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Physiological Aspects of Compatibility and Incompatibility in Grafted Cucumber Seedlings

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Abstract. The use of grafted seedlings in vegetable crops has increased in recent years to enhance the resistance to biological and abiotic stresses, and improve yields. However, incompatibility restricts the wide application of grafting. In this study, two pumpkin (*Cucurbita*) cultivars, with great differences in grafting affinity and symbiotic affinity, were used as rootstocks and cucumber (*Cucumis sativus*) seedlings were used as the scion. The effects of compatibility or incompatibility on histological aspects, antioxidant enzyme activities, phenylpropanoid contents, and chlorophyll fluorescence were studied. The results showed that compatible graft combinations present a stronger resistance to the oxidative damage resulting from grafting and had relatively weak phenylpropanoid metabolisms. The results also indicated that the chlorophyll fluorescence levels of incompatible combinations were lower, except compared with the original fluorescence. Finally, a necrotic layer existed earlier in compatible graft combinations. These differences at the morphological, physiological, and cellular levels may govern compatibility and incompatibility, and may provide valuable information for determining the symbiotic affinity of grafted seedlings at an early stage.

Grafting of vegetable seedlings is widely used in horticulture for various reasons, such as managing soil-borne disease and improving crop responses to abiotic stresses. Ling et al. (2013) suggested that watermelon wilt (*Fusarium oxysporum* f. sp. *niveum*) resistance in watermelon (*Citrullus lanatus*) may be enhanced when wilt-tolerant rootstocks are used in grafting. Rootstock grafting may not only enhance the vegetable's resistance to biotic stress, but also increase the plant's growth performance under abiotic stress. It has been reported that salt-tolerant rootstock grafting may improve the plant's growth performance by enhancing the photosynthetic properties of the vegetable plants under salt stress (Rouphael et al., 2012). Grafting can also increase the plant's resistance to low temperatures (King et al., 2010), high temperatures (Schwarz et al., 2010), and heavy metals (Savvas et al., 2010). In addition, grafting relieves the adverse impact on vegetable yield and quality induced by continuous vegetable cropping in greenhouses (Bletsos et al., 2003). In short, rootstock grafting has become an important means of improving the growth performance and quality of vegetable plants.

However, when grafting involves two different species or genera, a lack of affinity, known as graft incompatibility, may occur. Graft incompatibility can induce undergrowth or overgrowth of the scion, which can lead to decreased levels of water and nutrients flowing through the graft union, causing the plant to wilt (Davis et al., 2008). The survival rate of incompatible graft combinations, according to Yetisir and Sari (2003), was significantly lower than that of compatible combinations in watermelon. The growth performance of compatible combinations of coffee (*Coffea arabica*) were also reported as superior to that of incompatible combinations after grafting (Bertrand et al., 2001). Therefore, the compatibility of rootstock and scion greatly contribute to the effects of grafting. Affinity is composed of grafting affinity and symbiotic affinity. The former is the survival after grafting of rootstock and scion, and normally indicates the survival rate of graft combinations (Yetisir and Sari, 2003). The latter is the symbiotic ability after the grafting combinations' survival and often indicates the growth performance, yield, and quality of graft combinations. The graft combination with the highest grafting affinity may not always have the highest symbiotic affinity, as studies have demonstrated (Gisbert et al., 2011; Salehi-Mohammadi et al., 2009).

Many means have been used to analyze compatibility in grafted vegetable seedlings. Antioxidant enzymes were usually studied in the responses to abiotic and biotic stresses, but not often for graft stress. This was also true for the phenylpropanoid substances (Pereira et al., 2014). Many studies have suggested that certain antioxidases play roles in the xylem lignification of the newly differentiating vascular system after the graft assemblage between the cells of the rootstock and scion was developed (Quiroga et al., 2000; Whetten et al., 1998). Gulen et al. (2002) reported that grafted pear (*Pyrus communis*) plants showed more antioxidant enzyme activities in the graft union than controls. In experiments with a melon (*Cucumis melo*) scion and compatible and incompatible pumpkin rootstocks, Aloni et al. (2008) suggested that in

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comparison with incompatible graft combinations, the antioxidant enzyme activities were higher and the levels of reactive oxygen species were lower in the compatible rootstock–scion interface. In addition, as Deloire and Hebant (1982) have demonstrated, incompatibility in heterografts between tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) were both anatomical and biochemical. They described the anatomical incompatibility as a lack of, or a decrease in, the number of differentiated vascular bundles at the graft union, which inhibited the transport of nutrients between the rootstock and scion. Conversely, the accumulation of polyphenols at the graft union was termed biochemical incompatibility. Further, in a study by Calatayud et al. (2013), the maximal photosynthetic efficiency under dark conditions (F_v/F_m) was lower in compatible melon's graft union 10 or 15 d after grafting. However, as a biological process, the incompatibility reaction is difficult to study because of the wide range of different scion–rootstock interactions produced when grafting. Leonardi and Romano (2004) showed that it was difficult to form an acceptable formula for defining grafting compatibility and success in vegetables.

Cucumber is a major vegetable in Chinese cultivation. However, in greenhouse production, cucumber plants are often exposed to high and low temperatures and other abiotic stresses. Meanwhile, continuous cropping obstacles result in tremendous adverse effects on the yield and quality of cucumber plants. Therefore, Chinese cucumber producers have selected grafting as an efficient tool to overcome various biotic and abiotic stresses, and have had excellent results (Lee et al., 2010). However, even if a rootstock with high resistance is selected for grafting, failures caused by an incompatibility between the rootstock and scion sometimes occur (Yetisir et al., 2007). The graft combinations with high survival rates at the seedling stage do not always perform well with respect to symbiotic affinity as reflected in the growth, yield, and quality of the adult plant (Gisbert et al., 2011). If the symbiotic affinity of graft combinations could be estimated by determining certain physiological and biochemical indices, the above problem could be prevented. Nevertheless, there are few reports concerning the differences in physiological and biochemical properties between cucumber graft compatible combinations and incompatible combinations. Thus, in this research, two cultivars of pumpkin, with great differences in grafting affinity and symbiotic affinity, were used as rootstocks, and cucumber seedlings were used as the scion. Histological aspects, antioxidant enzyme activities, phenylpropanoid contents, and chlorophyll (Chl) fluorescence in the early developmental stages of grafted or nongrafted seedlings were measured to compare the physiological aspects of compatibility and incompatibility in grafted cucumber seedlings. This will provide valuable information for determining the symbiotic affinity of grafted seedlings at an early stage.

Materials and Methods

Plant materials and union establishment

The research was carried out from Sept. 2012 to June 2013 in the greenhouse of the Nanjing Agricultural University. Cucumber seedlings of cultivar Jinchun No.4 [J (Tian Kerun Cucumber Institute, Tianjin, China)] were used as scions, and pumpkin seedlings of two cultivars, Dongyangshenli [D *Cucurbita moschata* (Fengyuan Seed Co., Shouguang, China)] and Heizinangua [H *Cucurbita ficifolia* (Hongwei Seed Co., Shouguang, China)], were used as rootstocks. In previous experiments, grafted "H" showed much better growth potential than grafted "D."

Seeds of rootstocks were sown in 15-cell polystyrene trays filled with commercial organic substrates [2 vinegar waste compost:2 peat:1 vermiculite (by volume); Beilei, Zhenjiang, China], and scion seeds were sown in 72-cell trays when the rootstock seeds had just emerged. Seeds of nongrafted cucumber were sown 3 d later than the scion to produce seedlings with about the same size as the grafted cucumber seedlings. When the cotyledons of scions and first true leaves of rootstocks were just flat, "hole insertion grafting" was performed (Hassell et al., 2008). Nongrafted cucumber seedlings were used as controls. Each rootstock–scion combination was made three replications with 60 subsamples in each replication per experiment. All the grafting was performed by one operator. Grafted plants were transferred to a small plastic-arched shed where they were maintained at a temperature between 25 and 30 °C with a relative humidity of between 85% and 100% for ≈7 d until the graft union healed. The temperature of the small plastic-arched shed was regulated by shading and ventilating. The seedlings grew with sufficient light all the time.

At the three true-leaf stage, plants were transplanted in the greenhouse into plastic barrels containing 10 L of commercial organic substrate [2 vinegar waste compost:2 peat:1 vermiculite (by volume), Beilei], and each barrel contained two cucumber seedlings. Plants were in double rows spaced 60 cm apart, with plants spaced 30 cm within rows. During the culturing, the greenhouse was maintained at between 28 and 30 °C during the day and between 14 and 16 °C at night. The plants were irrigated with Hoagland nutrient solution. The amount of irrigation solution applied for each plant was 0.2 to 1 L each day, depending on the plant growth stage and environmental conditions.

Survival rate, growth, yield, and quality

Surviving plants were counted 25 d after grafting and the survival rate was expressed as a percentage of the total number of grafted plants. The growth measurements and all the physiological measurements were performed with the seedlings of the last repetition. Plant height, from the cotyledonary node of the rootstock to the growing point, was measured using a ruler. Root length, from the cotyledonary node of the rootstock to the root tip, was also measured using a ruler. Scion and rootstock shoot widths were measured 1 cm under the cotyledons of scions and rootstocks, respectively. The area of the second expanded leaf (from the top) and root volume were measured with a scanner (Expression 1680; Epson, Sydney, Australia) and image analysis software (WinRHIZO; Regent Instruments, Quebec, QC, Canada). After all the shoots and roots were washed with distilled water and topical moisture removed, their fresh weights were measured. Then, the dry weights were obtained after drying at 75 °C for 72 h.

Cucumber fruit was harvested two or three times every week between 5 May and 12 June 2013. The number and fresh weight in each fruit category was recorded. Fruit was harvested 15 d after anthesis. At middle harvest, four representative fruits from each barrel were analyzed for fruit quality. Titratable acidity was determined by potentiometric titration with 0.1 M NaOH up to pH 8.1 using 10 mL of juice, and the results were expressed as a percentage of fresh weight. The content of soluble sugar was determined using the anthrone method (Spiro, 1966), and the results were expressed as a percentage

of fresh weight. The vitamin C content was tested using the 2,6-dichloroindophenol titrimetric method (AOAC, 1984), and the results were expressed as milligrams of ascorbic acid per 100 g fresh weight. Fruit slices were weighed and placed into an oven at 105 °C for 15 min, followed by 70 °C for 5 d. Then, the dry weight was measured and the fruit dry matter content (percent) was calculated.

Histology

Six days after grafting, tissue samples from "D" and "H" grafting joints were fixed in formalin–acetic acid–alcohol fixative solution (63% ethanol, 5% glacial acetic acid, and 5% formaldehyde) for the histological analysis (Huber et al., 2005). After being dehydrated, cleaned, infiltrated, and embodied in wax, samples were sectioned to 8 μm vertically, stained with Fast Green, counterstained with Safranin and fixed with Neutral Balata. Sections were visualized using a microscope (BX53; Olympus Corp., Tokyo, Japan) and digitally photographed.

Chl fluorescence

Chl fluorescence parameters of grafted/nongrafted cucumber seedling plants were evaluated using a pulse amplitude modulation (PAM) fluorometer (IMAGING-PAM; Walz, Effeltrich, Germany). Chl fluorescence was measured at grafting joints when seedlings were at the three true leaves stage (25 d after grafting). Before measuring the Chl fluorescence, the samples had been dark-adapted for at least 20 min. The minimum Chl fluorescence yield in the dark-adapted state (F_0) was determined using light pulses at a low frequency (1 Hz). Maximum fluorescence (F_m) was determined by applying a blue saturation pulse (10 Hz). The maximum quantum yield of photosystem II (PSII) photochemistry, the F_v/F_m ratio, was determined as $(F_m–F_0)/F_m$. Actinic illumination (260 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was then switched on and saturating pulses were applied at 20 s intervals for 5 min to determine F_m' and Chl fluorescence during actinic illumination (F_s). The quantum efficiency of PSII photochemistry [$\Phi_{PSII} = (F_m'–F_s)/F_m'$] and the quantum yield of regulated energy dissipation in PSII ($\Phi_{NPQ} = 1–\Phi_{PSII}–1/[F_m/F_m'+qL(F_m/F_0–1)]$) were calculated according to Kramer et al. (2004).

Enzyme activities and sample contents

Samples were collected at 25 d after grafting, when the grafted and nongrafted plants were with three true leaves. All the root, stem (graft area), and leaf samples were immediately frozen in liquid nitrogen and stored at –80 °C until analyses.

Antioxidant enzyme activities assay. The frozen samples of roots, stems, and leaves were homogenized in an ice-bath with 100 mM phosphate-buffered solution [PBS (pH 7.0)] at a ratio of 1:10 (w/v). Next, samples were centrifuged at 11,000 g_n for 25 min at 4 °C, and a spectrophotometer was used to determine the activities of the antioxidant enzymes— superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX).

The activity of SOD was determined according to the method of Dhindsa et al. (1981). One unit of enzyme activity was taken as the activity to cause 50% inhibition. The nitroblue tetrazolium reduction rate was measured by monitoring the absorbance at 560 nm.

The activity of POD was determined in a reaction solution composed of 50 mM PBS (pH 7.0), 2 mM H_2O_2, 2.7 mM guaiacol, and 0.05 mL enzyme extract by monitoring the increase in absorbance at 470 nm due to guaiacol oxidation (Polle et al., 1994). One unit of POD activity was defined as an absorbance change of 0.01 $U \cdot min^{-1}$ and POD activity was expressed as units per milligram average fresh weight.

A modified method of Rao et al. (1996) was used to assay CAT activity. The reaction mixture contained 100 mM PBS (pH 7.0), 3 mL plant extract, and 10 μL of 30% (w/v) H_2O_2. The absorbance changes were recorded at 240 nm for 2 min. An absorbance change of 0.1 $U \cdot min^{-1}$ was defined as 1 unit of CAT activity, and CAT activity was expressed as units per milligram fresh weight.

APX was assayed following the method of Nakano and Asada (1981) with some modifications. The assay was carried out in a reaction mixture consisting of 50 mM PBS (pH 7.0), 0.5 mM ascorbate, 3 mM H_2O_2, and 100 mL of the enzyme extraction. The changes in the absorbance at 290 nm were recorded at 25 °C for 1 min after the addition of H_2O_2. One unit of APX activity was defined as an absorbance change of 0.1 $U \cdot min^{-1}$ and APX activity was expressed as units per milligram protein.

Phenylalanine ammonia-lyase activity assay. Phenylalanine ammonia-lyase (PAL) activity was measured using a modified method of Sanchez-Rodriguez et al. (2011). The reaction mixture was 0.4 mL of 100 mM Tris-HCl buffer (pH 8.8), 0.2 mL of 40 mM phenylalanine, and 0.2 mL of enzyme extract. The reaction mixture was incubated for 30 min at 37 °C, and the reaction was ended by adding 25% trichloroacetic acid. The absorbance of the supernatant was measured at 280 nm.

Polyphenol oxidase activity assay. The polyphenol oxidase (PPO) assay was performed in a mixture containing 2.85 mL of 50 mM PBS (pH 7.0), 50 μL of 60 mM catechol, and 0.1 mM of supernatant, and the increasing absorbance was read over 2 min at 390 nm (Aquino-Bolanos and Mercado-Silva, 2004).

Measurement of total phenol content. The total phenolic compound content was determined using the method of Ghimire et al. (2011). Ethanoic extract (100 mg) was added to 0.3% HCl in methanol. This mixture was added to 2 mL of 2% aqueous sodium carbonate. The mixture was incubated for 2 min, and then 50% Folin–Ciocalateu reagent was added. The absorbance was measured at 750 nm.

Measurement of lignin content. Samples of 50 mg were extracted once with 400 μL of 80% ethanol. Next, the pellets were re-extracted with 1 mL of 80% ethanol and resuspended in 10% thioglycolic acid. The samples were heated at 100 °C for 4 h. After centrifugation at 13,000 g_n for 10 min at room temperature (RT), the pellet was resuspended in 0.5 mL 2 N NaOH. The samples were centrifuged at 13,000 g_n for 10 min at RT, and the supernatants were transferred to fresh tubes and acidified with 0.2 mL 10 N HCl. After chilling on ice for 1 h, the tubes were centrifuged at 13,000 g_n for 10 min at RT. The supernatant was removed, and all pellets were dissolved in 1 mL 0.5 N NaOH. The solution was measured at 280 nm using a spectrophotometer (Taga et al., 1984).

Measurement of Chl content

The content of Chl was assayed using 0.1 g of sample in a 10-mL extraction solution of 4.5 isopropyl alcohol:4.5 acetone:1 water. After at least 1 d, the absorbance readings were measured using a spectrophotometer at three wavelengths, 440, 645, and 663 nm (Nagata and Yamashita, 1992), and the results, expressed in milligrams per gram, were calculated using the following formulae: $Chla = [(12.7 \times A_{663}) – (2.69 \times A_{645})] \times V/(1000 \times W)$,

Chlb = $[(22.9 \times A_{645}) - (4.68 \times A_{663})] \times V/(1000 \times W)$, and carotenoid (Car) = $[(4.7 \times A_{440}) - 0.27 \times \text{Chl(a + b)}] \times V/(1000 \times W)$; where V indicates the volume of the extraction solution and W indicates the fresh weight of leaves.

Statistical analysis

Statistical analysis was carried out with SPSS Statistics (version 20.0; IBM Corp., Armonk, NY). Statistical testing of growth indices, survival rates, yield, and fruit quality within each sampling data were performed with Student's *t* test at $P \leq 0.05$. Chlorophyll fluorescence parameters, antioxidant enzyme activities, enzyme activities, and substance contents involved in the phenylpropanoid metabolism, chlorophyll contents were analyzed with analysis of variance (ANOVA). The ANOVA was performed using Duncan's multiple range test at $P \leq 0.05$.

Results

Survival rate, growth, yield, and quality. Results of survival rate and plant growth are presented in Table 1. A higher grafted survival rate (100%) was observed between cucumber and "H," whereas a lower survival rate (88%) was observed between cucumber and "D." Seedling growth was significantly influenced by the different rootstocks. All the growth indices of the "H" combination, including plant height, scion shoot width, shoot fresh weight, and shoot dry weight, were much greater than those containing "D." Thus, in general terms, both combinations showed different compatibility behaviors with the "H" combination presenting a better compatibility than "D." Results of yield and quality are presented in Table 2. The fruit quality and fruit number of the "H" combination were higher than those of the "D." The yield per plant was significantly different between "D" and "H." In the "D" combination, the fruit dry matter content was greater than in "H," while the total soluble sugar content was lower. There was no obvious difference between the vitamin C and titratable acidity contents.

Necrotic layer. As shown in Fig. 1, the graft union formation was observed using paraffin sections. A necrotic layer was induced between the scion and the "H" rootstock 6 d after grafting, whereas a necrotic layer had not yet been induced in the graft combination containing the "D" rootstock.

Activities of antioxidant enzymes. The effect of compatibility on the activities of SOD, POD, CAT, and APX in roots, stems, and leaves of grafted and nongrafted seedlings is shown in Fig. 2. The activities of SOD, POD, CAT, and APX in the roots and stems of "J" were lower than in the graft unions "D" and "H." No significant differences were observed among the POD and CAT activities in the leaves of the three treatments. In the stems of grafted seedlings containing rootstock "D," the activities of SOD, POD, and APX were lower than those in the "H" graft union.

Enzyme activities and substance contents in phenylpropanoid metabolism. Certain enzymes and substances involved in phenylpropanoid metabolism during graft union development were analyzed in this study (Fig. 3). The PAL activity in stems and leaves was lower in "D" than in "H." However, the PPO activity was higher in "D" than in "H," but in roots it was higher in "H" than in "D." The phenolic and lignin quantities in roots, stems, and leaves of "D" were significantly higher than in "H" and "J." The results showed that the PAL activity in incompatible combinations of stem and leaves was lower than in the compatible combination, while the PPO activity, phenolic content and lignin content were higher.

Chl fluorescence parameters and Chl content. Our results (Table 3) showed that none of the Chl fluorescence parameter (F_0, F_v/F_m, Φ_{PSII}, F_v'/F_m', electron transport rates (ETR), $L_{(PFD)}$, photochemical quenching (qP), and Φ_{NPQ}) values at the grafting joint showed any significant differences between nongrafted seedling "J" and grafted seedling "H." In addition, the F_0 of graft union "D" was higher, whereas all the other values of the Chl fluorescence parameter obtained from "D" were lower than in "J" and "H." The Chla, Chlb, and Car contents (Fig. 4) were significantly different between "J," "D," and "H," with the maximums in "D" and the minimums in "J."

Discussion

It has been reported that grafting promotes vegetative growth at different levels depending on rootstock characteristics (Ito, 1992). In this study, survival rate, growth, production, and quality indices significantly varied based on rootstock genotypes, in agreement with the study of Yetisir et al. (2007). The grafted combination with better compatibility survived more easily, grew more strongly, and achieved a higher production rate and better quality of fruit

Table 1. Effect of different rootstocks on growth indices and survival rates of 'Jinchun No. 4' cucumber seedlings at 25 d after grafting on two pumpkin cultivars, Dongyangshenli (D) and Heizinangua (H).

								Shoot		Root	
Treatment	Survival rate (%)	Plant ht (cm)	Root length (cm)	Scion shoot width (cm)	Rootstock shoot width (cm)	Leaf area (cm^2)	Root vol (cm^3)	Fresh wt (g/plant)	Dry wt (g/plant)	Fresh wt (g/plant)	Dry wt (g/plant)
D	88 b[z]	2.80 b	13.23 b	2.50 b	5.17 b	18.77 b	0.75 b	4.43 b	0.37 b	0.57 b	0.02 b
H	100 a	4.50 a	27.83 a	4.25 a	5.84 a	73.14 a	1.73 a	12.49 a	1.08 a	1.22 a	0.07 a

[z]Different letters in columns indicate significant differences at $P \leq 0.05$ using Student's *t* test.

Table 2. Effect of different graft combinations on yield and fruit quality of 'Jinchun No. 4' cucumber plants grafted on two pumpkin cultivars, Dongyangshenli (D) and Heizinangua (H).

Treatment	Yield (g/plant)	Fruit wt (g/fruit)	Fruit (no./plant)	Titratable acidity (%)	Total soluble sugar (%)	Vitamin C (mg/100 g FW)	Fruit dry matter (%)
D	1131.82 b[z]	137.19 b	8.25 b	0.32 a	1.15 b	2.96 a	4.81 a
H	1871.13 a	185.26 a	10.10 a	0.29 a	1.59 a	3.02 a	4.18 b

[z]Different letters in columns indicate significant differences at $P \leq 0.05$ using Student's *t* test.

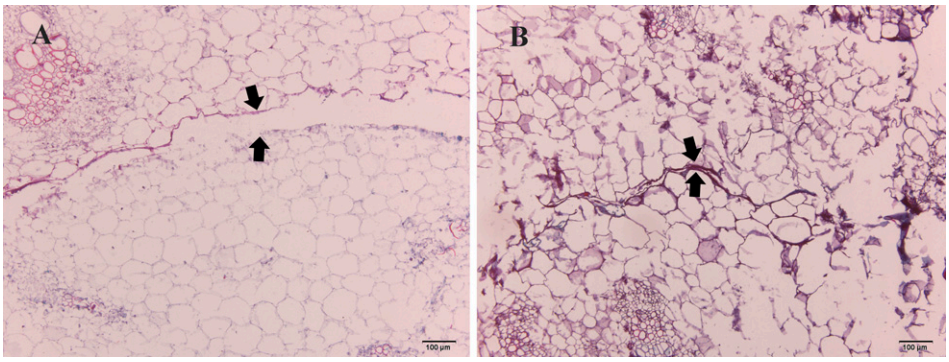

Fig. 1. Characteristics of the concrescence of a cucumber graft union with rootstocks of different compatibilities: (**A**) graft union with 'Dongyangshenli' pumpkin rootstock, (**B**) graft union with 'Heizinangua' pumpkin rootstock. The arrow indicates the necrotic layer. The samples are paraffin sections of the graft interface fixed at 6 d after grafting (bars = 100 μm).

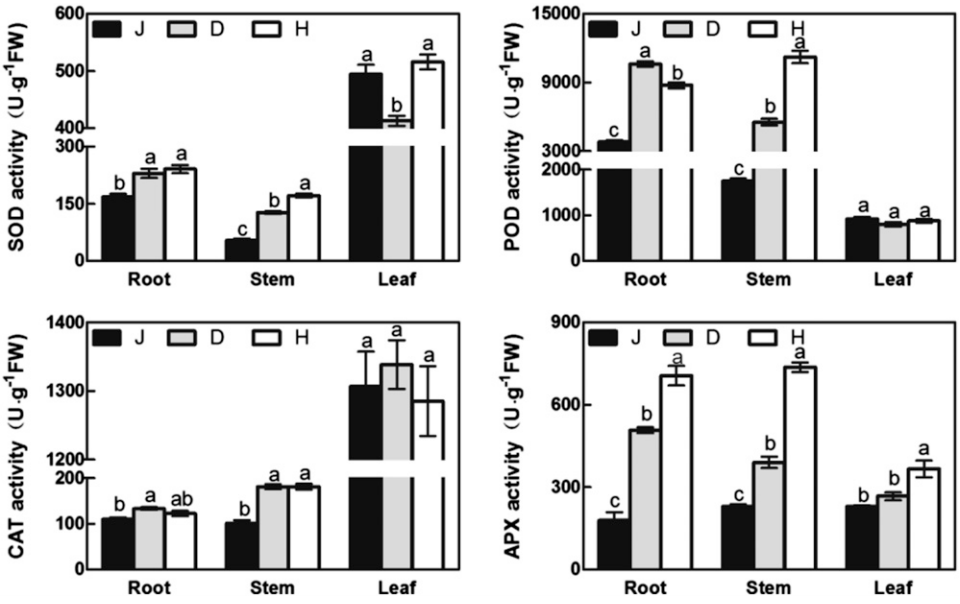

Fig. 2. Effect of different graft combinations on antioxidant enzyme activities of 'Jinchun No.4' cucumber seedlings and nongrafted plants (J) at 25 d after "hole insertion grafting" on two pumpkin cultivars, Dongyangshenli (D) and Heizinangua (H). Data represent mean ±SE (n = 3). Different letters within a column (plant part) indicate significant differences at $P \leq 0.05$ by Duncan's multiple range test. Stem = middle stem in nongrafted plants or the graft union in grafted plant; APX = ascorbate peroxidase; CAT = catalase; POD = peroxidase; SOD = superoxide dismutase.

both in our study and in those of others (Maršić and Osvald, 2004). In this work, the compatibility of the grafted combination using "H" as rootstock was better than that of the grafted combination using "D" as the rootstock, as expressed not only in grafting affinity but also in symbiotic affinity. To further illuminate the morphological, physiological, and biochemical differences in grafted cucumber seedlings with rootstocks of different compatibilities, the rootstocks above could be used.

In each plant, grafting formation process includes the generation of a necrotic layer and its subsequent reduction or elimination, growth of callus, cohesion of stok and scion, and the differentiation of graft-bridging vascular tissue and cambium (Stoddard and McCully, 1980). The formation of necrotic layer was attribute to the cells injured during cutting (Tiedemann, 1989), and was compartmentalized as a defensive mechanism to eliminate the invasion of pathogens. A necrotic layer was always formed ≈3 to 5 d after grafting in seedlings (Estrada-Luna et al., 2002), so it was studied at day 6. In our study, the necrotic layer of "H" already existed and made a close contact between scion and rootstock, while that of "D" had not appeared yet. The previous studies indicate that graft partners are held together at this stage by forces of cohesion and adhesion, possibly through the necrotic layer (Moore, 1984). It can promote the communication of graft partners and the formation of callus. And this phenomenon has also been found in many other plants' grafting (Fernandez-Garcia et al., 2004). The earlier appearance of necrotic layer may promote the communication, differentiation of callus, and establishment of new vascular tissue.

Reactive oxygen species (ROS) may be produced during regular physiological plant processes; however, the ROS level increases when plants are exposed to various stresses. Grafting may damage the dynamic balance of ROS metabolism in plants (Aloni et al., 2008). Irisarri et al. (2015) have shown that grafting may enhance the ROS level in pear seedlings, and this was also seen in grafted melon (Aloni et al., 2008). The increase in ROS may inhibit the growth of pathogenic bacteria at the injury site, further facilitating the lignification of the wound. This may be a positive response to the external damage of the plant itself. However, the excessive accumulation of ROS may be detrimental to plant cells. The defense capability of plants may produce certain amounts and kinds of protective enzymes to protect them from external injury. Complex defense antioxidative systems, including antioxidant enzymes such as SOD, POD, CAT, and APX, play an important role in alleviating peroxide stress. Production of defense antioxidases may eliminate the ROS induced by grafting stress and promote wound healing. Grafting has a direct influence on the stems of plants. The results of our study showed a regularity of antioxidant enzyme activities in the stems of the three kinds of seedlings. The SOD, POD, CAT, and APX activities in the stems and roots of nongrafted "J" seedlings were significantly lower than in the other two grafted seedling types. Perhaps nongrafted plants were not damaged, and thus did not need to increase antioxidant enzyme levels. Recent studies have shown that the activities of defense-activated enzymes in grafted seedlings in other species were higher than in nongrafted seedlings, and this may be

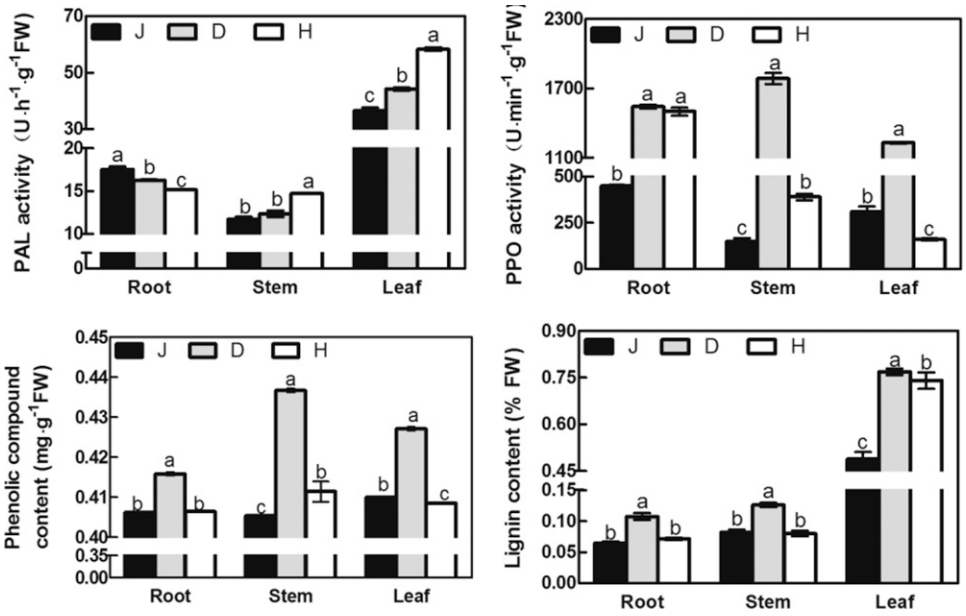

Fig. 3. Effect of different graft combinations on enzyme activities and substance contents involved in the phenylpropanoid metabolism of 'Jinchun No.4' cucumber seedlings and nongrafted plants (J) at 25 d after "hole insertion grafting" on two pumpkin cultivars, Dongyangshenli (D) and Heizinangua (H). Data represent mean ±SE (n = 3). Different letters within a column (plant part) indicate significant differences at $P \leq 0.05$ by Duncan's multiple range test. Stem = middle stem in nongrafted plant or graft union in grafted plant; PAL = phenylalanine ammonialyase; PPO = polyphenol oxidase.

beneficial in the removal of ROS, which was induced by the wounding and protects plants from grafting damage. In other species-incompatible combinations, the activities of SOD and POD were lower and the ROS levels were higher (Aloni et al., 2008). The plants suffered more damage from ROS, and their growth performance was inferior to compatible combinations. In our study, the activities of SOD, POD, and APX in stems of the compatible combination "H" were much higher than in the incompatible combination "D," suggesting that compatible combinations showed more resistance to the oxidative damage derived from grafting, in accordance with previous studies in other species. The higher activities of defense antioxidant enzymes in the "H" combination reflected that their scavenging capability for ROS was stronger and thereby accelerated wound healing, allowing plants to recover regular growth earlier. In addition, it also improved the combination's survival rate and growth performance, which was expressed as a better grafting affinity.

Phenylpropanoid metabolism is a biochemical factor that responds to plant stress. The resistance in plants to abiotic and biotic stresses is usually regulated by phenolic compounds. Therefore, a higher enzyme activity and accumulation of phenolic compounds is typically associated with plant stress resistance (Evrenosoglu et al., 2010). The enzymes involved in the phenylpropanoid pathway and lignification had not been studied in the graft union of pumpkin and cucumber. In this study, we analyzed activities of PAL and PPO, the contents of phenolic compounds and lignin. PAL is an important enzyme in phenylpropanoid metabolism and is responsible for the biosynthesis of many secondary metabolites. Its measurement is a promising strategy for predicting graft compatibility. The PAL activity of some incompatible *Prunus* combinations is higher (Pereira et al., 2014). However, in our research, the "H" combination showed a higher PAL activity in comparison with its activity in the other two combinations in stems and leaves, which is different from the results of Pereira et al. (2014). This might be due to differences in species and/or sampling period. A higher PAL activity in the "H" combination at our sampling stage may indicate that its ability to sense the wound caused by the grafting was stronger. This may have resulted in a quicker synthesis of phenolic compounds at the graft site by increasing PAL activity, further activating PPO earlier and eliminating phenolic compounds produced at the early stage, which are harmful to the primordium formation.

PPO is a protective enzyme in plants and an important enzyme in the phenylpropanoid metabolic pathway. When plants are cut, the induction of PPO activity can increase some black and brown substances that form a necrotic layer, produced by the oxidation of phenolics (Lopez-Gomez et al., 2007). In fruit trees, graft incompatibility link with the accumulation of phenolic compounds (Errea, 1998) synthesized by the phenylpropanoid pathway. These compounds are often induced under biotic or abiotic stresses and are involved in several metabolic processes (Jin et al., 2012). In addition, a high content of phenolic compounds is associated with damage during graft formation, limiting the proliferation and differentiation of calli, as well as the formation of the new vascular tissue in incompatible grafts (Feucht et al., 1988). In our study, incompatible graft combinations also had high contents of lignin and phenolics. The high content of phenolic substances in the "D" graft combination limits the formation of tubes and calli, thus producing a low grafting affinity.

Grafts cause plant stress, which can be measured by changes in Chl fluorescences (Calatayud et al., 2013). Chl fluorescence analysis has become a powerful and widely used technique available to plant physiologists and ecophysiologists. According to Calatayud et al. (2013), the F_v/F_m ratio in the graft area is the most sensitive to graft stress. Our results showed that, in the incompatible combination, the values of F_v/F_m, ΦPSII, F_v'/F_m', ETR, $L_{(PFD)}$, qP, and ΦNPQ were lower than in the compatible combination and nongrafted plants. The decrease of the F_v/F_m values in the "D" graft combination could be the result of an increase in the protective nonradioactive energy dissipation, photodamage to PSII centers, or both (Osmond, 1994). Calatayud et al. (2013) indicated that values of ΦPSII related to plant defense responses. The value of ΦPSII in the incompatible "D" combination was lower, indicating that this combination has a poor defense response, which is consistent with the measured changes in enzymatic activities. The observed decreases in effective quantum yield of PSII (F_v'/F_m'), in ETR and in qP indicated an over-excitation of the photochemical system. When this is the case, the accumulation of reduced electron acceptors may increase the probability of the generation of reactive radicals, which can damage PSII components (Barber and Andersson, 1992). In the incompatible combination, these

Table 3. Effect of different graft combinations on chlorophyll fluorescence parameters of 'Jinchun No. 4' cucumber seedlings and nongrafted plants (J) at 25 d after grafting on two pumpkin cultivars, Dongyangshenli (D) and Heizinangua (H); n = 3.

Treatment	Chlorophyll fluorescence parameter (mean ±SE) [z]						
	F_0	F_v/F_m	Φ_{PSII}	F_v'/F_m'	ETR	$L_{(PFD)}$	qP	Φ_{NPQ}
J	0.143 ± 0.004 b[y]	0.733 ± 0.001 a	0.278 ± 0.005 a	0.644 ± 0.003 a	15.300 ± 0.058 a	0.664 ± 0.008 b	0.433 ± 0.011 a	0.149 ± 0.003 a
D	0.215 ± 0.005 a	0.651 ± 0.013 b	0.175 ± 0.005 b	0.602 ± 0.012 b	9.700 ± 0.458 b	0.793 ± 0.006 a	0.285 ± 0.003 b	0.120 ± 0.005 b
H	0.151 ± 0.002 b	0.734 ± 0.003 a	0.283 ± 0.002 a	0.645 ± 0.015 a	15.833 ± 0.088 a	0.660 ± 0.009 b	0.438 ± 0.005 a	0.134 ± 0.002 a

[z]F_0 = original fluorescence; F_v/F_m = maximal photosynthetic efficiency in the dark condition; Φ_{PSII} = actual photochemical efficiency of photosystem II; F_v'/F_m' = maximal photosynthetic efficiency in the light condition; ETR = electron transport rate; $L_{(PFD)}$ = light photon flux density; qP = photochemical quenching; Φ_{NPQ} = regulation heat dissipation.
[y]Different lowercase letters within a column indicate significant differences at $P \leq 0.05$ by Duncan's multiple range test.

Fig. 4. Effect of different graft combinations on chlorophyll contents at the graft union of 'Jinchun No.4' cucumber seedlings and nongrafted plants (J) at 25 d after "hole insertion grafting" on two pumpkin cultivars, Dongyangshenli (D) and Heizinangua (H). Data represent mean ±SE (n = 3). Different letters within a column (Chl type or Car) indicate significant differences at $P \leq 0.05$ by Duncan's multiple range test. Chla = chlorophyll a; Chlb = chlorophyll b; Car = carotenoid.

decreased fluorescence parameters indicated that the grafted seedling's photosynthetic mechanisms appeared irreversibly photodamaged. We also note that the incompatible combination showed a higher F_0 value. There is a positive correlation between the value of F_0 and the Chl content. This may be due to the higher photosynthetic pigment contents in the incompatible combination (Maxwell and Johnson, 2000). Romero et al. (1997) indicated that the concentration of leaf pigments in grafted plants was higher than in the controls. Wang and Nii (2000) declared that a lower water content would further increase the content of Chl. In the incompatible combination, the synthesis of vascular bundles in the graft union decreased, and water and nutrient transport were inhibited (Trinchera et al., 2013). This may have caused an increase in the Chl content at the graft union, thus the value of F_0 would also have changed.

In our study, we discussed different behaviors between compatible seedlings and incompatible seedlings, such as within cells, antioxidant defenses, defense substances, and Chl fluorescence. Compatible graft combinations presented stronger resistance to oxidative damage resulting from grafting, a relatively weak phenylpropanoid metabolism and higher Chl fluorescences, except at F_0. In addition, the necrotic layer existed earlier in compatible graft combinations. These differences at the morphological, physiological, and cellular levels may be due to compatibility or incompatibility, and may provide valuable information for determining the symbiotic affinity of grafted seedlings at an early stage.

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