by NaCl were alike in both cultivars indicating that this enzyme was not effective in maize tolerance to salinity.

The kinetic parameters V_{max}, K_m and K_{cat} define the rate behaviour of an enzyme-catalysed reaction as substrate concentration is varied. Since V_{max} is a function of the enzyme concentration, a decrease in its value consequently suggests a decrease in the enzyme active sites participate in the reaction. Therefore, the increased V_{max} of PEPC, MDH and PPDK in treated Hybrid points to an increase in their concentrations by NaCl while the decreased values in treated Giza confirm an inhibition in biosynthesis rate. However, the Rubisco V_{max} was the least affected by salinity in both cultivars, indicating a state of balance between the enzyme biosynthesis and breakdown.

K_m reflects an enzyme's affinity for its substrate and can be related to isoenzyme distribution and enzyme–substrate affinity (Engel 1984; Nemat Alla and Hassan 1996; Tovar-Mendez and Munoz-Clares 2001; Nemat Alla et al. 2007, 2008). Tabatabai (1973) assumed that the increase in K_m is an indication for the interference with the enzyme–substrate affinity; the lesser the value, the better the affinity. Thus, the increase in K_m of PEPC, MDH and PPDK from treated Giza would suggest that NaCl interferes with the structural integrity of these enzymes in this cultivar. However, NaCl did not change the values of K_m of all enzymes extracted from Hybrid confirming that salinity had no effect on the enzyme–substrate affinity for the cultivar. Nonetheless, the increased K_m of PEPC, MDH and PPDK extracted from Giza points to an interference with their structural integrity. At the same time, the decrease in V_{max} of PEPC, MDH and PPDK obtained from treated Giza in addition to the increase in K_m values could confirm that NaCl reduced synthesis of enzymes and altered their affinity with substrates in this least tolerant cultivar. In contrast, the increase in the V_{max} values of enzymes extracted from treated Hybrid while K_m values unchanged suggest that this more tolerant cultivar can cope with harsh conditions through biosynthesis of C4 enzymes. In confirmation, the values of K_{cat}, K_{cat}/K_m and V_{max}/K_m for most enzymes obtained from Hybrid were higher than those obtained from Giza. These findings support that these enzymes were more efficient as a response to NaCl in Hybrid than in Giza. However, both cultivars tended to preserve Rubisco in salinity conditions as little interference with its synthesis was confirmed but without alteration in its structural integrity. However, both cultivars tended to preserve Rubisco under salinity conditions as there was few interference of NaCl with its synthesis but without alteration in structural integrity.

Conflict of interest The authors declare that they have no conflict of interest.

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