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Vol. 15(40), pp. 2243-2250, 5 October, 2016
DOI: 10.5897/AJB2016.15580
Article Number: 9A26B8160847
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Enzymatic regulation of organic acid metabolism in an alkali-tolerant halophyte *Chloris virgata* during response to salt and alkali stresses

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Received 22 July, 2016; Accepted 15 September, 2016

***Chloris virgata*, an alkali-tolerant halophyte, was chosen as the test material for our research. The seedlings of *C. virgata* were treated with varying salt and alkali stress. First, the composition and content of organic acids in shoots were analyzed and the results indicated that there was not only a significant increase in total organic acids, but there were also obvious changes in different components of organic acids under alkali stress. The increments in citrate were the largest, followed by malate. However, none of the organic acids showed significant alterations in the content and components under salt stress. Also, activity of some enzymes (citrate synthase, malate synthase, NADP-isocitrate dehydrogenase, and isocitrate lyase) associated with such organic acids did not change significantly under alkali stress, but malate dehydrogenase activity markedly decreased under a stronger alkali stress (80 mM). Under salt stress as well as increased malate synthase (MS) activity, however, there was no significant change for other enzymes. These results strongly demonstrated that the enzymatic regulation of organic acid metabolism may be the biochemical basis of alkali tolerance for *C. virgata*. Citrate synthase (CS), MS and isocitrate lyase (ICL) might be the key enzymes that determine the alkali tolerance of *C. virgata*.**

Key words: Salinity, ion balance, enzyme activity, *Chloris virgata*.

INTRODUCTION

More than 800 million hectares of land throughout the world are affected by salt level. This amount accounts for more than 6% of the world's total land area. The salinization of soil is a widespread environmental problem and an important factor in limiting plant growth and productivity (Allakhverdiev et al., 2000). The detrimental effects of high salinity on plants can be observed at the whole-plant level as the death of plants and/or decreases in productivity. Some reports have clearly classified natural salt stress, in terms of salt characteristics, into neutral, alkaline and mixed salt-stress. It has also been shown that alkaline and neutral salt-stress are two

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distinct kinds of stress for plants and should be called alkali and salt stress, respectively (Shi and Yin, 1993; Yang et al., 2008a, b, c; Wang et al., 2015b). Although some researches had focused on alkali tolerance of plants (Wang et al., 2012, 2015a, b; Gong et al., 2014; Sun et al., 2014), little is known about physiological mechanisms of plant alkali tolerance.

The presence of different concentrations of organic acids among various plants in their natural habitat is evident (Miyasaka et al., 1991); these organic acids play essential roles in interactions between the soil and various microorganisms (Yang and Crowley, 2000), making sparingly soluble soil Fe, P and other metals available to growing plants (Johnson et al., 1994). Many studies have demonstrated that plants secrete considerable amounts of citrate, malate and oxalate from roots in response to some abiotic stresses (Delhaize et al., 1993; Yang et al., 2007). The low-molecular weight organic acids may play important roles in protecting plants against abiotic stresses around the rhizosphere. Although some studies have shown that the total organic acids accumulated in shoots of *Kochia sieversiana* and other plants under alkali stress (Yang et al., 2007), only a few reports have demonstrated the organic acids metabolism under alkali stress. The total organic acids and oxalate content of shoots increased in *K. sieversiana* and *Suaeda glauca* under alkali stress (Yang et al., 2007) and the increase in the content of citrate was also detected in *Puccinellia tenuiflora* and sunflower (Shi et al., 2002; Shi and Sheng, 2005). The accumulation of organic acids might be responsible for coping with the imbalance of charges and high pH stress, but the biochemical basis and metabolic control mechanisms of accumulated organic acids is still unclear.

Some researchers have shown that the accumulation and exudation of citrate in root tips with aluminium (Al) treatments is associated with increased citrate synthase (CS) activity and decreased aconitase activity (Yang et al., 2004). There are also a few reports showing the influence of salt stress on organic acid metabolism. It had been indicated that NaCl salinity *in situ* causes an increase in all three MDH activities (total NAD-MDH, mitochondrial NAD-MDH, and chloroplastic NADP-MDH) in salt-tolerant cultivars CSR-1 and CSR-3, whereas 16 to 100% inhibition in activities was noted in salt-sensitive cultivars Ratna and Jaya (Kumar et al., 2000). In *Mesembryanthemum crystallinum* Linn, steady-state transcript levels for chloroplast NADP-MDH decreased transiently in the leaves after salt stress and then increased to levels greater than two-fold higher than levels in unstressed plants, whereas transcript levels in roots were extremely low and were unaffected by salt stress treatment (Cushman, 1993). Popova et al. (2002) reported that the activity of NADP-ICDH in plants adapted to high salinity increased in leaves and decreased in roots, and expression of Mc-ICDH1 was found to be stimulated in leaves in salt-adapted *M. crystallinum* by transcript analyses and western blot hybridizations. These results indicated that organic acid metabolism is influenced by salt stress due to the alteration of some enzyme activities under salt stress, and this kind of change might play a key role in enhancing salt resistance. However, the content of accumulated organic acids under alkali stress was greater than that under salt stress based on a few comparative tests of the two kinds of stress (Yang et al., 2007, 2008c). Therefore, studying organic acid metabolism in plants under alkali stress is becoming more important.

In this study, an alkali-resistant halophyte, *C. virgate*, a grass with high protein content, which makes it a high-quality forage plant (Zheng and Li, 1999) was used as material. The seedlings were treated with varying salt stress and alkali stress to explore the metabolic control mechanisms of organic acid accumulation in *C. virgata* during responses to alkali stress.

MATERIALS AND METHODS

Plant

In this study, an alkali-resistant halophyte, *C. virgata,* a grass with high protein content, which makes it a high-quality forage plant (Zheng and Li, 1999) was used as material. Seeds of *C. virgata* were collected from native grassland in Changling County, Jilin Province, Northeast China, and sown in 17-cm diameter plastic pots containing washed sand. Each pot contained 13 seedlings and seedlings were sufficiently watered with Hoagland nutrient solution every 2 days. Quantity of evaporation was evaluated with weight method (weight of each pot was recorded two times at 8:00 and 16:00). Evaporation was compensated for with distilled water at other times. The research was carried out at Northeast Normal University, Changchun, China during April to July. All pots were placed outdoors and protected from rain. Temperatures during the experiment were 22 to 26°C during the day and 19 to 22°C at night.

Design of simulated salt and alkaline conditions

Salt and alkaline solutions were prepared with Hoagland nutrient solution. Two neutral salts were mixed in a 1:9 molar ratio ($NaCl:Na_2SO_4$), and applied to the salt stress group. Two alkaline salts were mixed in a 1:9 molar ratio ($NaHCO_3:Na_2CO_3$), and applied to the alkali stress group. Within each group, two total salt concentrations (40 and 80 mM) were applied. Therefore, in the 80 mM solution for salt stress, a mixture of 8 mM NaCl and 72 mM Na_2SO_4 would result in total ion concentrations of 152 mM Na^+ + 8 mM Cl^- + 72 mM SO_4^{2-}. In the 80 mM solution for alkali stress, a mixture of 8 mM $NaHCO_3$ and 72 mM Na_2CO_3 would result in total ion concentrations of 152 mM Na^+ + 8 mM HCO_3^- + 72 mM CO_3^{2-}. The pH ranges in the salt stress and alkali stress groups were 6.70 to 6.72 and 10.46 to 10.62, respectively.

Stress treatment

The seedlings of *C. virgata* were treated with varying salt stress (1:9 molar ratio of NaCl to Na_2SO_4; pH 6.70 to 6.72; 40, 80 mM) and alkali stress (1:9 molar ratio of $NaHCO_3$ to Na_2CO_3; pH 10.46 to 10.62; 40, 80 mM) to explore the metabolic control mechanisms of organic acid accumulation as a response to these abiotic stress

factors. When the seedlings of *C. virgata* were 3 weeks old, 15 pots with seedlings growing uniformly were selected and randomly divided into 5 sets, 3 pots per set. One set was used as a control, and the remaining 4 sets were used as various stress treatments. Each pot was considered a single replicate; therefore, there were three replicates per set. Stress treatments were performed once every day around 8:00 am, with the application of nutrient solutions containing the appropriate salts. All pots were watered thoroughly with 500 cm^3 treatment solution applied in three portions. Control plants were maintained by watering with nutrient solution. The entire treatment duration was 3 days.

Organic acid analysis

The composition and content of organic acids in shoots was analyzed and compared to determine the characteristics of organic acids accumulation in response of *C. virgata* to salt and alkali stresses. All plants were harvested in the morning after the final treatment. The plants were first washed with tap water, then with distilled water. Roots and shoots were separated. The samples were oven-dried at 80°C for 15 min, then vacuum-dried at 40°C to constant weight. Shoots were then crushed, and used for testing organic acid. Dry samples (100 mg) were treated with 10 ml deionized water at 100°C for 60 min, and the extract was used to determine the contents of organic acids. Oxalic acid was determined by ion chromatography (DX-300 ion chromatographic system; AS4A-SC ion-exchange column, CD M-II electrical conductivity detector, mobile phase: $Na_2CO_3/NaHCO_3$ = 1.7/1.8 mM; DIONEX, Sunnyvale, USA). The other organic acids were determined by ion chromatography (DX-300 ion chromatographic system; ICE-AS6 ion-exclusion column, CDM-II electrical conductivity detector, AMMS-ICE II suppressor, mobile phase: 0.4 mM heptafluorobutyric acid; DIONEX, Sunnyvale, USA).

Determination of enzyme activity

According to the analysis of organic acids, the relevant enzymes for study were selected and assayed. The main enzymes of the organic acid metabolism should include phosphoenolpyruvate carboxylase (PEPCase, EC 4.1.1.31), citrate synthase (CS, EC 4.1.3.7), aconitase, NADP-isocitrate dehydrogenase (NADP-ICDH, EC 1.1.1.42), Malate dehydrogenase (MDH, EC 1.1.1.37), isocitrate lyase (ICL, EC 4.1.3.1) and malate synthase (MS, EC 4.1.3.2). Following this, *C. virgata* plants were cultivated and treated in exactly the same way as mentioned earlier. After the final treatment, about 3 g leaves were randomly removed with scissors and cut as fresh samples.

Enzyme extraction

250 mg fresh samples were homogenized in an ice-cold pestle and mortar with 4 ml 50 mM HEPES-NaOH buffer (pH 7.5) containing 5 mM $MgCl_2$, 5 mM EDTA, 10% (v/v) glycerol, and 0.1% (v/v) Triton X-100 (Yang et al., 2004). The homogenate was then centrifuged at 15,000 *g* at 4°C for 5 min, and the supernatant was used as the enzyme sources of PEPCase, CS, aconitase, NADP-ICDH and MDH. Another 0.3 g fresh leaves were homogenized in an ice-cold 3 ml 167 mM Tris-HCl buffer (pH 7.5) containing 10 mM KCl, 1 mM EDTA and 1 mM $MgCl_2$. The homogenate was then centrifuged at 10, 000 *g* at 4°C for 5 min and the supernatant was used to measure the activities of ICL and MS (Gerhardt and Beevers, 1970).

Enzyme assay

The final volume of each assay was 1 ml. The activity of CS was spectrophotometrically measured using method of Li et al. (2000). The NADP-ICDH activity was assayed by monitoring the increase of NADPH at 340 nm for 3 min, and according to method of Yang et al. (2004). The activities of PEPCase and MDH were spectrophotometrically assayed by monitoring the decrease of NADH at 340 nm for 3 min using method of Johnson et al. (1994). For the MDH activity measurement, the reaction mixture contained 100 mM Tris–HCl buffer (pH 7.8), 1 mM EDTA, 0.25 mM NADH, 1.25 mM oxaloacetate and 25 µl crude enzyme extract diluted 20 times. The PEPCase reaction mixture contained 100 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM $NaHCO_3$, 1.5 mM PEP, 1 mM NADH and 2 units of MDH. The activities of MS were assayed by a DTNB method at 30°C (Hock and Beevers, 1966). The ICL assay measured the formation of glyoxylate-phenylhydrazone by following the increase of absorbance at 324 nm at 30°C (Ebel and Schwienbacher, 2006). The protein in the enzyme extract was quantified by the method of Bradford (Bradford, 1976). Each sample was repeated at least five times and the error could not exceed 5%.

Data analysis

Each result was the mean of at least three replicated treatments. Values indicate mean ± standard error (SE). Statistical analyses were performed by one-way analysis of variance (ANOVA) using the statistical program SAS. The treatment mean values within the same stress type were compared by post hoc least significant difference (LSD) test at 0.05 level.

RESULTS

Qualitative and quantitative changes of organic acids under salt and alkali stress

The analysis of organic acid composition showed that there were no changes in composition of organic acids in the shoots of *C. virgata* under salt and alkali stress compared with the control. Citrate, malate, succinate, acetate, oxalate and lactate were detected in *C. virgata* shoots (Figure 1). However, the content of total organic acids and the percentages of six organic acids to total organic acid in the shoots of *C. virgata* were significantly different between salt stress and alkali stress. The content of total organic acids increased remarkably with increasing salinity under alkali stress (Figure 1, $p<0.01$), and they increased to 2.3 and 3.3 times that of control for 40 and 80 mM alkali stress, respectively. Of these, citrate content was the greatest, with 26.2 and 44.9% of total organic acids, and increased to 4.6 and 11.3 times than that of the control in shoots (Table 1) for 40 and 80 mM alkali stress, respectively ($p<0.01$). For malate, they were 40.3 and 26.9% and increased to 2.9 and 2.8 times, respectively ($p<0.001$). In addition, the alkali stress increased the content of lactate, succinate and acetate weakly to different extents (Figure 1).

Enzyme activity responses to salt and alkali stress

The activities of CS in leaves of *C. virgata* increased significantly with increasing salinity under alkali stress

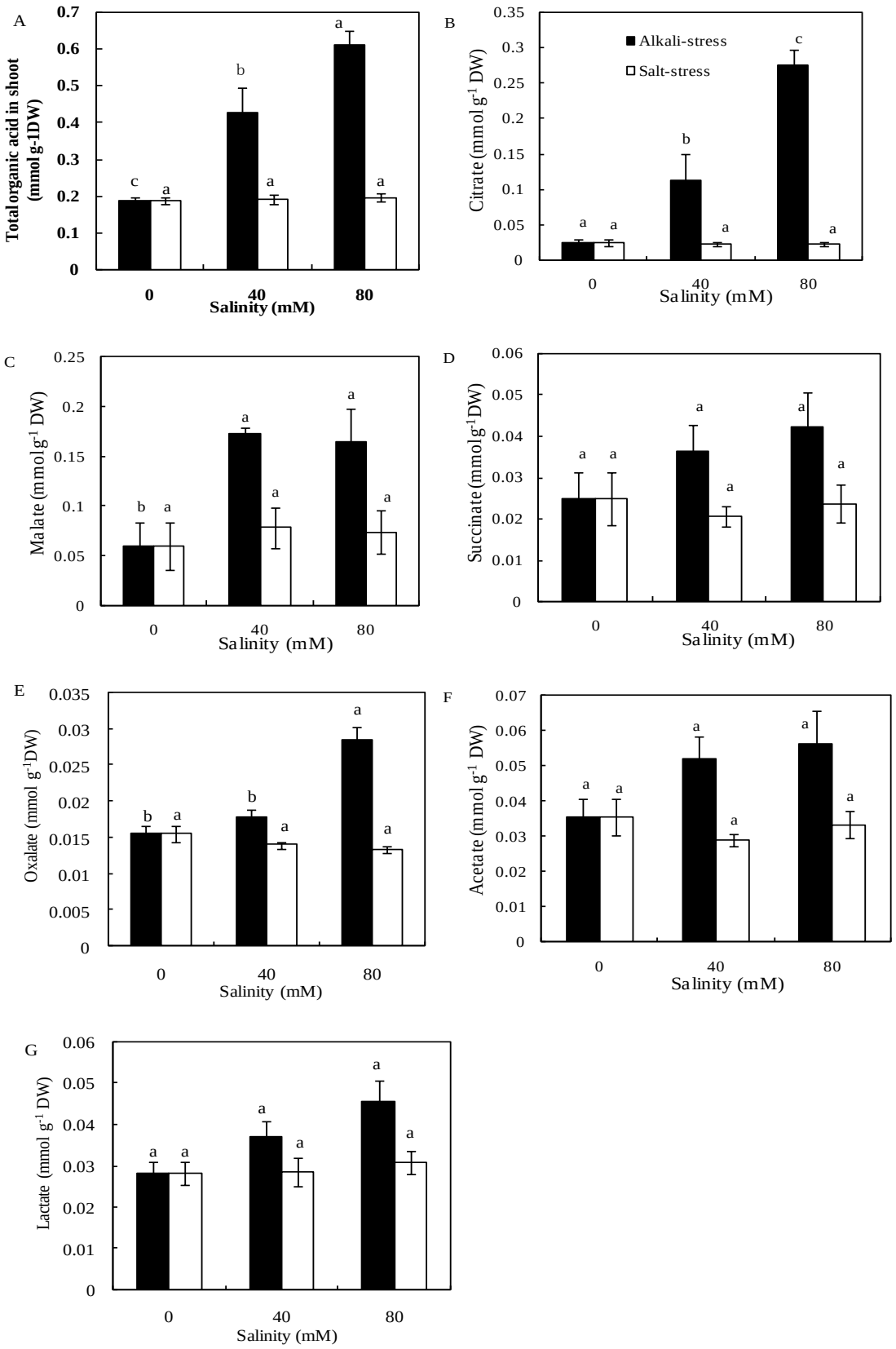

Figure 1. Effects of alkali and salt stresses on contents of organic acids in shoots of *C. virgata*. The values are means ± S.E. of three replicates. Means followed by different letters within the same stress type are significantly different at P ≤ 0.05, according to post hoc least significant difference (LSD) test.

Table 1. Percentage contribution of six organic acids to total moles of organic acid in *C. virgata* shoots under salt and alkali stresses. Percentage is calculated according to the means of three replications.

Parameter	Salinity (mM)	Citrate (%)	Malate (%)	Succinate (%)	Acetate (%)	Oxalate (%)	Lactate (%)
Control	0	13	31.6	13.2	18.9	8.3	15
Alkali stress	40	26.2	40.3	8.5	12.2	4.2	8.6
	80	44.9	26.9	6.9	9.2	4.7	7.4
Salt stress	40	11.8	40.6	10.6	15	7.2	14.8
	80	11.3	37.4	12	16.8	6.8	15.7

($p<0.01$); they increased by 10.4 and 14.9%, respectively, compared to the control under 40 and 80 mM alkali stress (Figure 1). PEPCase activity showed little change or decreased weakly. In the meantime, the activity of aconitase decreased slowly under 80 mM alkali stress, MDH activity markedly decreased, but the activity of NADP-ICDH increased significantly. However, the activities of PEPCase, CS, aconitase, NADP-ICDH and MDH in leaves of *C. virgata* were unaffected or increased weakly by exposure to 40 and 80 mM salt stress.

Under salt stress, the activity of ICL had no significant change, but its activity increased markedly under alkali stress ($p<0.0001$). Salt and alkali stress all increased MS activity, but it was greater under alkali stress ($p<0.0001$) than under salt stress ($p<0.05$); it increased to 1.51 and 1.55 times as against the control for 40 and 80 mM alkali stress, respectively, but it was greater only 1.16 and 1.17 times for 40 and 80 mM salt stress, respectively.

DISCUSSION

Accumulation of organic acids is the key physiological response for *C. virgata* during adaptation to alkali stress

Some reports have clearly demonstrated that alkali salt and salt stress are two distinct kinds of stress for plants and that the effects of alkali stress on plants are more severe than those of salt stress (Yang et al., 2008a, b, c). An explanation for the different effects of the two stresses might be their different mechanisms of action on plants. The injurious effects of salt stress may be a result of low water potentials and ion toxicities. Alkali stress consists of the same stress factors as salt stress, but the influence of high pH is added. The high pH environment surrounding the roots not only may have effect on the ion activities in the nutrient solution, but also may directly cause metal ions, such as Ca^{2+}, Mg^{2+}, Fe^{2+} and Cu^{2+}, to precipitate (Shi and Zhao, 1997), which may lead to depletion of the nutrient supply and disturbance of ion balance around the roots.

The previous studies have also indicated that accumulation of organic acids in plants was an adaptive response to the influx of sodium ions and an important mechanism to maintain stable tissue pH and ion balance for the internal environment (Yang et al., 2007; Shi et al., 2002). Our results also show that the stable tissue pH and ion balance maintained in *C. virgata* is primarily due to the significant accumulation of organic acids. Results shown in Figure 2 indicate that various organic acids and the ratio of each component, do not change much under salt stress. However, a large number of organic acids accumulated under alkali stress, especially for citrate and malate.

Although, an accumulation of organic aids in shoots is a widespread physiological response to plants under alkali stress, the characteristics of organic acid accumulation, especially the kinds of organic acids among various species, is obviously different. For instance, it was reported that the oxalate represented about 90% of total organic acids in the stems and leaves of *K. sieversiana* (Yang et al., 2007) and *S. glauca* (Yang et al., 2008c); oxalate was also the dominant accumulated component under alkali stress. However, *C. virgata* was found to predominantly accumulate citrate under alkali stress; the contents of 40 and 80 mM alkali stress treatments were greater 4.6 and 11.3 times compared with the control, respectively. The gramineous halophyte *P. tenuiflora*, which is in the same family as *C. virgata,* also mainly accumulated citrate under alkali stress (Shi et al., 2002). This may indicate that the plants of different families and genus (or even from the same family), have different metabolic control mechanism of organic acids under alkali stress.

The metabolic control regulation mechanism of organic acids under alkali stress in *C. virgata*

Organic acid metabolism exerts key roles in the plant adapting to various adversity stresses (Ryan et al., 1995; Wang et al., 2007; Ligaba et al., 2004; Rauser, 1999; Yang et al., 2007). A large amount of published data has shown the formation and transport of organic acids in plant cells, characteristics of related enzymes and the exudation of roots (Wang, 2001; López-Bucio et al., 2000; Li et al., 2000). The characteristics of organic acid

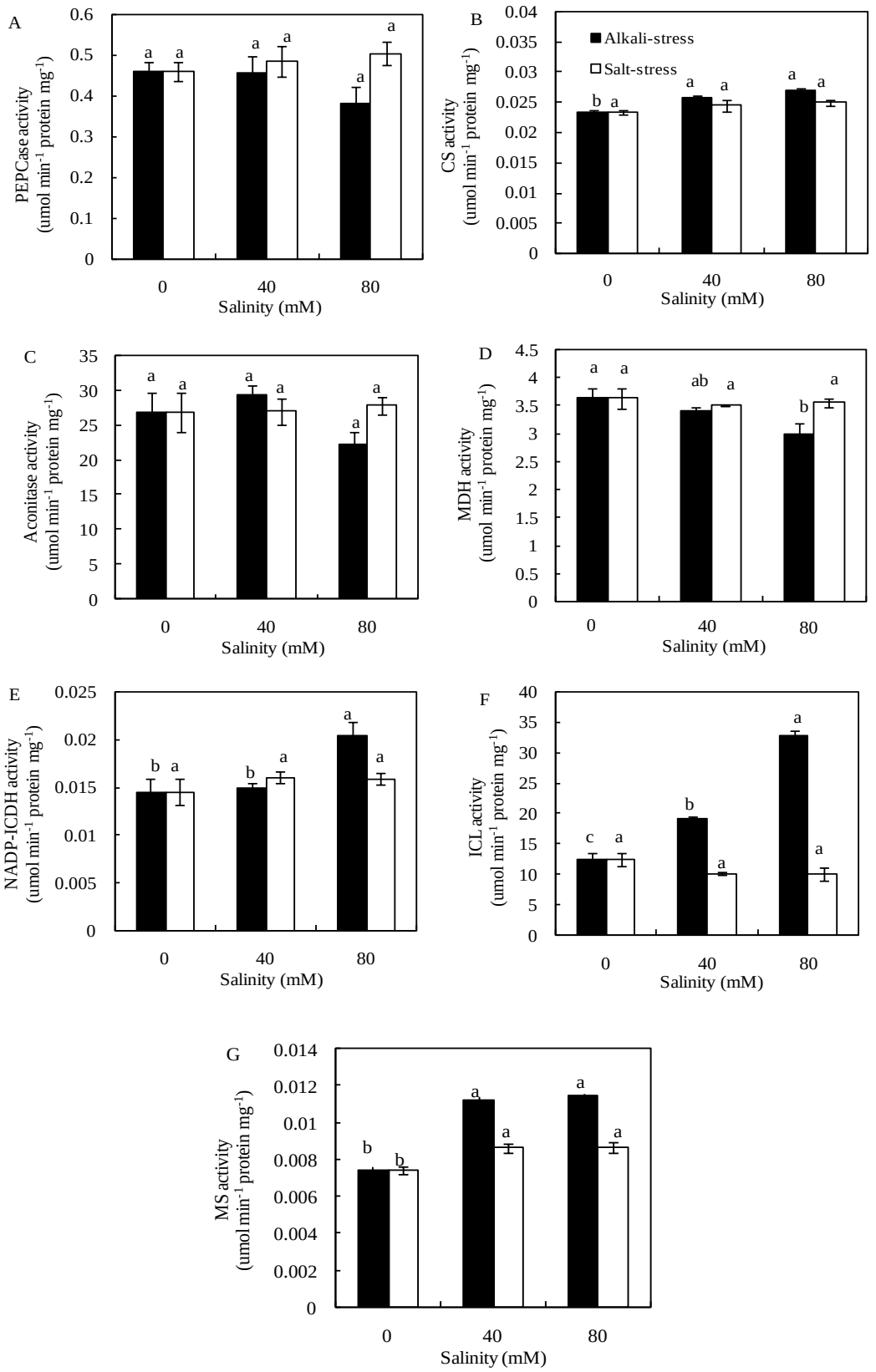

Figure 2. Effects of alkali and salt stresses on activities of key enzymes involved in organic acid metabolism in leaves of *C. virgata*. The values are means ± S.E. of three replicates. Means followed by different letters within the same stress type are significantly different at $P \leq 0.05$, according to post hoc least significant difference (LSD) test.

metabolism at the enzyme level should be revealed under alkali stress by monitoring the key enzymes that determine the rates of relevant reactions under alkali stress. Furthermore, the key genes of plant alkali tolerance would be easily identified by finding the key enzymes of plant alkali tolerance. According to the dominantly accumulated organic acids under alkali stress, citrate and malate, the activities of relevant enzymes were determined and assayed and were expected to reveal the organic acid metabolism in *C. virgata* under alkali stress.

The enzymes, such as CS, MDH, PEPCase, aconitase, and NADP-ICDH involved in synthesis or degradation of citrate, all affect the accumulation of citrate in plants. Yang et al. (2004) reported that aluminium-induced exudation of citrate from roots of *C. tora* was mainly due to the increase in CS activity and the decrease in aconitase activity, but NADP-ICDH, MDH and PEPCase were not directly involved in this physiological process. The P-stressed proteoid roots of *Lupinus albus* also largely excreted citrate, but the activities of CS, MDH and PEPCase were all increased. Meanwhile, western blot analysis also showed that the PEPCase enzyme protein was more highly expressed in –P proteoid roots compared to other tissues (Johnson et al., 1994). The organic acid metabolism is extremely complex in plants and various stress-induced metabolism control mechanisms may also be different. Our results proved this point and showed that the activities of enzymes involved in organic acid accumulation did not alter much under salt stress; however, some enzymes changed significantly under alkali stress (Figure 2).

Our results showed that the CS activity was significantly increased under alkali stress, which may be the main reason for the accumulation of citrate. In addition, the cytosolic form of NADP-ICDH is probably the major pathway for the catabolism of citrate after its transport out of mitochondria and conversion to isocitrate by aconitase in the cytosol (Delhaize et al., 2003; Gálvez et al., 1999). It had been shown that carrot cell lines with a high citrate efflux have reduced NADP-ICDH activity, along with enhanced CS activity, which is consistent with a role for the cytosolic NADP-ICDH in contributing towards the control of citrate concentrations in plant cells (Takita et al., 1999; Kihara et al., 2003). However, the NADP-ICDH activity was significantly increased under 80 mM alkali stress; although this was not conducive to the explanation of accumulated citrate, it was beneficial to interpreting the accumulation of malate simultaneously. Interestingly, alkali stress did not affect the PEPCase activity, indicating that this enzyme was not the limiting factor for the substrate of synthesizing citrate.

The experimental data showed that, although MS activity significantly increased under salt stress, ICL, NADP-ICDH, PEPCase and MDH activities showed little change; this may be one of the reasons for the unchanged malate content. However, MS activity increased significantly under 40 mM alkali stress; when the alkali stress intensity increased to 80 mM, the activities of MS and ICL increased significantly, whereas MDH activity decreased. These results indicated that the changes in these enzyme activities facilitated the accumulation of malate. However, the activities of two specific enzymes in the glyoxylate cycle, namely, MS and ICL, were significantly increased under alkali stress, which might contribute to the malate accumulation. Although, ICL and NADP-ICDH compete for the same substrate, isocitrate, the two enzymes may be expressed together. Malate accumulated sharply due to the high-level expression of glyoxylate-cycle enzymes under alkali stress. The isocitrate, citrate and oxaloacetate may be transported to glyoxysome to support the glyoxylate-cycle activity. When plants become senescent or are kept in the dark for a longer period of time, they start to degrade their endomembrane system and the glyoxylate cycle becomes important in exploiting the end product of fatty acid β-oxidation (Cornah and Smith, 2002). The malate accumulation may also be associated with lipid metabolism under alkali stress.

The organic acid accumulation response to alkali stress was a comprehensive regulated result of two or more enzymes for *C. virgata*: CS, MS and ICL might play key roles in this process. However, the regulation of enzyme activity was likely to be a complex process that occurred at different levels or at different stages, such as transcription, translation, zymoprotein assembly or modification. It could also be the result of interactions of enzyme inhibitors or activators with other regulating substances. Thus, the organic acid metabolic control mechanism at the enzyme level remains to be further studied.

Intracellular organic acid accumulation was not only related to the organic acid metabolism, but also to the organic acid transport. Organic acids synthesized in mitochondria or other parts entered into the certain sites through carriers or specific channels for storage or other specific physiological functions. Under alkali stress, organic acids were mainly transferred to the vacuole and accumulated in order to balance the positive charge and regulate intracellular pH (Yang et al., 2007; Shi et al., 2002). The transport route and efficiency are also closely related to the accumulation of organic acids, and this is worth studying further. Some research had concerned the gene expression and protein interaction in response of plants to alkali stress (Wang et al., 2015a, 2012; Gong et al., 2014; Sun et al., 2014). Metabolism regulation of organic acid should be considered in model plants such as rice and Arabidopsis, which may further facilitate the identification of gene involved in alkali tolerance.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This study was supported by 13th Five-Year Science and Technology Project of Educational Department of Jilin Province (No. 2016195), Initial Project of Jilin Agricultural University (No. 201508), and Key Project of Science and Technology Development in Jilin Province (No. 20126033). We thank anonymous reviewer for the critical and constructive comments for further improving of the manuscript.

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