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Physiological and molecular mechanisms of salinity tolerance in grafted cucumber

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ABSTRACT

Salinity is considered as one of the main stress factors, which impact the horticultural crops. Thus, the objective of this research was to study the molecular, biochemical, and physiological mechanisms of salinity tolerance in grafted cucumber. Cucumber seedlings *Cucumis sativus*, L cv 1010 was either grafted onto four rootstocks (bottle gourd, pumpkin, winter squash, and nubian watermelon) or was self-grafted. Three different concentrations (0, 50, 100 mM) of NaCl solution was applied on the grafted seedlings at an early stage, and throughout the growth, the salinity level was maintained. The grafted plants were observed to maintain higher photosynthetic activity which can be attributed to the improved membrane stability achieved by active non-photochemical quenching (NPQ) and antioxidant enzyme activities compared to self-grafted plants under salinity stress. Moreover, grafting treatments considerably increased auxin (IAA), gibberellin (GA), cytokinin (CK), and salicylic acid (SA) concentrations over the control, whereas, abscisic acid (ABA) decreased under salinity stress. qRT-PCR analysis of gene expression indicated that the expression levels of some stress and photosynthesis-related genes, (*HSP17.8, HSP22, DHN1, LEA2, CAB,* and *CDKG2*) were significantly correlated with stress tolerance. According to the final yield, cucumber with pumpkin rootstock showed a better performance in comparison to other studied grafts, which can be due to the improved status of non-photochemical quenching (NPQ), phytohormones, active enzymatic reactive oxygen species (ROS) quenching, and expression of stress genes in pumpkin rootstock. The obtained results indicated an increase in free radical scavenging capacity in grafter cucumber under salinity stress, which helps in the maintenance of the cellular membrane under stress conditions. The effect can be correlated with better protection mechanisms in the grafted plant due to increased activity of antioxidant enzymes, endogenous phytohormones, and stimulated by the deviation in the expression level of studied genes which improved the defense mechanism against oxidative damage due to salinity.

1. Introduction

Among different abiotic stresses, high salinity is considered to negatively impact the yield of horticultural crops around the globe (Arzani, 2008). The majority of horticultural crops which are highly sensitive to salinity stress are classified as glycophytes (Shannon and Grieve, 1999). The adverse effects of salinity on plant production and growth are status (Colla et al., 2010). There are various mechanisms of salinity tolerance in different plant species, which includes two main strategies: (1) modification of the morphological characteristics of root system *viz*., higher surface area, root length, root density, and root hairs which can participate in the uptake of water molecules and different ions (Krasilnikoff et al., 2003), and/or (2) configuration of the physiological and biochemical mechanisms against salinity stress (Colla et al., 2013; Zhu, 2016). These two strategies comprise of the exclusion of salt from the shoot, whereas salt ions are retained by the root (Zhu et al., 2008a) for the better preservation of potassium homeostasis (Huang et al., 2009). The salt ions were also observed to compartmentalize in vacuoles, whereas, osmoregulator solutes

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accumulate in the cytosol (Munns and Tester, 2008). Salinity also stimulates the enzyme activities in the plant (Helaly et al., 2014, 2017; Helaly 2018) and induces the plant hormones, which may result in the changes in plant growth (Wei et al., 2010). Even after several attempts to improve the salinity stress in crops through traditional plant breeding, the rate of success was observed to be very low, which can be due to the complexity of the salt tolerance traits (Flowers, 2004). For solving this problem, several new cultural practices were implemented in addition to the development of tolerant cultivars, but the results obtained were not promising (Cuartero and Fernández-Muñoz, 1999). One of the methods which were considered to improve the salinity tolerance in plants was the technique of grafting the targeted crop to rootstocks, which was proven to be capable of ameliorating salinity stress in several crops including horticultural crops. Over the years the grafting of horticultural crops from different families for the development of different abiotic stress tolerance in plants has been increased dramatically. For instance, grafting has been performed for the purpose of developing resistance against high temperatures (Rivero et al., 2003), improving the water use efficiency by different plants (Cohen and Naor, 2002) and raising salt tolerance (Estañ et al., 2005).

Nowadays, grafting is a common process for the purpose to develop salt-tolerant plants, in which, both rootstock and scion influences salt tolerance in grafted plants (Etehadnia et al., 2008). The significance of the root system and root characteristics in the regulation of salinity tolerance has been reported in potato genotypes with salt sensitivity or tolerance (Shaterian et al., 2005). Different studies have shown that one of the important mechanisms involved in salt tolerance is antioxidative machinery (Hernández et al., 2000). Additionally, several investigations have shown that the plant hormones (phytohormones) such as cytokinins (CKs), auxin (IAA), gibberellins (GAs), abscisic acid (ABA), and salicylic acid (SA) can prove to be an important factor in metabolic engineering targets for the purpose to produce crops with abiotic stress tolerance (Wani et al., 2016) Furthermore, the plant growth and their response to different abiotic stress conditions are controlled by relevant genes. In previous studies, less attention was given to understand the molecular changes associated with the grafting process and molecular mechanism of graft union development (Mo et al., 2018). Several studies have shown that growth of grafted plants can be modulated in rootstocks and scion by changing its gene expression patterns (Prassinos et al., 2009; Jensen et al., 2010). The study of gene regulatory networks may identify important genes in rootstocks, which may induce growth differences in the scion (Liu et al., 2017). Associated changes in the expression pattern of scion genes with changes in the rootstock induced the growth vigor and fire blight resistance in grafted apple plants (Jensen et al., 2003). In grapevine, the upregulation of genes involved in stress responses was observed when compared between self-grafted and hetero-grafts plants (Cookson et al., 2014).

Cucumber is one of the most important horticultural crops, which is considered sensitive to salinity (Zhu et al., 2008a). However, successful identification of rootstocks for cucumber (family *Cucurbitaceae*) is rare except for the study by Zhu et al. (2008b). To our knowledge, there is no data published until now about the effect of salinity on the antioxidant activities, gene expression, and changes in endogenous phytohormones in grafted cucumber.

Therefore, this investigation aimed to understand the mechanisms by which grafting can improve salinity resistance. The cucumber was chosen for grafting onto four Cucurbita rootstocks to evaluate the photosynthetic activity, membrane stability, mineral status, compatible solutes, antioxidant natural defense system, and plant phytohormones. The changes in the level of expression of stress-related genes within the plant tissues were also examined. The performed study gives an excellent opportunity to systematically understand the salt-tolerant mechanisms in grafted horticultural crops, which may further be used to improve the productivity of crops.

2. Materials and methods

2.1. Plant material

The experiments were conducted in pots at the greenhouse of the Agriculture Botany Department, Faculty of Agriculture, Tanta University, Egypt during autumn of the two growing seasons of 2016 and 2017. In both growing seasons, the average of the daily temperature ranged between 19–34 °C and relative humidity between 16–24%. Hybrid 1010; cucumber (*Cucumis sativus*, L) obtained from "Enza Zaden Company" transplants were grafted onto four rootstocks, pumpkin (*Cucurbita moschata* L), bottle gourd (*Lagenaria siceraria*, L), nubian watermelon (*Citrullus lanatus* L var colocynthoide) and winter squash (*Cucurbita maxima* L, commercial cultivar Flexil). The first three rootstocks were obtained from Vegetable Research Institute, Ministry of Agriculture, Egypt, whereas the last rootstock was obtained from Enza Zaden Company, Holand. Self-grafted hybrid cucumber 1010 transplants were used as a control to eliminate the effects of wound-related hormones. In both seasons on 1st September, seeds of rootstocks were sown separately in seedlings trays (84 cells) 6–7 days earlier than the seed of scion.

Seedlings with first true leaf (15–18 days old) were picked and grafting was performed on the rootstock of scion according to the method described by Lee (1994), and Oda et al. (1994). After 30–32 days, the grafted seedlings were transplanted in the experimental pots of 45 cm depth and 40 cm inner diameter. The pots were filled with equal quantities of vermiculite and compost, then salinized with three concentrations of NaCl (0, 50 and 100 mM). The chemical analysis of the applied compost were weight - 6.8 Kg, moisture content - 16.6%, pH - 7.8), EC - 4.46 dS m^{-1}, total N - 1.03%, O.M. - 31%, OC - 17.5%, ashes - 69%, C/N – 1:17%, total P - 1.20%, and total K - 1.34%.

Each pot contained of 4 seedlings. The seedlings were further thinned after 7 days to leave only one plant per pot. Salted irrigation water was made as recommended by Strogonov (1962). Drip irrigation was used to irrigate the pots with the salinized water, with one emitter per pot and the flow rate was 6 drops per hole per hour. Leaching requirement was established (Richards, 1954) and the amount of irrigation water was adjusted for the purpose to drain at least 35% of the saline solution from the pots.

2.2. Experimental design and sampling data

Differently treated plants were arranged in groups, where the different rootstocks were randomly organized within.

Each treatment was performed with 10 replications (pots), five of them were kept for fruit components and the rest for the physiological analysis. Samples were taken after 60 days of transplantation for physiological characterization and analysis of gene expression.

2.3. Physiological and biochemical characterization

The 3rd Leaf from the plant tip was taken for the purpose to determine the physiological and biochemical characteristics. All the chemicals used for the determination were obtained from Sigma-Aldrich (Sigma-Aldrich, Missouri, USA).

2.3.1. Photosynthetic parameters

Pigments analysis:

Chlorophyll contents (Chlorophyll a, Chlorophyll b, and carotenoids) were determined by the spectroscopic method as per its description from Lichtenthaler and Wellburn (1983).

Net Assimilation Rate (NAR) and Stomatal Conductance (gs): The measurements were performed by a portable photosynthetic system (LI–6200; LI–COR Inc., Lincolin NE, USE). The NAR and stomatal conductance were determined from the 3rd fully expanded leaf of grown plants. The system was equipped as described by Tennessen et al. (1994).

Table 1
The list of designed primers for selected gene sequences as expressed sequence tags (ESTs) on the database of the National Center of Biotechnology Information (NCBI) using primer 5 software.

Gene	Accession no.(NCBI)	Primer sequence 5′———3′	Tm	GC%	Product leangth (bp)
Actin3	GH571733.1	F-TTGACGACTTCGCCCTTAGC R-TCCTTCATCCTCCAACGCAC	*59.90 60.32*	*60 55*	*173*
Hsp 17.8	XM_004138391.2	F-CGGAGCATGGGGAAGTTCAT R-GCCTTAACTTGTGGCCTCCT	*60.11 59.96*	*55 55*	*137*
HSP 22	XM_004136912	F-CGGGCAGAGAGGATGAGTTC R-CTTGAGGCAACTTTGGCACG	*59.90 60.32*	*60 55*	*133*
DHN1	XM_011654848	F-CTCCTCGGAAGATGATGGGC R-CGTATAACCTCCCGGTGTCG	*59.97 59.97*	*60 60*	*130*
LEA2	XM_004136034.2	F-TTGGCCAGGCTCAGGTTAAG R- TGTTCTTCACTGCCTCAGCG	*59.90 60.60*	*55 55*	*174*
CAB	DQ641131	F-GCTTGACGACTTCGCCCTTA R- TCCTTCATCCTCCAACGCAC	*60.39 60.4*	*55 55*	*175*
CDKG2	XM_011657279	F-ATTCCGAACTCTAGGCACGC R-GGTCACCAAATGAGGCCAGA	*60.18 59.18*	*55 55*	*138*

Chlorophyll fluorescence was measured at pre-dawn and mid-day by using Opti-Sciences OS-5P pulse amplitude fluorimeter (Opti-Sciences INC., Hudson, NH, USA). Formula Fv/Fm = (Fm − Fo)/Fm was used to calculate the maximum quantum yield of PSII as described in Genty et al. (1989). Nonphotochemical quenching (NPQ), which represents the absorbed light energy dissipated as the heat was calculated according to Bilger and Bjorkman (1990). Measurements were conducted on 10 different leaves (replications) for each treatment.

2.3.2. Electrolyte leakage (EL)

Electrolyte leakage (EL) was estimated following the protocol described in Lutts et al. (1995).

Lipid peroxidation: Malondialdehyde (MDA) concentration was determined for the determination of lipid peroxidation. The MDA extraction was performed as per protocol in Madhava-Rao and Sresty (2000) from 1 g fresh weight of leaf material.

Total carbohydrate and sugars were determined according to the method described in Dubois et al. (1956).

Total phenol content: The phenol content from leaf material was determined according to the Folin–Ciocalteu colorimetric method (Singleton and Rossi, 1965), where gallic acid was used as the standard. Proline contents and total free amino acid contents from leaf were measured according to Bates et al. (1973) and Rosed (1957), respectively. The total protein content in leaf was estimated by following the protocol from A.O.A.C (2000).

2.4. Antioxidant enzymes activities

The 3rd fully expanded leaf from the plant tip was used to calorimetrically determine antioxidant enzyme activities and stored at −80 °C after harvesting. Antioxidant enzymes such as Catalase (CAT) and peroxidase (POX) were assayed at 570 and 430 nm respectively using the methods described by Urbanek et al. (1991), whereas, the activity of superoxide dismutase (SOD) was measured by using the protocol described in Beauchamp and Fridovich (1971). The activity of ascorbate peroxidase (APOX) was measured by following the protocol from Nakano and Asada (1981). The activity of glutathione reductase (GR) was according to the description in Foyer and Halliwell (1976), whereas, polyphenol oxidase (PPO) activity was measured as described by Kunwar and Khan (1982).

2.5. Endogenous phytohormones analysis

Plant hormones such as auxin (IAA), abscisic acid (ABA), and gibberellins (GA) were measured by using high performance liquid chromatography (HPLC) method as per its description in Koshioka et al. (1983). Cytokinins were also measured using HPLC, for which the protocol from Nicander et al. (1993) was used. Salicylate (SA) was measured spectrophotometrically according to the methods described in Warrier et al. (2013).

2.6. Gene expression analysis

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to determine the changes in the expression patterns of selected genes. Leaves of grafted cucumber on various rootstock were used to extract total RNA using RNeasy plant kit (BioFlux Cat#BSC52S1) according to the instructions of the manufacturers. DNase I (TaKaRa, Japan) was used to remove DNA contamination. RNA was analyzed in 1.2% agarose gel using RNase-free buffers and treated equipment to asses RNA integrity. RNA extracts were diluted 1:10 in DEPC-treated water and RNA concentration was determined using NanoDrop spectrophotometer (BioDrop μLITE.UK). RNA purity values were relatively higher by 1.8, which is considered acceptable.

First-strand cDNA was synthesized using total extracted RNA for each sample according to the protocol provided by SensiFAST ™ cDNA Synthesis Kit (two steps) with oligo $(dT)^{15}$ primer. After that, 1 μl cDNA was used as a template in a 20 μl reaction volume according to the instructions provided by Eva-green RT-PCR mix (SOLIS BIO DYNE). Specific primers were designed for selected gene sequences as expressed sequence tags (ESTs) on the database of National Center of Biotechnology Information (NCBI) using primer 5 software. Used primers are presented in Table 1. Actin gene (ACT3: accession no. GH571733) was used as an internal constitutively expressed control (reference gene). qRT-PCR for each sample was performed in triplicate, and the data were presented as the mean and their standard errors. Comparative $^{\Delta\Delta}$CT experiments on Applied Biosystems StepOne™ Real-Time PCR Systems were used to perform the qRT-PCR reactions. The melt curve analysis (from 55 °C to 94 °C) was used to check the specificity of the PCR amplification.

2.7. Statistical analysis

All obtained data were tabulated and subjected to the statistical analysis of variance (ANOVA) according to Gomez and Gomez (1984). To compare the significant differences between mean and new least significant differences (New LSD) at 5% level was done by MSTAT–C program version 7 (1990).

3. Results

3.1. Physiological responses

3.1.1. Photosynthetic activity and chlorophyll fluorescence parameters

The obtained result shows that the salinity impacted the total chlorophyll concentration, net assimilation rate (NAR), and stomatal conductance (gs) in studied plants. The grafting combination and their interactions are presented in Fig. 1. Data indicated that salinity

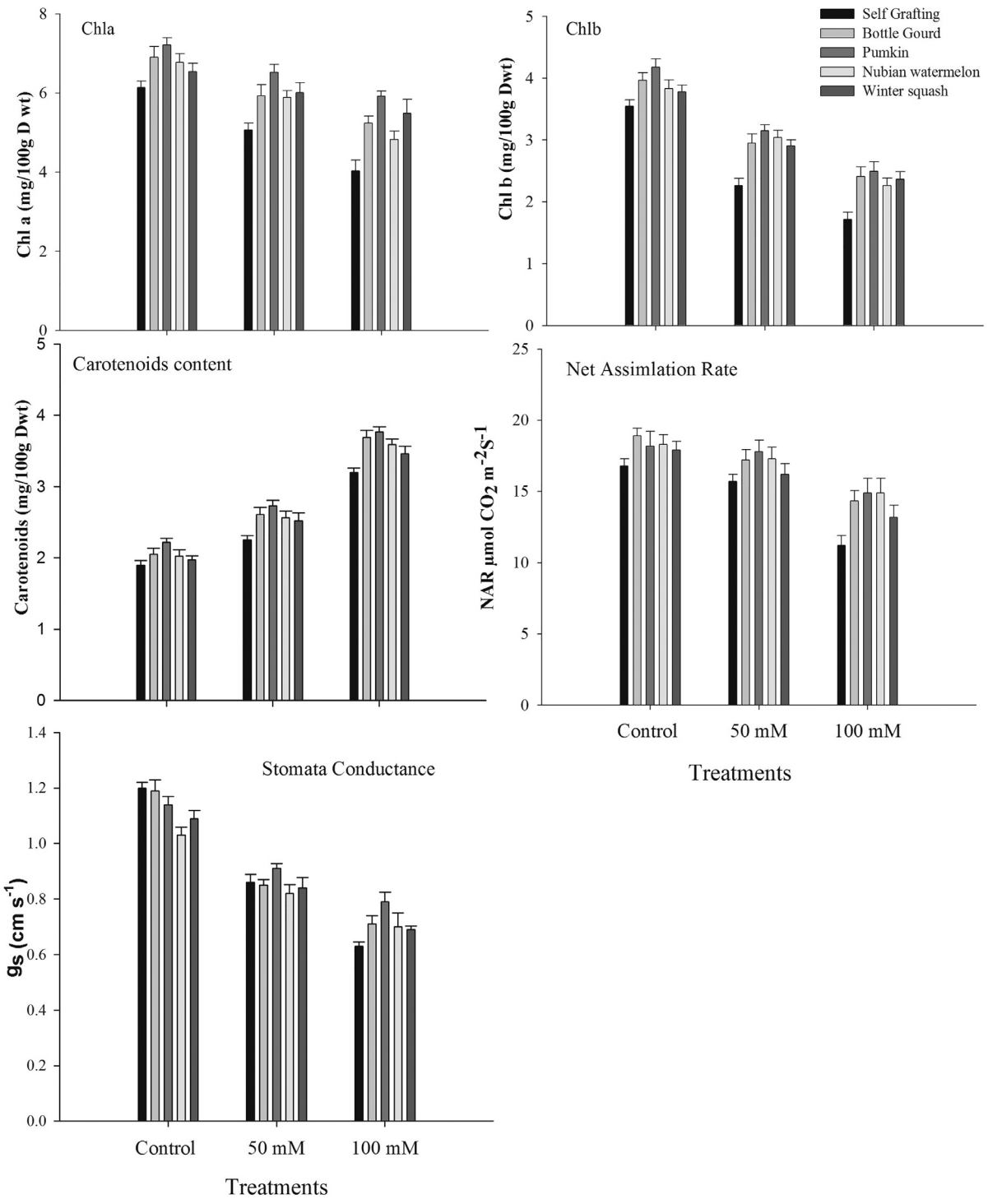

Fig. 1. Effect of rootstocks on the Chl a, b,carotenoids net assimilation rate (NAR), and stomatal conductance (gs in four rootstocks compared with self-grafted of cucumber plants grown under salinity stress (0, 50 and 100 mM) during the two consecutive seasons (2016 and 2017). Each number represents the average of 10 replicate of both season, and different letters above the columns indicate significant differences at the $p < 0.05$ level.

resulted in a significant loss of chlorophylls concentration, especially among self-grafted plants. The grafted cucumber plants onto pumpkin followed by bottle gourd showed higher Chl a, Chl b and lower carotenoid contents, which was followed by nubian watermelon and winter squash rootstocks in comparison to control. The highest increased in NAR, gs, Chl a, Chl b and carotenoid were 33.1%, 25.4%,46.8%, 45.2%, and 17.8%, respectively, in pumpkin compared with self-grafted plants under 100 mM of salinity level. The interaction treatments indicated that NAR in self-grafted plants grown under salinity levels of 50 mM and 100 mM were drastically decreased in comparison to non-salinized conditions. Under different salinity levels, the Fv/Fm values were observed to be significantly decreased ($P < 0.05$) for self-grafted plants in comparison to rootstocks, whereas the NPQ values significantly increased ($P < 0.05$) in rootstocks in comparison to self-grafted plants under salinity stress (Fig. 2). The highest Fv/Fm and NPQ values were obtained in grafted cucumber plants on pumpkin (12.1% and 39.4%) compared with self grafted cucumber.

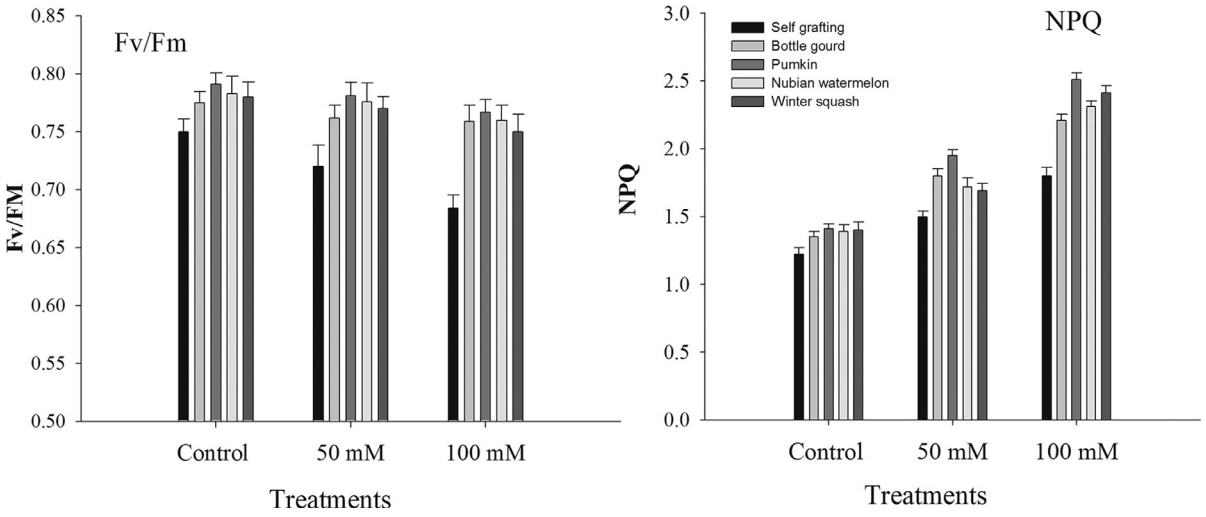

Fig. 2. Effect of rootstocks on chlorophyll fluorescence parameters (Fv/Fm and NPQ) in four rootstocks compared with self-grafted of cucumber plants grown under salinity stress (0, 50 and 100 mM) during the two consecutive seasons (2016 and 2017). Each number represents the average of 10 replicate of both season, and different letters above the columns indicate significant differences at the $p < 0.05$ level.

3.1.2. Membrane stability and MDA

Membrane stability, as indicated with the percentage of electrolyte leakage (EL%) was observed to be significantly affected by different salinity concentrations, the combination of grafting and their interactions (Fig 3). Irrespective of grafting, plants grown under salinity had high levels of EL%. The increase in EL% was dependent on salinity concentration. Moreover, the EL% was observed to be significantly high in self-grafted plants compared with other grafted plants, especially under saline conditions. The grafted cucumber plants onto bottle gourd showed higher membrane stability followed by pumpkin, nubian watermelon, and squash rootstocks respectively. The highest EL% was obtained in self-grafted plants compared with grafted ones, especially under the high level of salinity (100 mM). The lowest decreased in EL% and MDA was 9.3% and 24.3% in pumpkin plants compared with self grafted plants.

3.1.3. Compatible organic solutes and osmolytes

Concentrations of total phenols, total protein (TSP), total sugars (TS), and proline as affected by salinity, grafting, and their interaction are presented in Fig 3. Salinity stress regardless of grafting resulted in the increase of total phenol (TSpH), TS, TSP and proline concentrations of the cucumber shoots in both seasons. The highest value for the above parameters was recorded at 100 mM of salt concentration. Grafted cucumber accumulated more organic solutes compounds and osmolytes in comparison to self-grafted plants irrespective of salinity level. Thus grafted plants had a higher concentration of TSpH, TS, TSP, and proline than self-grafted plants under the salinized and non-salinized conditions in both seasons. Rootstocks have an insignificant increase in this respect. However, cucumber plants grafted onto Bottle gourd and Pumpkin rootstocks gave the highest value followed by those grafted on nubian watermelon and winter squash, respectively. The highest increased for total phenols total sugars and praline were 2.9%, 48.2%, and 14.4% in pumpkin while bottle gourd was 7.2% higher in total protein over self-grafted plants. The grafting under salinity stress has shown the high compatible organics solutes and osmolytes for bottle gourd and pumpkin, followed by nubian watermelon and winter squash rootstocks, which was observed to be high for 50 mM salinity stress in comparison to 100 mM.

3.2. Oxidative enzymes

The activities of CAT, POX, APOX, SOD, and GR, as well as PPO of self-grafted and grafted cucumber plants under the influence of the different concentrations of salinity, are shown in Table 2. The SOD activity was observed to be decreased due to an increase in salinity levels for both self-grafted and grafted cucumber plants. The rate of decrease in SOD activity for all conditions was higher under 100 mM salinity stress in comparison to 50 mM. Unlike SOD a significant increase in POX and CAT activities was observed under salinity, and the highest activities were observed from 100 mM salt-treated plants. However, SOD, POX and CAT activities were lower in self-grafted than grafted plants, especially for salinity treated plants. No significant difference in enzymatic activity in grafted plants was observed among the four rootstocks. The interaction treatments showed that grafted plants grown under the salinity concentration of 100 mM (highest) were observed with the highest activities of CAT and POX for both the growing seasons. In addition, grafting counteracted the depression effects of salinity by increasing SOD activity.

3.4. Endogenous phytohormones

The data in Fig. 4 shows that in both growing seasons, salinity caused a reduction in IAA, GA, CK levels while ABA and SA increased in the leaves of cucumber plant for grafting treatment. However, a significant increase in IAA, GA, CK, and SA concentration was observed for the grafted plant in comparison to control, whereas a decrease in ABA concentration was observed. When grown in salinized conditions, the interaction treatment (salinity x grafting) for all four combinations does not cause any significant differences in phytohormones. The depression effects of salinity on the concentrates of phytohormones (IAA, GA, and CK) were observed to be inhibited, whereas, an enhancing effect of salinity was detected for SA. The highest increased in GA, CK, and SA were 12.3%, 12.6% and 55.1% in pumpkin while the highest increased in IAA was 31.2% in bottle gourd compared with self-grafted plants. Whereas the highest decreased in ABA was 42.6% in pumpkin compared with self-grafted plants under 100 mM NaCl concentration.

3.5. Stress-related genes

qRT-PCR analysis was performed to investigate the role of some stress-related genes which are known for their protection function such as dehydrins (DHNs), heat shock proteins (HSPs) and late embryogenesis abundant proteins (LEA). Other genes related to

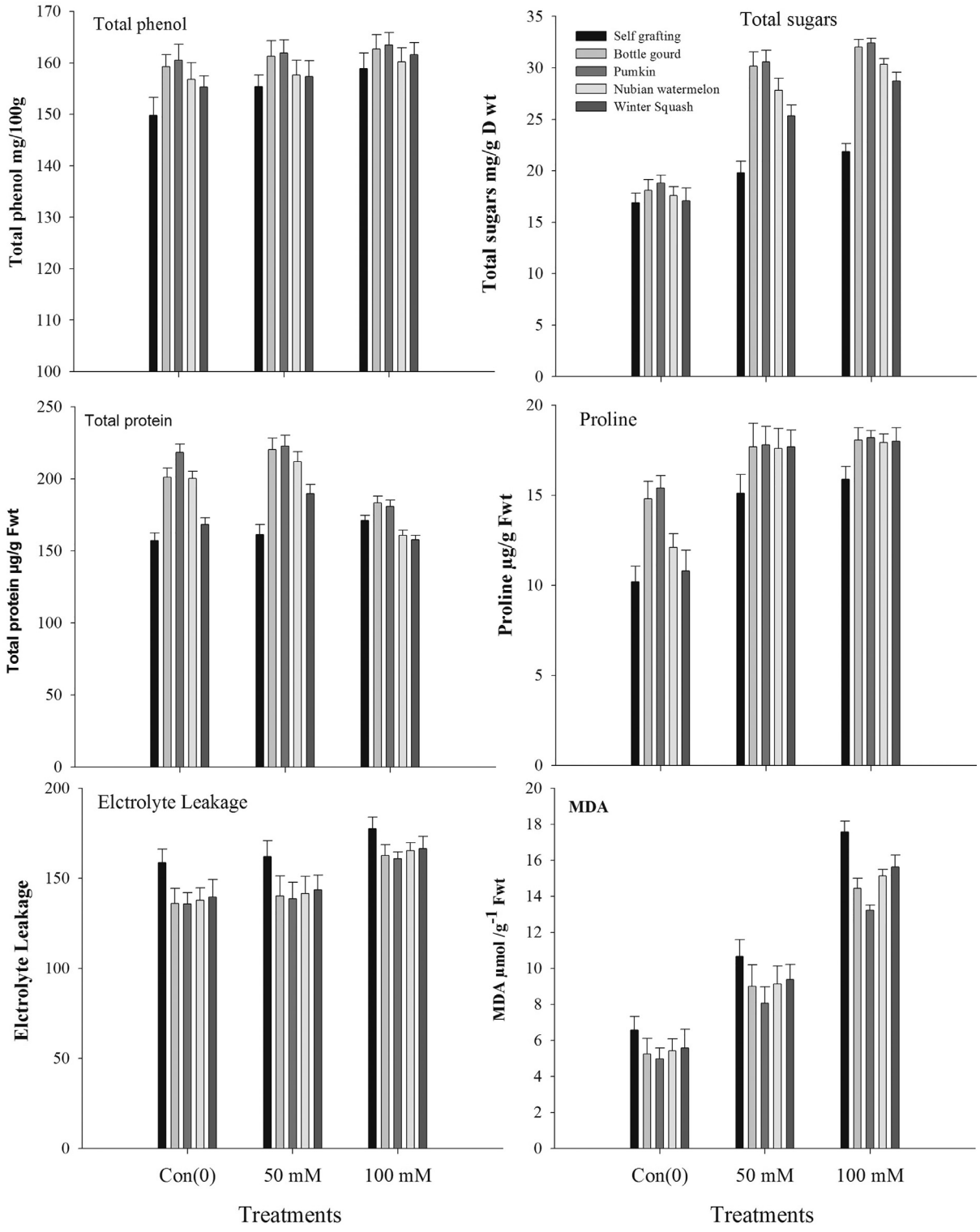

Fig. 3. Effect of rootstocks grafting on the total phenol (mg/100 g Fwt), total sugars (mg/g D wt), total protein (µg/g Fwt), proline (µg/g Fwt), electrolyte leakage (EL), and MDA (μmol/gFwt) in four root stock compared with self-grafted of cucumber plants grown under salinity stress (0, 50 and 100 mM) during the two successive seasons (2016 and 2017). Each number represents the average of 10 replicate of both season. Numbers written in italic represent LSD at $P < 0.05$.

photosynthesis process [chlorophyll a/b binding protein (CAB)] and cell division (CDKG2) were also considered in this study. All studied genes showed an increase in their expression level in all hetero-grafted plants in comparison to self-grafted plants under both studied conditions (Fig. 5). The highest increased *HSP17.8, HSP22, DHN1, LEA2*, CAB and CDKG2 were 61.8%, 37.5%, 170.4%, 98.9% and 75.2% in pumpkin over grafted plants.

4. Discussion

4.1. Physiological responses

4.1.1. Photosynthetic activity and chlorophyll fluorescence parameters

A decrease in chlorophyll concentration could induce a reduction in net photosynthetic rate, which attributed to the reduction in leaf

Table 2
Effect of rootstocks on the CAT min/g F wt, POX min/g F wt, SOD min/g F wt, GR min/g F wt, PPO min/g Fwt, and APOX min/g F wt in four rootstock compared with self-grafted of cucumber plants grown under salinity stress (0, 50 and 100 mM) during the two successive seasons (2016 and 2017). Each number represents the average of 10 replicate of both season. Numbers written in italic represent LSD at $P < 0.05$.

Salinity L	Rootstock	CAT min/g Fwt	POX min/g Fwt	SOD min/g Fwt	GR min/g Fwt	PPO min/g Fwt	APOX min/g Fwt
Cont(0)	Self-grafted	11.45	2.18	16.10	24.05	2.52	4.72
	Bottle gourd	12.23	2.36	16.80	21.21	2.86	4.51
	Pumpkin	11.97	2.38	16.88	21.18	2.87	4.34
	Nubian watermelon	11.89	2.42	16.39	21.23	2.81	4.32
	Winter squash	11.91	2.38	16.30	22.71	2.77	4.42
	Mean	11.88	2.34	16.49	22.1	2.76	4.46
50 mM	Self-grafted	13.09	2.29	15.54	23.35	2.78	4.91
	Bottle gourd	15.36	2.54	16.80	20.26	3.02	4.82
	Pumpkin	15.43	2.57	16.88	20.23	3.07	4.71
	Nubian watermelon	15.38	2.48	16.40	20.24	2.96	4.82
	Winter squash	15.03	2.49	16.11	22.05	2.89	4.51
	Mean	14.85	2.47	16.34	21.22	2.94	4.76
100 mM	Self-grafted G	17.09	2.37	13.53	20.14	3.12	6.31
	Bottle	17.71	2.74	15.31	17.21	3.26	6.15
	Pumkin	17.28	2.79	15.56	17.29	3.26	6.01
	Nubian	17.21	2.49	14.77	17.21	3.12	6.11
	Squash	17.19	2.50	14.80	20.11	3.01	6.02
	Mean	17.3	2.58	14.79	18.39	3.15	6.12
Mean	Self-grafted	13.88	2.28	15.06	22.51	2.82	5.31
	Bottle gourd	15.1	2.55	16.30	19.56	3.05	5.16
	Pumpkin	14.89	2.58	16.44	19.57	3.07	5.02
	Nubian watermelon	14.83	2.46	15.85	19.55	2.98	5.08
	Winter squash	14.71	2.46	15.74	20.29	2.97	4.98
N.LSD 5%	Salinity	*1.72*	*0.13*	*0.76*	*0.86*	*0.12*	*0.33*
	Rootstock	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
	SxR	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

area in response to salinity, especially in the self-grafted plants (data not shown). The reduction of photosynthetic pigments activity under salinity was previously reported, which could induce peroxidation of chloroplast membranes mediated by salinity (Helaly et al., 2017). Several investigators found that chlorophyll pigments were reduced with an increase in salinity level, which may be due to the disruption of the fine structure of chloroplasts and pigment-protein complex or chlorophyll stability which can result in chlorophyll oxidation (Meng et al., 2016). Salinity stress can produce ROS, which is highly active molecules, therefore can easily disrupt cell membrane and induce oxidation of photosynthetic pigments and protein content. In cucumber under stress conditions, Hattori et al. (2008) observed the formation of proteolytic enzymes such as chlorophyllase, which can induce the reduction of chlorophyll content as a result of ROS. Thus our study suggested that grafting may improve the adverse impact of salinity by protecting chlorophyll.

The Fv/Fm and NPQ results can suggest that rootstock grafted plants may delay photoinhibition under salinity. The observation may partially be attributed to the higher rate of NPQ which suggests that more energy is dissipated safely as heat (Elsheery and Cao, 2008). It appears to be related to the capacity of maintaining higher net CO_2 assimilation under salt stress. The decrease in NAR was associated with a paralleled decrease in g_s. Moreover, NAR can be suppressed as a result of the constraint of leaf area that reduces the available assimilation rate for the growth of the leaf. Previously, in several studies, it has been demonstrated that under salinity, the CO_2 assimilation rate of plant decreases, which is the main energy source for plant growth and development (Wu, 2013). Similarly, a significant decrease in photosynthetic activity and stomatal conductance of plan in response to salinity was previously reported (Martre et al., 2002). Gupta and Huang (2014) observed that salinity inhibits plant growth as a result of stomatal closure, which reduces the CO_2 fixation and may lead to the leaf elongation and a decrease in cell division.

An increase in NAR and g_s was observed in grafted plants in comparison to self-grafted plants; this may be due to the accumulation of sugars and proline in the leaves. Similarly, Sperling et al. (2014) have observed that sugars have dual roles in plant metabolism, which includes sucrose metabolism and its function as osmoprotectants during drought stress. The drought stress can affect plant – water relations, and reduced stomatal closure, which further results in a decrease in photosynthetic rate and plant growth (Gong and Chen, 2012). Moreover, underwater deficient conditions the stomata closure may lead to decreases in CO_2 assimilation rate and then the accumulation of NADPH + H^+. In photosynthesis, the $NADP^+$ acts as a limiting factor, whereas, the oxygen acts as an alternative acceptor site for electrons generated through photorespiration, which results in the production of superoxide anion radicals (O_2^-) as stated by Cadenas (1989). In plants, many factors can induce a strong reduction in photosynthetic rates such as the loss of protein and chlorophyll and decreased membrane permeability as a result of oxidative stress and sign of senescence. Therefore, the PSII activity was measured in this study through active chlorophyll fluorescence measurement techniques. One of the photosynthetic factors Fv/Fm (an indicator of the quantum efficiency of photosystem II) is an established indicator of the photoinhibition or the biotic/abiotic stress damage to the PSII (Calatayud and Barreno 2004). In this study, the value for Fv/Fm was observed to be significantly reduced in the self-grafted plants under the studied salinity concentrations of 50 and 100 mM, this result indicated the occurrence of photoinhibition and damage to PSII system (Demmig-Adams and Adams, 1992). Under salinity stress, a decrease in Fv/Fm values was observed in the rootstock-grafted plants in comparison to self-grafted plants. The result suggested that the grafting process may reduce the chances of photoinhibition under salinity stress. Similar results have been previously observed in tomatoes (He et al., 2009), and in grafted cucumber with different rootstock under saline conditions (Liu et al., 2012 and Colla et al., 2013). Higher

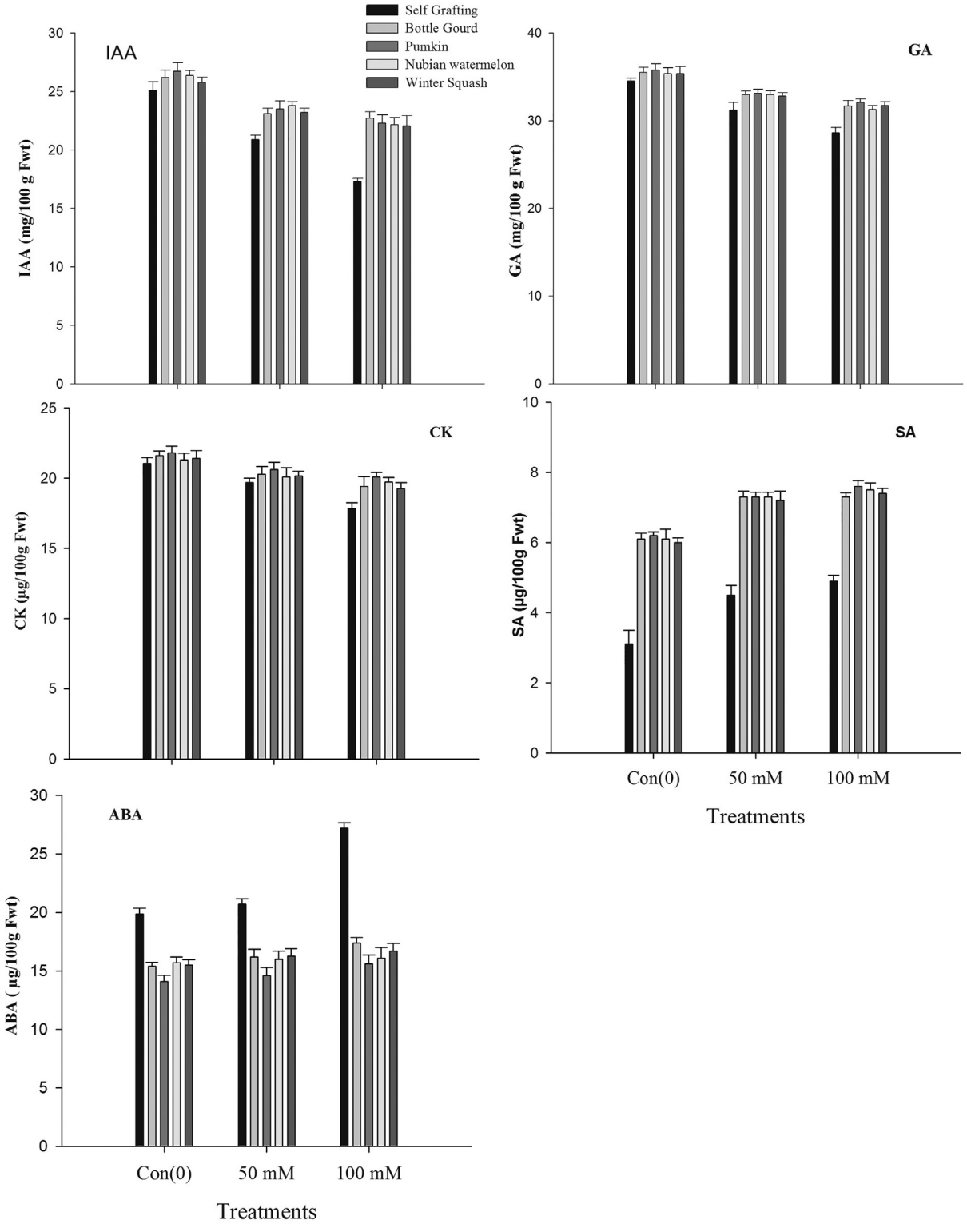

Fig. 4. Effect of rootstocks on the endogenous phytohormones (IAA, GA, CK, SA, and ABA) in four rootstocks compared with self-grafted of cucumber plants grown under salinity stress (0, 50 and 100 mM) during the two consecutive seasons (2016 and 2017). Each number represents the average of 10 replicate of both season, and different letters above the columns indicate significant differences at the $p < 0.05$ level.

NPQ in grafted conditions may protect the plant plastids from high light stress and stomatal closure (Ghanem et al., 2008). The above-mentioned mechanisms were less effective in both non-grafted and self-grafted plants, which may be due to the higher rate of stomatal closure in leaves due to growth impairment of the plants under salinity (Perez et al., 2010).

4.1.2. Membrane stability and MDA

Data in the present investigation indicated that grafting reduced the amount of ion leakage in salt-affected cucumber plants indicating that grafting has facilitated the maintenance of membrane functions i. e. semipermeable. Bor et al. (2003) indicated that the decreases in resistance to salinity may be due to an increase in membrane

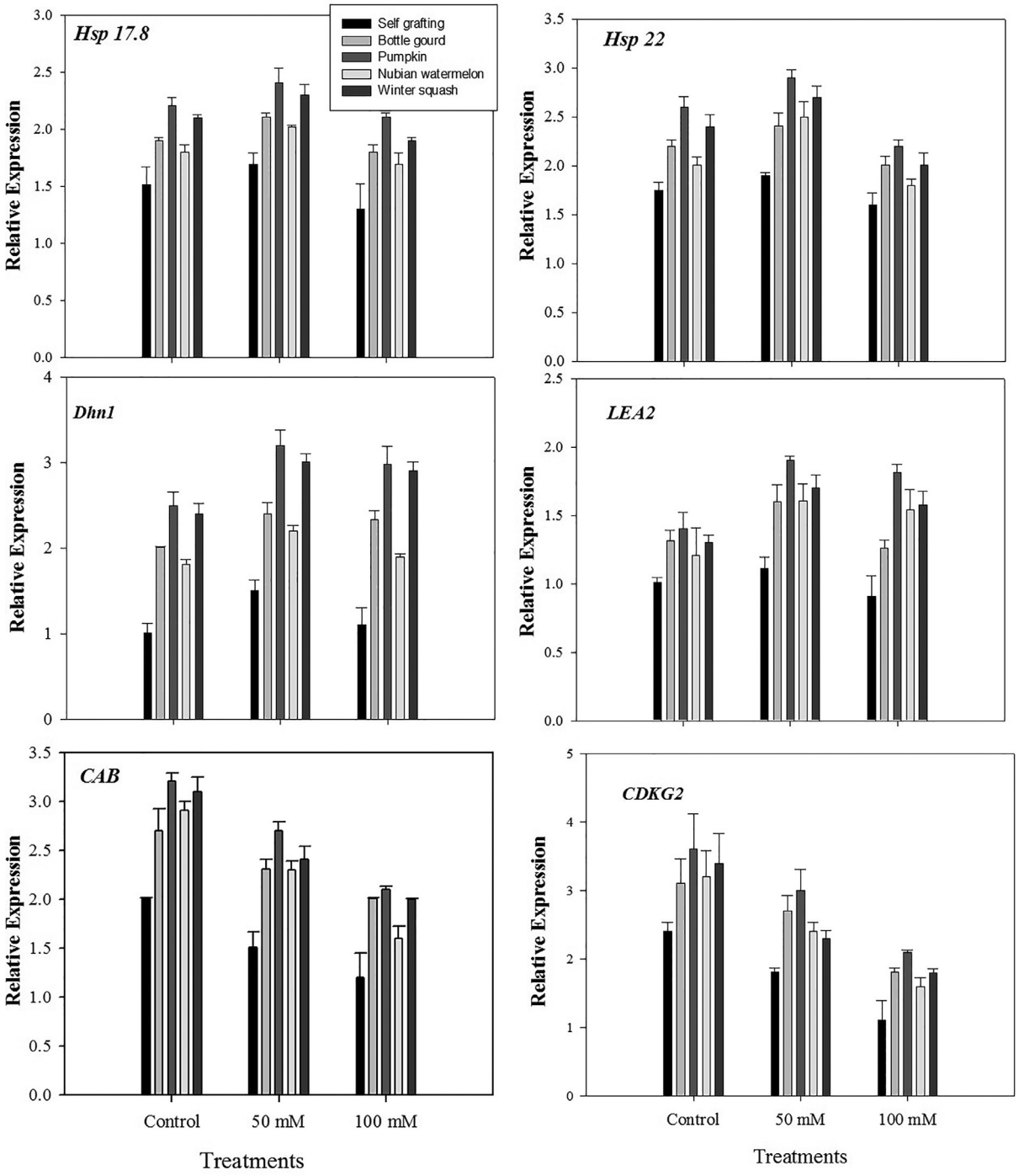

Fig. 5. qRT-PCR analysis of studied genes. Total RNA was extracted from scion leaves, converted to cDNA and subjected to comparative real-time RT–PCR quantification. Relative transcript level from qRT-PCR is means ± SE of three replicates. Expression levels were calculated and normalized to actin mRNA (Ac. no. GH571733.1). Different letters above the columns indicate significant differences at the $p < 0.05$ level.

permeability, which may occur due to an increase in solute leakage. In this context, Sairam et al. (2005) found that under salinity, the membrane stability index from tolerant wheat genotypes was lower than the sensitive wheat genotypes. The reduction in EL% in grafted plants, particularly under saline conditions indicated grafting may provide better protection from oxidative damage as a result of salinity. The enzymatic antioxidant defense system may protect plant cells from injury due to salinity. In grafted plants, an increase in the constitutive levels and activities of antioxidant enzymes can cause an increment of tolerance to salt stress (Helaly et al., 2017; Helaly, 2018). For releasing lipid peroxidation in the plant cell, it requires the active uptake of oxygen molecules which results in the production of superoxide anion radical (O_2^-) while the other ROS resulted in the formation of singlet oxygen ($^1O_2$), H_2O_2, and hydroxyl free radical (OH^-), all of which may involve in the initiation process of lipid peroxidation (Fridovich, 1986; Rastogi and Pospisil, 2010; Rastogi et al., 2014). Therefore, for the protection from oxidative damage, the constitutive and/or induced activity of different antioxidant enzymes is essential (Bor et al., 2003). Ashraf and Foolad (2007) reported that the proline can play an important role in the stability of cell membrane osmotic potential and the detoxification of toxic ions from the plants under salinity. In response to the deficiency of water stress, Helaly et al. (2017) found that there is

a correlation between the proline and accumulation of total sugars. The authors attributed this relation either to a reduction in proline degradation and/or to an increase in proline synthesis. The higher reduction in EL% was observed in grafted plants especially under saline conditions, which indicated that grafting may induce high membrane stability under the saline condition and thus can improve the protection from oxidative stress. Chen and Wang (2008) have previously reported that cell membrane stability index was reduced in the grafted plants leaves under salinity stress.

4.1.3. Compatible organic solutes and osmolytes

The study has indicated that an increase in salt tolerance in grafted plants may be due to an increase in the organic solutes accumulation, which may be effective in controlling the sensitivity to salt stress. Similar results were observed by Gu et al. (2008) on cucumber. They attributed these increases in grafted plants to the strong root system of the rootstocks, which increases mineral uptake capacity of grafted plants. El-Beltagi et al. (2013) observed an increase in the total concentration of plant protein, which may be due to an increase in the synthesis of adaptive protein under salinity. However, the increase in TSP in salinized plants can be a result of degradation or synthesis of protein. Differences were observed between self-grafted and grafted plants in response to salinity regarding the accumulation of osmoprotectants solutes can improve the ability of grafted plants under stress condition.

Some specific protein types related to the adaptation of the grafted plant was observed to be synthesized in grafted condition (Al-Mulla et al., 2013). In addition, the hydrophilic protein was observed to protect the plasma colloids from coagulation (Sperling et al., 2014). Stress condition may cause hormonal modification of protein metabolism (Dawood et al., 2012). Increasing sugar content in grafted plants under saline conditions was observed in our investigation, which was in agreement with several studies where a strong correlation between osmotic stress tolerance and sugar accumulation in the plant was observed (Bartels and Sunkar, 2005). Misra and Saxena 2009 obtained a high accumulation of osmoprotectants solutes in tolerant cultivars of wheat, which can also be responsible for high relative water content and proline (Misra and Gupta 2006). Thus, salt tolerance in grafted plants may be due to an increase in total phenol accumulation, which may be very useful in controlling the adverse impact of salinity.

Chand et al. (1999) found a positive relation between phenolics and IAA content in stressed plants. Thus in parts, tolerance to salinity in cucumber grafted plants may be the result of the high level of phenolic compounds in these plants. The authors also observed the less deviation in phytohormones and a lack of chlorosis and necrosis symptoms of salinity in cucumber grafted plants. Our results showed that grafting can increase polyphenol oxidase (PPO) activity in the shoot (Table 3). It is well known that PPO is the main enzyme in the synthesis of phenolics. Thus, it is well documented that there is a correlation between PPO activity and phenolic content, suggesting that higher the PPO activity higher will be the level of phenolic content (Wada et al., 1995). Also, PPO was observed to influence several physiological processes within plant tissues, which helps in the regulation of biotic and abiotic stresses (Hatung 2004). It was also found that PPO might be involved in the defense system against biotic stress (Najafi et al., 2007). Therefore, higher activity of PPO and a higher level of total phenolic compound content in grafted cucumber plants could play a positive effect on any type of stress like salinity stress. The high phenolic compounds may also influence the antioxidant system in the plants. The elevated CAT and POX activities in these plants (Table 2) might be due to the accumulation of high phenolic compounds.

4.2. Oxidative enzymes

These results indicate that SOD was a dominant antioxidant enzyme in protecting cellular damage (Scandalios 1993; Omar et al., 2012). Basically, SOD was observed to be significantly lower in self-grafted cucumber than the grafted one. Under saline conditions, an increase in values of CAT, POX, and SOD activities was observed for grafted cucumber compared with self-grafted plants, which suggested that grafted plants have the highest dis-mutating capacity. Our observations are in agreement with the results of Abd El-Gawad et al. (2016) where the authors stated that a high level of SOD was observed in tolerant maize and barley cv (s) subjected to salt stresses. Similarly, an increase in POX activity in grafted plants rather than self-grafted ones could be an indicator that the grafting process can rapidly induce a higher capacity to break dawn H_2O_2 in the plant cell. POX is one of the enzymes that scavenge H_2O_2 in chloroplasts, where the H_2O_2 are produced through dismutation of O_2^- catalyzed by SOD (Asada and Takahashi 1987; Mbarki et al., 2018). Increased POX activity under saline condition may be the result of increased activity of genes encoding POX encoding or the increased enzymatic activities as proposed by Dionisio-Sese and Tobita (1998). The ascorbic acid peroxidase (APOX) is an enzyme which uses ascorbate as the electron donor for the reduction of H_2O_2; thus it plays an essential role in the detoxification of H_2O_2. An increase in APOX activity was observed in both self-grafted and grafted cucumber plants with elevating salinity levels (Table 3). However, salinity induced APOX activity in grafted plants at a higher rate than self-grafted plants. Besides, self-grafted plants had higher APOX activity than grafted ones in salinized and non-salinized conditions. Bor et al. (2003) suggested that H_2O_2 in the leaves of salt tolerant beet plants is highly removed by ascorbate-glutathione cycle in which APOX performs as an active catalyst with DHAR, MDHAR, and GR. The GR activity (Table 3) was observed to be significantly reduced due to the effect of salinity in both grafted and self-grafted cucumber plants. In 50 and 100 mM salinity levels, the reduction of GR activity was lower in self-grafted in comparison to grafted one, and the differences in GR activity was significant between the self-grafted and grafted plants under high salinity stress. These results indicated that a higher rate of the active ascorbate-glutathione cycle might be related to an increase in salt tolerance in grafted cucumber plants. Ascorbate glutathione cycle, redox reaction of ascorbate, glutathione, and NADPH that are catalyzed by APOX, dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), and GR are the main ways to eliminated H_2O_2 (Foyer and Halliwell 1976).

Concerning the effects of rootstocks overall salinity levels, the data in this study indicated that there are no significant differences between grafted plants on any of the four rootstocks examined in both seasons. However, an insignificant increase was detected in grafted plants on bottle gourd, pumpkin, and nubian watermelon compared with those grafted on squash rootstocks. The least activities of all stress oxidative enzymes were obtained in self-grafted plants grown under normal and salinized conditions.

4.3. Endogenous phytohormones

The beneficial effect of grafting was considered to be its capacity to balance endogenous phytohormones within the plant tissues. The balance in phytohormones may help in the enhancement of nutrients uptake, which helps in the reduction of harmful effects of salt stresses. Zn, Fe, and Mn regularly released as an intercellular second messenger which mediate different regulatory path of phytohormones in the plant (Jeffrey, 1987). GA and CK were found to decrease under stress conditions. Basu et al. (2016) reported a change in plant metabolism and enhancement of senescence in the shoot of stressed plants, which may be partially involved in the decrease of CK supply to the root. Besides, they reported that GA caused an increase in TR when treated with GA, which can be sufficient to counter the higher level of endogenous ABA that alleviated the stress response. It has been reported that CK or CK-like growth regulators can prevent the activity of free radical groups such as H_2O_2 and superoxide anion (O^-_2), which may cause the degradation of chlorophyll molecules in leaves (Marschner 2011). In this

study, our results showed that grafting process could affect the osmolytes, ionic balance, and protein stabilization. These compounds controlled the endogenous phytohormone metabolism and currently impressed stress condition, consequently, the yield and its components (Wani et al., 2016).

It has been established that under drought stress, the ABA increased in plant tissues (Zhang et al., 2005). The authors observed a rapid increase in the concertation of free ABA in stressed tissues. The increase in the amount of ABA in the leaves or a marked increase in the ABA-cytokinin ratio or both can cause a reduction in transpiration rate (TR), which was subsequent to the reduction of the osmotic potential of the root medium (Pessaraki 2002). However, ABA application can play an important role in the regulation of stomatal closer and the reduction in TR. Auxins, GA, and CK are growth stimulants that can endogenously promote the plant morphogenesis and regulate the correlation between different metabolism of different organs in the plant (Peleg and Blumwald, 2011). In addition, Hayat et al. (2010) assessed the role of phenolic compounds especially salicylates such as salicylic acid as an antioxidant and a potent plant hormone which play an important role in the regulation of plants response to different environmental stresses along with plant growth and development (Naseem et al., 2015). Similarly, Posmyk et al. (2009) reported that phenolics are considered as a good antioxidant, which plays an important role in plant defense mechanisms against environmental stresses.

4.4. Stress-related genes

Studied genes such as *HSP17.8, HSP22, DHN1,* and *LEA2* are known with its protective function and their role in the maintenance of membrane stability under oxidative stresses in different plant species (Omar et al., 2011; 2013; Omar, 2018). To achieve successful grafting, an effective antioxidant defense system in plants could be an important factor (Mo et al., 2018). Increasing the expressions of *HSPs* and *DHNs* can guarantee good performance of a number of enzymes such as SOD, CAT, and APOX (Table 2). The *HSPs* also work as chaperons and prevent proteins aggregation and protect cells against oxidative stress (Omar et al., 2011). Also, most of *DHNs* possess antioxidant activity (Hara et al., 2003) and stabilizing the plasma membranes (Sun et al., 2009; Omar et al., 2018). Preservation of membrane integrity and stability associated with increasing the expression of these proteins could be noticed in decreasing the leakage rate of electrolyte and MDA content in hetero-grafted plants (Table 2). Changes in the expression pattern of different genes as a result of grafting on different rootstock was reported in citrus (Lui et al. 2017), in Pecan (Mo et al., 2018) and in cherries (Prassinos et al., 2009). Expression levels of all studied genes were higher in hetero-grafted plants in comparison to self-grafted plants under both of salinity levels, although it decreased with an increase in the salinity from 50 to 100 mM (Fig. 4). An increase in the expression of *CAB* in hetero-grafted plants was associated with improvement in chlorophyll contents. Also, its decrease under high salinity level (100 mM) was accompanied by a reduction in the chlorophyll contents in both self and hetero-grafted plants (Fig. 1). In a similar pattern, the expression level of *CDKG2* was higher in hetero-grafted than self-grafted plants under control and salinity condition. Changes in expression levels of CAB and CDK genes were reported in apple tree scions as a result of using different rootstocks (Jensen et al., 2003). Considering the role of plant hormones in graft union development (Mauriat and Moritz, 2009), it is acceptable to suggest that changes in the expression pattern of some genes associated with grafting process are a result of the changes in regulatory signals accompanied with changes in phytohormones contents (Table 3). Our results showed that grafting with different rootstocks stimulate changes in gene expression of studied genes which could improve the salinity tolerance of cucumber plants

5. Conclusion

Grafting onto four Cucurbita rootstocks plants can tolerate the salinity and had better growth and good yield parameters than those of self-grafted (control) plants, grown under salinized and non-salinized conditions. The salt tolerance mechanisms in the grafted plants are associated with the improvement in growth strategy, which is a consequence of several morphological modifications, physiological configurations, and changes in gene expression to cope with salt stress. Grafted plants grown in salinized media are capable of maintaining high photosynthetic activity and high membrane stability as well as oxidative enzymes (SOD, POX, CAT, APOX, and GR). Moreover, grafting treatments increased a considerable amount of IAA, GA, CK, and SA concentration over the control, whereas it decreases ABA under salinity stress. qRT-PCR analysis of gene expression indicated that the expressions level of some stress and photosynthesis-related genes (*HSP17.8, HSP22, DHN1, LEA2, CAB,* and *CDKG2*) were remarkably correlated with stress tolerance. These data suggested that the studied biochemical components and molecular changes might help the grafted plants defense against salinity induced oxidative injury and/or increased capacity of grafted cucumber plants for oxygen radical scavenging in addition to the participation of stimulated protective proteins in the maintenance of cellular membranes.

Declaration of Competing Interest

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

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Supplementary materials

Supplementary material associated with this article can be found in the online versio at doi:10.1016/j.sajb.2019.12.014.

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