

## Article

# Hibiscus hamabo Rootstock-Grafting Improves Photosynthetic Capacity of Hibiscus syriacus under Salt Stress

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**Abstract:** *Hibiscus syriacus*, a woody ornamental plant with great economic value, is vulnerable to salinity. Hence, its cultivation in saline areas is severely restricted. Although grafting *H. syriacus* onto *H. hamabo* rootstock can greatly improve *H. syriacus*'s salt resistance, the photosynthetic response of *H. syriacus* to grafting and salt stress remains largely unknown. To address this question, self-rooted (Hs), self-grafted (Hs/Hs), and *H. hamabo*-grafted (Hs/Hh) *H. syriacus* were exposed to 0 or 300 mM NaCl. Salt significantly reduced the net and maximum photosynthetic rates, chlorophyll content, and maximum (Fv/Fm) and actual (ΦPSII) photochemical quantum yield of photosystem II (PSII), as well as the apparent electron transport rate, in Hs and Hs/Hs. However, these reductions were largely alleviated when *H. syriacus* was grafted onto *H. hamabo*. In line with the changes in the chlorophyll fluorescence parameters, the expression of genes encoding subunits of PSII and PSI in Hs/Hh was higher than that in Hs and Hs/Hs under saline conditions. Moreover, *H. hamabo* rootstock grafting upregulated the genes involved in the Calvin–Benson–Bassham cycle in *H. syriacus* under salt conditions. These results indicate that grafting can ameliorate the inhibition of salinity on the photosynthetic capacity of *H. syriacus*, mainly resulting from alleviated limitations on photosynthetic pigments, photochemical efficiency, and the Calvin–Benson–Bassham cycle.

**Keywords:** grafting; salt stress; gas exchange; chlorophyll fluorescence; *Hibiscus syriacus* Linn.; *Hibiscus hamabo* Sieb. et Zucc



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## 1. Introduction

Salinity is one of the major critical environmental constraints that limits the growth, yield, and quality of trees, crops, and horticultural plants [1–3]. Nowadays, over 20% of the arable land throughout the world is salt influenced [4], and improper fertilization and irrigation further exacerbate salinization [5]. It is estimated that approximately half of the cultivated land will be affected by salinity in 2050 [6]. The declining productivity of plants grown in saline environments is usually correlated with a reduction in their photosynthesis. The reduction in gas exchange is mainly due to the following factors: (i) stomatal closure, which restricts the intercellular CO<sub>2</sub> concentration (C<sub>i</sub>) for carboxylation; (ii) impairment of the photosynthetic apparatus, resulting in the limitation of photosystem II (PSII) efficiency; and (iii) reduction in the activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) for the Calvin–Benson–Bassham (CBB) cycle [7]. Photosynthesis is recognized as a good indicator of a plant's response to salt stress [8]. Thus, exploring the response of photosynthesis to NaCl stress could lay a physiological foundation for promoting plants' salt tolerance.

Grafting is a well-established agronomic practice to promote the salt tolerance of plants by transferring its branch or bud to the stem or root of a salt-tolerant species [2,9]. Owing to its low cost and ease of operation, grafting is widely applied in vegetable, fruit, and woody crop production to improve their salt resistance [5,10,11]. For instance, grafting the scions of Thompson Seedless, a salt-sensitive variety of grapevine (*Vitis vinifera* L.), onto the rootstocks of the 110R variety, which has a higher salt-tolerant capacity, can greatly enhance the growth and salt resistance of the scions [10]. Grafting can also alleviate the damage of salt stress to mulberry (*Morus alba* L.) and avocado (*Persea americana* Mill.) [12,13]. Grafting the scions of citrus (*Citrus aurantium* L.) onto salt-tolerant rootstocks reveals higher salinity tolerance than grafting onto salt-sensitive rootstocks [14]. Moreover, previous studies have revealed that salt stress significantly decreases the net photosynthetic rate in self-rooted and/or self-grafted salt-sensitive watermelon (*Citrullus lanatus* Matsum. Et Nakai) and cucumber (*Cucumis sativus* L.), whereas salt-tolerant rootstock-grafted plants exhibit a much higher net photosynthetic rate under NaCl stress [7]. Currently, little information is available on the mechanisms underlying grafting that improve the photosynthetic capacity of plants under salt stress.

*Hibiscus syriacus* L., which belongs to the genus *Hibiscus*, is an important woody ornamental plant. There are more than 350 varieties with distinct flower colors and forms distributed around the world [15]. The flowers of this species have a long blooming period. Aside from its ornamental value, *H. syriacus* also has certain medicinal properties, including antifungal, antihypertensive, and anti-inflammatory [16]. However, *H. syriacus* plants grown in saline areas exhibit leaf shrinkage and plant withering, indicating their susceptibility to salt stress. This seriously restricts the cultivation and application of *H. syriacus* in saline areas. *H. hamabo* Sieb. et Zucc., also a member of the genus *Hibiscus*, is a significant semi-mangrove shrub with golden yellow flowers [17]. However, its flowers are sparse and short-lived, which severely limits its ornamental value [18]. This plant is halophytic and can survive in habitats where the NaCl concentration varies from 1.1 to 1.5% [18]. Owing to its superior salt tolerance, *H. hamabo* has been used as a rootstock to graft *H. syriacus*. The grafted *H. syriacus* plants can grow well in coastal and inland saline lands, indicating that grafting has greatly enhanced the salt tolerance of *H. syriacus*. However, there is currently little information available regarding the photosynthetic response of grafted *H. syriacus* to soil salinity.

In this study, self-rooted (Hs), self-grafted (Hs/Hs), and *H. hamabo*-grafted (Hs/Hh) *H. syriacus* are exposed to either 0 (−Na, serving as the control) or 300 (+Na) mM NaCl, respectively. We hypothesize that grafting *H. syriacus* scions onto *H. hamabo* rootstocks would markedly enhance the photosynthetic capacity and induce the transcriptional regulation of key genes that participate in photosynthesis under salt stress. To test this hypothesis, we characterize the changes in the photosynthetic rates, photosynthetic pigment contents, chlorophyll fluorescence, and expression of key genes (genes encoding subunits of PSII and PSI and involved in the CBB cycle) involved in photosynthesis in the leaves of Hs, Hs/Hs, and Hs/Hh in response to salt stress.

## 2. Materials and Methods

### 2.1. Plant Cultivation and Salt Treatment

Cuttings (length, 15 cm; diameter, 0.5 cm) of *Hibiscus syriacus* Linn. and *Hibiscus hamabo* Sieb. et Zucc. were planted in plastic pots (2.6 L) containing soil (categorized as Alfisol based on USDA soil taxonomy; total nitrogen, 842 mg kg<sup>−1</sup>; available phosphorus, 42 mg kg<sup>−1</sup>; available potassium, 152 mg kg<sup>−1</sup>; 1.30% organic matter; pH, 6.9). When the stems of the one-year-old cuttings were 6 mm thick, 48 *H. syriacus* and 24 *H. hamabo* plants were selected for grafting. Among them, 24 *H. syriacus* plants were selected as self-rooted plants (Hs). The remaining *H. syriacus* and *H. hamabo* plants were used for grafting using the splice-grafting technique to obtain self-grafted *H. syriacus* (Hs/Hs) and heterografted *H. syriacus* (*H. syriacus* grafted on *H. hamabo* rootstock, Hs/Hh) plants, respectively. All plants were grown in a greenhouse (natural light and temperature; relative humidity, 75%). Seventy-

five days after grafting, the growth of Hs/Hs and Hs/Hh became vigorous, and each of the three types of plants (Hs, Hs/Hs, and Hs/Hh) was equally divided into two groups. The plants in each group were irrigated with either 0 (−Na, serving as control) or 300 (+Na) mM NaCl. The irrigation was performed every other day. To avoid salt shock, NaCl was added to obtain the desired concentration (300 mM, 29.2 dS m<sup>−1</sup>) in increments of 50 mM at a time.

## 2.2. Harvesting

After 23 days of salt treatment, distinct morphological differences appeared among the groups. The leaf samples (leaf plastochron index = 5 to 10) were collected and frozen immediately in liquid nitrogen. Frozen samples were ground into fine powder in liquid nitrogen using a ball mill (GT300, Beijing Grinder Instrument Co. Ltd., Beijing, China) and stored at −80 °C for further analysis. For further biochemical analysis, equal amounts of frozen samples acquired from two plants that received the same treatment in each plant type were pooled and thoroughly mixed. As a result, six mixed samples were obtained for each treatment per plant type.

## 2.3. Determination of Chlorophyll and Carotenoids

The concentrations of photosynthetic pigments were determined using the ethanol extraction procedure, as described elsewhere [19]. Briefly, about 50 mg of the sample was extracted using 10 mL of 95% ethanol. The mixture was left to soak in the dark overnight until the samples turned white. The concentrations of chlorophyll a, chlorophyll b, and carotenoid in the supernatant were determined spectrophotometrically at 665, 649, and 470 nm, respectively.

## 2.4. Determination of Gas Exchange

Prior to harvesting, two mature leaves (LPI = 7–8) from six plants per group were selected for photosynthetic measurements. The net photosynthetic rate (Pn), intercellular CO<sub>2</sub> concentration (Ci), transpiration rate (E), stomatal conductance (Gs), and photosynthetic water-use efficiency (WUE, calculated as Pn/E) were measured using a portable photosynthesizer (CIRAS-3, PP-Systems, Amesbury, MA, USA) according to the manufacturer's instructions. The photosynthetic active radiation gradient (PAR) was set at 1000 μmol m<sup>−2</sup> s<sup>−1</sup>, and the external CO<sub>2</sub> concentration was maintained at 400 ± 10 μmol mol<sup>−1</sup>. The environmental temperature was maintained at 32 ± 5 °C.

For the diurnal variation of photosynthesis, the gas exchange was measured every 2 h from 8:00 to 18:00. The measured leaves were marked to ensure that the same leaf was measured at different time points.

For the determination of the light-response curves of Pn, the following PAR levels were set: 2000, 1800, 1600, 1400, 1200, 1000, 800, 600, 400, 300, 200, 100, 50, 0 μmol m<sup>−2</sup> s<sup>−1</sup>. The Pn value under each PAR was recorded every 2 min.

## 2.5. Determination of Chlorophyll Fluorescence Parameters

The chlorophyll fluorescence parameters of the leaves were determined using a portable pulse-modulated fluorometer (FMS-2, Hansatech, King's Lynn, Norfolk, UK) between 9:00 and 11:00 am according to the manufacturer's instructions. Briefly, endogenous actinic light was activated to measure the minimum fluorescence (Fs), maximum fluorescence (Fm'), and actual photochemical quantum yield of PSII (ΦPSII) after light adaptation, as suggested by Lu et al. [20]. After dark adaptation for 0.5 h, the initial fluorescence (Fo), maximum fluorescence (Fm), steady-state fluorescence (Fs), and maximum photochemical quantum yield of PSII (Fv/Fm) were determined, as described previously [20]. The non-photochemical quenching coefficient (NPQ), photochemical quenching coefficient (qP), and apparent electron transport rate (ETR) were calculated according to the following formula: NPQ = Fm/Fm' − 1, qP = ΦPSII/(Fv/Fm), ETR = ΦPSII × PPFD × f × α [21,22].

## 2.6. Analysis of Transcript Levels

Total RNA was isolated from the fine, powdered fresh samples (ca. 50 mg) using an OminiPlant RNA Kit (CW2598S, CVBIO, Taizhou, China). RNA concentration was determined using a Colibri ultramicro spectrophotometer (LB915, Corrine Technology, Beijing, China). RNA integrity was checked using 1% agarose gel electrophoresis. The RNA (ca. 1 µg) was used to synthesize the first strand of cDNA using a PrimeScript RT reagent kit (RR047A, TaKaRa, Dalian, China). The synthesized cDNA was used for quantitative real-time PCR (qRT-PCR), as described elsewhere [23]. qRT-PCR was conducted using a TB Green Premix Ex Taq II (RR820, TaKaRa) in a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Primers were designed specifically for each gene, and 18S rRNA was selected as the reference gene (Table S1).

## 2.7. Statistical Analysis

Statistical tests were performed using Statgraphics (STN, St Louis, MO, USA), as described elsewhere [24], with minor modifications. Briefly, prior to statistical analysis, the data were tested for normality. Two-way ANOVAs were used with grafting and NaCl as two factors. Differences in means were considered significant if the *p*-value was less than 0.05. The light-response curve was fitted using the rectangular hyperbola model, as described elsewhere [25]. Principal component analysis (PCA) was performed using the command `prcomp` in R (<http://www.r-project.org/>, accessed on 9 January 2023).

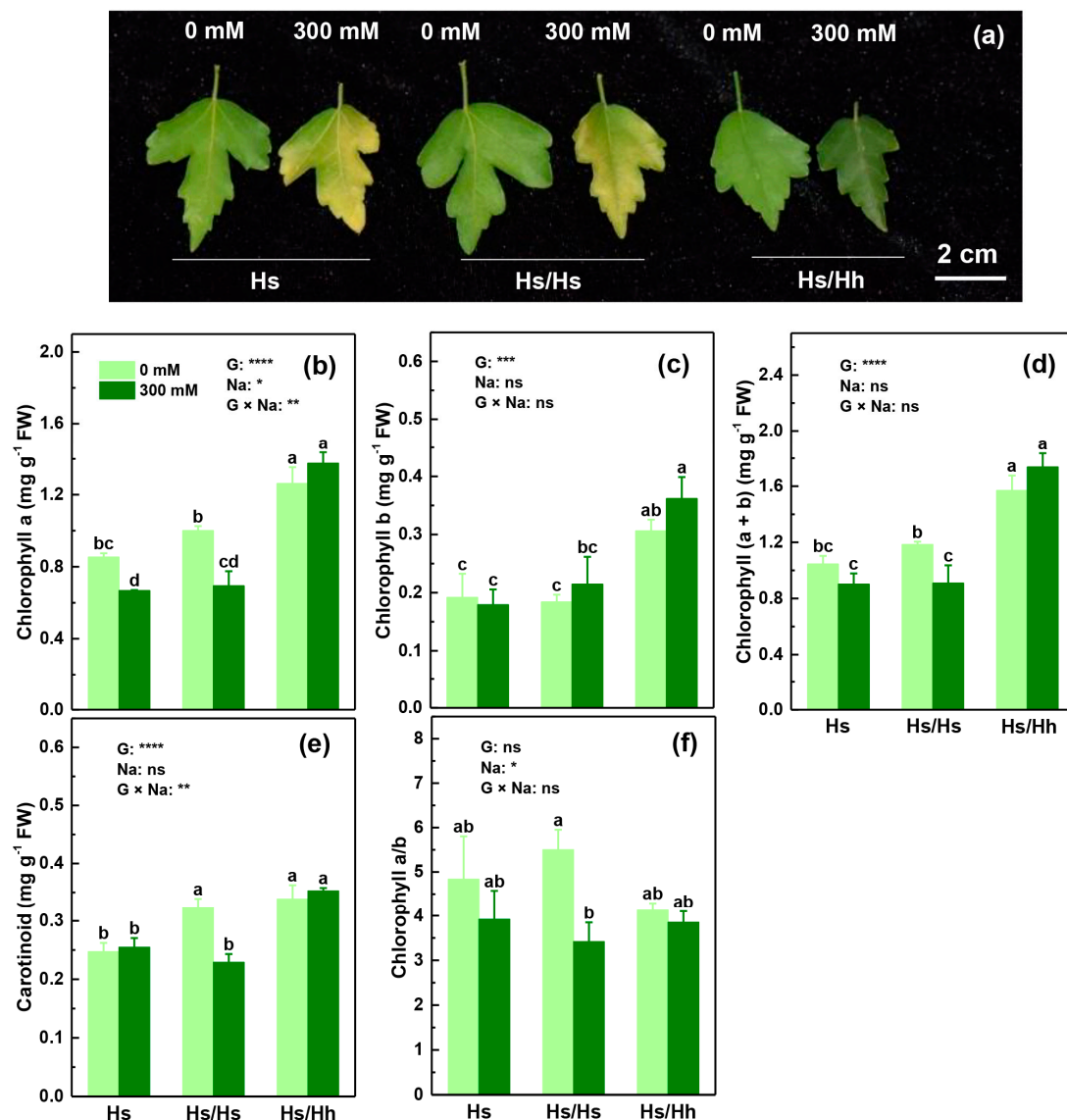
## 3. Results

### 3.1. Morphology and Photosynthetic Pigments

After 23 days of 300 mM NaCl treatment, the leaves of Hs and Hs/Hs turned yellow but the leaves of Hs/Hh remained green (Figure 1a). Consistently, the chlorophyll *a* concentration was decreased by 22% and 31%, and the chlorophyll (*a* + *b*) concentration was reduced by 19% and 23% in the leaves of Hs and Hs/Hs, whereas no marked differences were found for these two parameters in Hs/Hh leaves grown under NaCl treatment compared to the control (Figure 1b,d). The carotenoid and chlorophyll *a/b* concentrations were reduced by 29% and 38% in the leaves of Hs/Hs, but no marked differences were found in Hs/Hh leaves grown under NaCl conditions compared to those grown under control conditions (Figure 1e,f). Except for the carotenoid content in Hs/Hs under control conditions, the concentrations of chlorophyll *a*, chlorophyll *b*, chlorophyll (*a* + *b*), and carotenoid were all significantly higher in Hs/Hh than in Hs and Hs/Hs, regardless of the NaCl treatment (Figure 1b–e).

### 3.2. Gas-Exchange Parameters

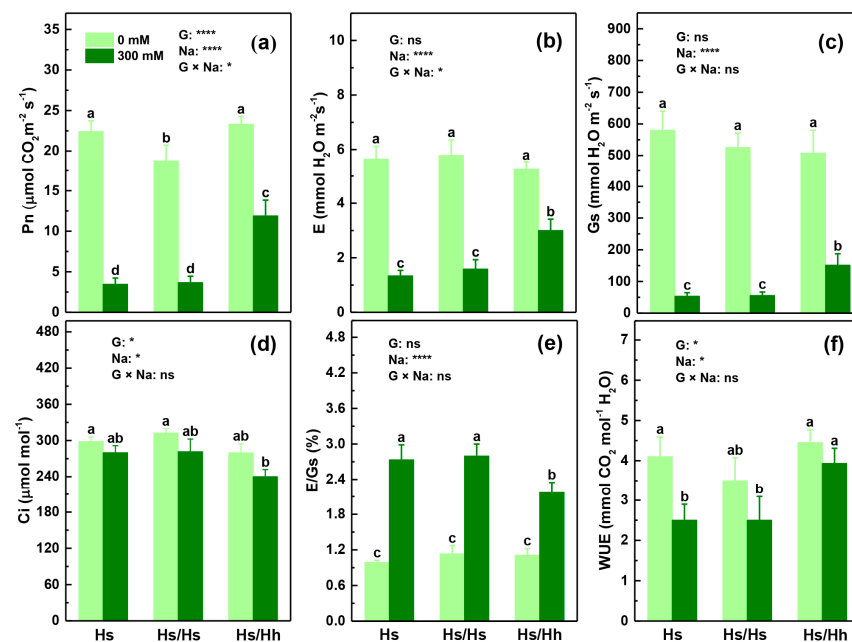
The *P<sub>n</sub>*, *E*, and *G<sub>s</sub>* were significantly decreased in the leaves of all the plants exposed to NaCl compared to those grown under control conditions. In addition, the reduced levels of these parameters observed in Hs/Hh were significantly lower than those in Hs and Hs/Hs (Figure 2a–c). The *C<sub>i</sub>* was not markedly altered by salt in the leaves of any of the plants (Figure 2d). The *E/G<sub>s</sub>* was increased by 177%, 147%, and 95%, respectively, in the leaves of Hs, Hs/Hs, and Hs/Hh treated with NaCl compared to those under control conditions (Figure 2e). The WUE was reduced by 39% in the leaves of Hs, but no significant differences were observed in the leaves of Hs/Hs and Hs/Hh treated with NaCl compared to those grown under control conditions (Figure 2f). Except for the *P<sub>n</sub>* in Hs/Hs, no significant differences were found in the *P<sub>n</sub>*, *E*, *G<sub>s</sub>*, *C<sub>i</sub>*, WUE, and *E/G<sub>s</sub>* in all plants under control conditions (Figure 2a–f). However, the *P<sub>n</sub>*, *E*, *G<sub>s</sub>*, and WUE in Hs/Hh leaves were all significantly higher, and the *E/G<sub>s</sub>* was lower compared to the levels observed in Hs and Hs/Hs under salt conditions (Figure 2a–f). For instance, the *P<sub>n</sub>* in Hs/Hh (11.90 µmol CO<sub>2</sub> m<sup>−2</sup> s<sup>−1</sup>) leaves was, respectively, 243% and 225% higher than that in Hs (3.47 µmol CO<sub>2</sub> m<sup>−2</sup> s<sup>−1</sup>) and Hs/Hs (3.67 µmol CO<sub>2</sub> m<sup>−2</sup> s<sup>−1</sup>) leaves under salt treatment (Figure 2a).



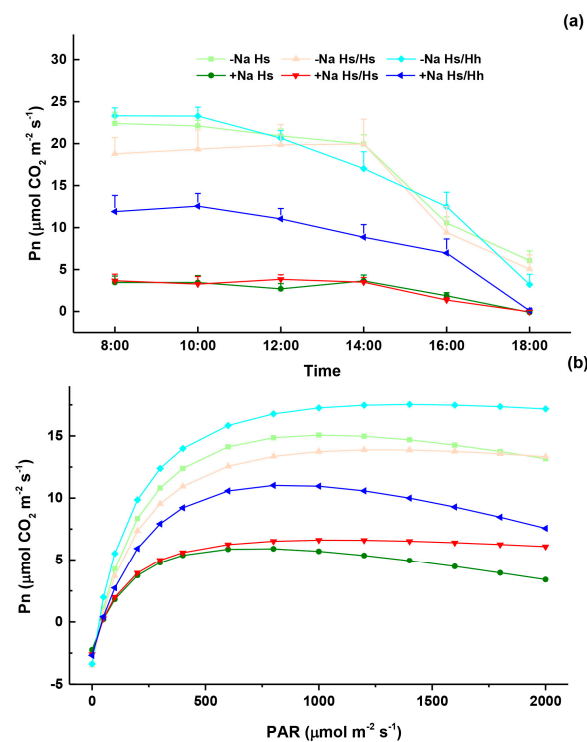
**Figure 1.** Morphology (a), and concentrations of chlorophyll a (b), chlorophyll b (c), chlorophyll (a + b) (d), carotenoid (e), and chlorophyll a/b (f) of grafted *H. syriacus* leaves grown under either 0 (control) or 300 mM NaCl conditions. Hs: self-rooted *H. syriacus*; Hs/Hs: self-grafted *H. syriacus*; Hs/Hh: heterografted *H. syriacus* with *H. hamabo* as the rootstock. Data indicate means  $\pm$  SE ( $n = 6$ ). Different letters on the bars indicate significant differences between the treatments.  $p$ -values based on the ANOVA tests for grafting (G), NaCl (Na), and their interaction ( $G \times Na$ ) are indicated: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ ; ns: not significant.

The diurnal changes in the Pn in all the plants showed single-peak curve change patterns, and no mid-day depression was evident (Figure 3a). The highest daily Pn observed in Hs/Hh (10:00) grown under control and salt conditions occurred later than that in Hs (14:00) grown under control and salt conditions, as well as Hs/Hs grown under salt conditions (Figure 3a). The Pn was decreased in all the plants grown under NaCl conditions compared to those grown under control conditions at each time point (Figure 3a). The Pn in the leaves of Hs/Hh was consistently higher than that of Hs and Hs/Hs grown under salt treatment between 8:00 and 16:00 (Figure 3a).





**Figure 2.** (a) Net photosynthetic rate (Pn), (b) transpiration rate (E), (c) stomatal conductance (Gs), (d) intercellular CO<sub>2</sub> concentration (Ci), (e) E/Gs, and (f) photosynthetic water-use efficiency (WUE) of grafted *H. syriacus* leaves grown under either 0 (control) or 300 mM NaCl conditions. Hs: self-rooted *H. syriacus*; Hs/Hs: self-grafted *H. syriacus*; Hs/Hh: heterografted *H. syriacus* with *H. hamabo* as the rootstock. Data indicate means  $\pm$  SE (n = 6). The information about the treatments and statistical analysis is the same as that mentioned in Figure 1.



**Figure 3.** Diurnal variation of photosynthesis (a) and light-response curves (b) of grafted *H. syriacus* leaves grown under either 0 (–Na, serving as the control) or 300 mM NaCl (+Na) conditions. Hs: self-rooted *H. syriacus*; Hs/Hs: self-grafted *H. syriacus*; Hs/Hh: heterografted *H. syriacus* with *H. hamabo* as the rootstock. In (a), bars indicate means  $\pm$  SE (n = 6); in (b), numbers represent the averages of six biological replicates.

Figure 3b shows the fitted light-response curves of the Pn in the leaves of the three plants. The Pn–PAR curves of all the plants are parabolic. The patterns of variation in the curves are quite similar for Hs grown under both control and salt conditions and Hs/Hh grown under salt conditions. In other words, as the PAR increased, the Pn exhibited a trend of first increasing and then slowly decreasing. The light-response curves of the Pn in Hs/Hs grown under control and salt conditions and Hs/Hh grown under control conditions exhibited a trend of first increasing and subsequently plateauing. Specifically, when the PAR was less than 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , the Pn increased rapidly as the PAR increased. When the PAR ranged from 200 to 800 (for plants under NaCl treatment) or 1000 (for plants under control conditions)  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , the rate of increase of the Pn gradually slowed down. When the PAR further increased, the Pn in the Hs grown under control and salt conditions and the Hs/Hh grown under salt conditions gradually decreased, and the curves in the Hs/Hs grown under control and salt conditions and the Hs/Hh grown under control conditions became flat. Except for 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , the Pn in the three plants grown under NaCl conditions decreased compared to those grown under control conditions, and the Pn in Hs/Hh was higher than in Hs and Hs/Hs grown under salt treatment.

The maximum net photosynthetic rate (Pnmax) and apparent quantum efficiency (AQY) were significantly reduced in the leaves of all the plants grown under NaCl conditions compared to those grown under control conditions (Table 1). The Pnmax value was markedly higher in Hs/Hh (11.30  $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ ) than in Hs (5.97  $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ ) under salt treatment (Table 1). The AQY value in Hs/Hh (0.14  $\text{mol mol}^{-1}$ ) was 27% and 21% higher than in Hs (0.11  $\text{mol mol}^{-1}$ ) and Hs/Hs (0.12  $\text{mol mol}^{-1}$ ) under control conditions (Table 1). The dark respiration rate (Rd) was decreased by 34% in the leaves of Hs grown under NaCl conditions compared to those grown under control conditions (Table 1).

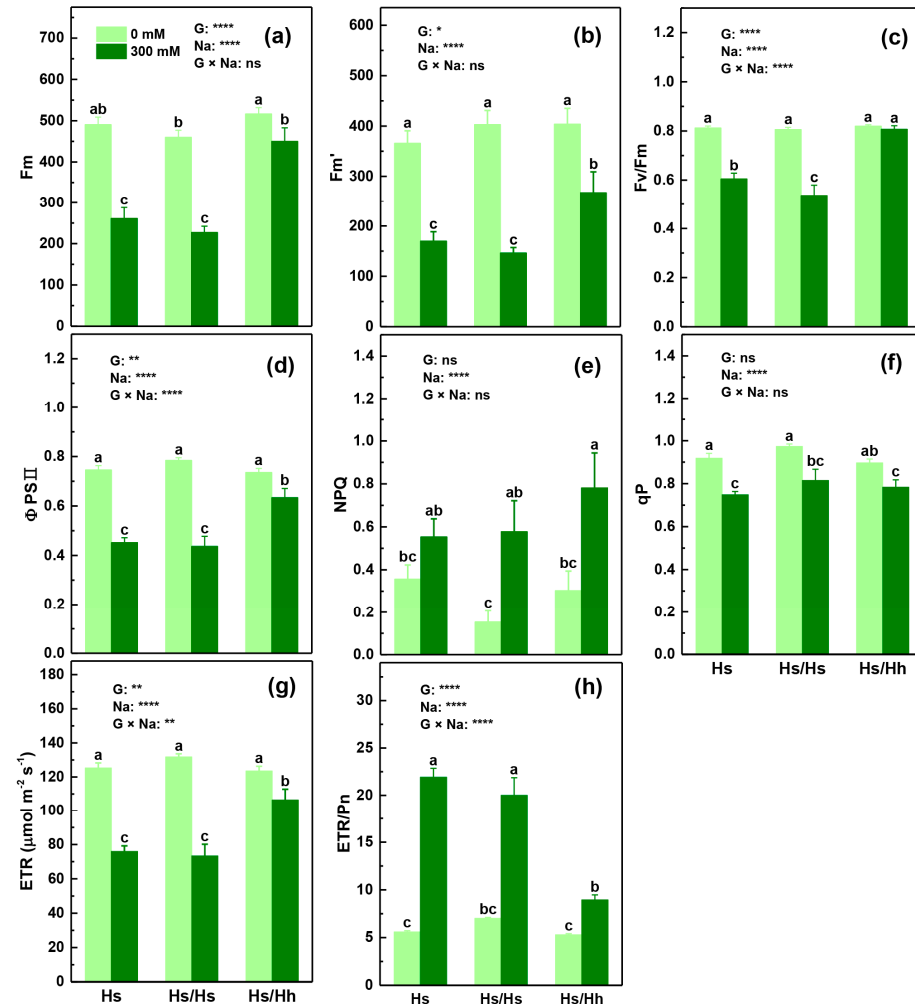
**Table 1.** Simulated parameters in light-response curves of grafted *H. syriacus* leaves grown under either 0 (control) or 300 mM NaCl conditions. Hs: self-rooted *H. syriacus*; Hs/Hs: self-grafted *H. syriacus*; Hs/Hh: heterografted *H. syriacus* with *H. hamabo* as the rootstock. Data indicate means  $\pm$  SE (n = 6). The different letters beside the values indicate significant differences. The *p*-values based on the ANOVA tests for grafting (G), NaCl (Na), and their interaction (G  $\times$  Na) are indicated: \* *p* < 0.05; \*\*\* *p* < 0.001; \*\*\*\* *p* < 0.0001; ns: not significant. Pnmax: the maximum net photosynthetic rate; LSP: the light saturation point; LCP: the light compensation point; AQY: the apparent quantum efficiency; Rd: the dark respiration rate.

Grafting	NaCl	Pnmax ( $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ )	LSP ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	LCP ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	AQY ( $\text{mol mol}^{-1}$ )	Rd ( $\text{mmol CO}_2 \text{m}^{-2} \text{s}^{-1}$ )
Hs	0 mM	15.32 $\pm$ 1.42 a	1057.32 $\pm$ 186.86 ab	36.09 $\pm$ 4.26 a	0.11 $\pm$ 0.01 b	3.39 $\pm$ 0.15 a
	300 mM	5.97 $\pm$ 0.99 d	713.27 $\pm$ 63.24 b	45.17 $\pm$ 9.88 a	0.06 $\pm$ 0.01 c	2.25 $\pm$ 0.34 b
Hs/Hs	0 mM	12.99 $\pm$ 1.30 ab	1274.99 $\pm$ 366.86 a	35.11 $\pm$ 2.46 a	0.12 $\pm$ 0.00 b	3.36 $\pm$ 0.09 a
	300 mM	7.47 $\pm$ 1.74 cd	818.93 $\pm$ 29.76 ab	47.97 $\pm$ 0.20 a	0.08 $\pm$ 0.00 c	2.98 $\pm$ 0.05 ab
Hs/Hh	0 mM	15.28 $\pm$ 0.44 a	1051.27 $\pm$ 6.70 ab	44.86 $\pm$ 4.28 a	0.14 $\pm$ 0.00 a	3.53 $\pm$ 0.52 a
	300 mM	11.30 $\pm$ 1.26 bc	899.88 $\pm$ 149.53 ab	43.00 $\pm$ 6.96 a	0.08 $\pm$ 0.01 c	2.79 $\pm$ 0.12 ab
<i>p</i> -values	G	ns	ns	ns	*	ns
	Na	****	*	ns	****	***
	G $\times$ Na	ns	ns	ns	ns	ns

### 3.3. Chlorophyll Fluorescence Parameters

Although the Fm, Fm',  $\Phi\text{PSII}$ , qP, and ETR were significantly decreased in the leaves of Hs, Hs/Hs, and Hs/Hh exposed to NaCl compared to those grown under control conditions, the reduced levels of these indexes in Hs/Hh were significantly lower than those in Hs and Hs/Hs (Figure 4a,b,d,f,g). The Fv/Fm was decreased by 26% and 33% in the leaves of Hs and Hs/Hs, respectively, whereas it was not significantly altered in Hs/Hh leaves grown under NaCl conditions compared to those grown under control conditions (Figure 4c). The NPQ was increased by 55% and 276% in the leaves of Hs/Hs and Hs/Hh, and the ETR/Pn was increased by 292%, 185%, and 69%, respectively, in the leaves of Hs, Hs/Hs, and Hs/Hh treated with NaCl compared to those grown under control conditions (Figure 4e,g). The Fm, Fm', Fv/Fm,  $\Phi\text{PSII}$ , qP, and ETR in Hs/Hh were markedly higher than those in Hs and Hs/Hs under NaCl treatment, but no such differences were found in

the leaves grown under control conditions (Figure 4a–d,f,g). The ETR/Pn in Hs/Hh (8.95) leaves was lower than that in Hs (21.92) and Hs/Hs (20.01) leaves under salt treatment, whereas no such difference was observed in the leaves grown under control conditions (Figure 4h). Grafting and salt stress had no effect on the  $F_o$  and  $F_s$  (Figure S1).

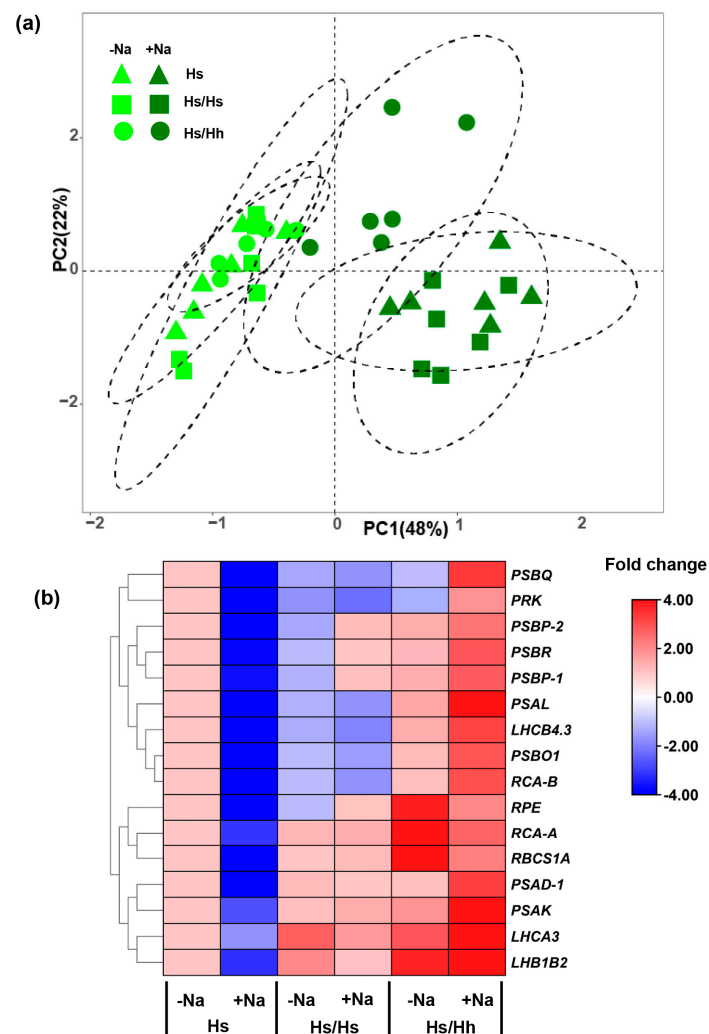


**Figure 4.**  $F_m$  (a),  $F_m'$  (b),  $F_v/F_m$  (c),  $\Phi_{PSII}$  (d), NPQ (e),  $q_P$  (f), ETR (g), and ETR/Pn (h) of grafted *H. syriacus* leaves grown under either 0 (control) or 300 mM NaCl conditions. Hs: self-rooted *H. syriacus*; Hs/Hs: self-grafted *H. syriacus*; Hs/Hh: heterografted *H. syriacus* with *H. hamabo* as the rootstock. Data indicate means  $\pm$  SE ( $n = 6$ ). The information about the treatments and statistical analysis is the same as that mentioned in Figure 1.

### 3.4. PCA Analysis of Photosynthetic Parameters

In order to investigate the relationship between varying the Pn and grafting and saline treatments, PCA was performed (Figure 5a, Table S2). PC1 and PC2 accounted for 48% and 22% of the total variation, respectively. PC1 clearly distinguished the effects of salt, and PC2 revealed the effects of grafting when the plants were exposed to NaCl treatments. Moreover, the differences between the control and salt-stress treatments in Hs/Hh were smaller than those in Hs and Hs/Hs, which indicated that Hs/Hh was more tolerant to saline stress than Hs and Hs/Hs. The variables  $F_o$ ,  $F_s$ ,  $q_P$ , Gs, and E/Gs were key contributors to PC1, and the variables  $F_m$ ,  $\Phi_{PSII}$ , ETR, chlorophyll a, chlorophyll (a + b), and  $F_m'$  were essential factors for PC2. These results demonstrate that grafting induced a higher Pn capacity under salt conditions, which can be attributed mainly to the chlorophyll concentration,  $F_m$ ,  $\Phi_{PSII}$ , and ETR.





**Figure 5.** Principal component analysis (PCA) plots of photosynthetic parameters (a), cluster analysis of transcriptional fold changes of photosynthetic genes (b) of grafted *H. syriacus* leaves grown under either 0 (−Na, serving as control) or 300 mM NaCl (+Na) conditions. Hs: self-rooted *H. syriacus*; Hs/Hs: self-grafted *H. syriacus*; Hs/Hh: heterografted *H. syriacus* with *H. hamabo* as the rootstock.

### 3.5. Changes in mRNA Levels of Key Genes Participating in Photosynthesis

The transcript levels of representative genes involved in photosynthesis were assessed in the three plants (Figure 5b). These genes included *Photosystem I subunit D-1* (PSAD-1), *PSAK*, and *PSAL*, which encode complexes of PSI, and *Photosystem II oxygen-evolving complex 1* (PSBO1), *Photosystem II subunit P-1* (PSBP-1), *PSBP-2*, *PSBQ*, and *PSBR*, which encode the oxygen-evolving complexes of eukaryotic PSII. The expression of these genes was markedly lower in Hs leaves but was upregulated in Hs/Hh leaves treated with salt compared to those grown under control conditions. The expression of these genes was significantly higher in the leaves of Hs/Hh compared to Hs and Hs/Hh compared to Hs/Hs grown under salt treatment.

*Photosystem I light-harvesting complex gene 3* (LHCA3), *Photosystem II light-harvesting complex gene B1B2* (LHB1B2), and *light-harvesting complex Photosystem II* (LHCB4.3) are involved in capturing light energy in PSI and PSII. Except for LHCB4.3 in Hs/Hs, the expression of these three genes was significantly lower in the leaves of Hs and Hs/Hs but was markedly higher in Hs/Hh leaves grown under salt conditions compared to those grown under control conditions. The mRNA levels of LHCA3 and LHB1B2 were upregulated in the leaves of Hs/Hh compared to Hs grown under control conditions. The

transcript levels of *LHCA3*, *LHB1B2*, and *LHCB4.3* were markedly higher in the leaves of Hs/Hh compared to Hs and Hs/Hh compared to Hs/Hs grown under salt conditions.

*Ribulose biphosphate carboxylase small subunit 1A* (*RBCS1A*), *rubisco activase-A* (*RCA-A*), *RCA-B*, *ribulose-phosphate 3-epimerase* (*RPE*), and *phosphoribulokinase* (*PRK*) participate in the Calvin–Benson–Bassham (CBB) cycle, which is a vital pathway of photosynthesis in plants. The expression of these five genes was markedly lower in Hs leaves grown under salt conditions compared to those grown under control conditions. The mRNA levels of *RPE*, *RCA-A*, and *RBCS1A* were downregulated, but the transcript levels of *RCA-B* and *PRK* were significantly higher in Hs/Hh leaves grown under salt conditions compared to those grown under control conditions. The expression of *RPE*, *RCA-A*, and *RBCS1A* was markedly higher in the leaves of Hs/Hh compared to Hs and Hs/Hh compared to Hs/Hs, regardless of the NaCl treatment. The mRNA levels of *RCA-B* and *PRK* were higher in the leaves of Hs/Hh compared to Hs and Hs/Hh compared to Hs/Hs grown under salt conditions.

#### 4. Discussion

##### 4.1. Grafting onto *H. hamabo* Rootstock Ameliorates the Inhibition of Saline on the Photosynthetic Capacity of *H. syriacus* Leaves

Photosynthesis provides the primary material and energy for plant growth. The photosynthetic capacity of most plants has been reported to be reduced by salt stress [5,20,26], and saline-tolerant plants can better acclimate to NaCl stress with a lesser decrease in the Pn [27]. Consistently, the Pn and Pnmax were markedly decreased in the leaves of Hs, Hs/Hs, and Hs/Hh exposed to salt compared to those grown under control conditions. More importantly, Hs/Hh exhibited a higher Pn and Pnmax than Hs and Hs/Hs under saline conditions, demonstrating that grafting onto *H. hamabo* rootstock could largely enhance the salt-tolerance of *H. syriacus* by alleviating the limitations of salt stress on its photosynthetic capacity. This result is consistent with previous studies on grafting in salt-treated watermelon, cucumber, and tomato (*Lycopersicon esculentum* Mill.) [5,28,29].

In general, salt-induced restrictions on the Pn can result from either stomatal closure or non-stomatal-related limitations [30]. For instance, a marked decrease in the Pn, Gs, and Ci has been observed in NaCl-exposed non-grafted and grafted apple (*Malus pumila* Mill.), tomato, and cucumber, implying that stomatal limitation is mainly responsible for the salt-induced reduction in the Pn [28,30,31]. In contrast, we found that the Pn and Gs were markedly decreased, whereas the Ci was not altered by salt in Hs, Hs/Hs, and Hs/Hh, indicating that non-stomatal limitation was likely the dominant factor for the reduced Pn under salt stress. Similar results were observed in 200 mM NaCl-treated *H. syriacus* [20]. Moreover, Hs/Hh exhibited a higher WUE than Hs and Hs/Hs when grown under saline treatment. Since the WUE is calculated as the ratio of Pn to E, the higher WUE in the salt-exposed Hs/Hh may have been caused by the grafting-induced improvement in photosynthetic performance. Having a higher WUE is significant for a plant's salt tolerance because it helps reduce the absorption of salt and alleviates water deficiency under salt stress [32].

##### 4.2. Grafting Alleviates the Inhibition of Salt Stress on Photosynthetic Capacity through Amelioration of Photosynthetic Pigment Reduction

Chlorophyll is the main photosynthetic pigment in higher plants. The light energy harvested by chlorophyll is converted to stable chemical energy under a series of complex reactions in the chloroplast, and plant photosynthesis will be limited due to the damage and degradation of chlorophyll [20]. A reduced chlorophyll content has been observed in many plants suffering from salt stress [20,33,34]. In this study, the concentrations of chlorophyll a and chlorophyll (a + b) were significantly decreased by salt stress in Hs and Hs/Hs. Since chlorophyll b was not markedly altered by salt stress, the decreased chlorophyll (a + b) content can mainly be attributed to the reduced concentration of chlorophyll a in salt-exposed Hs and Hs/Hs. Notably, chlorophyll a is the primary photosynthetic pigment involved in determining the photosynthetic rate [8]. Thus, the reduction in the Pn was

likely mainly due to the decreased concentration of chlorophyll a in salt-treated Hs and Hs/Hs. Moreover, the chlorophyll a and chlorophyll (a + b) concentrations were not altered in Hs/Hh leaves exposed to salt compared to those grown under control conditions. This indicates that grafting effectively ameliorated the salinity-induced reduction in chlorophyll concentrations. Similar findings of grafting alleviating the salt-induced decline in the chlorophyll content have also been reported in watermelon, tomato, and apple [5,7,9,30]. These results suggest that the inhibition of salt stress on photosynthetic capacity through grafting is caused by the alleviation of reductions in the concentrations of chlorophyll a and chlorophyll (a + b).

#### 4.3. Grafting Alleviates the Inhibition of Salt Stress on Photosynthetic Capacity through Amelioration of Limitations on Photochemical Efficiency

The decline in photochemical activity is known to be one of the non-stomatal factors that limits photosynthesis [35]. Therefore, the photochemical efficiency was determined in this study. The  $F_v/F_m$ , the maximum quantum use efficiency of PSII, is a classic parameter related to PSII functioning [36]. In the current study, the  $F_v/F_m$  was decreased by 26% and 33% in Hs and Hs/Hs under 300 mM NaCl treatment, indicating that photoinhibition occurred, which could be the result of damage to PSII [37]. Nonetheless, the constant  $F_v/F_m$  in Hs/Hh demonstrates that the salt-induced photoinhibition of PSII was repaired by the *H. hamabo* rootstock. Similar results were observed in salt-exposed grafted watermelon, cucumber, and tomato [7,28,38]. In this study, salt stress led to significant reductions in the  $\Phi PSII$  and ETR in Hs, Hs/Hs, and Hs/Hh, likely owing to the decrease in the open PSII reaction centers (qP). Since the  $\Phi PSII$  and ETR represent the photochemical conversion efficiency of PSII [38], their reductions indicate that the electron transporting for carbon fixation was limited by salt in the leaves of Hs, Hs/Hs, and Hs/Hh. This would harm the PSII complexes and cause the production of excess reactive oxygen species [7]. However, Hs/Hh exhibited a higher  $\Phi PSII$  and ETR than Hs and Hs/Hs under saline treatment, suggesting that the *H. hamabo* rootstock can alleviate the salt-induced inhibition of the photochemical conversion efficiency of PSII in *H. syriacus*. Moreover, the  $\Phi PSII$  and ETR were reduced, whereas the  $F_v/F_m$  was not altered in Hs/Hh, which was likely a result of the improvement in the NPQ under saline conditions (Figure 4e), implying that more energy was safely dissipated as heat in PSII [28].

In fact, photosynthesis is a very complex process that depends on the synergistic functioning of large multi-subunit pigment-protein complexes of PSII and PSI, which are embedded in specific regions of the thylakoid membrane [39]. The genes responsible for encoding the subunits of PSII and PSI are closely associated with the photochemical efficiency in plants [40,41]. For instance, the suppression of *PSBO1* and *PSBP2* through RNA interference (RNAi) led to a marked reduction in the  $F_v/F_m$  and  $\Phi PSII$  and an accumulation of PSII core subunits [42–44]. Arabidopsis *psad1-1* mutant plants exhibited retarded growth and lower PSI activity,  $F_v/F_m$ , and  $\Phi PSII$ , as well as downregulation of most genes participating in the light phase of photosynthesis compared to wild-type (WT) plants [41]. The transcript levels of *PSBO1*, *PSBP-1*, *PSBP-2*, *PSBQ*, *PSBR*, *PSAD-1*, *PSAK*, and *PSAL* were significantly reduced by salt in Hs, and the transcript levels of these genes in Hs/Hh were higher than in Hs and Hs/Hs under saline conditions, suggesting that *H. hamabo* rootstock can alleviate the salt-induced inhibition of mRNA levels of genes encoding the subunits of PSII and PSI in *H. syriacus*. Thus, these results indicate that the amelioration of the inhibition of salt on photosynthetic capacity through grafting can be attributed to the *H. hamabo* rootstock-enhanced expression of genes encoding the subunits of PSII and PSI, as well as the stimulation of the photochemical efficiency in *H. syriacus* under saline conditions.

#### 4.4. Grafting Alleviates the Limitation of Salt Stress on Photosynthetic Capacity through Amelioration of Inhibition on mRNA Levels of Genes Involved in the CBB Cycle

The CBB cycle is responsible for CO<sub>2</sub> fixation in photosynthesis [45]. In this cycle, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is a primary rate-limiting

enzyme that catalyzes the binding of CO<sub>2</sub> to ribulose-1,5-bisphosphate (RuBP) to form 3-phosphoglycerate [46,47]. Ribulose-phosphate 3-epimerase (RPE) catalyzes the conversion of xylulose-5-P to form ribulose-5-phosphate (Ru5P) [48], and phosphoribulokinase (PRK) catalyzes the ATP-dependent phosphorylation of Ru5P to form RuBP [45]. The de-activation of the Rubisco and a reduced Pn have often been observed in long-term NaCl stress [29]. The mRNA levels of *RBCS1A*, which encodes a Rubisco small subunit that constitutes Rubisco [49], as well as *RCA-A* and *RCA-B*, which code for Rubisco activase responsible for activating Rubisco [50], and *RPE* and *PRK* were downregulated by saline in Hs. The transcript levels of these genes in Hs/Hh were significantly higher than those in Hs and Hs/Hh under salt conditions. Similar results have been found in salt-exposed grafted watermelon [7]. T-DNA insertion mutants of *rbcS1a* revealed significant reductions in the Rubisco activity and net photosynthetic rates compared to wild-type (WT) *Arabidopsis* [49]. RCA uses the energy from ATP hydrolysis to restore catalytic competence to Rubisco [47]. A previous study revealed that the reduction in the net CO<sub>2</sub> assimilation was related to Rubisco deactivation owing to the inhibition of RCA under moderate heat stress in *A. thaliana* [51]. Therefore, these results suggest that the amelioration of the inhibition of salt stress on photosynthetic capacity through grafting can be attributed to the higher expression of *RBCS1A*, *RCA*, *RPE*, and *PRK* under saline conditions.

## 5. Conclusions

Taken together, our results show that after 23 days of NaCl treatment, the leaves of Hs and Hs/Hs exhibited chlorosis, whereas the leaves Hs/Hh remained green. Saline stress markedly decreased the Pn, Pnmax, E, Gs, WUE, chlorophyll a, chlorophyll (a + b), Fm, Fm', Fv/Fm, ΦPSII, and ETR in the leaves of Hs and Hs/Hs. In contrast, the reductions in these parameters were greatly alleviated when *H. syriacus* was grafted onto *H. hamabo* rootstock. Since the Ci was not markedly altered by 300 mM NaCl in Hs, Hs/Hs, and Hs/Hh, it indicates that these three plants experienced significant non-stomatal limitations to photosynthesis under salt conditions. Consistent with the changes in the Fv/Fm, ΦPSII, and ETR, genes encoding subunits of PSII and PSI were downregulated by salt in Hs, and the transcript levels of these genes were higher in Hs/Hh than in Hs and Hs/Hs under salt conditions. Moreover, *H. hamabo* rootstock grafting upregulated *RBCS1A*, *RCA*, *RPE*, and *PRK*, which were involved in the CBB cycle in *H. syriacus* scions under saline treatment. These results demonstrate that grafting can alleviate the inhibition of salt on the photosynthetic capacity of *H. syriacus*, which is mainly attributed to ameliorated limitations on photosynthetic pigments, photochemical efficiency, and the CBB cycle.

**Supplementary Materials:** The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/f14061226/s1>, Figure S1. Fs and Fo of grafted *H. syriacus* leaves; Table S1. Primers used for RT-qPCR; Table S2. PCA loadings of physiological parameters.

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