

Effects of Different Pumpkin Rootstocks on Grafted Cucumber Resistance to Powdery Mildew

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Abstract: Powdery mildew (PM) is a major fungal disease in cucumber (*Cucumis sativus* L.) cultivation. Grafting serves as an important agricultural practice for improving disease resistance and stress tolerance in scions. This study aimed to determine the effects of different pumpkin rootstocks on PM resistance in grafted cucumber plants. Susceptible 'Xintai Mici' cucumber scions were grafted onto 10 different pumpkin rootstock varieties, with self-grafted plants serving as the experimental control. Grafting significantly promoted plant biomass accumulation compared to the self-grafted control, and this enhancement was positively correlated with the rootstock's root system size. However, grafted plant growth was still negatively affected by PM infection. Among the 10 rootstocks, seedlings grafted onto rootstock GP8 exhibited the lowest disease index and the slowest spore development, and the strongest PM resistance. While some resistant pumpkin rootstocks failed to confer significant PM resistance to their grafted cucumber scions, rootstock GP8 provided consistent PM resistance to its grafted plants. Furthermore, cucumber grafted onto rootstock GP8 showed a significantly enhanced net photosynthetic rate and increased activities of antioxidant enzyme (superoxide dismutase, ascorbate peroxidase, and glutathione reductase) activities. Concurrently, these plants accumulated lower levels of superoxide anions and exhibited the smallest increases in malondialdehyde content among all the grafted combinations. Additionally, during PM infection, the expression levels of salicylic acid biosynthesis-related genes (*CsICS1* and *CsPAL*) and downstream disease resistance genes (*CsPR1*, *CsPR5*, and *CsNPR1*) were significantly higher in scions grafted onto rootstock GP8 compared to self-grafted cucumbers. These results suggest that the enhanced PM resistance in grafted cucumber is significantly influenced by the rootstock, potentially through the regulation of photosynthetic performance, reactive oxygen species metabolism, and the expression of genes associated with the salicylic acid signaling pathway in the scion.

Keywords: pumpkin; cucumber; grafting; powdery mildew; disease resistance; *Podosphaera xanthii*

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1. Introduction

Cucumber (*Cucumis sativus* L.) is an economically significant vegetable crop in China. Powdery mildew (PM) is one of the three major diseases affecting cucumber and represents the most prevalent fungal disease in both greenhouse and field production. This disease is primarily caused by *Podosphaera xanthii* [1]. Disease development is favored by high humidity, elevated temperatures, and low light conditions. The infection process in plants typically follows the following four fundamental stages: (i) Within 1–2 h after spore deposition on leaves, spores germinate and form primary germ tubes; (ii) These tubes penetrate plant epidermal cells via appressoria, leading to the invagination of the host plasma membrane and the formation of primary haustoria. At this stage, no visible symptoms appear, and infected leaves are indistinguishable from healthy ones; (iii) After approximately 48 h, secondary hyphae develop from the primary hyphae on the epidermides of stems and leaves, producing new appressoria that form secondary haustoria within adjacent epidermal cells and (iv) Approximately 5 d post-infection, new conidiphores and spores are produced, initiating a renewed cycle of dispersal and infection [2–5]. PM can infect cucumber plants throughout their lifecycle, colonizing nearly all above-ground tissues, including the hypocotyl, cotyledons, stems, petioles, leaves, and fruit [1,2]. This results in leaf yellowing and brittle necrosis, a compromised photosynthetic capacity, and a shortened harvesting window, collectively leading to significant reductions in both crop yield and fruit quality [6].

Grafting represents an effective approach to enhancing disease resistance in horticultural crops and is generally more safer than chemical or biological control measures [7]. The choice of rootstock variety is a key factor influencing scion resistance to PM, as the scion's disease response can be modulated by the rootstock's own resistance profile. For example, in watermelon, grafting susceptible scions onto different resistant rootstocks improves PM resistance in certain scion-rootstock combinations [8]. Similar findings have been reported in pepper grafting studies [9]. Conversely, grafting susceptible scions has been observed to increase the vulnerability of otherwise disease-resistant rootstocks to the soil-borne bacterium. Conversely, grafting susceptible scions can increase the vulnerability of otherwise disease-resistant rootstocks to the bacterium *Rallstonia solanacearum* [10]. In potato, the use of disease-resistant varieties as rootstocks can enhance scion resistance to late blight [11].

The role of rootstocks in enhancing scion disease resistance is primarily achieved through the regulation of long-distance transport processes, including the translocation of water and nutrients. Such rootstock-mediated physiological adjustments lead to significant modifications in scion traits, including alterations in biomass accumulation, photosynthetic capacity, enzymatic activity, hormonal profiles, and the expression of genes associated with disease resistance [12,13]. Generally, grafting can enhance disease resistance by improving physiological traits of the scion, such as water and nutrient uptake capacity [14,15]. Moreover, different propagation methods can alter the plant's physiological state and promote its growth and development [16]. Grafting can induce the thickening of the scion leaf epidermis [17], forming a physical barrier that impedes pathogen invasion and reduces subsequent attachment and infection. Beyond structural defenses, rootstocks enhance plant stress tolerance by regulating water uptake and nutrient transport, which in turn improve the photosynthetic capacities of the scions [18]. This enhancement of photosynthesis under stress conditions has been observed across various grafted systems. For example, grafting increases stomatal conductance and photosynthetic capacity in grapevines [19]. In cucumber, grafted plants exhibit higher net CO₂ assimilation rates and transpiration rates, along with altered chlorophyll fluorescence parameters, collectively contributing to greater stress tolerance [20]. Similarly, tomato plants grafted onto eggplant rootstocks show improved chlorophyll fluorescence, photosynthetic performance, and enhanced tolerance to waterlogging [21]. Moreover, the use of salt-tolerant rootstocks in

sweet pepper helps maintain photosynthetic capacity and nutrient transport under saline conditions, thereby boosting overall stress tolerance [22].

Grafting itself can be considered a form of traumatic stress that activates the plant's antioxidant defense system [23]. This response typically involves the generation of reactive oxygen species (ROS) and the accumulation of defense-related and antioxidant enzymes [15,23,24]. Grafting modulates antioxidant enzyme activity in tobacco [25] and, under drought stress, enhances photosynthetic capacity while reducing ROS accumulation in tomato scions and grapevines [26]. It also decreases malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels in scions, thereby improving drought tolerance [27]. Furthermore, the post-grafting accumulation of NADPH oxidase has been linked to enhanced PM resistance in pepper scions [9]. At the hormonal and proteomic levels, grafting significantly increases the diversity of proteins released from roots, many of which are associated with resistance to biotic and abiotic stresses [28]. The overexpression of *SINCE1* in tomato rootstocks elevates abscisic acid levels in the scions, thereby improving salinity tolerance [29]. Upon infection with PM, plants typically exhibit a rapid burst of ROS, which stimulates antioxidant capacity and triggers a cascade of defense responses [30–32]. Pathogenesis-related (PR) proteins are encoded by a major class of genes associated with disease resistance. In grapevines, PR genes (such as *PR-1*, *PR-4*, and *PR-5*) are markedly upregulated following PM infection, thereby enhancing plant resistance [33]. Among them, *PR-1*, *PR-2*, and *PR-5* are widely recognized as marker genes for the salicylic acid (SA)-signaling pathways, which function in part through the binding and activation of NPR1 [34]. In cucumber, the upregulation of *CsCYP82D102* enhances *PR-5* expression and improves PM resistance [35].

PM poses a serious threat to cucumber crop production. However, previous studies have provided limited insights into how different rootstocks influence PM susceptibility in cucumber scions, and the physiological and molecular mechanisms underlying PM resistance in grafted plants remain unclear. In this study, the physiological and biochemical responses of cucumber scions grafted to different pumpkin rootstock varieties were investigated following PM inoculation. Our aim was to elucidate the mechanisms by which rootstocks confer PM resistance to grafted scions.

2. Materials and Methods

2.1 Plant materials and growth conditions

The cucumber (*C. sativus* L. 'Xintai mici') served as the scion. Ten pumpkin (*C. moschata* D.) varieties (GP3-10 and GP13-14, high-generation inbred squash rootstock germplasm resources provided by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences) were used as rootstocks in the grafting combinations. Plants were grown in a growth chamber under a 16/8 h (day/night) photoperiod with temperatures of 25°C/18°C and relative humidity maintained at 60–70%. Seeds of scions and rootstocks were sown in 50-cell (one cell: 45×45×45 mm, length×width×height) and 32-cell (one cell: 60×60×110 mm, length×width×height) polystyrene trays, respectively, containing a commercial organic substrate mixture ($V_{\text{peatmoss}}:V_{\text{vermiculite}}:V_{\text{perlite}} = 1:1:1$). Cucumber scions were grafted onto pumpkin rootstocks using hole insertion grafting. For the control group, cucumber plants were self-grafted using the spliced grafting method. Similarly, pumpkin plants were also self-grafted following the same procedure. The hole insertion grafting, specific grafting procedures, and subsequent seedlings management during graft union formation followed established protocols [36]. A total of 45–60 plants of each rootstock variety were grafted in our study, with three replicates, and all grafting procedures were performed by the same person.

2.2 Inoculation with PM

PM inoculation was performed when grafted cucumber plants reached the two-true-leaf stage. Fresh spores were collected from severely diseased cucumber plants in a greenhouse and suspended in an aqueous solution containing 0.01% Tween-20 [37]. The spore suspension was adjusted to 10^4 spores per mL and evenly sprayed onto the true leaves using a hand-held sprayer. At least 5 ml was applied per grafted plant, ensuring uniform distribution of powdery mildew spores. After inoculation, plants were maintained in a growth chamber set at 25°C day/20°C night with a 16-h light/8-h dark photoperiod. Disease severity was assessed 15 days after inoculation. The percentage of leaf area covered with pathogen colony on each leaf was visually rated on a 0-5 scale: 0 = no symptoms; 1 = <30% infection; 2 = 30-60%; 3 = >60-80%; 4 = >80%; 5 = leaf senescence. A disease index (DI) was calculated according to the following formula [38]:

$$DI = [\sum (\text{disease scale} \times \text{number of leaves at that scale}) / (\text{total leaves inoculated} \times \text{highest disease grade})] \times 100.$$

The experiment was arranged in a randomized design with three replications, each consisting of 45-60 plants per rootstock variety.

2.3 Determination of biomass

15 days after PM inoculation, the aboveground fresh weight of scion plants and control plants was measured after rinsing with deionized water. The samples were then oven-dried at 105°C for 30 min, followed by drying at 75°C until a constant weight was achieved to determine the dry weight [39]. The daily growth rate was calculated as follows: Absolute Growth Rate (AGR) = $(W_{15d} - W_{0d}) / 15d$, where W_{15d} is the biomass weight at 15 days after PM inoculation; W_{0d} is the biomass weight at 0 days after PM inoculation. The mean value of 8-12 plants was obtained for each rootstock [36].

2.4 Observation of the growth of PM mycelium

At 24 hours, 72 hours, and 8 days after inoculation with PM, leaf samples were collected from the scions of cucumber plants grafted onto different pumpkin rootstocks using a punch. The leaf discs were immersed in a 0.1% (w/v) 3,3'-diaminobenzidine (DAB) solution and incubated at room temperature in the dark for 8 hours. Following incubation, the staining solution was discarded, and an appropriate volume of decolorizing solution (anhydrous ethanol: sterile water: glacial acetic acid: glycerol = 8:1:1:1) was added. The samples were then left to decolorize at room temperature for 3 days. Subsequently, a 0.4% toluidine blue solution was applied dropwise to the leaf discs. After a 30 min incubation at room temperature, the leaf discs were rinsed seven times with distilled water. The stained leaf tissues were then dissected using forceps, mounted on glass slides with coverslips, and examined under a light microscope at 10× to 40× objective magnification to observe the germination of powdery mildew mycelium on the cucumber leaves.

2.5 Measurement of photosynthetic parameters

Photosynthetic parameters were measured on the second fully expanded leaf using a portable photosynthesis system 7 days after PM inoculation. The parameters included chlorophyll content (Chlorophyll Meter SPAD-502 Konica Minolta, Osaka, Japan), net photosynthetic rate [$\mu\text{mol}/(\text{m}^2 \cdot \text{s})$] and intercellular CO_2 concentration [$\mu\text{mol}/\mu\text{mol}$]. Three independent biological samples were analyzed, each consisting of eight grafted plants inoculated with PM.

2.6 ROS scavenging enzyme activity and MDA content

Fresh leaf samples (0.3 g) were homogenized in an ice bath with 3 mL of 50 mM phosphate buffer (pH 7.8). The buffer contained 0.2 mM ethylenediaminetetraacetic acid, 2% (w/v) polyvinylpyrrolidone, and 2 mM reduced ascorbic acid (35.2 mg added to 100 mL of buffer). The homogenate was centrifuged at 10,000×g for 25 min at 4°C, and the resulting supernatant was collected as the crude enzyme extract for subsequent assays of reactive oxygen species (ROS) scavenging enzyme activities and malondialdehyde (MDA) content. Superoxide dismutase (SOD) activity was measured based on its ability to inhibit the photochemical reduction of nitroblue tetrazolium [36]. Peroxidase (POD) activity was determined using the guaiacol method [40]. The activities of catalase (CAT) and ascorbate peroxidase (APX) were assayed according to established protocols [41,42]. Glutathione reductase (GR) and dehydroascorbate reductase (DHAR) activities were determined as described previously [43,44]. Malondialdehyde (MDA) content was quantified by the thiobarbituric acid method [32,45]. Hydrogen peroxide (H₂O₂) content was measured using the titanium chloride precipitation method [46]. Superoxide anion (O₂⁻) production rate was assayed following a previously described procedure [47].

2.7 RNA extraction and expression analysis

On the third day post-inoculation with PM, leaf samples were collected from self-grafted plants and from plants grafted onto pumpkin rootstocks GP4, 6, 8, 9, and 10. Leaf samples were collected from 3-5 plants per cultivar, pooled into a mixed sample, and equally divided into three replicates. Total RNA was extracted and first-strand cDNA was synthesized following established protocols [48]. The expression levels of key defense-related genes were analyzed via RT-qPCR. This included genes involved in the phenylpropanoid pathway (*CsPAL* and *CsPPO*) [49] and the salicylic acid (SA) signaling pathway (*CsNPR1*, *CsPR1*, *CsPR2*, *CsPR5*, and *CsICS1*) [50]. The RT-qPCR procedure was performed according to standard protocols, using the primer sequences listed in Table 1. Gene expression data were analyzed using the 2^{-ΔΔCT} method [51].

Primer name	Primer Sequences (5'-3')
<i>Actin</i>	F: GTGGTGGTGAATGAGTAGCC
	R: TTGGATTCTGGTGATGGTGTC
<i>ICS1</i>	F: GCATTCACCTCCGGGATTAT
	R: AAGGTGCGAGGAAGATG
<i>NPR1</i>	F: CGCTTTGGTGAGGAGTTT
	R: CAAGATTACAACAAGGGT
<i>PR1</i>	F: AACTCTGGCGGACCTTAC
	R: GACTTCCTCCACACTACT
<i>PR2</i>	F: TCTTGGTCTTCTTGTGCC
	R: GAGCATCAAGTGAACCTC
<i>PR5</i>	F: CTTCTGCTAGTTGTGTTG
	R: GCAGTCACCCGTCTGGCA
<i>PAL</i>	F: AACTTCTCCTCAATGGCTTGGT
	R: TGAAACATCAATCAAAGGGTTG
<i>PPO</i>	F: CTAGCCGTGGAAACCGA
	R: TGATTGGCTCACAGTGGA

Table 1. Primer sequences used for gene expression analysis.

2.8 Statistical analysis

Data processing was performed using Excel 2021 for data organization and DPS 9.5 software for statistical analysis of significant differences [35]. All treatments and assays were replicated three times. Data were analyzed by one-way ANOVA, followed by the LSD post-hoc test. All results are presented as means $\pm$ standard error. Statistical significance was defined as $P < 0.05$.

3. Results

3.1 Effects of rootstock on the biomass and PM resistance of grafted cucumber

Investigating the relationship between plant biomass and PM resistance, the results indicate: among the 10 rootstocks evaluated, cucumber grafted onto GP4 exhibited the highest biomass under non-inoculated conditions (Figure S1A). Following PM inoculation, the biomasses of most grafted combinations were significantly higher than that of the self-grafted control, except for plants grafted onto GP14 (Figure S1B, 1B, 1D). Following powdery mildew inoculation, the fresh weight and dry weight of GP9 plants exhibited the highest biomass accumulation (Figure S1B, 1B). The plant growth rate followed a trend similar to that of biomass accumulation (Figure 1C, D). Biomass and growth rates of the grafted plants showed no consistent positive correlations with root system size across both non-inoculated and PM-inoculated conditions, as exemplified by rootstocks GP9 and GP10 (Figure 1). Compared to the self-grafted control, the biomass and growth rates of grafts on GP10, which possessed a larger root system than the other rootstocks, did not differ significantly after PM inoculation. In contrast, grafts on GP9, with a comparatively smaller root system, accumulated the highest biomasses under PM stress (Figure 1E). These results indicate that the influence of rootstock on graft biomass and growth rate differed between non-inoculated and PM-inoculated conditions, and that root system size was not the sole determining factor in this process.

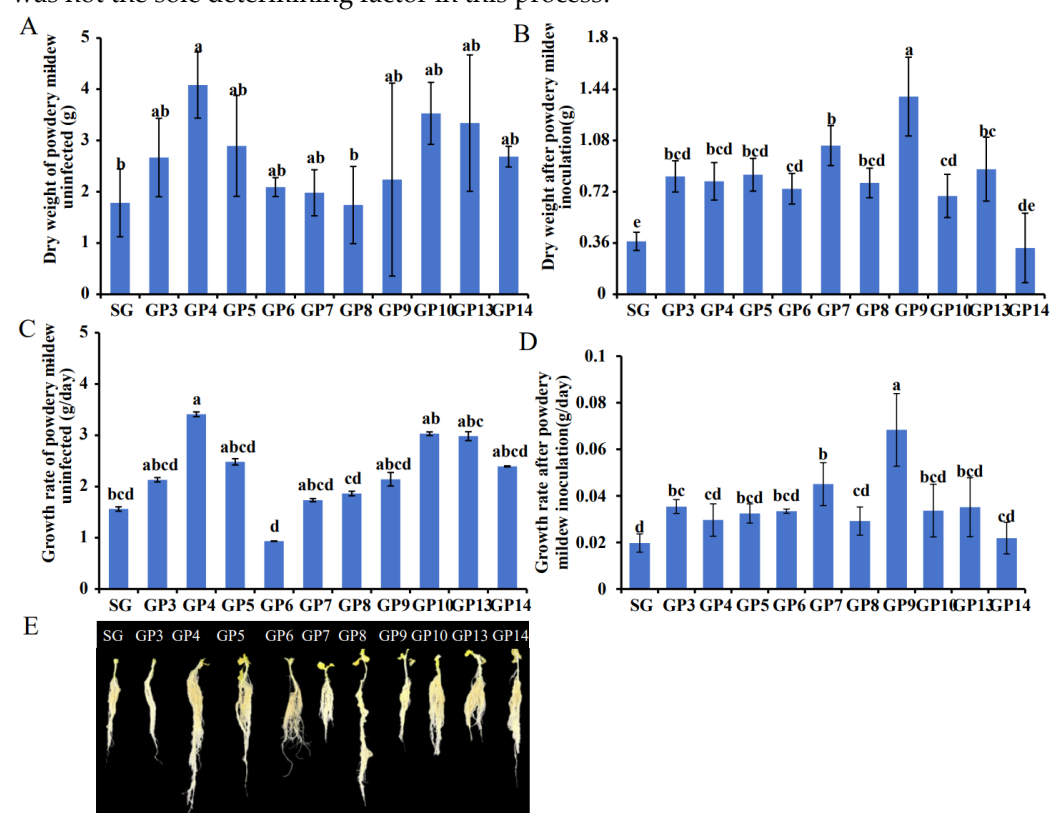


Figure 1. Biomass, growth rates, and root phenotypes of self-grafted cucumber seedlings and seedlings grafted onto different pumpkin rootstocks under uninoculated and powdery mildew inoculated conditions: (A) Dry weight of powdery mildew-uninfected seedlings; (B) Dry weight at 15 d

after powdery mildew inoculation; (C) Growth rate of powdery mildew-uninfected seedlings; (D) Growth rate at 15 d after powdery mildew inoculation; Data were analyzed by one-way ANOVA, followed by the LSD post-hoc test. All results are presented as means $\pm$ standard error. Statistical significance was defined as $P < 0.05$. (E) Root phenotypes of cucumbers self-grafted and those grafted onto various pumpkin rootstocks after 15 d under conditions without powdery mildew.

3.2 Effects of rootstocks on cucumber PM resistance and mycelium growth

A further assessment of the disease index following PM inoculation (Figure 2A) revealed that grafting increased PM severity in the susceptible 'Xintai mici' cucumber when using certain rootstocks, including GP3, 6, 10 and 14. In contrast, grafting onto rootstock GP8 conferred significant PM resistance. Notably, the PM resistance of the grafted cucumber scion did not correspond directly to the resistance of the self-grafted rootstock itself. For instance, while self-grafted GP5, 8, and 10 plants exhibited the lowest disease indices among the 10 evaluated rootstocks, only scions grafted onto GP8 maintained a similarly low index. Conversely, grafting onto rootstocks GP5 and GP10 resulted in significantly higher PM severity levels compared to their self-grafted counterparts. Based on these results, five rootstock varieties were selected to examine leaf disease severity levels and root growth condition, as well as fungal mycelium growth, after PM inoculation (Figure 2B–D). Pronounced differences in PM severity were observed on the second true leaves of cucumber plants grafted onto the five selected rootstocks. Compared to the self-grafted control, grafting onto the PM-resistant rootstock GP8 markedly reduced disease severity on leaves. In contrast, grafting onto the PM-resistant rootstocks GP6 and GP10 increased leaf PM severity compared to the control, as did grafting onto PM-susceptible rootstocks GP4 and GP9 (Figure 2D). In addition, after inoculation with powdery mildew, root growth is inhibited (Figure 1 and 2B). Consistent with these observations, mycelial development on leaves of plants grafted onto GP8 was slower and lower, respectively, at both 72 h and 8 d after inoculation compared to the control. The other rootstock combinations, particularly those of GP4 and GP9, supported relatively faster mycelium growth (Figure 2D).

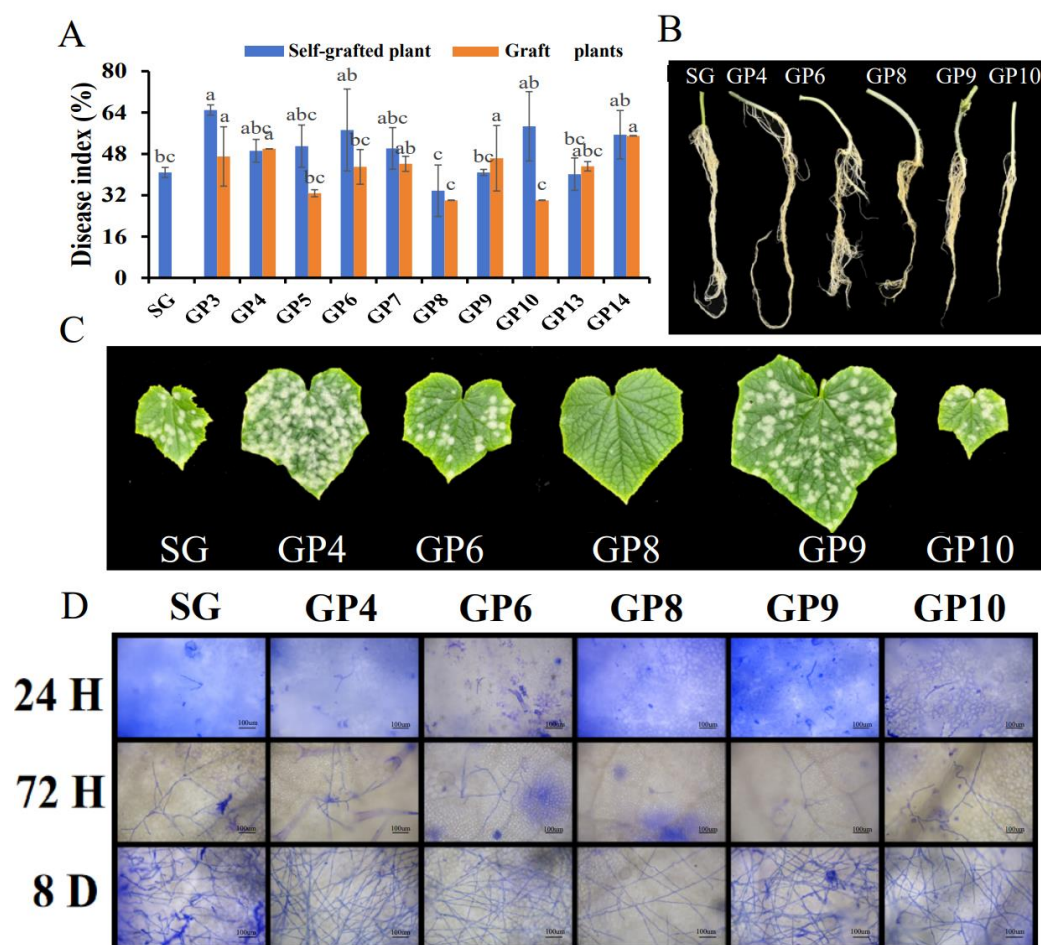


Figure 2. Powdery mildew disease index and phenotypic characteristics of cucumbers grafted onto different pumpkin rootstocks: (A) PM disease indices of cucumber plants grafted onto different pumpkin rootstocks at 15 d after PM inoculation; Data were analyzed by one-way ANOVA, followed by the LSD post-hoc test. All results are presented as means $\pm$ standard error. Statistical significance was defined as $P < 0.05$. (B, C) symptomatic root (B) and leaf (C) phenotypes of grafted seedlings onto different rootstocks at 15 d after PM inoculation; (D) Development of PM mycelium on leaves of cucumber seedlings grafted onto different rootstocks at 24 h, 72 h, and 8 d post-inoculation.

3.3 Effects of rootstocks on grafted cucumber photosynthesis after PM inoculation

Following PM inoculation, chlorophyll content was highest in cucumbers grafted onto rootstocks GP6 and GP8. In contrast, plants grafted onto rootstocks GP4 and GP10 showed no significant differences compared to the self-rooted control (Figure 3A). The net photosynthetic rate was highest in plants grafted onto rootstock GP8, whereas the rates of plants grafted onto rootstocks GP4, 6, 9, and 10 did not differ significantly from the control (Figure 3B). Similarly, only grafting onto rootstock GP4 resulted in a significantly higher intercellular CO_2 concentration compared to the control, with no significant differences observed among the other rootstock combinations (Figure 3C).

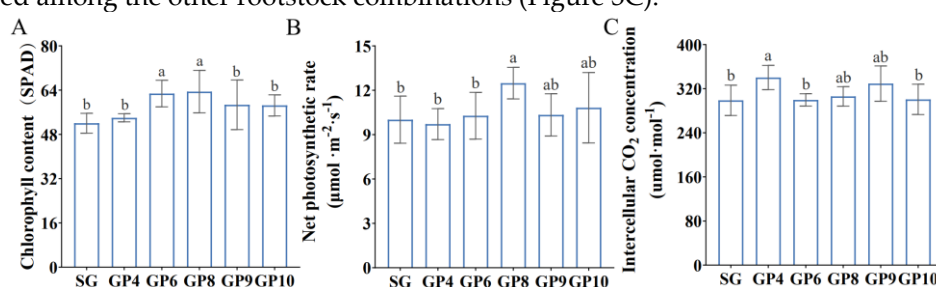


Figure 3. Photosynthetic characteristics of the cucumber seedlings grafted onto different pumpkin rootstocks and self-grafts at 7 days post-inoculation with powdery mildew: (A) Chlorophyll content; (B) Net photosynthetic rates of seedling; (C) Intercellular CO₂ concentration. Data were analyzed by one-way ANOVA, followed by the LSD post-hoc test. All results are presented as means $\pm$ standard error. Statistical significance was defined as $P < 0.05$.

3.4 Activities of reactive oxygen defense enzymes and lipid peroxidation contents

Based on measurements of ROS-scavenging enzyme activities performed 7 d after PM inoculation (Figure 4), grafting significantly increased superoxide dismutase activity (Figure 4A) but reduced catalase activity (Figure 4C) in most grafted combinations compared to the self-grafted control. Guaiacol peroxidase activity was significantly decreased in all the grafted plants, except those on rootstock GP8 (Figure 4B). In addition, plants grafted onto rootstock GP8 exhibited increases in ascorbate peroxidase and glutathione reductase activities by approximately 20% and 80%, respectively, relative to the control (Figure 4D, E). Dehydroascorbate reductase activity showed no significant difference among grafted plants, except for those on rootstock GP4, compared to the self-grafted control (Figure 4F). The aforementioned alterations in antioxidant enzyme activities contributed to seedlings grafted onto rootstock GP8 exhibiting the lowest levels of MDA, H₂O₂, and superoxide anion (O₂⁻) among all the treatments (Figure 4G-I), which likely constitutes a primary mechanism for its enhanced PM resistance. In contrast, seedlings grafted onto rootstock GP4 accumulated the highest O₂⁻ content, those on GP9 showed the highest MDA levels, and those on GP10 contained the highest H₂O₂ concentration, with these factors likely being associated with their increased PM susceptibility levels (Figure 4G-I). Seedlings grafted onto rootstock GP6 displayed moderate levels of MDA, H₂O₂, and O₂⁻, corresponding to their intermediate PM resistance (Figure 4G-I).

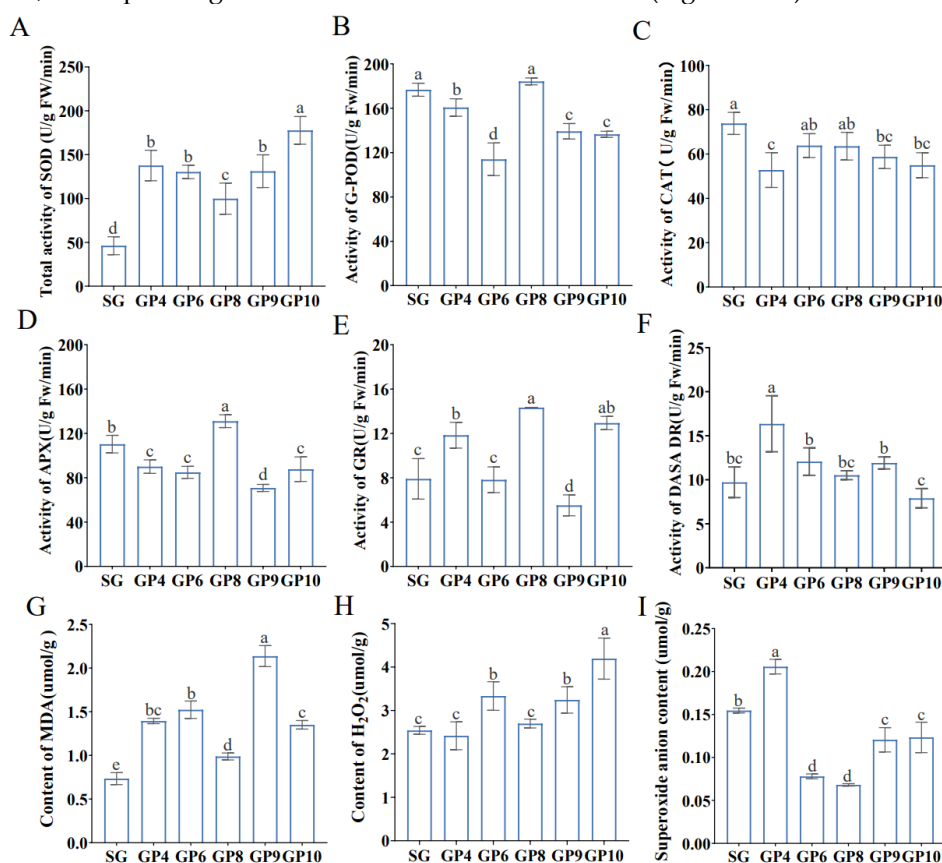


Figure 4. Antioxidant enzyme activities in the leaves of cucumber seedlings grafted onto different rootstocks and self-grafts at 7 d post-inoculation with powdery mildew: (A) Superoxide dismutase

(SOD) activity; (B) Guaiacol peroxidase (G-POD) activity; (C) Catalase (CAT) activity; (D) Ascorbate peroxidase (APX) activity; (E) Glutathione reductase (GR) activity; (F) Diaminobenzidine peroxidase (DASA) activity; (G) Malondialdehyde (MDA) content; (H) H₂O₂ content; (I) Superoxide anion content. Data were analyzed by one-way ANOVA, followed by the LSD post-hoc test. All results are presented as means $\pm$ standard error. Statistical significance was defined as $P < 0.05$.

3.5 Expression levels of defense genes in cucumber grafted onto different rootstocks and self-grafts

Compared to self-grafted cucumbers, grafting significantly upregulated the expression of *CsICS1* and *CsNPR1* in leaves 3 d after PM inoculation (Figure 5A, B). In particular, seedlings grafted onto rootstock GP8 showed 3.9 fold and 1.3 fold increases in *CsICS1* and *CsNPR1* expression, respectively. The expression patterns of *CsPR1*, *CsPR2*, and *CsPR5* varied across different rootstock-grafted seedlings (Figure 5C–E). Overall, relative to the self-grafted control, seedlings grafted onto rootstocks GP4 and GP6 displayed downregulated expression levels of these PR genes, whereas those grafted onto rootstocks GP8, 9, and 10 exhibited upregulated expression levels. Notably, the expression of *CsPR5* was markedly upregulated following PM inoculation in seedlings grafted onto rootstocks GP8, 9, and 10, with increases of 2.5, 4.5, and 7.0 fold, respectively, compared to the self-rooted control (Figure 5E). In contrast, the expression levels of *CsPAL* and *CsPPO* in seedlings grafted onto rootstock GP4 did not differ significantly from those in the self-grafted plants (Figure 5F, G). Conversely, these two genes were significantly upregulated in seedlings grafted onto rootstocks GP8, 9, and 10, with *CsPAL* expression in the GP8 combination being 58 fold greater than that of the control (Figure 5F). Collectively, PM infection strongly induced the expression of *CsICS1*, *CsNPR1*, *CsPR1*, *CsPR5*, *CsPAL*, and *CsPPO* in seedlings grafted onto rootstock GP8 (Figure 5).

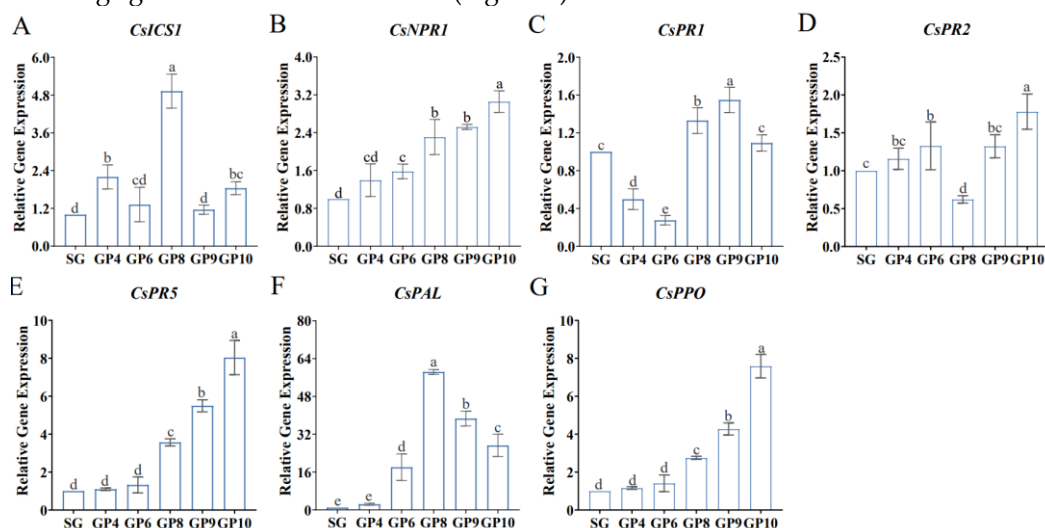


Figure 5. Expression levels of defense genes in the leaves of cucumber seedlings grafted onto different rootstocks and self-grafts at 3 d post-inoculation with powdery mildew: (A–G) RT-qPCR analysis revealed the expression levels of *CsICS1*, *CsNPR1*, *CsPR1*, *CsPR2*, *CsPR5*, *CsPAL*, and *CsPPO* genes at 3 d post-inoculation in cucumber seedlings grafted onto different pumpkin rootstocks. The expression levels of each gene in the self-grafted plants were normalized to 1.0. Data were analyzed by one-way ANOVA, followed by the LSD post-hoc test. All results are presented as means $\pm$ standard error. Statistical significance was defined as $P < 0.05$.

4. Discussion

4.1 Rootstocks are not the sole factor determining grafted cucumber growth and PM resistance

PM remains one of the three major diseases affecting cucumber, significantly compromising both yield and quality [6]. Grafting is a well-established technique for improving resistance to PM in crops like watermelon [8] and chili pepper [9]. Numerous studies highlight that screening rootstock varieties for disease resistance is essential in obtaining tolerant germplasms, which is crucial for breeding new grafted cultivars to manage PM in both field and greenhouse cucumber production [52]. In this study, 10 pumpkin rootstock varieties were grafted with a susceptible cucumber scion. Grafting generally promoted plant biomass accumulation compared to self-grafted plants, with rootstock GP4 (which possesses a larger root system) showing a particularly pronounced effect. This is consistent with previous reports that grafting enhances biomass accumulation [53]. Following PM infection, this trend became more distinct. The biomasses of all the grafted combinations were significantly greater than that of the self-grafted control, with the exception of plants grafted onto rootstock GP14, B). However, we observed that the growth rates of plants grafted onto different rootstocks were affected by PM infection, and these rates were not necessarily positively correlated with final plant biomass. For example, while plants grafted onto rootstock GP4 exhibited significantly increased biomasses compared to the self-grafted control under non-inoculated conditions, no significant difference in biomass accumulation or growth rate was detected between the two groups after PM inoculation. In contrast, plants grafted onto rootstock GP9 displayed the opposite trend, with PM infection promoting their biomass accumulation. Overall, post-grafting biomass accumulation did not show a direct relationship with rootstock root system size, a phenomenon that may be attributed to variations in root vitality and nutrient uptake capacity among rootstocks [54]. Additionally, studies indicate that the transport and signaling of photosynthetic assimilates, hormones, and miRNAs between the scion and rootstock can induce modifications in root architecture, nutrient uptake efficiency, and rhizosphere microbial composition, ultimately shaping the growth and stress resistance levels of grafted plants [55–57]. In this study, the PM resistance of a rootstock itself was not consistently correlated with the PM resistance of the grafted plant. Notably, grafting onto PM-resistant pumpkin rootstocks GP5 and GP10 significantly increased PM severity on the susceptible cucumber scion. This contrasts with findings for watermelon, in which grafting a susceptible scion onto a PM-resistant rootstock generally reduces disease severity compared to using a susceptible rootstock, although some resistant rootstocks also failed to confer significant resistance to the scion [8]. In our study, mycelium development was markedly limited on the second leaves of cucumber grafted onto rootstock GP8, whereas abundant mycelium chains were visible in other graft combinations at both 72 h and 8 d post-inoculation. The trends in powdery mildew index recorded for GP8 fungal hyphae were consistent with those observed in cucumbers grafted onto different rootstocks. This suppression of pathogen colonization and reproduction directly drove the superior PM resistance conferred by the GP8 rootstock. Structural and biochemical barriers, such as papillae, cell-wall proteins, lignin-like deposits, penetration-resistance proteins, ROS, act as physical defenses against pathogen invasion [58,59]. Additionally, altered water relations in the cucumber scion suppress PM colony development during graft healing [17].

4.2 PM-resistant grafting combinations have high net photosynthetic rates and antioxidant enzyme activities

In plant defense, reactive oxygen species (ROS) play a dual role in plant defense. In cucumber, resistance to PM has been linked to ethylene-mediated ROS metabolism [60]. For example, a knockdown of *CsMLO8* and *CsMLO11* increases ROS levels and improves PM resistance [61]. In contrast to the enhanced PM resistance in cherry pepper grafted onto susceptible sweet pepper, which was attributed to elevations in ROS accumulation

and NADPH oxidase activity [9], our study demonstrated a different mechanism in cucumber. The highly PM-resistant rootstock GP 8 was associated with lower levels of ROS and MDA. Such a reduced ROS accumulation likely functions as a localized signal to induce antioxidant defenses and pathogenesis-related gene expression near infection sites [62,63]. The lower ROS levels in plants grafted onto rootstock GP8 are likely a consequence of the significantly higher activities of ROS-scavenging enzymes, including superoxide dismutase, peroxidase, ascorbate peroxidase, and glutathione reductase. A similar association between enhanced antioxidant enzyme activity and improved PM resistance has been reported in pumpkin [49]. PM conidia can cover leaf surfaces and significantly suppress photosynthesis [64]. In contrast, our results demonstrated that cucumbers grafted onto rootstock GP8 maintained higher net photosynthetic rates after PM infection. This effect can be attributed to the following two main factors: their higher chlorophyll contents and their lower levels of oxidative damage (as indicated by reduced MDA and ROS) coupled with elevated activities of ROS-scavenging enzymes. These findings align with observations in highly PM-resistant melon, in which PM inoculation significantly increases net photosynthetic rate, transpiration rate, leaf water-use efficiency, and chlorophyll levels [65]. It is important to note that excessive or misregulated ROS can damage cellular components, such as DNA, proteins, and lipids, ultimately leading to cell death.

4.3 SA-signaling-associated and phenylpropanoid pathway genes were significantly activated in PM-resistant grafted cucumbers

The SA pathway is typically associated with defense against biotrophic pathogens, whereas JA and ethylene (ET) are more frequently involved in responses to necrotrophic pathogens [66]. SA biosynthesis proceeds via two routes: the phenylalanine ammonia-lyase (PAL) pathway and the isochorismate synthase (ICS) pathway, with PAL and ICS serving as the key enzymes in their respective routes [67]. In *Arabidopsis*, *AtICS1* mediates SA accumulation in leaf tissues under both biotic or abiotic stress [68,69]. Notably, the conversion of chorismate to isochorismate catalyzed by ICS1 is the only chloroplast-localized step in this biosynthetic pathway [70]. For example, in melon, red light promotes the activation of *CmWRKY42*, which directly binds to the promoter of *CmICS* to upregulate its expression. This leads to increased SA accumulation and enhanced PM resistance, a transcriptional activation that can be inhibited by *CmPIF* proteins [71]. Phenylalanine is then transformed into trans-cinnamic acid by PAL, from which salicylic acid is generated through the sequential action of ABNORMAL INFLORESCENCE MERISTEM 1 (AIM1) and a yet-to-be-identified benzoic acid hydrolase [72]. In rice, loss-of-function mutations in *OsPAL4* and *OsPAL6* reduce SA accumulation and increase susceptibility to multiple pathogens, suggesting that these genes play a dominant role in pathogen-induced SA biosynthesis [73,74]. Our results showed that the expression of *CsICS* was highest in cucumber grafted onto rootstock GP8 compared to both self-rooted plants and grafts on other rootstocks. The *NPR1* gene, known to promote SA accumulation in *Arabidopsis* [75] was also significantly activated in grafts using rootstock GP8. Furthermore, the SA pathway marker genes *PR1* and *PR5* exhibited similar upregulation patterns in these grafts. This coordinated induction aligns with reports that the transactivation of *PR1a*, *PR5*, and *NPR1* depends on transcription factors, such as *CmbHLH87*, which integrates H₂O₂ and SA signaling in tobacco following PM infection [76]. Approximately 90% of pathogen-induced SA biosynthesis occurs via the ICS pathway [77]. Our results suggest that the enhanced PM resistance in cucumber grafted onto rootstock GP8 is mediated through the upregulation of *ICS* and *NPR1*, leading to increased SA accumulation. Both PAL and polyphenol oxidase (PPO) are key enzymes in the phenylpropanoid pathway and contribute to PM resistance in cucumber [78]. *PePAL* knockdown, PAL activity and total lignin content were significantly reduced, while flavonoid levels were markedly decreased [79]. In our study,

the expression levels of *PAL* and *PPO* were significantly higher in PM-resistant grafts on rootstock GP8 than on other rootstocks. This upregulation may promote lignin deposition and elevate the total phenolic content through enhanced *PAL* and *PPO* activities. As the first rate-limiting enzyme in the phenylpropanoid pathway, *PAL* is involved in the synthesis of phytoalexins, lignins, and phenolic compounds [80]. Consistently, *PAL* activity has been positively correlated with PM resistance in *Poa pratensis* [81]. *CpVQ20* enhances tobacco resistance to PM by upregulating the expression of *PAL*, *C4H*, and *F5H*, leading to elevated flavonoid and lignin biosynthesis [82]. In contrast, *CmbHLH87* can activate PM resistance primarily through SA-dependent defense pathways without significantly altering *PAL* expression [82]. These examples highlight the diversity of molecular mechanisms underlying PM resistance. Therefore, further studies are needed to elucidate the biological functions of key genes that confer PM resistance to cucumber scions when grafted onto different pumpkin rootstocks.

5. Conclusions

Grafting is an effective agricultural technique for controlling both soil-borne and foliar diseases in plants. In grafted cucumber, the effect of rootstocks on PM resistance varied considerably. The susceptible cucumber cultivar ‘Xintai mici’ when grafted onto the tolerant pumpkin rootstock GP8, exhibited the strongest PM resistance among all the tested rootstock varieties. The underlying mechanism likely involves the activation of the PM defense system in the scion through the rootstock’s own tolerance, slower fungal mycelium development, and higher net photosynthetic rates in the grafted plants. These traits were not positively correlated with plant biomass accumulation or root system size. Concurrently, the elevated activities of ROS-scavenging enzymes reduced ROS accumulation, thereby alleviating oxidative damage. Additionally, the upregulated expression of genes in the SA-signaling pathway likely promoted the accumulation of phenolic compounds and lignin, further enhancing PM resistance in grafted cucumbers. Therefore, selecting suitable rootstocks represents an effective approach for improving PM resistance in cucumber grafting. Further studies are needed to elucidate the precise mechanisms by which rootstocks confer PM resistance, and key regulatory genes should be identified and functionally validated.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Fresh weight of self-grafted cucumber seedlings and seedlings grafted onto different pumpkin rootstocks under uninoculated and powdery mildew inoculated conditions.

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