

Mechanisms of tolerance differences in cucumber seedlings grafted on rootstocks with different tolerance to low temperature and weak light stresses

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Abstract: Stresses of low temperature and weak light are major environmental limiters for horticultural production in greenhouses in northern China during winter and early spring. Grafting is regarded as a promising approach to enhance cucumber tolerance to these environmental stresses. However, it is still unclear why cucumber plants grafted on different rootstocks usually exhibit different tolerance. The responses of cucumber (*Cucumis sativus* L. cv. 'Xintaimici') seedlings that were grafted on three rootstock cultivars ('Kilameki', 'Tielizhen', and 'Figleaf gourd') to low temperature (5 °C) and weak light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were investigated in this study. The results showed that, compared to ungrafted seedlings, the electrolytic relative leakage rate and malondialdehyde (MDA) content were significantly reduced in grafted cucumber, especially in 'Figleaf gourd'. Seedlings grafted on 'Figleaf gourd' had the highest proline content, as well as activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), followed by 'Tielizhen' and 'Kilameki'. Relative expression of *Cu/Zn-SOD*, *Mn-SOD*, and *CAT* mRNAs was consistent with activities of the corresponding enzymes, and higher levels were found in grafted seedlings, especially in 'Figleaf gourd'. APX activities and the relative expression of *APX* were also stimulated in grafted seedlings, although their changes were not completely consistent. All evidence indicated that the differences in rootstock-mediated tolerance improvement in cucumber were mainly attributed to the different stimulation of antioxidative defense systems under low temperature and weak light stresses.

Key words: Cucumber, grafting, rootstock, low temperature, weak light

1. Introduction

Environmental stresses represent the most limiting conditions for horticultural productivity (Schwarza et al., 2010). Although greenhouses have been developed for use in modern agriculture and may alleviate some damage to crops grown inside, stresses such as low temperature and weak light still occur frequently. Previous studies indicated that stresses of low temperature and weak light resulted in growth inhibition, fruit drop, and disease aggravation and subsequently reduced productivity of horticultural crops (Adams et al., 2001; Allen and Ort, 2001; Ai et al., 2006; Tazoe et al., 2006). Solar greenhouse is one of the main facility types for protected horticulture in northern China, where occurrence of low temperature and weak light stresses in winter and early spring is more frequent due to the simple structure, extensive management, and low environmental control level (Meng et al., 1999; Liang et al., 2009).

Cucumber (*Cucumis sativus* L.) is the main of thermophilic vegetables cultivated in solar greenhouses

in China. Low temperature and weak light usually have damaging impacts on growth and production of cucumber (Zhang and Ma, 1995; Yan et al., 2011). Grafting is nowadays widely used to reduce susceptibility to environmental stress (Edelstein, 2004). Several studies have demonstrated that stress-tolerant rootstocks could improve the growth and early yield of cucumber by reducing the degree of lipid peroxidation and electrolyte leakage under stress conditions (Lee and Chung, 2005; Zhou et al., 2007; Guy et al., 2008), and activities of some antioxidative enzymes, such as SOD and APX, were observed to increase under low temperature and weak light conditions (Feng et al., 2002; Li and Yu, 2007).

'Figleaf gourd' has long been the main rootstock for cucumber grafting in China, but there was an increasing proportion of white-seed or yellow-seed squash employed to improve the commodity and nutritional quality of grafted cucumber in the past few years, and numerous rootstock cultivars were applied in production. Moreover, there was a great difference in tolerance to stresses among

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rootstock cultivars (Li et al., 2008; Sakata et al., 2008). It is still unclear why cucumber grafted on different rootstocks usually show different tolerance levels to low temperature and weak light stresses. Therefore, several rootstocks with different tolerance levels were used in the present work, and the biochemical and molecular responses of the antioxidative defense system in grafted cucumber under low temperature and weak light stresses were investigated to elucidate the reasons for tolerance differences among various rootstocks.

2. Materials and methods

2.1. Plant material and experimental design

Based on our previous study, three cultivars with different tolerance levels to low temperature and weak light, i.e. 'Kilameki' (*Cucurbita moschata* × *Cucurbita moschata*), 'Tielizhen' (*C. maxima* × *C. moschata*), and 'Figleaf gourd' (*C. ficifolia* Bouche.), were selected.

Experiments were conducted from May to July 2010 in a greenhouse situated on the campus of Shandong Agricultural University, P. R. China. Cucumber cv. 'Xintaimici' was bred by Xiangyun Seed Co., Ltd. of Xintai. Rootstock seeds were germinated at 26 °C for 2 days, and then were sown in plastic pots (8 cm × 10 cm) filled with sand and daily watered with full-strength Yamazaki's nutrient solution from cotyledon unfolding. When rootstock seedlings had developed one true leaf, grafting was performed by using the hole insertion method described by Lee (1994).

Fifty uniform seedlings from each treatment were selected at the three-true-leaf stage and transferred into a growth chamber (RH = 70%) with a photoperiod of 12-h light (100 μmol m⁻² s⁻¹)/12-h darkness, pretreated at 15 °C/10 °C (day/night) for 2 days with the aim of cold acclimation, and then treated at 5 °C/5 °C for another 2 days (T5 + L100). The same number of ungrafted cucumber seedlings was used as controls (CK). After that, the second fully expanded leaves from treated seedlings were sampled for physiological measures. Each treatment was replicated three times with 5 plants per replicate.

Another thirty uniform seedlings at the four-true-leaf stage were treated under the same conditions mentioned above. The third functional leaves were sampled 0, 24, and 48 h after treatment, frozen immediately in liquid nitrogen, and stored at -80 °C for RNA isolation.

2.2. Plant growth

Plant height and leaf length (L) and width (W) were measured with a ruler, and leaf area (LA) was calculated as follows: $LA = 14.16 - 5.0 \times L + 0.94 \times L^2 + 0.47 \times W + 0.63 \times W^2 - 0.62 \times (L \times W)$. The dry weight (DW) was determined after deactivation at 105 °C for 15 min, and then weighed after oven-drying at 75 °C for 48 h and calculated as follows: relative increasing of DW (%) = $(FW - DW)/FW \times 100\%$.

2.3. Measurement of electrolytic relative leakage rate and MDA content

Electrolytic relative leakage rate was determined according to the method described by Dionisio-Sese and Tobita (1998). The initial electrical conductivity (EC1), the final electrical conductivity (EC2), and the electrical conductivity when the tissues were killed (ECt) were measured with an electrical conductivity analyzer (DDS-307; Shanghai Precision Scientific Instrument Co., Ltd., China). It was calculated as follows: electrolytic relative leakage rate (%) = $(EC1 - EC2)/(ECt - EC1) \times 100$. Malondialdehyde (MDA) content was measured by the method described by Cakmak and Marschner (1992).

2.4. Measurement of proline content

Free proline content was estimated by using the second functional leaves following the method of Zhang et al. (1990).

2.5. Extractions and assays of antioxidative enzymes

SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT), according to the methods of Dhindsa et al. (1981). The 1-mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 6.5 mM methionine, 50 μM NBT, 10 μM EDTA, 20 μM riboflavin, and 20 μL of enzyme extract. A reaction mixture lacking enzyme served as the control. All mixtures were stirred under darkness in small glass test tubes, and then irradiated for 5 min by fluorescent lamps (160 μmol m⁻² s⁻¹). After the reaction mixture turned from yellow to blue-black, its absorbance was measured at 560 nm. The mixture lacking enzyme and not being irradiated was used to zero the absorbance at 560 nm. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of NBT reduction under assay conditions.

CAT activity was measured as the drop in absorbance at 240 nm due to the decomposition decline of extinction of H₂O₂. The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 10 mM H₂O₂, and 0.1 mL of enzyme extracts. (Cakmak and Marschner, 1992).

APX activity was evaluated by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized (extinction coefficient: 2.8 mM⁻¹ cm⁻¹; Nakano and Asada 1981). The 1-mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.5 mM AsA, 1 mM H₂O₂, and 20 μL of enzyme extract.

Protein concentration of enzyme extracts was determined using Coomassie brilliant blue with bovine serum albumin as the standard (Bradford, 1976).

2.6. RNA isolation and quantitative RT-PCR

Total RNA was extracted from cucumber leaves using Trizol (Fermentas, Shenzhen, China), and then treated with RNase-free DNase to remove contaminating DNA. First strand cDNA of the total RNA was synthesized using

M-MuLV reverse transcriptase and oligo-(dT) 18 as the primers (Fermentas, Shenzhen, China). Quantitative RT-PCR was performed with the primers in Table 1 to detect the level of expression of the target genes and the control gene β -*Actin*. The specific primers (Table 1) used for RT-PCR were designed based on GenBank accession Nos. AAZ74666.1, AY274258, D88649, and EF530128 using Primer express 3.0 software. The β -*Actin* gene was used as an internal control. RT-PCR was performed using a Light Cycler 480 SYBR Green I master kit (Roche Diagnostics, Mannheim, Germany). PCR amplification included a 5-min preincubation step at 95 °C, followed by 40 cycles of 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 20 s. PCR products were quantified by the Light Cycler 480 RT-PCR detection system with the SYBR Green I master kit.

2.7. Statistical analysis

The data were processed using Origin 7.5 (Microcal Software Inc., Northampton, MA, USA). Analysis of variance was performed using SPSS 11.0 Software (SPSS Inc., Chicago, IL, USA) and presented as means \pm standard error (SE) of three replicates. Different letters indicate a significant difference at a 0.05 level.

3. Results

3.1. Plant growth

The relative increases in plant height from the three rootstock-grafted seedlings were significantly higher ($P < 0.05$) than those of ungrafted ones after the stresses (Table 2), being much higher in 'Figleaf gourd' and 'Tielizhen', and slightly higher in 'Kilameki'. There was a similar variation trend in LA. The relative increase in DW was significantly higher in grafted seedlings than in ungrafted ones ($P < 0.05$); moreover, 'Figleaf gourd' had the highest increase, significantly greater than the other rootstocks.

3.2. Electrolytic relative leakage rate and proline content

The electrolytic relative leakage rate in ungrafted seedlings reached 24.8% in response to T5 + L100 stresses, being remarkably higher than those in grafted plants (Figure 1). Cucumber grafted on 'Figleaf gourd' was the lowest (12.6%), followed by 'Tielizhen'. The electrolytic relative leakage rates in both rootstocks were significantly lower when compared to those in 'Kilameki' and the control ($P < 0.05$).

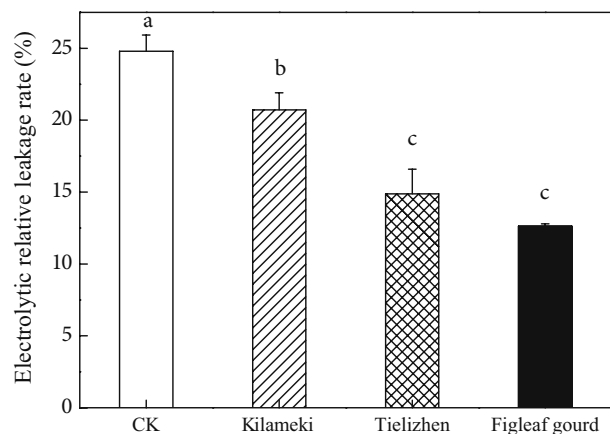
As shown in Figure 2, no significant difference in proline content was found before treatment among grafted

Table 1. Primers for detecting every enzyme gene expression by real-time quantitative RT-PCR.

Name	Cas	Oligonucleotide sequence	Size (bp)
APX	Csa019442	cAPXF:GACTTCTGCAGTTGGCATCA	131
		cAPXR:CTCGGAAAGCTTCTGGTGAG	
CAT	Csa013194	cCATF:ATCACAGTCACGCCACTCAG	128
		cCATR:CGTATCCAACGGCTCAAAT	
Cu/Zn-SOD	Csa001740	cCuZnSODF:ACGCCTTAGGTGACACAACC	138
		cCuZnSODR:TGCCATCTCACCAACGATA	
Mn-SOD	Cas004428	cMnSODF:CCTCTGCTTGGGATTGATGT	117
		cMnSODR:AATGTCGCCAGCATATTTCC	
β -ACTIN	AB00192	cACTINF:gctggcatatgttctcttg	121
		cACTINR:gaatctctcagctccgatgg	

Table 2. Changes in plant growth in grafted and ungrafted cucumber seedlings under low temperature (5 °C) and weak light intensity (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) stresses. Different letters indicate significant differences between the treatments at the 0.05 level.

Materials	Relative height increase (%)	Relative leaf area increase (%)	Relative dry weight increase (%)
CK (control)	5.95d	4.22c	20.49d
Kilameki	7.58c	8.74b	30.75c
Tielizhen	11.38a	14.87a	54.24b
Figleaf gourd	11.43a	16.96a	60.86a



Different rootstocks grafted and ungrafted cucumber seedlings

Figure 1. Changes in electrolytic leakage rate in grafted and ungrafted cucumber seedlings under low temperature (5 °C) and weak light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) stresses. Vertical bars represent the standard errors (n = 3). Different letters indicate significant differences between the treatments at the 0.05 level.

and ungrafted seedlings, although the former was slightly higher. However, proline content increased significantly in both grafted and ungrafted seedlings after stresses ($P < 0.05$). The increments in plants grafted on 'Figleaf gourd', 'Tielizhen', and 'Kilameki' were 172%, 135%, and 127%, respectively, and only 108% in the control. There were great differences among ungrafted seedlings and seedlings grafted onto rootstocks with different tolerance levels.

3.3. MDA content

MDA content was increased during stress treatment in both ungrafted and grafted plants (Figure 3), especially in the first 24 h after treatment. Grafted seedlings always showed lower MDA content compared to ungrafted ones, ranked as 'Figleaf gourd' < 'Tielizhen' < 'Kilameki'.

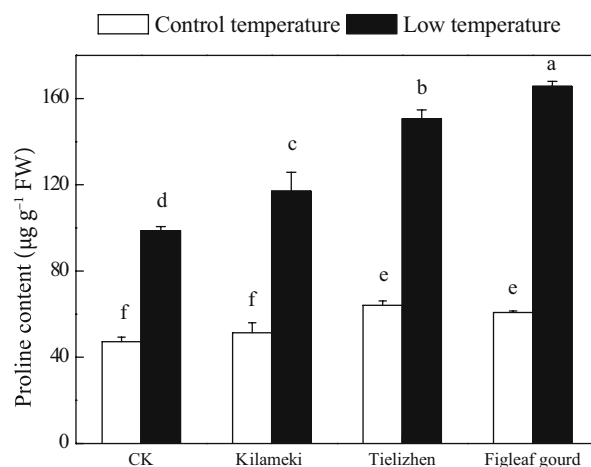
3.4. Antioxidative enzymes activity and relative expression of related genes

3.4.1. Total activities of SOD

SOD activities rose significantly in both grafted and ungrafted seedlings during the stress treatment, being greater in the grafted ones (Figure 4). The enzyme activities of 'Figleaf gourd', 'Tielizhen', and 'Kilameki' were increased by 128.9%, 90.4%, and 84.7% at 24 h after treatment, respectively, and only 42.7% in the control. The increment became smaller in grafted and ungrafted cucumber at 48 h, when 'Figleaf gourd' had the highest activity.

3.4.2. Relative expression of *Cu/Zn-SOD* mRNA and activities of *Cu/Zn-SOD*

The expression of *Cu/Zn-SOD* mRNA was increased at the start of treatment, and then declined after 24 h, especially at 48 h (Figure 5), with a significantly higher level in grafted



Different rootstocks grafted and ungrafted cucumber seedlings

Figure 2. Changes in proline content in grafted and ungrafted cucumber seedlings under low temperature (5 °C) and weak light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) stresses. Vertical bars represent the standard errors (n = 3). Different letters indicate significant differences between the treatments at the 0.05 level.

seedlings than in ungrafted ones ($P < 0.05$). Transcript level of *Cu/Zn-SOD* in 'Figleaf gourd', 'Tielizhen', and 'Kilameki' was increased to 2.27-, 2.37-, and 1.95-fold at 24 h, respectively, while that of ungrafted plant was only 1.32-fold.

The variations in *Cu/Zn-SOD* activities were similar to those in relative expression of *Cu/Zn-SOD* mRNA during stress treatment, but a lower reduction was detected in both grafted and ungrafted seedlings at 48 h. 'Figleaf gourd' exhibited the greatest activity, followed by 'Tielizhen' and 'Kilameki'. Compared to the control, the stronger *Cu/Zn-SOD* activities in grafted seedlings were due to the higher expression of *Cu/Zn-SOD* mRNA.

3.4.3. Relative expression of *Mn-SOD* mRNA and activities of *Mn-SOD*

The relative expression of *Mn-SOD* mRNA was increased significantly in both grafted and ungrafted seedlings after stress treatment (Figure 6). More expression was observed in 'Figleaf gourd' and 'Tielizhen' at 24 h, i.e. 132.5% and 149%, respectively, but just 108.8% and 97% in 'Kilameki' and ungrafted seedlings.

Mn-SOD activities changed less in both grafted and ungrafted seedlings at 24 h, but rose sharply at 48 h. *Mn-SOD* activity in grafted plants was higher than that in ungrafted ones, of which 'Figleaf gourd' exhibited the highest, followed by 'Tielizhen' and 'Kilameki'. These results indicated that higher activities of *Mn-SOD* in grafted cucumber were probably correlated with the greater transcript levels.

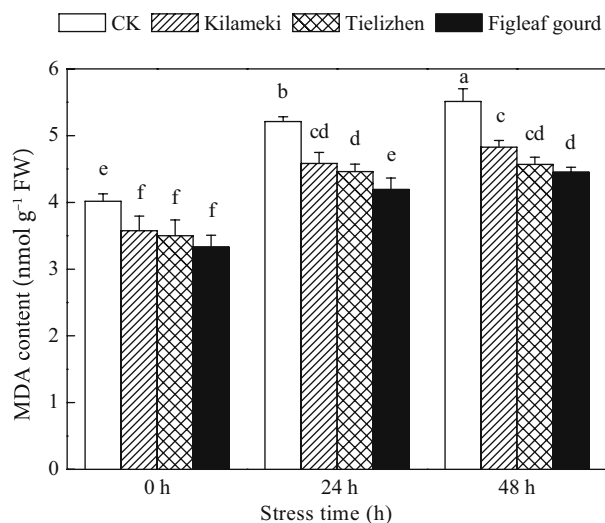


Figure 3. Changes in MDA content in grafted and ungrafted cucumber seedlings under low temperature (5 °C) and weak light (100 μmol m⁻² s⁻¹) stresses. Vertical bars represent the standard errors (n = 3). Different letters indicate significant differences between the treatments at the 0.05 level.

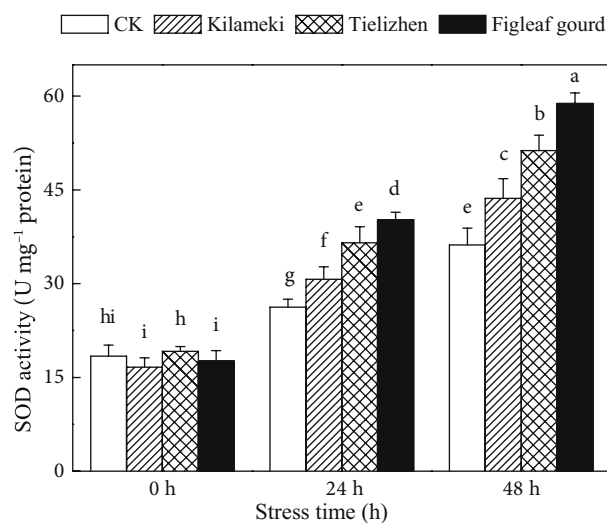


Figure 4. Changes in SOD activities in grafted and ungrafted cucumber seedlings under low temperature (5 °C) and weak light (100 μmol m⁻² s⁻¹) stresses. Vertical bars represent the standard errors (n = 3). Different letters indicate significant differences between the treatments at the 0.05 level.

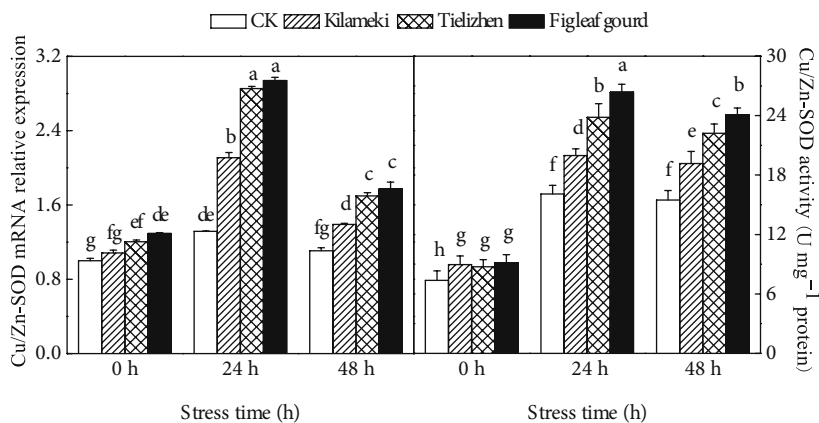


Figure 5. Changes in Cu/Zn-SOD mRNA relative expression and Cu/Zn-SOD activities in grafted and ungrafted cucumber seedlings under low temperature (5 °C) and weak light (100 μmol m⁻² s⁻¹) stresses. Vertical bars represent the standard errors (n = 3). Different letters indicate significant differences between the treatments at the 0.05 level.

3.4.4. Relative expression of CAT mRNA and activities of CAT

As revealed in Figure 7, a reduction in CAT transcript was observed in both grafted and ungrafted plants under stresses; ‘Kilameki’ and ungrafted seedlings decreased more when compared to ‘Figleaf gourd’ and ‘Tielizhen’. At 48 h after treatment, the lowest expression of CAT mRNA was found in ungrafted seedlings, followed by ‘Kilameki’; there was no significant difference between ‘Figleaf gourd’ and ‘Tielizhen’.

CAT activities followed similar variations with its mRNA expression, and the activities were greater in grafted seedlings than in ungrafted ones.

3.4.5. Relative expression of APX mRNA and activities of APX

Transcript level of APX went up at 24 h, and then fell at 48 h in both grafted and ungrafted seedlings (Figure 8). The expression was significantly higher in grafted plants than in the control, with the greatest level in ‘Figleaf gourd’. Compared with the transcript levels at 24 h, 25.7% and

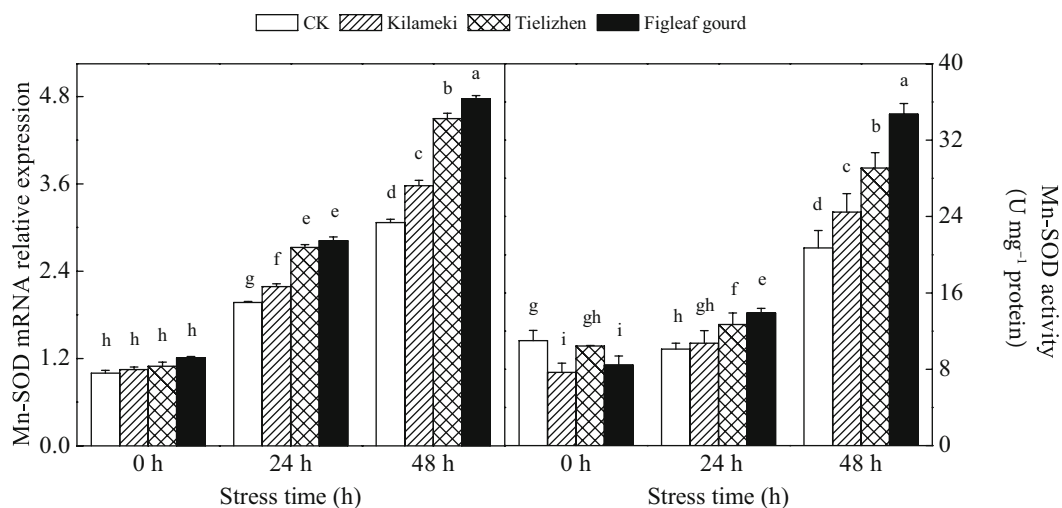


Figure 6. Changes in *Mn-SOD* mRNA relative expression and Mn-SOD activities in grafted and ungrafted cucumber seedlings under low temperature (5 °C) and weak light (100 μmol m⁻² s⁻¹) stresses. Vertical bars represent the standard errors (n = 3). Different letters indicate significant differences between the treatments at the 0.05 level.

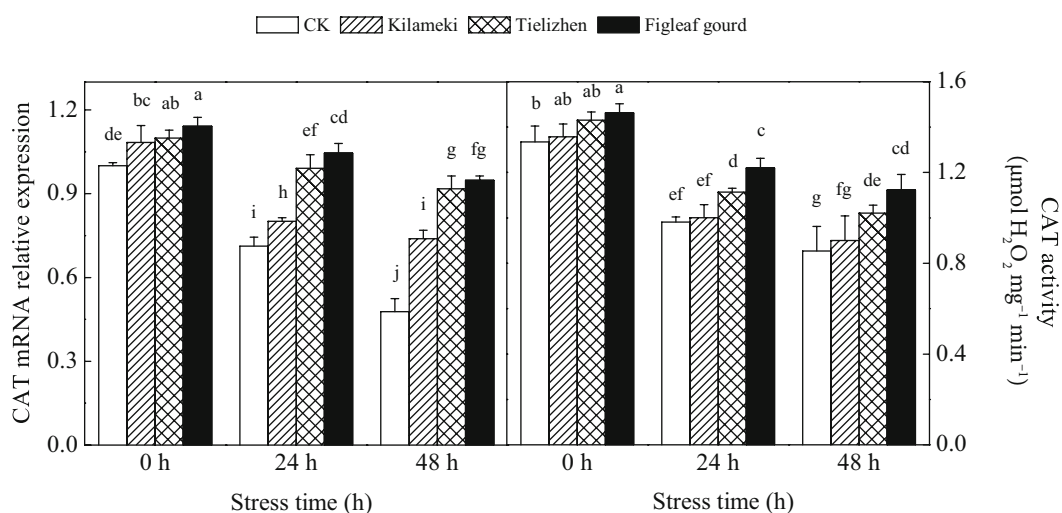


Figure 7. Changes in *CAT* mRNA relative expression and *CAT* activities in grafted and ungrafted cucumber seedlings under low temperature (5 °C) and weak light (100 μmol m⁻² s⁻¹) stresses. Vertical bars represent the standard errors (n = 3). Different letters indicate significant differences between the treatments at the 0.05 level.

21% reductions were detected in ungrafted seedlings and ‘Kilameki’, and 15.4% and 10.5% in ‘Figleaf gourd’ and ‘Tielizhen’, respectively.

The APX activity was not consistent with the expression of *APX* in grafted and ungrafted plants under stress conditions. The variations in APX activities were consistent with expression in ‘Kilameki’ and ungrafted seedlings. However, expression of *APX* mRNA declined significantly in ‘Figleaf gourd’ and ‘Tielizhen’ at 48 h, while APX activities increased continuously.

4. Discussion

Compared with ungrafted cucumber, the growth of grafted ones was less inhibited when subjected to low temperature and weak light stresses in this study. Seedlings grafted on ‘Figleaf gourd’ exhibited the highest stress tolerance. Many studies have shown a correlation between adaption to environmental stress and efficiency of osmotica (Shi et al., 2007; Khan and Panda, 2008; Yang et al., 2010). Proline, as a major osmoprotectant, increased significantly in grafted cucumber compared with ungrafted cucumber under the

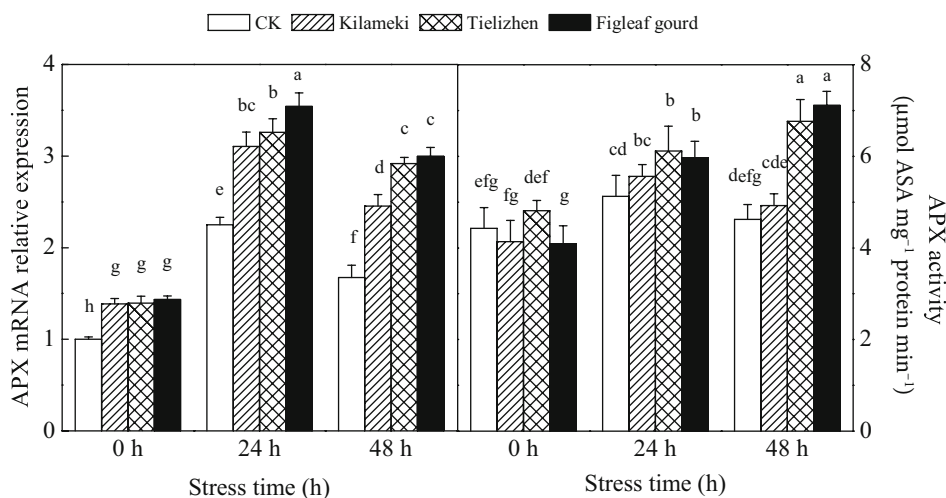


Figure 8. Changes in APX mRNA relative expression and APX activities in grafted and ungrafted cucumber seedlings under low temperature (5 °C) and weak light (100 μmol m⁻² s⁻¹) stresses. Vertical bars represent the standard errors (n = 3). Different letters indicate significant differences between the treatments at the 0.05 level.

stresses of low temperature and weak light, and ‘Figleaf gourd’ exhibited the highest level, followed by ‘Tielizhen’ and ‘Kilameki’, indicating that cucumber seedlings grafted on high stress-tolerant rootstocks possess strong ability of osmotic regulation.

Large amounts of ROS were induced and lipid peroxidation was aggravated when plants suffered from abiotic stresses, which resulted in damage to the membrane systems, and consequently an increment in both electrolytic relative leakage rate and MDA (Karabal et al., 2003; Liu et al., 2013; Li et al., 2014). In the present study, electrolytic relative leakage rate and MDA content were significantly lower in grafted seedlings than they were in ungrafted ones, similar to previous reports (Tajvar et al., 2011; Zhen et al., 2011). Moreover, seedlings grafted on ‘Kilameki’ exhibited significantly a higher electrolytic relative leakage rate and MDA content, followed by ‘Tielizhen’, and those on ‘Figleaf gourd’ were the lowest, which meant that grafting with rootstocks could improve the stress tolerance of cucumber by alleviating membrane lipid peroxidation.

Antioxidative enzymes are an important ROS detoxification system in plant cells, and the induced increment in enzyme activity was considered as the plant’s mechanism against oxidative stress (Xu et al., 2008; Zhang et al., 2012). In the present study, despite the reduction in CAT activity following the prolonged low temperature and weak light stresses, the activities of SOD, Cu/Zn-SOD, Mn-SOD, CAT, and APX in grafted cucumber seedlings were always significantly higher than those in ungrafted ones, and the seedlings grafted on ‘Figleaf gourd’ exhibited the highest level, followed by ‘Tielizhen’ and ‘Kilameki’.

Based on the results, the lower damage to the membrane system in grafted cucumber was attributed to the higher activities of defense enzymes under low temperature and weak light stresses.

The increment in expression of some antioxidant enzyme genes could enhance the cold tolerance of plants (Ke, 2007). In the present study, the relative expression levels of *Cu/Zn-SOD* and *Mn-SOD* mRNAs were higher in grafted cucumber than they were in the ungrafted one, especially in ‘Figleaf gourd’, and their expression levels were consistent with the activities of Cu/Zn-SOD and Mn-SOD. These data suggested that strengthened stress tolerance in grafted cucumber was caused by increased expression of *Cu/Zn-SOD* and *Mn-SOD* mRNAs, which enhanced the activities of corresponding enzymes. Furthermore, compared with the relative expression of *Cu/Zn-SOD* at 48 h during stress treatment, a lower reduction in activity of Cu/Zn-SOD was detected in both grafted and ungrafted seedlings; this might be due to the delaying of translation, and it needs further study. Although more *CAT* and *APX* transcripts were detected in grafted seedlings compared to ungrafted ones, the expression of *CAT* and enzyme activity decreased gradually during the stress treatment, indicating that other pathways besides *CAT* enzyme, such as catalysis of *APX*, may play a more important role in ROS scavenging in cucumber (Miyake and Asada, 1994; Noctor and Foyer, 1998). *APX* mRNA expression and *APX* activity were not completely consistent in seedlings grafted on ‘Figleaf gourd’ and ‘Tielizhen’ at 48 h after treatment, implying that other factors might be involved in the regulation of *CAT* and *APX* activities.

In conclusion grafted cucumber had improved tolerance to low temperature and weak light stresses by strengthening the activities of antioxidative defense systems, and the differences in rootstock-mediated tolerance improvement in cucumber were mainly attributed to the different stimulation of antioxidative defense systems.

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