

Comparative Analysis of Amino Acid Between Transgenic and non Transgenic Egyptian Cotton (*Gossypium barbadense*) Lines under Different Salt Stress Conditions

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Abstract: Based on transgenic cotton lines carrying the bacterial mtID gene and non-transgenic, conventional cotton variety, a greenhouse experiment was conducted to assess the effect of different levels of salt stress on the amino acid content of both transgenic and non transgenic seeds. The amino acid profile was determined by GC mass-spectrum. Transgenic and non-transgenic cotton seeds were grown to maturity under different concentrations of salt stress after determining the field capacity. Seeds obtained from transgenic and non transgenic plants showed increasing concentration of amino acids with increasing the level of salt stress. However, the transgenic cotton seeds accumulated significant amounts of amino acids compared with non-transgenic seeds. There were some differences in mean content of some individual amino acids between transgenic and non transgenic seeds, with some significant differences at higher level of salt stress. Amongst the amino acids that showed significant differences at high level of salt stress were Alanine, Proline, Glutamine, Asparagine and histidine. The difference in amino acid content between transgenic and non transgenic plants was also high for tryptophane, lucine and tyrocine under low level of salt stress. Accumulation of amino acids in seeds from both transgenic and non transgenic plants as a result of salt stress appeared to play an important role in the acclimation to salt stress of cotton plants. However, the higher accumulation of total and individual amino acid in the seeds obtained from transgenic lines under salt stress compared with non transgenic seeds may be a result of the expression of the mtID gene into the genome, which might prove that transferring the mtID gene could be considered one of the effective strategies to produce salt tolerance cotton variety. The results indicated that the insertion of mtID gene in the transgenic cottons had some influence on the synthesis and accumulation of amino acids in transgenic seeds under salt stress.

Key words: Salt stress • amino acids • transgenic cotton • mtld gene • gc mass-spectrum • gossypium barbadense

INTRODUCTION

Salinity is the major stress factor, which delimit crop plants cultivation, especially in developing countries. The adverse effect of salinity on plants may lead to disturbances in plant metabolism, which consequently lead to a reduction of the plant growth and productivity [1, 2]. The ability of plants to cope with salinity stress is an important determinant of crop distribution and productivity in many areas, so it is important to understand the mechanisms that confer tolerance to saline environments. Many trials have been made to help the plants to overcome these disturbances using

various treatments in the laboratory, for future application in the field.

Salt stress involves both osmotic stress, by limiting absorption of water from soil and ionic stress, resulting from high concentrations of potentially toxic salt ions within plant cells. A variety of protective mechanisms have evolved in plants to allow them to acclimatize to these unfavorable environmental conditions for survival and growth. Although the effect of salinity has been studied in a variety of plants, the mechanisms of salt tolerance are not well understood [3].

Biochemical studies have shown that plants under salinity stress accumulate a number of metabolites, which

are termed compatible solutes because they do not interfere with biochemical reactions [4, 5]. These metabolites include carbohydrates, such as mannitol, sucrose and raffinose oligosaccharides and nitrogen-containing compounds, such as amino acids and polyamines. The function of compatible solute accumulation is often associated with osmotic adjustment, by lowering the water potential to improve the uptake of water against the external gradient. It is well established that the intracellular accumulation of these solutes prevents water loss and maintains the turgor pressure of the cell essential for the cell growth. However, a number of other roles for these compounds have been hypothesized [6-8].

Amino acids have been shown to accumulate in plants grown under salinity [9-13]. These amino acids include proline, arginine, alanine, glycine, serine, leucine and valine. Among increased amino acids, proline accumulates in larger amounts compared with other amino acids. In many plant species a remarkable increase in proline content under salt stress was observed [14, 15].

Increased accumulation of amino acids in salt stressed plants could be due to protein degradation [16], or inhibition of protein synthesis [17], or decreases in amino acids and amide export [18], or could be due to growth inhibition of leaves [19]. Stress-induced protein degradation may be essential in providing amino acids for synthesis of new proteins suited for growth or survival under the modified conditions and also substrates for energy metabolism [20].

Plants vary greatly in their capacity to accumulate amino acids under salinity. When plants subjected to salt stress, salt tolerant species/genotypes accumulate more amino acids than sensitive ones [21-25]. Furthermore, contents of amino acids increases also during cell adaptation *in vitro* in cultures treaded with NaCl [26].

Bacterial genes, engineered into plants and resulting in accumulation of proline, mannitol and other osmoregulatory solutes can increase ability to tolerate salinity [27-29].

Cotton is an especially attractive crop for genetic engineering because of its worldwide importance as a crop plant [30]. Various areas of interest are receiving attention; including fiber quality modification, stress tolerance and herbicide and pest resistance [31]. In Egypt, cotton is considered one of the major fiber crops and an essential economic asset.

In previous study [28], a bacterial gene encoding mannitol-1-phosphatase dehydrogenase was used in the transformation of two Egyptian cotton varieties (Giza 86

and Giza 87) by particle bombardment with the aim of producing an accumulation of the sugar alcohol mannitol.

The work reported here represents a contribution to this approach aiming at studying the effect of different salt stress levels on the amino acid content of transgenic and non transgenic cotton seeds, in an attempt to compare the transgenic salt tolerant cotton lines with the non-transgenic, conventional cotton plants in response to different concentrations of salt stress.

MATERIALS AND METHODS

Plant material: The effect of different salt stress conditions on the content of total and individual amino acid in cotton seeds was investigated using both transgenic and non-transgenic seeds (Giza 87).

Transgenic materials: Transgenic cotton (*Gossypium barbadense*) seeds were obtained from gene expression and regulation technology lab-Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza. In a previous work, a bacterial gene, the *mtlD* that encodes mannitol-1-phosphate dehydrogenase, was used to transform an extra long staple cotton variety (Giza 87). The transformation was carried out by particle bombardment of mature, dissected embryos using the Bio-Rad PDS/1000/He gun and the expression of *mtlD* gene and mannitol accumulation were confirmed [28].

Non-transgenic materials: Non-transgenic conventional cotton seeds were obtained from the Cotton Research Institute, Agricultural Research Center (ARC), Giza, Egypt.

Experimental design: Transgenic and non transgenic seeds were grown in pots (30 cm diameter and 30 cm height) containing a mixture of soil, peat and sand (1: 1: 1) with a field water capacity of 35%. Mechanical and chemical analysis of soil samples from each pot were performed according to the method of Black [32]. A Completely Randomized Design was used with four replicates per treatment. An individual pot containing one plant represented a replicate. The experiments were replicated twice and the data presented in this study represent the mean among them.

Fertilization: Fertilization was carried out according to the recommendation of Ministry of Agriculture; each pot received 2.2 g calcium superphosphate (15.5% P_2O_5) and

0.7 g potassium sulphate (48% K₂O) before planting and 3.0 g ammonium nitrate (33.5% N) before the first irrigation.

Salt treatment: A salt mixture consists of sodium chloride, calcium chloride and magnesium sulfate (1: 1: 0.5) was added to the soil to give the desired concentration of salt stress in ppl (0, 1000, 2000, 3000, 4000, 6000, 8000 and 10000). Pots were then irrigated with water to balance the water lost by evapotranspiration. Evapotranspiration was estimated by the pot weight loss before each irrigation and the water content of the soil was always adjusted to the field capacity. The average daily evapotranspiration from the pots was approximately 15% of field water capacity. Adding powder of salt mixture direct into the soil and adjusting water irrigation to the field capacity exhibits a real simulation of salt stress condition and prevents accumulation of salt in soil that could happens if soil is irrigated with different concentration of salt solutions.

Germination conditions: Transgenic and non-transgenic cotton seeds were grown to maturity in a greenhouse under the same conditions of temperature, light and humidity (28°C; 16/8 h day/night and 50-70% relative humidity) and seeds were collected from all plants and subjected to amino acid analysis, however, non-transgenic plants did not survive and no seeds were obtained under 10000 ppl salt stress.

Extraction and identification of amino acids: Extraction and identification of amino acids in seeds were performed according to the method of Gary [33].

A. Extraction of amino acid: 0.2 g of dried seeds were hydrolyzed by 6 M HCl then placed in oven at 110°C for 20 h. After filtrating the mixture and evaporating the supernatant to dryness, 10 ml distilled water were added and the pH was adjusted to 1.8. Few grams of cation exchange resin were added; the mixture was shaken for 10 min on mechanical shaker and then filtrated. Five ml of 10% ammonia solution were added to cation exchange and shaken then filtrated. The ammonia solution was evaporated to dryness under stream of N₂ at 40°C.

B. Derivatization procedure: After extraction of amino acids the residue was mixed with 1 ml methanolic HCl reagent and incubated in oven for 30 min at 70°C then the residue was evaporated to dryness under stream of N₂ at 40°C. Two hundred and fifty microliter

trifluoroaceticanhydrie were added and incubated in oven for 10 min at 140°C then the solvent was evaporated to dryness under stream of at 40°C. Fifty microliter of methanol were added just before injection into gas liquid chromatography apparatus (Hewlett-Pakard 59801) under the following conditions:

- Column: HP-5 M.S. [cross-linked 5% Phenyl Methetyl Silicone] 30×0.250 mm
- Carrier gas: Helium, at flow rate 0.90 ml min⁻¹
- Injector Temp. Program: 100°C/2 min (6°C/min) to 260°C/1 min
- Detector: Mass selective detector

Concentration of total and individual amino acids in cotton seeds from transgenic and non transgenic plants was estimated in (µg g⁻¹) for each salt stress treatment (Table 2 and Figs. 1&2) and the percentage of the difference in amino acid content between transgenic and non transgenic seeds was calculated (Fig. 3)

RESULTS

Mechanical and chemical analysis of the soil: The results of soil analysis are presented in Table 1. Clay was the most abundant element in the soil (37.6%) followed by sand (34.4%) and silt (28%). The total soluble salt was 0.17% and the calcium carbonate was 2.30. 18.2% of the soil was for Ca, Mg, Na, K, HCO₃, SO₄ and Cl collectively. The analysis was performed for soil samples from each pot to ensure the homogeneity of the soil used in this study along the different treatments. The amount of total soluble salt in soil (0.17%) did not appear to have significant effect on accumulation of extra salt in soil; however the homogeneity of soil in all pots assured same condition and salt concentration for all plants.

Analysis of individual amino acids: The content of the individual amino acids (µg g⁻¹) for both transgenic and

Table 1: Mechanical and chemical properties of the soil used for seed germination

Property	Value	Property	Value
Clay (%)	37.60	EC (ds m ⁻²) 1:5	0.54
Silt (%)	28.00	Ca meq l ⁻¹	3.20
Sand (%)	34.40	Mg meq l ⁻¹	2.00
Texture class	Clay loam	Na meq l ⁻¹	2.00
Total soluble salts (%)	0.17	K meq l ⁻¹	1.00
Organic matter (%)	0.31	HCO ₃ meq l ⁻¹	1.20
Calcium carbonate (%)	2.30	SO ₄ meq l ⁻¹	4.50
pH	7.70	Cl meq l ⁻¹	4.30

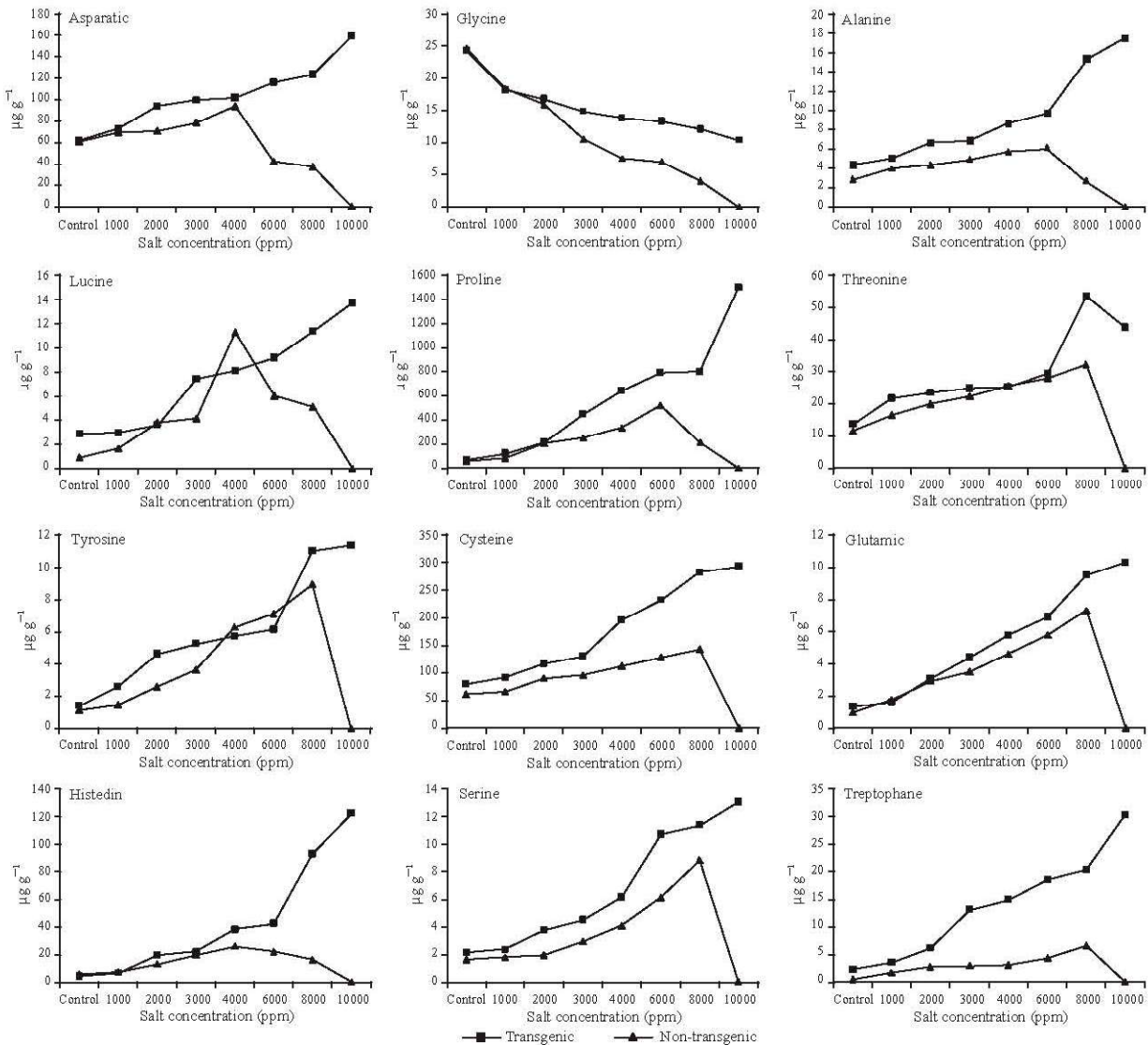


Fig. 1: Amino acid contents ($\mu\text{g g}^{-1}$) of transgenic and non transgenic cotton seeds obtained from plants grown under different salt stress condition

non transgenic seeds obtained from plants grown under different salt stress conditions is presented in Table 2 and (Fig. 1). In non transgenic seeds, some amino acids (Thr, Tyr, Cys, Glu, Tre and Sei) increased with increasing the salt concentration from 0 to 8000. In transgenic seeds these amino acids also increased with increasing salt concentration, however, the content of these amino acids was higher in transgenic seeds (Fig. 1).

The content of Asp and Luc increased with increasing salt stress from 0 to 4000 ppl then a dramatic decrease was observed at 6000 and 8000 ppl. This was not the case for transgenic seeds, where the content of Luc

continued to increase to 1.5 fold and 2.2 fold at 6000 and 8000 ppl, respectively and Asp increased to 2.7 fold and 3.7 fold at 6000 and 8000 ppl, respectively.

The content of Ala and Pro in non transgenic seeds increased with increasing salt stress from 0 to 6000 ppl then decreased at 8000 ppl. While in transgenic seeds, the content of Ala and Pro continued to increase to 5.68 fold and 3.76 fold at 8000 ppl, respectively.

The content of Gly in transgenic and non transgenic seeds decreased with the increasing of salt stress from 0-8000 indicating that Gly is the most negatively affected amino acid during salt stress or it has no significant role in salt tolerance.

Table 2: Concentration ($\mu\text{g g}^{-1}$) of amino acids in cotton seeds from Transgenic (T) and non transgenic (C) plants grown under different concentrations of salt stress (ppm)

Salt	0		1000		2000		3000		4000		6000		8000		10000	
Con.	-----		-----		-----		-----		-----		-----		-----		-----	
(ppm)	C	T	C	T	C	T	C	T	C	T	C	T	C	T	C*	T
Asp.	60.10	61.90	69.50	72.90	69.90	93.30	78.10	98.90	93.60	100.80	41.20	114.70	37.40	122.60	0	158.30
Gly.	24.50	24.20	18.30	18.00	15.80	16.80	10.50	14.80	7.58	13.72	6.91	13.23	4.01	12.09	0	10.27
Ala.	2.80	4.30	3.90	5.10	4.30	6.60	4.90	6.90	5.70	8.70	6.00	9.60	2.70	15.30	0	17.40
Luc.	1.00	2.80	1.60	3.00	3.80	3.70	4.20	7.40	11.30	8.10	6.00	9.20	5.10	11.40	0	13.80
Pro.	54.10	66.40	86.90	122.00	200.00	213.00	251.00	446.00	338.00	639.70	522.70	793.50	213.00	801.10	0	1501.00
Thr.	11.60	13.40	16.30	21.70	20.00	23.40	22.20	24.60	25.30	24.97	27.86	29.15	31.90	53.37	0	43.75
Tyr.	1.30	1.60	1.70	3.00	3.00	5.40	4.20	6.10	7.30	6.70	8.30	7.20	10.40	12.90	0	13.20
Cys.	60.20	80.20	65.40	91.30	88.80	116.00	95.60	130.00	111.00	194.40	128.90	232.10	141.00	283.10	0	293.20
Glu.	0.95	1.30	1.80	1.60	2.90	3.10	3.50	4.40	4.60	5.80	5.80	6.90	7.30	9.50	0	10.30
His.	5.90	3.80	7.10	6.70	13.10	19.70	19.60	21.70	26.30	38.20	21.80	42.70	16.50	93.00	0	122.40
Ser.	1.60	2.13	1.80	2.30	1.90	3.70	2.90	4.50	4.10	6.10	6.10	10.70	8.80	11.30	0	13.00
Tryp.	0.47	2.34	1.67	3.38	2.58	6.17	2.81	12.98	3.06	14.93	4.30	18.45	6.43	20.44	0	30.41
Total	224.50	264.30	275.90	350.90	426.00	510.80	499.50	778.28	637.80	1062.10	785.87	1287.40	484.50	1446.10	0	2227.03

*Non transgenic plants grown under 10000 ppm salt did not survive and no seeds were obtained

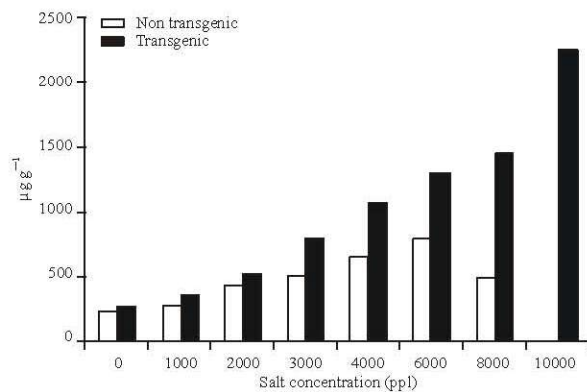


Fig. 2: Total amino acid content of transgenic and non transgenic cotton seeds obtained under different concentration of salt stress (0, 1000, 2000, 3000, 4000, 6000 and 8000 ppm)

At 10000 ppl salt stress, non transgenic plants did not survive and no seeds were obtained while transgenic plants survived and produced seeds with high level of amino acid content (Table 2). This result suggests that the over expression of the bacterial mtID gene in transgenic plants have a significant role in salt tolerant under high salt concentration (10000 ppl) due to mannitol accumulation and other amino acids. In general, transgenic seeds showed more accumulation of individual amino acids compared with non transgenic seeds under different salt stress conditions. There were some differences in mean content of some amino acid

between the transgenic and non-transgenic seeds, with some significant differences at higher level of salt stress. Amongst the amino acids that showed significant increase at 8000 ppl salt stress were Ala (5.68 fold) then followed by His (5.63 fold), Pro (3.7 fold), Asp (3.27 fold), Try (3.17 fold), Gly (3 fold) (Table 2 and Fig. 1). The 3.7 fold increase in proline concentration found in this study is in the range of values reported for other species (summarized by Delauney and Verma [34]).

Analysis of total amino acid: The data for total amino acids concentration in transgenic and non transgenic seeds were consistent with those for individual amino acids. The total amino acids, as determined by summation, progressively increased in transgenic and non transgenic seeds as the salt level increased (Table 2). However, the transgenic seeds accumulated significantly amino acids in seeds compared with non-transgenic plants. The total amino acid content was slightly higher in transgenic seeds compared with non transgenic seeds at low salt concentrations (0, 1000, 2000, 3000 ppl) and clearly built up in transgenic seeds at high salt concentrations (4000, 6000, 8000 ppl) (Fig. 2).

DISCUSSION

Indeed, transgenic seeds showed higher concentration of amino acids compared with non transgenic seeds. It is reported that salt stress provokes reduction in nitrate assimilation process, which supplies

ammonia for synthesis of new amino acids mainly via the GS/GOGAT pathway [35]. Furthermore, salinity inhibits drastically the protein synthesis in the plant tissues [36]. On this basis, the amino acids content is a consequence, at least in part, of the balance between these two processes. Moreover, the salt stress can induce an increment in the de novo synthesis of some particular amino acid [37].

In this study, Ala, His, Glu and Pro were accumulated largely in transgenic seeds compared with non transgenic seeds. These results agree with the findings of Silveira *et al.* [37] who reported a high accumulation of these amino acids in salt tolerant cowpea plants compared with salt sensitive plants under salt stress. It is reported that when plants subjected to salt stress, salt tolerant species/genotypes accumulate more amino acids than sensitive ones [21-25]. Significant differences in Asp, Glu, Pro, Arg, contents were found between the salt-tolerant *Triticum aestivum* L. cv. Sakha and the salt-sensitive *T. aestivum* cv. Regina [38]. The effect of salt stress on the amino acid content of rice varieties differing in salt tolerance is also investigated [39]. Amino acids were differentially accumulated according to the degree of salt tolerance and a relationship between changes in the concentration of amino acids and the accumulation of toxic ion Na⁺ were identified. This accumulation might be a consequence effect of salt stress on nitrogen metabolism in the plant, probably due to an increase of protein degradation [40, 41] and an inhibition of protein synthesis [42, 43]. The same result and conclusion were obtained for sorghum genotypes differ in their ability to tolerate salt stress [44] and two legumes varieties differing in salt tolerance [45]. Ashraf and Fatima [46] also reported that salt-tolerant accessions of safflower (*Carthamus tinctorius* L.) accumulated significantly greater amino acids in the leaves than the salt-sensitive accessions.

In some species, however, both salt tolerant and sensitive genotypes have similar concentration of some amino acids [10]. This was clearly demonstrated in this study for Asp, His and Glu where their content was almost the same in both transgenic and non transgenic seeds under low salt stress condition (0 and 1000 ppl). Supporting the view that no change occurs in protein synthesis or degradation when salt accumulation is mild [47].

Negative correlation between amino acid content and salt tolerant has been shown (e.g. in soybean and blackgram by Moftah and Michel [48], Ashraf [49], Ashraf [14]. These findings support our results that

showed a decrease of the content of Gly in both transgenic and non transgenic seeds with increasing salt concentration from 0 to 8000 ppl. Catello *et al.* [50] reported a decrease in Gly content in Spanish plants grown under severe salt stress condition. This decrease is due to the usage of Gly in the synthesis of glycine betaine, which is common in salt stressed plants. Accordingly, Catello *et al.* [50] suggested that glycine betaine substituted Gly as an osmolyte in salt stressed tissues. Although there was a decrease in Gly content in both transgenic and non transgenic seeds, the content of this amino acid was higher in transgenic seeds compared with non transgenic. This could be explained as the transgenic seeds accumulated more Gly to be used in synthesis of more glycine betaine as an adaptive mechanism for salt tolerant.

Comparing the content of individual amino acids, five amino acids with quantitative importance, namely Ala, Pro, Glu, Asp and His showed a significant increase with salinity, especially in transgenic seeds, at high level of salt stress. The increase of Ala suggests that glycolysis and thus, respiration were increased to sustain the higher energy demand to confine salt to the vacuoles and to furnish carbon skeletons for the photorespiratory cycle [51].

The accumulation of both Pro and Glu in response to salinity is well established in literature (reviewed by Delauney and Verma [34]. Their accumulation is caused by both the activation of its biosynthesis from glutamate and by inactivation of its degradation [52]. However, Pro was shown to minimize cellular damage by enhancing the stability of proteins and membranes [53]. Thereby, it can be of protective value also at the low concentration of salt used in this study. A number of results was published which support the hypothesis of a positive correlation between the ability for Pro accumulation and the degree of salt tolerance [34, 54-56]. Proline accumulation may be interpreted as a symptom of injury caused by stress [57] or some type of adaptive response [58]. Osmoregulation has been attributed to Pro accumulation in tissues of the plants in response to salt stress [59]. On the other hand, Pro would stabilize enzymes as RUBISCO, allowing its efficient functioning even in the presence of NaCl [60].

The amino acid Asp has been shown to accumulate in a number of salt tolerant species (reviewed by Rabe [5]) and in some cases it is the most commonly accumulating nitrogen containing compounds that contain at least two amino groups, this suggest that it may be preferentially synthesized in response to stress and serve as important nitrogen sources for metabolic pathways.

Mansour [61] reported that accumulation of nitrogen containing compounds, especially amino acids, is usually correlated with plant salt tolerance. In our experiment, transgenic seeds responded to increasing salinity merely with double to triple fold increase of total and some individual amino acids especially at high level of salt stress. This variation might be a result of the over expression of the bacterial gene *mtlD* and the accumulation of mannitol in transgenic seeds used in this study. As found for wheat [27], tobacco [29], cotton [28] and other species, expression of the *mtlD* gene increased the tolerance level for salt stress due to accumulation of mannitol. On the other hand, the results obtained in this study suggest that there might be a correlation between mannitol accumulation and amino acid accumulation under critical salt stress conditions as we believe. It is reported that amino acid accumulation under salt stress need amount of carbon which may be a limiting resource in plants in saline environment, because of stomatal closure which reduces the flux of CO₂ to the leaves [62, 63]. Mannitol is a sugar alcohol, which is an intermediate of carbohydrate metabolism [64]. Many studies suggested physiological roles of sugar alcohols including osmoregulation and storage of reduced carbon and energy [65], service as compatible solutes [66], regulation of coenzymes [65, 67] and neutralization of hydroxyl radicals [8]. These studies support our suggestion that mannitol accumulation have a role in the significant accumulation of amino acids in transgenic seeds compared with non transgenic seeds under different salt stress conditions

Transgenic cotton varieties have provided new tools for abiotic stress tolerance but raised concerns about the relative performance of these varieties compared to conventional varieties. This work presents a comparative analysis of amino acid between transgenic and non transgenic Egyptian cotton lines under different salt stress conditions, in an attempt to investigate the performance of transgenic lines compared to conventional variety under salt stress.

Our results support the hypothesis that accumulation of amino acids is one of the adaptive mechanisms for salt stress condition. From the results obtained in this study, we suppose that transgenic seeds were more tolerant to different salt stress condition, specially at high level of salt, due to over expression of *mtlD* gene and accumulation of mannitol, which might play an important role in accumulation of amino acids. We conclude that transgenic seeds accumulated larger amount of individual and total amino acids compared with non transgenic seeds. Therefore, transgenic seeds have better chance

of tolerating salt stress than non transgenic seeds. This conclusion is clearly demonstrated at high level of salt stress applied in this work.

Despite the evidences that transgenic seeds showed higher accumulation of individual and total amino acids compared with non transgenic seeds under different salt stress conditions and survived at 10000 ppl salt stress level while non transgenic seeds did not, further studies are necessary to compare the performance of transgenic and non transgenic cotton seeds under salt stress such as hormones content, growth and some other biochemical characteristics.

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