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Study on the Mechanism of Exogenous 5-Aminolevulinic Acid (ALA) in Regulating the Photosynthetic Efficiency of Pear Leaves

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Abstract: To provide a theoretical basis for the application of ALA in pear production, the effects of exogenous 5-aminolevulinic acid (ALA) treatment on leaf photosynthetic gas exchange parameters, chlorophyll fast fluorescence properties, and relative expression of the related genes were investigated using pear (*Pyrus pyrifolia* Nakai cv. 'Whasan') as a material in the study. The results show that exogenous ALA treatment improved the photosynthetic gas exchange parameters of pear leaves, upregulated the expression of multiple key genes which are related to ALA biosynthesis, metabolism, and transformation into chlorophylls. GUS staining in tobacco leaves showed that exogenous ALA activated the promoter activity of *PypHEMA* and *PypCHLH* genes, implying that the synthesis of endogenous ALA and chlorophylls was promoted by exogenous ALA. Furthermore, ALA promoted the expression of the genes encoding photosystem II (PSII) reaction center proteins, such as core protein D1, inner light-harvesting pigment proteins CP43 and CP47, and cytochrome b559. This led to increased PSII reaction center activity. In addition, ALA alleviated the donor side oxygen-evolving complex inhibition and reduced the closure rate on the receptor side, allowing for increased photochemical electron transfer and reduced heat dissipation while improving the photosynthetic performance index PI_{abs} and PI_{total}. The findings of this study contribute to a better understanding of ALA's promotion of plant photosynthetic efficiency, providing valuable insights for further research and potential applications in pear production.

Keywords: ALA; chlorophyll; pear; photosynthesis; photosystem II; photosynthetic electron transport

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1. Introduction

Pears are the second largest deciduous fruit tree in China after apples, with a total cultivated area of 921.61 × 1000 hm^2 in 2021, resulting in a total output 19.88 million tons. Although the pear cultivation area has slightly decreased due to national policy adjustments, the total output continues to grow because of increased unit yield. Cultivation of pear trees is the main income source for many farmers, and the pears are a major fresh fruit consumed by most Chinese residents. However, improper management practices in pear production, such as the overuse of inorganic fertilizers to compensate for insufficient organic fertilizer, can lead to fruit quality issues [1]. As is well known, photosynthesis is a process of matter and energy conversion. It involves the H_2O photolysis-induced release of oxygen, conversion of solar energy into electrochemical energy, and the transformation of atmospheric CO_2 into carbohydrates with which to store energy in organic compounds. Xu et al. have pointed out that photosynthesis is the material basis and energy source of all life activities in the Earth's ecosystem [2]. The stronger the photosynthesis of plants, the more organic matter synthesized, the more conducive to the growth and development of plants and the production of higher quality fruits [3]. Therefore, the question of how to improve the photosynthetic performance of plant leaves and how to promote plants to produce more organic matter is a major issue to which producers and researchers pay attention.

5-aminolevulinic acid (ALA) is a key precursor for the biosynthesis of porphyrins such as chlorophylls and heme in all organisms [4]. Recent studies have shown that it is not only a simple biochemical metabolite, but also a multifunctional natural plant growth regulator that can promote seed germination [5,6], seedling growth [6,7], crop yield [8–10], and fruit quality [11–15] as well as plant salt tolerance [16], cold tolerance [17–19], high temperature tolerance [20] and low light tolerance [17,21,22]. These studies indicate that ALA has important application potential in agricultural and forestry production. In terms of promoting leaf photosynthesis, ALA can significantly increase the chlorophyll content of plants [17,23,24]. At the same time, it can also improve the photochemical efficiency of photosynthetic system II reaction center (PSII-RC) in leaves [25,26], and alleviate photosynthetic system II reaction center (PSI-RC) photosynthetic photoinhibition [12,27]. The effect of ALA on plant photosynthesis is found not only in the normal temperature and light environment, but also when plants encounter different adverse conditions. Recently, Chen et al. proposed that ALA reversed the stomatal closure of apple leaves induced by plant hormone abscisic acid (ABA). They demonstrated that ALA upregulated expressions of protein phosphatase 2A (PP2A) genes, especially the C subunit gene, with a result that the PP2A protein abundance, holoenzyme activity and phosphatase phosphorylation were promoted [28]. The phosphorylated PP2AC interacted with sucrose non-fermenting 1-related protein kinase 2.6 (SnRK2.6), a key component of ABA signaling pathway, causing the latter to become dephosphorylated and block ABA signaling and ABA-induced stomatal closure. Because ABA is a plant stress hormone, which is often accumulated when plants are subjected to stressful conditions, while ALA can reverse the stomatal closure induced by ABA, ALA enables plants to trap more atmospheric CO_2 for photosynthetic accumulation, growth, and development of plants under stress [29].

ALA application in pears has been reported to have various effects, such as flower thinning, improved fruit quality, and enhanced anthocyanin accumulation. Firstly, Shen et al. sprayed more than 600 mg L^{-1} ALA in the blooming period of 'Hosui' pear trees and found that it significantly thinned the flowers that opened that day. Consequently, the fruit setting rate was depressed, with increases in fruit size and quality [30]. In this study, the effect of ALA on the promotion of leaf photosynthesis extended at least three months. In another study, when an amino acid foliar fertilizer containing less than 5 mg L^{-1} ALA was sprayed on pear leaves of 8 cultivars, the leaf chlorophyll content was significantly increased, with higher photosynthetic electron transfer performance [31]. In 'Akimizu' pear, a week after ALA spray, the net photosynthetic rate (P_n) and photosynthetic electron transport efficiency were increased, companied with upregulation of the expression of *Rubisco* (*ribulose-1, 5-bisphosphate carboxylase oxygenase*) *small subunit* gene and Rubisco initial activity. Additionally, ALA also increased the activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate specific peroxidase (APX), and prevented the accumulation of malondialdehyde (MDA) from membrane lipid peroxidation [32]. Recently, ALA was proposed to alleviate the chilling damage caused to pear flowers by temperatures of −2 °C [33]. In red-skin pears, such as the 'Yunhongli 2' [34] and 'Nanhong' pears [35], ALA can induce anthocyanin accumulation and promote skin coloring. Despite the potential benefits, the mechanisms behind ALA's effects on pear biological processes are not yet fully understood.

Recently, our team has reported that ALA promotes peach leaf photosynthesis and photosynthate transport from the source to sink, contributing to high-quality fruit production [15]. Based on this study, we formulated a technical regulation for the application of 5-aminolevulinic acid in peach trees (T/JAASS 88-20 [36]), in which we proposed that ALA should be applied at several specific stages of growth and development of fruit trees. The first is rhizosphere application of ALA solution more than one week before blooming, which can promote floral differentiation and prevent chilling harm from the late spring cold. The second is to spray ALA solution at the late blooming stage to thin flowers, reducing the cost of fruit thinning. The third is to spray ALA solution at the leaves of fruit trees, which can promote cell division of young fruits and leaf development. These three applications of

ALA in fruit trees are fundamental for the leaf function and fruit quality. After that, ALA can be applied at 20–30-day intervals until 20–30 days before fruit harvest. However, the procedure has not been tested in the other species of fruit trees. To investigate ALA's effects on the photosynthetic capacity of pear trees, we conducted a study involving ALA solution application at specific growth stages, including root irrigation before flowering, flower spraying at the end of the full flowering stage, and foliar application at young fruit stage. One week later, we measured the leaf gas exchange, chlorophyll fast fluorescence, expressions of the genes related to chlorophyll synthesis and photosynthetic electron transport carriers. Through some key gene manipulation analyses, we found that exogenous ALA treatment might regulate the biosynthesis of endogenous ALA and chlorophylls. These findings provide a novel insight for ALA application in pear production.

2. Materials and Methods

2.1. Experimental Materials and Treatments

The experiment was conducted in the Jinmei Family Pear Garden, Hushu Street, Nanjing from March to September 2022. This is in the middle and lower reaches of the Yangtze River Plain, with brown–yellow soil, a subtropical monsoon climate, sufficient precipitation, rain and heat in the same season, and four distinct seasons. The 10-year-old pear (*Pyrus pyrifolia* Nakai cv. 'Whasan') trees were planted in the north–south direction, 2 m × 4 m plant spacing, and multi main branches within an open shape. The control and ALA treatment were set up with the trees. A single plant was set as a plot with 10 repeats and random arrangement. The pear trees were treated with ALA solution (provided by Nanjing Hejiachun Biot. Co., Ltd., Nanjing, China) on March 14 (the petal white stage), March 27 (the late blooming stage) and May 8 (six weeks after the fall of flowers). Among these, the treatment on March 14 was pre-flowering root irrigation, the main purpose being to prevent the late spring cold. During the operation, each plant was irrigated with 5 L 10 mg L^{-1} ALA solution. At the end of the blooming stage on March 27, the same concentration of ALA solution was sprayed on the flowers to remove the late flowering and reduce the fruit thinning cost in the later stage. May 8 was the fruit-set period. One fruit was retained in each inflorescence while the others were artificially thinned. Afterwards, leaves were sprayed with 10 mg L^{-1} ALA until water dripped from the leaf surface. For each ALA treatment, the control was applied with an equal amount of fresh water. One week after the leaf spraying, the mature functional leaves in the middle of the new peripheral shoots were randomly selected in order to measure the photosynthetic performance and chlorophyll fast fluorescence characteristics on the morning of sunny days. Meanwhile, the leaves were detached and treated with liquid nitrogen, and transported back to the laboratory in ice bottles and stored in a −80 °C refrigerator for subsequent analysis. The field measurements were performed weekly and continued for five weeks.

2.2. Determination of Pigment Content in Leaves

The pigment content of leaves was determined by acetone extraction method [37] and calculated according to the following formulas:

$$\text{Chlorophyll a (Chl a)} = (12.21\ OD663 - 2.81\ OD646) \times V/1000\ W$$

$$\text{Chlorophyll b (Chl b)} = (20.13\ OD646 - 5.03\ OD663) \times V/1000\ W$$

$$\text{Carotenoid (Car)} = (4.4\ OD470\text{-}0.01 \times \text{Chl a} - 0.45 \times \text{Chl b}) \times V/1000\ W$$

where V is the volume of extracted liquid (mL) and W is the fresh weight of leaves (g). Biological repeats were performed 3 times per treatment.

The relative chlorophyll content (SPAD) was determined using the SPAD-502PLUS instrument of Konica Minolta, Japan, and the mature leaves were selected in the middle of the peripheral shoots. Each data measurement was repeated 60 times and averaged.

2.3. Determination of Endogenous ALA Content and ALA Biosynthesis and Metabolism in Leaves

According to the method of Harel and Klein [38], the pear leaves (0.1 g) were ground and extracted with 200 mol L^{-1} acetic acid buffer (pH 4.6). After centrifugation at 5000× *g* for 15 min, the supernatant was taken and condensed with acetylacetone at 100 °C for 10 min. After cooling to room temperature, 1 mL of newly prepared Ehrlish's reagent (42 mL glacial acetic acid +8 mL 70% perchloric acid, 1 g dimethylaminobenzaldehyde) was added for color development. After shaking and standing for 10 min, the OD_{553} value was measured. The standard curve was prepared with ALA standard (Sigma-Aldrich, St. Louis, MO, USA).

To determine the biosynthesis and metabolism of ALA, a certain amount of pear leaves was immersed in a Petri dish containing 20 mmol L^{-1} levulinic acid (LA, a metabolic inhibitor of ALA) solution, and induced under 240 μmol m^{-2} s^{-1} light for 6 h to block ALA metabolism. Then, the accumulated ALA content of leaves was determined according to the method in the above paragraph, which was used as the ALA biosynthesis ability. The difference between the ALA accumulation determined by LA induction and the endogenous ALA content obtained by direct determination was the ALA metabolic capacity.

2.4. Determination of Photosynthetic Gas Exchange Parameters

The gas exchange parameters of pear leaves were measured using a Li-6800 portable photosynthetic system analyzer (Li-COR, Lincoln, NE, USA) between 9:00 and 11:00 on sunny days. The conditions were as follows: leaf chamber temperature 27.0 °C, CO_2 concentration 400 μmol mol^{-1}, air humidity 60%, light intensity 1800 μmol m^{-2} s^{-1}, fan speed 10,000 rpm, gas flow rate 500 μmol s^{-1}, and chamber pressure 0.1 kPa. The instantaneous carboxylation efficiency and water use efficiency were calculated according to the following formulas: instantaneous carboxylation efficiency = P_n/C_i and water use efficiency (WUE) = P_n/T_r. Each treatment was repeated 20 times and the average value was taken.

2.5. Determination of Chlorophyll Fast Fluorescence

Chlorophyll fast fluorescence was measured using the M-PEA multi-function plant efficiency meter (Hansatech, Norfolk, UK). During the determination, the leaves were exposed to saturated pulsed light (3000 μmol m^{-2} s^{-1}) for 1 s after dark treatment for more than 15 min. The obtained fluorescence induction curves were analyzed by JIP test and MR_o/MR according to the methods of Li et al. [39] and Wang et al. [40]. The measurements were repeated 15 times for each treatment and averaged.

2.6. RNA Extraction and RT-qPCR Analysis

According to the kit instructions, the total RNA of pear leaves frozen in liquid nitrogen was extracted by plant RNA extraction kit V1.6 (Biofit, Shanghai, China). The genomic DNA was removed with the Evo M-MLV Reverse Transcription Premix Kit (Accurate, Hunan, China) and reverse transcribed into cDNA. Primers (Table 1) were designed using Primer 5.0 (Premier, Vancouver, Canada). The SYBR Green Pro Taq HS premix qPCR kit (Accurate, Hunan, China) was used to perform qPCR amplification with cDNA as a template and *Pyrus bretschneideri actin* (LOC103931062) as an internal reference gene. The relative expression levels of genes encoding electron carriers related to chlorophyll synthesis and photosynthetic electron transport were calculated by $2^{-\Delta\Delta CT}$ method [41]. Each gene was subjected to 3 biological replicates and averaged.

Table 1. Primer sequences used in this experiment.

Name (Gene ID)	Primer	Amplicon Size
Actin (LOC103931062)	Fw:cccagaagtgctcttccaac Rv:ttgatcttcatgctgcttgg	1550
PsbA (ON478189.1:308-1369)	Fw:cattttctgtggtttccctgat Rv:cgcccttggattgctgtt	1061
PsbB (ON478189.1:75738-77264)	Fw:tttcgggcttgtgcttttt Rv:cccacgctggatttacgg	1526
PsbC (ON478189.1:36669-38088)	Fw:ggcgggggagatgtaagaa Rv:ccaaacaaagcaacaagcaata	1419
PsbD (ON478189.1:35660-36721)	Fw:atgggagttgctggggtg Rv:ccaaaaacggttagcggtg	1061
PsbE (ON478189.1:67489-67740)	Fw:gaattccttgtcggctttctg Rv:ccttatttattgcgggttggt	251
PsbF (ON478189.1:c67479-67360)	Fw:gtacctaccgtttctttttaggg Rv:ttggatgaactgcattgctga	119
PsbH (ON478189.1:77867-78091)	Fw:gatctggccgcccaaaa Rv:tgcgagacccatcaaagga	224
PsbJ (ON478189.1:c67094-66972)	Fw:acgacacaatcaaacccgaa Rv:caaaaatgagtaataaccccag	122
PsbL (ON478189.1:c67337-67221)	Fw:acgacacaatcaaacccgaa Rv:caaaaatgagtaataaccccag	116
PsbP (LOC103965639)	Fw:ctgagtccgagggtggctt Rv:tccttcatctccgtctgctgt	807
PsbQ (LOC103933887)	Fw:cttgctgtctgaaacaaccctc Rv:ttctctccacgactcgaactct	674
PsbW (LOC103946003)	Fw:tggaaggaaaaaaggagagca Rv:cgagggaggaagtatagataaagtaga	623
PsbY (LOC103949857)	Fw:caaccgcatgacaaacaagag Rv:ccaccacaaacagcaacaact	887
Psb27 (LOC103947836)	Fw:ccacccccacaatccca Rv:ccttatttatcacctcctccgtt	874
Psb28 (LOC103959875)	Fw:tcaggcttgttatgttgggg Rv:tctggtattgtctgttcatcggt	987
HEMA (LOC103957838)	Fw:acaaggaggataggctaaggaag Rv:ccattggaccatgaaggagtt	2115
GSA (LOC103936176)	Fw:taatggtatccgcaagctcac Rv:aacttcctggctccctcaac	1716
HEMB (LOC125472965)	Fw:gttgaggcaaatgaagacgagt Rv:tgcagctataggcagaggagag	1537
HEMD (LOC103957047)	Fw:tgctatgacaggagcggtga Rv:cctaagaatgggattgaaaaatg	1367
HEMF (LOC125474519)	Fw:caccgccccgatacctt Rv:tgacaccagccttctccca	1485
HEMG (LOC103967516)	Fw:atgatggtggactgtggtttga Rv:gacctggaggaggcgga	2010
CHLH (LOC103946503)	Fw:ggaggcaagaggggcg Rv:gggcaaaaccagtaagcga	4452
CHLM (LOC103931332)	Fw:ccaagaccgttgagaatgtga Rv:gccctgccgagaagcc	1288
CRD1 (LOC103938880)	Fw:agacaaatcgcactacagcca Rv:ttcaagtttttcaccacggg	1424
DVR (LOC103958557)	Fw:cgtggcaaaaacccaaaag Rv:aacacccaaatcagtcacatcc	1531
POR (LOC103949278)	Fw:ccctccgactgcctctca Rv:tactggcttccacttctgcttat	1843
CHLG (LOC103965133)	Fw:ttgaaggagatagagcaatggg Rv:tggaaaaagacttggggagc	1470
CAO (LOC103940306)	Fw:cccttctcccagcacttctac Rv:gacacatccaggtttcccatc	2157

Table 1. *Cont.*

Name (Gene ID)	Primer	Amplicon Size
NtActin (LOC107830629)	Fw:cctgaggtccttttccaacca Rv:ggattccggcagcttccatt	1574
GUS (LOC103930738)	Fw:cctgaggtccttttccaacca Rv:tcattgtttgcctccctgct	2038
Promoter of *PsbA*	Fw:accatgattacgccaagcttgatatccaccatcttgacttggtgatgg Rv:accacccggggatcctctagaatggagagagagagatattggagttgaagg	254
Promoter of *PsbB*	Fw:accatgattacgccaagcttctaattgctacacgcattgcagaatg Rv:accacccggggatcctctagatgccaatcagcttctctccacc	502
Promoter of *PsbJ*	Fw:accatgattacgccaagcttcgtaccaataacgtccaaatgattggtaca Rv:accacccggggatcctctagaaccaggactttagacaacggattacgtag	1032
Promoter of *HEMA*	Fw:accatgattacgccaagcttggacttttttttttttgggagaggagct Rv:accacccggggatcctctagaagggagggatatggaattgggataagg	2000
Promoter of *GSA*	Fw:accatgattacgccaagcttccccctgtagtattacaaattaaaaatttt Rv:accacccggggatcctctagaagactttggtcttagtatatatgagttctt	2000
Promoter of *HEMF*	Fw:accatgattacgccaagcttgtactatatctcactttgatgtgaaagcg Rv:accacccggggatcctctagatggaaataccccttttatcaatgaaaaatag	2000
Promoter of *CHLH*	Fw:accatgattacgccaagcttgatctgcggatttatcaacatcaagctc Rv:accacccggggatcctctagaataatcggatgaactgagttgtagacatga	2000

2.7. DNA Extraction and Promoter Sequence Cloning from Plant Materials

The genomic DNA of pear leaves frozen in liquid nitrogen was extracted by Genesand (Beijing, China), an efficient plant genomic DNA extraction kit. The nucleotide sequence of the target gene promoter (about 2 kb upstream the start codon) was retrieved from the National Center for Biotechnology Information (NCBI) database, and specific primers were designed according to the sequence at both ends of the promoter (Table 1). The promoter sequence was cloned using 2 × Phanta Flash Master Mix (P520) high fidelity enzyme (Vazyme, Nanjing, China) using pear genomic DNA as a template. The amplified products were detected by 1.0% agarose gel electrophoresis and the bands containing the target fragment were cut off. The purified bands were then recovered using the DNA gel recovery kit (Genesand, Beijing, China).

2.8. Construction of Plant Gene Expression Vectors

The construction of plant gene expression vectors was undertaken using binary expression vector pBI121. The double enzyme digestion of the carrier was carried out by rapid endonuclease (Transgen, Beijing, China) FlyCut® BamHI (JB101) and FlyCut® HindIII (JB101). After separation by agarose gel electrophoresis, the target fragment was recovered using the DNA gel recovery kit (Genesand, Beijing, China).

The recombination of the target gene promoter and the double enzyme digestion linearized vector was carried out according to the ClonExpress II One Step Cloning Kit (C112) (Vazyme, Nanjing, China) instruction. After ligation, the ligation product was transformed into DH5α competent *Escherichia. coli* (CAT#: DL1001) (Weidibio, Shanghai, China) at a ratio of 1:10 (v/v) in an ultra-clean bench. After shaking the culture at 37 °C for 6–9 h at 200 rpm until the solution was turbid, the normal *E. coli* monoclonal colonies were picked and cultured in LB liquid medium (KanR 50 mg/L). PCR reaction was performed using 2 × Rapid Taq Master Mix (Vazyme, Nanjing, China) to detect positive clones. The PCR reaction products were separated by 1% agarose gel electrophoresis, and the bacterial liquid group containing the target band was sent to General Biol. Co., Ltd. (Chuzhou, Anhui, China) for sequencing. The bacterial solution with correct sequencing results was extracted and collected with a high-purity plasmid DNA small extraction kit (Genesand, Beijing, China).

2.9. Agrobacterium Transformation, Tobacco Infection and GUS Qualitative and Quantitative Experiments

According to the requirements of Tsingke (Beijing, China) product specification, the recombinant plasmid was transformed into Agrobacterium GV3101 (TSC-A01). The cells were cultured on a 200 rpm shaker at 28 °C for 24 h until the solution was turbid and inoculated on LB liquid medium containing antibiotics (KanR 50 mg L^{-1} + Rif 50 mg L^{-1}). The normal Agrobacterium monoclonal colonies were picked, and the Agrobacterium positive clones were detected according to the method in the previous section for subsequent infection.

Using HindIII and BamHI enzymes, the cloned target gene promoter was inserted into the pBI121 vector to obtain a recombinant plasmid. Once confirmed by sequencing, it was transformed into an Agrobacterium (GV3101) competent state in order to obtain the Agrobacterium solution containing the recombinant plasmid. At the same time, the Agrobacterium solution containing the pBI121 empty plasmid, which contained the *35S* strong promoter was prepared to produce *35S::GUS*. According to the method of Wu et al., the Agrobacterium solution containing different plasmids was injected into the leaves of four-week-old *Nicotiana benthamiana*, and either water or 1 mg L^{-1} ALA solution was sprayed. This was repeated three times [42]. After being cultured in the dark for 24 h, the leaves were transferred to the light for 48 h before being collected and the expression level of *GUS* reporter gene qualitatively and quantitatively detected by the method of Jefferson et al. [43].

In the qualitative detection, the tobacco leaves were completely immersed in the GUS dye solution (X-Gluc mother liquid: X-Gluc base liquid = 1:9) and placed in a constant temperature incubator at 37 °C for 24 h. Then, the leaves were decolored with anhydrous ethanol at 37 °C until they became white, and were then observed and photographed.

In the quantitative detection, tobacco *NtActin* (*LOC107830629*) was used as an internal reference. The total RNA of tobacco leaves injected with Agrobacterium was extracted by the method described in 2.6, cDNA was obtained by reverse transcription, and RT-qPCR reaction was performed. The relative expression of GUS gene was calculated by $2^{-\Delta\Delta CT}$ method [41]. Each treatment was performed with three biological replicates. Primer names and sequences are shown in Table 1.

2.10. Data Analysis

All data were calculated and processed using Microsoft Excel. The charts were drawn using GraphPad Prism 9.0.1, and Photoshop CC (2015) was used to assist modification. SPSS 17.0 was used for statistical analysis. Two-way analysis of variance (F-test) and Duncan's test were used to analyze the different times after ALA treatment and the significant effect of ALA treatment (* $p < 0.05$, ** $p < 0.01$).

3. Results

3.1. Effects of Exogenous ALA Treatment on Photosynthetic Gas Exchange Parameters in Pear Leaves

Figure 1 shows the changes of photosynthetic gas exchange parameters in pear leaves over five weeks (May 15 to June 12) after exogenous ALA foliar application. Regardless of control or treatment, the net photosynthetic rate (P_n) of pear leaves was stable and had an upward trend overall, indicating that the photosynthetic performance of pear leaves was stable and gradually increased at this stage. However, in week 2 (May 22), the P_n of both leaves decreased. This may be the result of high temperature stress caused by a sudden rise in temperature, from the 30–34 °C recorded in the other weeks to 39 °C. The P_n of leaves in ALA treatment was significantly higher than that of control, which lasted for at least five weeks. During this period, ALA-induced *Pn* increased by 29.28% ($p < 0.01$, n = 100).

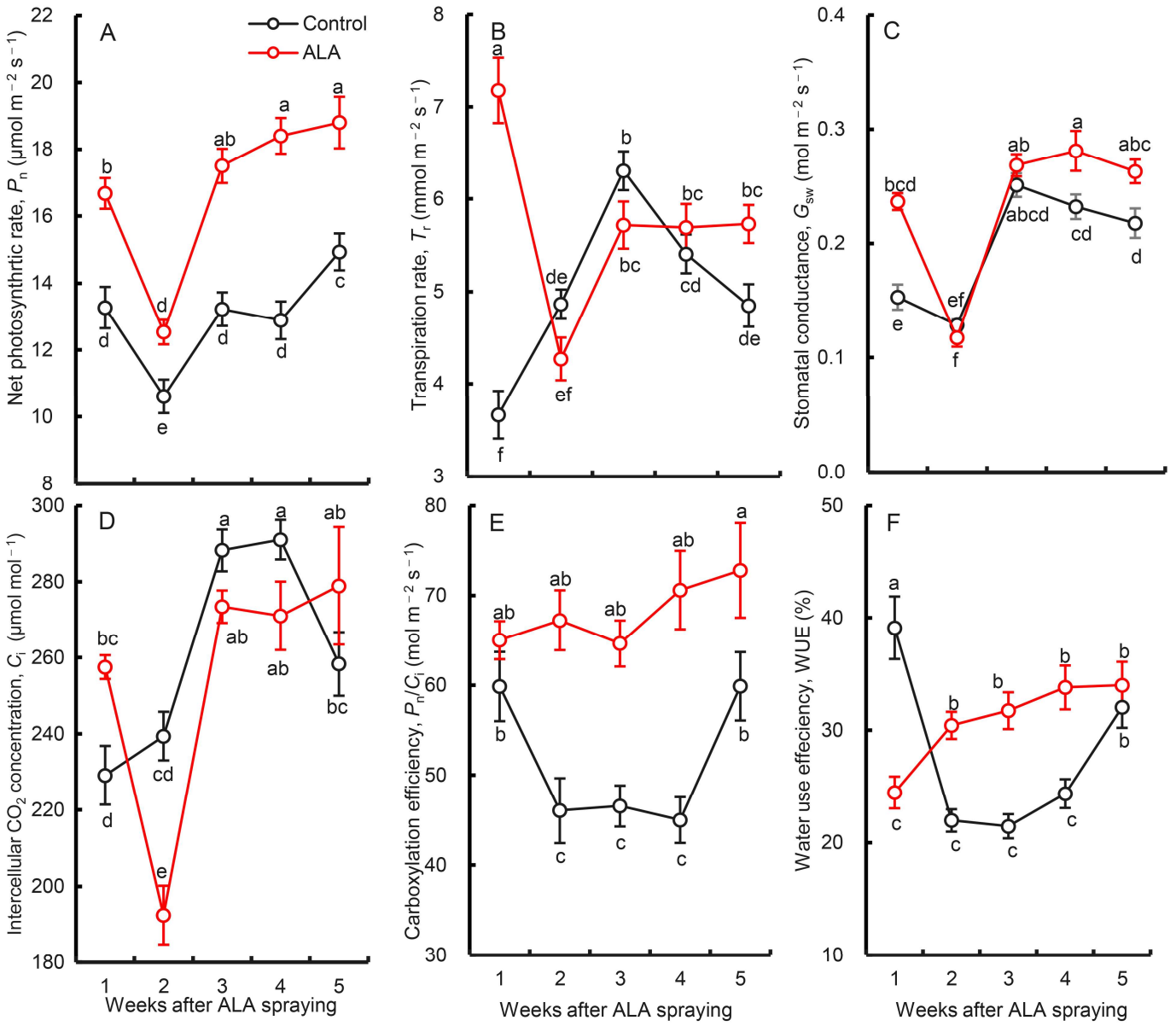

Figure 1. Effect of ALA treatment on photosynthetic gas exchange parameters of pear leaves. (**A**) P_n: net photosynthetic rate. (**B**) T_r: transpiration rate. (**C**) G_{sw}: stomatal conductance to water vapor. (**D**) C_i: intercellular CO_2 concentration. (**E**) P_n/C_i: instantaneous carboxylation efficiency. (**F**) WUE: water use efficiency. The data are the means of twenty biological repeats ± SE. The same lowercases in each panel represent no significant difference at $p = 0.05$.

The transpiration rate (T_r) of pear leaves one week after ALA spraying was significantly higher than that of the control, but was significantly decreased at the second week while that of the control was increased slightly. After the third week, T_r of the treated leaves was gradually higher than that of control. At five weeks, the T_r of ALA treated leaves was 13.94% higher than that of control ($p < 0.01$, n = 100). Similarly, the stomatal conductance (G_{sw}) of the pear leaves treated with ALA was also decreased significantly at the second week after ALA treatment. Overall, however, the G_{sw} of leaves treated with ALA was 18.73% higher than that of control ($p < 0.01$, n = 100). In contrast, there was no overall significant difference in CO_2 concentration between pear leaves due to ALA ($p > 0.05$), though the instantaneous carboxylation efficiency (P_n/C_i) of pear leaves was significantly increased by 32.30% ($p < 0.01$) due to ALA treatment. Water use efficiency

in ALA treatment was also increased by 11.16% ($p < 0.01$). In conclusion, ALA treatment significantly improved the photosynthetic performance of pear leaves.

3.2. Effects of Exogenous ALA Treatment on Chlorophyll Fluorescence Characteristics of Pear Leaves

Figure 2A–E show the effects of exogenous ALA on the chlorophyll fast fluorescence induction kinetic curves of pear leaves. After the pear leaves were darkened for 15 min and then irradiated with strong light, the fluorescence intensity rapidly increased from O point (0 ms) to J point (3 ms). At this time, the growth rate slowed slightly, and then increased rapidly again. After I point (30 ms), it gradually reached the highest point P (t ≈ 300 ms), and then stabilized, showing a typical OJIP curve. Within 1–5 weeks of the foliar spraying of ALA solution, the initial fluorescence (F_o) of the treated leaves decreased slightly, while the maximum fluorescence yield (F_m) at P point increased significantly, resulting in a significant increase in F_v and photochemical efficiency ($F_v/F_m \equiv \varphi P_o$).

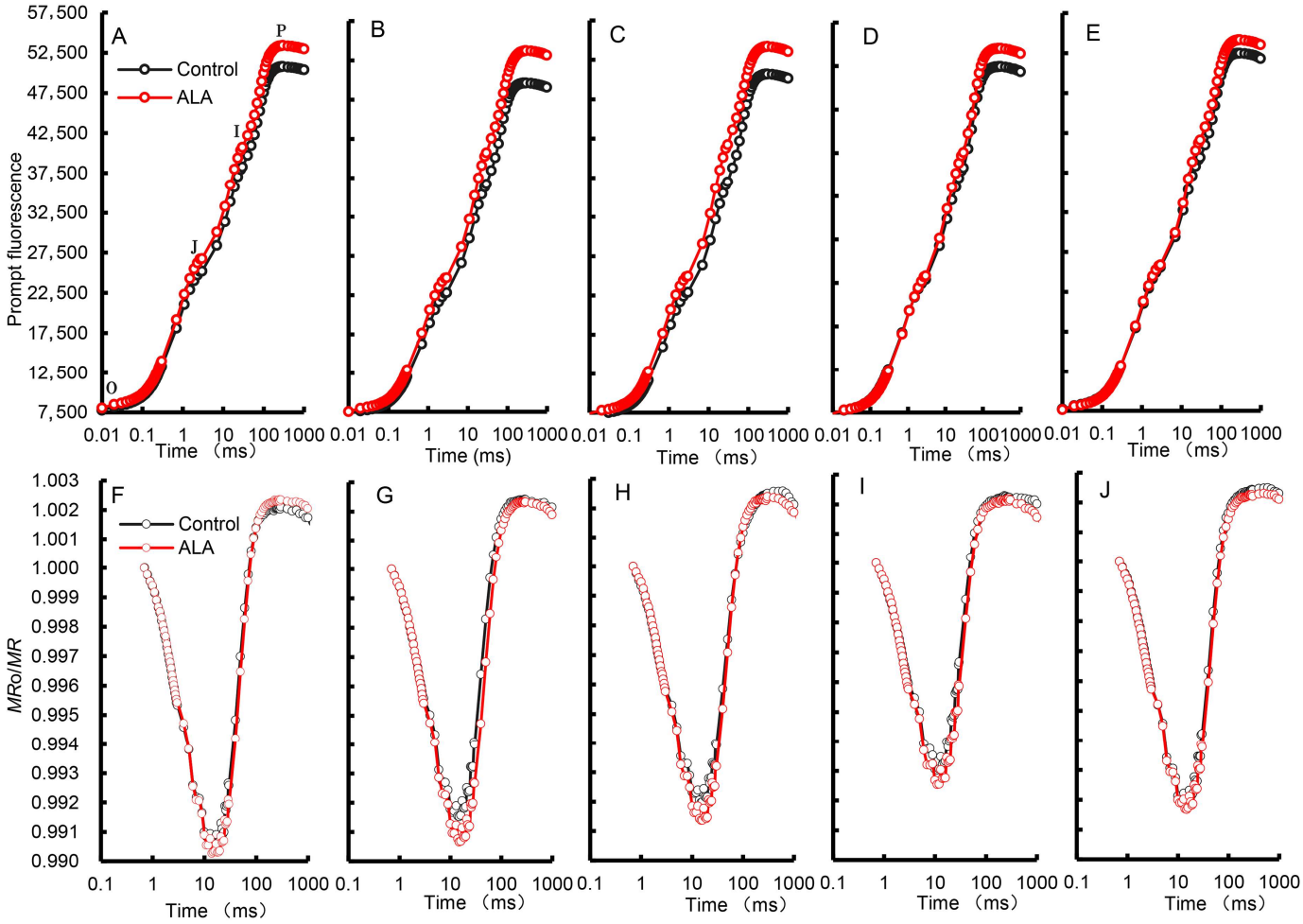

Figure 2. Effect of ALA treatment on the chlorophyll fast fluorescence curves (**A**–**E**) and the 820 nm modulated reflection fluorescence curves (**F**–**J**) of pear leaves. The capitals O, J, I, and P in the A represent the fluorescence at 0 ms, 3 ms, 30 ms, and 300 ms, respectively. The data are the means of fifteen biological repeats.

Figure 2F–J shows the 820 nm reflection fluorescence absorption curve of pear leaves within five weeks of the foliar spraying of ALA. The decrease stage of MR_o/MR represents the oxidation activity of PSI-RC, and the increase stage represents the reduction process of PSI-RC. The MR_o/MR ratio began to decrease from 0.7 ms (JIP time) and reached its lowest value between t = 10–30 ms. Then, PSI-RC was gradually reduced by the electrons

transferred from PSII-RC, so that the reflected fluorescence gradually increased and reached its peak at about 300 ms. At this time, PSI-RC was completely restored. The results show that the MR_o/MR of the treated leaves was slightly lower than that of the control at 2–4 weeks after ALA treatment, and that the difference was significant at $p = 0.05$ at the second week. However, it was not significant at other times ($p > 0.05$), indicating that the promotion of ALA treatment on PSI-RC activity in pear leaves appeared two weeks after ALA treatment, but soon disappeared. In addition, among the weekly MR_o/MR minimum, the first week was the lowest, and then gradually increased until, in the fourth week, it reached its maximum. There was no difference between the other three weeks. This result indicates that the redox activity of PSI-RC decreased from May 15 to June 12 during the development of pear leaves.

The chlorophyll fluorescence parameters calculated according to the chlorophyll fluorescence curve in Figure 2 are shown in Table 2. W_k represents the degree of inhibition of the PSII-RC donor-side oxygen-evolving complex (OEC). The W_k of ALA-treated leaves was significantly lower than that of the control in the first three weeks ($p < 0.05$), indicating that ALA can increase OEC activity and reduce the inhibitory effect of internal and external factors on the OEC. M_o is the maximum rate of Q_A reduction on the PSII-RC receptor side. The larger the value, the faster the PSII-RC is closed. Within three weeks of ALA treatment, the M_o of the treated leaves was significantly lower than that of the control ($p < 0.05$), indicating that ALA can delay the closure of PSII-RC and facilitate the transfer of photosynthetic electrons from PSII to Q_A^-. S_m represents the energy required for Q_A to be completely reduced, reflecting the capacity of the PQ reservoir on the PSII-RC receptor side. From Table 2, the S_m of the first three weeks after ALA treatment were significantly higher than those of the control ($p < 0.05$), indicating that ALA increased PQ storage capacity. Φ_o and φP_o represent the probability of the trapped excitons transferring electrons to other electron acceptors downstream of the electron transport chain Q_A^- and the maximum photochemical efficiency of PSII, respectively. Within five weeks of ALA treatment, the φ_o and φP_o in the ALA treatment were significantly higher than those in the control ($p < 0.05$). Similarly, φE_o, the quantum yield of light energy absorbed by PSII-RC for electron transfer after ALA treatment was significantly higher than that of the control within five weeks, while φD_o, the quantum yield for heat dissipation was significantly lower than that of the control ($p < 0.05$). RC/CS represents the density of active PSII reaction centers per unit area, while PI_{ABS} and PI_{total} represent the photosynthetic performance index based on light energy absorption and the photosynthetic performance index including PSI and PSII, respectively. The RC/CS of ALA-treated leaves was significantly higher than that of the control within five weeks, the PI_{abs} was significantly higher than that of the control at three weeks, and the PI_{total} was significantly higher than that of the control within four weeks ($p < 0.05$). In summary, the promotion of ALA on the photosynthetic electron transport performance of pear leaves can be maintained for at least three weeks, and some parameters can be maintained for five weeks. In addition, V_{PSI} is the maximum slope of the descending section of the 820 nm modulated reflection fluorescence curve (t = 0.7 to 3 ms in this study). This reflects the maximum rate of oxidation of the PSI reaction center. ALA treatment had no significant effect on the V_{PSI} of pear leaves, but significantly increased the $V_{PSII\text{-}PSI}$ of pear leaves ($p < 0.01$). This indicates that ALA had no effect on the PSI reaction center of pear leaves, but that it promoted the transfer of PSII-RC electrons to PSI-RC and the reduction of PSI-RC.

Table 2. Effects of ALA treatment on chlorophyll fluorescence parameters of pear leaves.

Weeks after Foliar Spraying of ALA	Treatment	W_k	M_o	S_m	φ_o	φP_o	φE_o	φD_o	RC/CS ($\times 10^3$)	PI_{ABS}	PI_{total}	V_{PSI} ($\times 10^{-4}$)	$V_{PSII\text{-}PSI}$ ($\times 10^{-5}$)
1	Control	0.37 ± 0.01 ab	0.53 ± 0.01 a	28.21 ± 0.74 b	0.63 ± 0.01 f	0.80 ± 0.01 d	0.54 ± 0.00 f	0.16 ± 0.00 a	4.48 ± 0.06 bc	5.77 ± 0.15 c	4.37 ± 0.23 c	20.27 ± 0.19 a	3.52 ± 0.10 abc
	ALA	0.35 ± 0.00 d	0.49 ± 0.01 bc	30.71 ± 0.69 a	0.65 ± 0.01 de	0.85 ± 0.00 b	0.56 ± 0.01 de	0.15 ± 0.00 bc	4.73 ± 0.03 a	6.18 ± 0.13 abc	5.04 ± 0.17 ab	19.90 ± 0.31 ab	3.86 ± 0.16 a
2	Control	0.37 ± 0.00 b	0.50 ± 0.01 b	27.25 ± 0.64 b	0.66 ± 0.01 cd	0.80 ± 0.01 d	0.56 ± 0.00 cd	0.15 ± 0.00 bc	4.35 ± 0.03 c	5.90 ± 0.22 bc	4.53 ± 0.13 c	19.13 ± 0.33 bc	3.11 ± 0.70 d
	ALA	0.35 ± 0.00 cd	0.46 ± 0.01 cd	30.81 ± 0.71 a	0.69 ± 0.01 a	0.85 ± 0.00 ab	0.59 ± 0.00 a	0.14 ± 0.00 d	4.54 ± 0.05 b	6.61 ± 0.21 a	5.22 ± 0.13 a	19.85 ± 0.26 ab	3.57 ± 0.14 ab
3	Control	0.35 ± 0.01 d	0.49 ± 0.01 bc	28.23 ± 0.64 b	0.67 ± 0.01 bc	0.81 ± 0.01 d	0.58 ± 0.00 bc	0.14 ± 0.00 d	4.49 ± 0.02 bc	5.75 ± 0.17 c	4.67 ± 0.12 bc	18.17 ± 0.37 de	3.18 ± 0.19 bcd
	ALA	0.33 ± 0.00 e	0.45 ± 0.01 d	30.26 ± 0.42 a	0.69 ± 0.01 a	0.86 ± 0.00 a	0.59 ± 0.00 a	0.14 ± 0.00 e	4.70 ± 0.07 a	6.49 ± 0.18 a	5.25 ± 0.15 a	18.34 ± 0.27 cde	3.45 ± 0.12 bcd
4	Control	0.39 ± 0.00 a	0.50 ± 0.01 b	23.70 ± 0.50 c	0.66 ± 0.01 cd	0.83 ± 0.00 c	0.56 ± 0.01 d	0.16 ± 0.00 b	4.11 ± 0.05 d	5.95 ± 0.18 bc	4.97 ± 0.13 ab	17.56 ± 0.43 e	2.04 ± 0.10 e
	ALA	0.37 ± 0.00 b	0.48 ± 0.01 bcd	24.69 ± 0.60 c	0.68 ± 0.01 ab	0.85 ± 0.00 ab	0.58 ± 0.00 ab	0.15 ± 0.00 c	4.35 ± 0.07 c	6.34 ± 0.18 ab	5.41 ± 0.09 a	18.35 ± 0.22 cde	2.33 ± 0.09 e
5	Control	0.36 ± 0.01 bc	0.50 ± 0.01 b	26.70 ± 0.68 b	0.64 ± 0.01 ef	0.82 ± 0.01 c	0.55 ± 0.01 ef	0.15 ± 0.00 bc	4.51 ± 0.07 b	5.75 ± 0.16 c	4.75 ± 0.08 bc	18.29 ± 0.24 cde	3.04 ± 0.15 d
	ALA	0.35 ± 0.01 cd	0.48 ± 0.01 bc	27.86 ± 0.62 b	0.66 ± 0.01 cd	0.85 ± 0.01 ab	0.56 ± 0.01 bcd	0.14 ± 0.00 d	4.69 ± 0.05 a	6.09 ± 0.17 abc	5.04 ± 0.13 ab	18.58 ± 0.25 cd	3.13 ± 0.13 cd

Note: The data are the means ± SE of 15 biological replicates. The same lowercases in each column represent no significant difference at $p = 0.05$.

3.3. Effects of Exogenous ALA Treatment on Endogenous ALA Biosynthesis and Metabolism in Pear Leaves

Figure 3 shows that the endogenous ALA content and the synthesis ability of pear leaves increased first and then decreased during the test period, while the ALA metabolic decomposition ability was at a low level except for May 15. Exogenous ALA treatment significantly increased endogenous ALA content in pear leaves. This difference appeared from the first week of leaf spraying and remained until the fifth week. The endogenous ALA content induced by ALA increased by 41.99% ($p < 0.01$) within five weeks. When the isolated pear leaves were treated with levulinic acid and then exposed to light, the endogenous ALA biosynthesis was normal but the metabolism was blocked. In this way, the ALA synthesis ability of pear leaves can be assessed, with the difference between ALA synthesis and the endogenous content being the amount of ALA metabolic decomposition. From Figure 3B, the endogenous ALA synthesis ability of pear leaves increased first and then decreased from May 15 to June 12. This trend is consistent with the endogenous ALA content. Exogenous ALA treatment significantly improved the ability of endogenous ALA synthesis. The mean value within five weeks was increased by 54.54% ($p < 0.01$). Similarly, exogenous ALA treatment promoted the metabolic decomposition of endogenous ALA in pear leaves ($p < 0.01$), but this effect was mainly reflected in the second week after ALA treatment. At other time points, although there were differences, they were not statistically significant. This means that spraying pear leaves with exogenous ALA in early May not only promotes endogenous ALA synthesis, but also promotes ALA metabolism. The promotion of exogenous ALA on endogenous ALA synthesis was maintained for at least five weeks, but the promoting effect on ALA metabolism was found mainly in the second week after treatment.

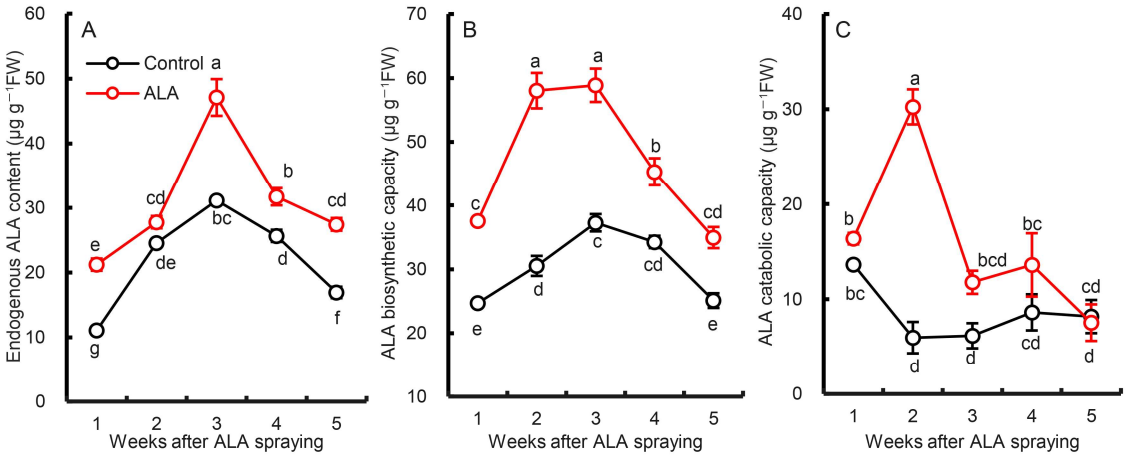

Figure 3. Effect of exogenous ALA spraying on the endogenous ALA content (**A**) and the biosynthetic (**B**) and catabolic capacity (**C**) of pear leaves. The data are the means of three biological repeats ± SE. The same lowercases in each panel represent no significant difference at $p = 0.05$.

3.4. Effects of Exogenous ALA Treatment on Photosynthetic Pigment Content in Pear Leaves

Figure 4 shows the effect of exogenous ALA treatment on photosynthetic pigment content in pear leaves. Between May 15 and June 12, the photosynthetic pigments of pear leaves increased first and then decreased. One can see, from the results of the relative chlorophyll content (SPAD) (Figure 4A), that, one week after ALA treatment, the SPAD of the treated leaves was slightly higher than that of the control but not within significance ($p > 0.05$). Thereafter, it was significantly higher than the control. Figure 4B–D show that the chlorophyll a, chlorophyll b and total chlorophyll content of pear leaves after one week of ALA treatment were significantly higher than those of the control. Although there was no difference in Chl b content between the two groups at the second week, the differences at other time points were significant $p = 0.05$, indicating that ALA treatment promoted chlorophyll accumulation in pear leaves and alleviated the decline.

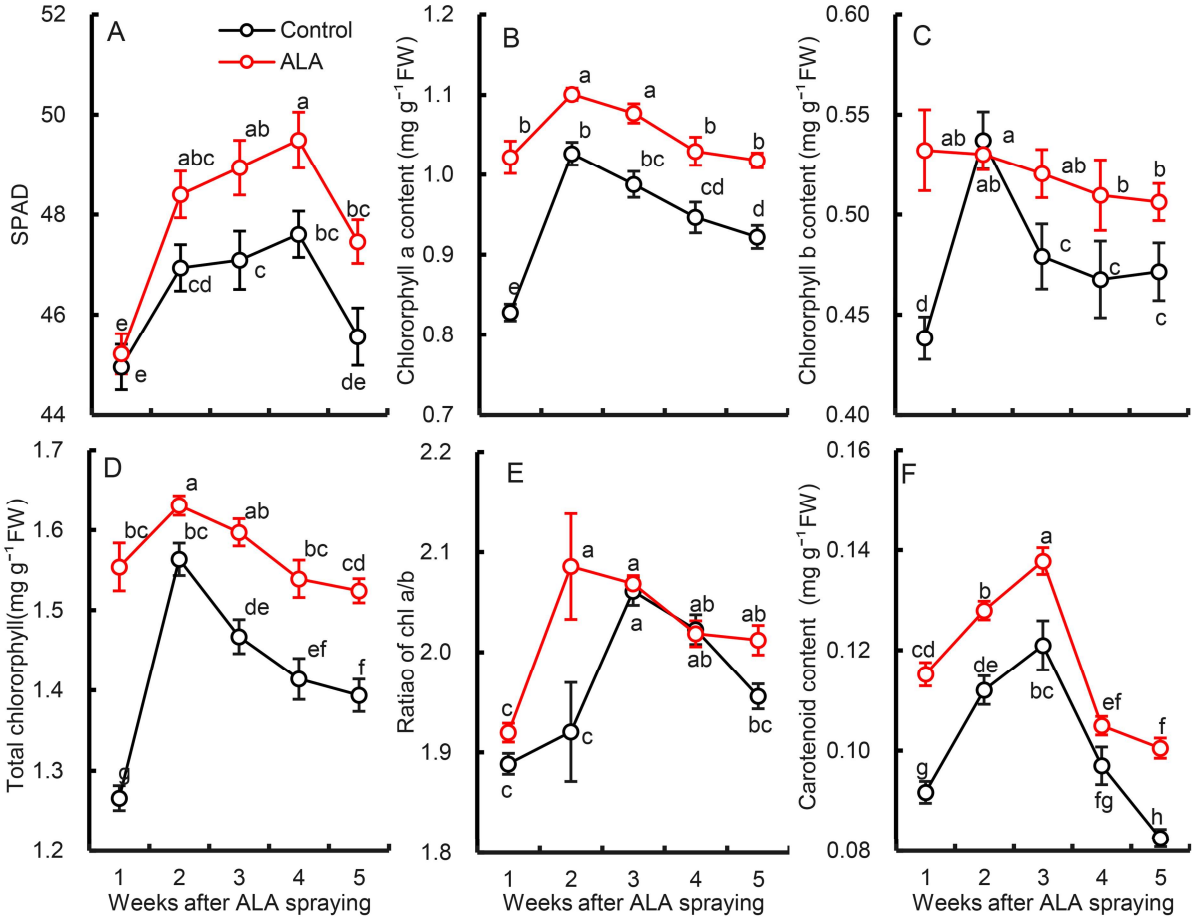

Figure 4. Effect of ALA treatment on the content of photosynthetic pigment in pear leaves. (**A**) Relative content of chlorophylls (SPAD), (**B**) chlorophyll a, (**C**) chlorophyll b, (**D**) total chlorophyll content, (**E**) ration of Chl a/b, (**F**) carotenoid content. The data are the means of nine biological repeats ± SE. The same lowercases in each panel represent no significant difference at $p = 0.05$.

Specifically, the average values of Chl a, Chl b and total chlorophyll in ALA-treated leaves within five weeks were 11.38%, 8.59% and 10.44% higher than those in the control, respectively ($p < 0.01$). In addition, ALA was seen to promote the increase of Chl a/b ratio in pear leaves ($p < 0.01$), indicating that ALA promoted the accumulation of chlorophyll a in leaves, but was not conducive to the conversion of Chl a into Chl b. Similarly, the carotenoid content of pear leaves also showed a trend of 'first rise and then fall' within five weeks after treatment, and ALA treatment promoted the increase of Car content in leaves (Figure 4F). The five-week average was 16.25% higher than the control ($p < 0.01$).

3.5. Effects of Exogenous ALA Treatment on the Gene Expressions Related with Chlorophyll Synthesis

To explore the mechanism by which exogenous ALA increases chlorophyll content in pear leaves, we analyzed the relative expression of the primary genes related to ALA and chlorophyll biosynthesis after the spraying of ALA. With the exception of encoding porphobilinogen deaminase (*HEMC*) and encoding uroporphyrinogen III decarboxylase (*HEME*), which were not detected, the relative expressions of other genes are shown in Figure 5. The relative expressions of two key genes, *HEMA* and *GSA*, which are involved in ALA synthesis, were significantly upregulated after exogenous ALA treatment. Among these, *HEMA* expression had the largest increase in the first week after ALA treatment, and was still significantly higher than the control in the second and third weeks; however, in the fourth and fifth week, it was significantly lower than the control. *GSA* expression was also significantly higher than that of the control from the first week to the fourth week

after ALA treatment, although it was significantly lower than that of the control at the fifth week. The expression of *HEMB*, which encodes the ALA dehydrase and catalyzes the ALA metabolism to produce porphobilinogen, was significantly higher than the control at the second and fourth week after ALA treatment, but no significant difference was found at other times. The expression of *HEMD*, which encodes the enzyme catalyzing the formation of uroporphyrinogen III from hydroxymethylbilane, was significantly lower than that of the control in the first two weeks of ALA treatment, nevertheless, it was significantly higher than the control at the third and fourth weeks. Overall, ALA treatment downregulated the gene expression ($p < 0.05$). The enzyme encoded by *HEMF* catalyzes the conversion of coproporphyrinogen III to protoporphyrin IX. From Figure 5, the expression of the *HEMF* gene in ALA-treated leaves was significantly higher than that in the control, at least within four weeks ($p < 0.05$). A similar situation occurred in *HEMG* (encoding the enzyme that catalyzes protoporphyrinogen IX to protoporphyrin IX).

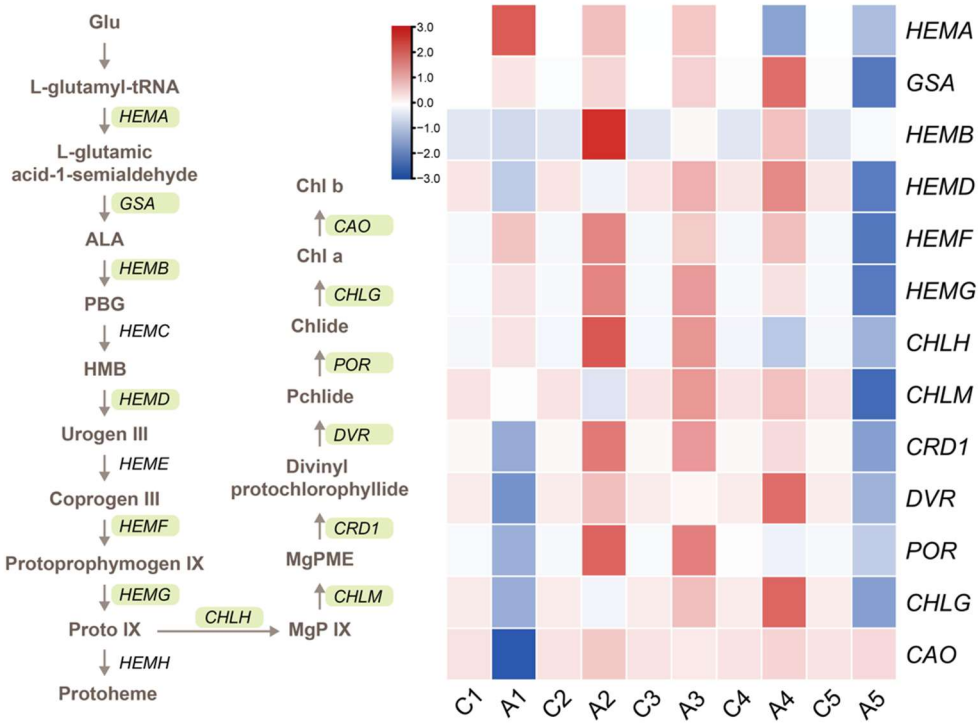

Figure 5. Chlorophyll biosynthesis pathway and the effect of ALA treatment on the expression of related genes in pear leaves. C1 to C5 and A1 to A5 represent the control and ALA treatment collected from 1 to 5 weeks after ALA foliar spraying, respectively. The color gradient from blue to red corresponds to fold changes from low to high. Glu: glutamic acid; ALA: 5-aminolevulinic acid; PBG: porphobilinogen; HMB: hydroxymethylbilane; Urogen III: uroporphyrnogen III; coprogen III: coproporphyrnogen III; Proto IX: protoporphyrnogen IX; MgP IX: mg-protoporphyrin IX; MgPME: Mg-protoporphyrin IX methyl ester; Pchlide: protochlorophyllide; Chlide: chlorophyllide; Chl a: chlorophyllide a; Chl b: chlorophyllide b. The genes depicted in the heatmap correspond to enzymes marked with green highlights in the chlorophyll biosynthesis pathway.

The *CHLH* encoding enzyme catalyzes the chelation of protoporphyrin IX with Mg^{2+} to produce Mg-protoporphyrin IX. This is the first gene to control the branch of chlorophyll biosynthesis. From Figure 5, the relative expression of *CHLH* in leaves treated with ALA was significantly higher than that of control at week 1, which lasted up to three weeks. Thereafter, the gene expressions were lower than controls. The *CHLM* encoding enzyme catalyzes the formation of Mg-protoporphyrin IX into Mg-protoporphyrin IX methyl ester. The relative expression of this gene was significantly higher than that of the control at the third and fourth week after ALA treatment but was lower than that of the control at other time points. Overall, ALA treatment downregulated the expression of this gene ($p < 0.05$).

The *CRD1* encoding enzyme catalyzes the conversion of Mg-protoporphyrin IX methyl ester to divinyl protochlorophyllide. ALA treatment promoted the upregulation of *CRD1* expression, which was manifested in the 2–4 weeks after treatment ($p < 0.05$). The *DVR* gene is different, the encoding enzyme catalyzes the conversion of divinyl protochlorophyllide to pchlide and was significantly upregulated at the second and fourth weeks after ALA treatment; however, overall, ALA treatment downregulated *DVR* gene expression ($p < 0.05$). The enzyme encoded by *POR* catalyzes the conversion of pchlide into chlide. At the second and third weeks after ALA treatment, the expression of *POR* was significantly higher than that of the control. The enzyme encoded by *CHLG* catalyzes the conversion of chlide into chlorophyll a. Its expression profile is like that of *DVR*, which was significantly upregulated at 3–4 weeks after ALA treatment, but lower than that of the control at other times. Overall, the gene expression was downregulated by ALA ($p < 0.05$). The enzyme encoded by *CAO* catalyzes the conversion of chlorophyll a into chlorophyll b. After ALA treatment, the relative expression of *CAO* was lower than that of the control ($p < 0.05$), indicating that ALA treatment inhibited the conversion of chlorophyll a to chlorophyll b.

Overall, ALA promoted the expression of genes encoding its own synthesis, metabolism, and transformation into chlorophyll. The effects were significant within four weeks after ALA treatment, the expression of most genes was upregulated to varying degrees.

3.6. Effect of Exogenous ALA Treatment on Promoter Activity of Genes Related to ALA and Chlorophyll Synthesis

To further explore the mechanism by which ALA regulates chlorophyll synthesis, we selected five genes, *HEMA*, *GSA*, *HEMF*, *HEMG* and *CHLH*, which were continuously upregulated from the first week after ALA treatment, in order to analyze the activation of ALA on the promoters of these genes by GUS staining.

The structural map of the pBI121 recombinant vectors carrying the promoters of the target genes was shown in Figure 6A. The Agrobacterium solution carrying the recombinant plasmid was injected into tobacco leaves, and some leaves were selected to spray 1 mg L^{-1} exogenous ALA solution. Then, the tobacco plants were cultured under dark conditions for 24 h, and transferred to light for 48 h. Leaves were collected and GUS staining was performed with the results in Figure 6B (*HEMG* gene expression vector was not successfully constructed). The GUS staining degree of tobacco leaves injected with the *35S::GUS* empty vector was highest, while those of *proPypHEMA::GUS* and *proPypCHLH::GUS* after ALA treatment were significantly higher than that of the control; however, those of *proPypGSA::GUS* and *proPypHEMF::GUS* were not significantly stained with the control. Figure 6C shows the relative expression of the *GUS* gene in tobacco leaves after transient infection. The relative expressions of the *GUS* gene in tobacco leaves injected with *proPypHEMA::GUS* and *proPypCHLH::GUS* were significantly higher than that of the control after ALA treatment, and significantly lower than that of the leaves injected with the empty vector. The relative expressions of the *GUS* gene in the leaves treated with *proPypGSA::GUS* and *proPypHEMF::GUS* bacterial solution injection and the control were not significantly different. Therefore, exogenous ALA treatment may promote gene expression by upregulating the promoter activity of endogenous ALA synthesis key gene *HEMA* and chlorophyll synthesis key gene *CHLH*, thus leading to the increase of endogenous ALA and chlorophyll content in pear leaves.

3.7. Expression Analysis of PSII-Related Genes in Pear Leaves Treated with Exogenous ALA

From the results of the chlorophyll rapid fluorescence curve, the promoting effect of ALA on photosynthetic electron transport in pear leaves is mainly manifested in PSII-RC. To explore the mechanism, we further measured the relative expressions of the genes encoding the main members of PSII-RC in pear leaves within five weeks of ALA treatment. From Figure 7, the genes *PsbB* and *PsbC* encoding the main members of the inner light-harvesting pigment protein complex CP43 and CP47 were significantly up-regulated at one week after ALA treatment, but this effect disappeared at two weeks. The gene *PsbA* encoding PSII-RC

core protein D1 was significantly higher than that of the control after two weeks of ALA treatment. Similarly, ALA continuously promoted the expressions of *PsbE* and *PsbF* genes encoding the Cyt b_{559} branch core protein for at least four weeks. In addition, *PsbY*, *Psb27*, *psbH*, *PsbJ* and *PsbL* were upregulated to varying degrees in the second week after ALA treatment, where *PsbY* is related to the photoprotection of Cyt b_{559}. *Psb27* and *psbH* are involved in the assembly of PSII-RC by binding to antenna proteins CP43 and CP47, and *PsbJ* and *PsbL* may be involved in the light energy conversion process. However, there were no obvious regular changes in the expression of other genes.

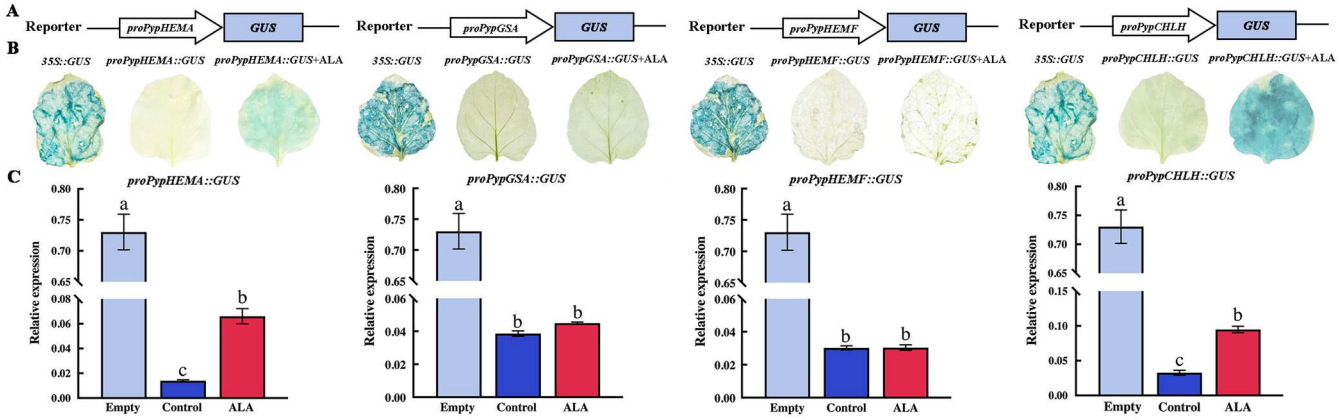

Figure 6. Effect of exogenous ALA on the promoter activities of genes associated with ALA and chlorophyll synthesis. (**A**) Construction of gene vector atlas for GUS staining analysis. (**B**) Analysis of *GUS* gene expression in tobacco leaves after injection with different agrobacterium liquids. (**C**) Analysis of *GUS* expression in tobacco leaves after injection of different Agrobacterium solutions. Empty represents the *GUS* expressions of tobacco injected with *35S::GUS* Agrobacterium solution, while the control and ALA respectively represent the *GUS* expressions in the tobacco injected with Agrobacterium liquids with or without ALA. Different lowercase letters in each histogram represent significant differences at $p = 0.05$.

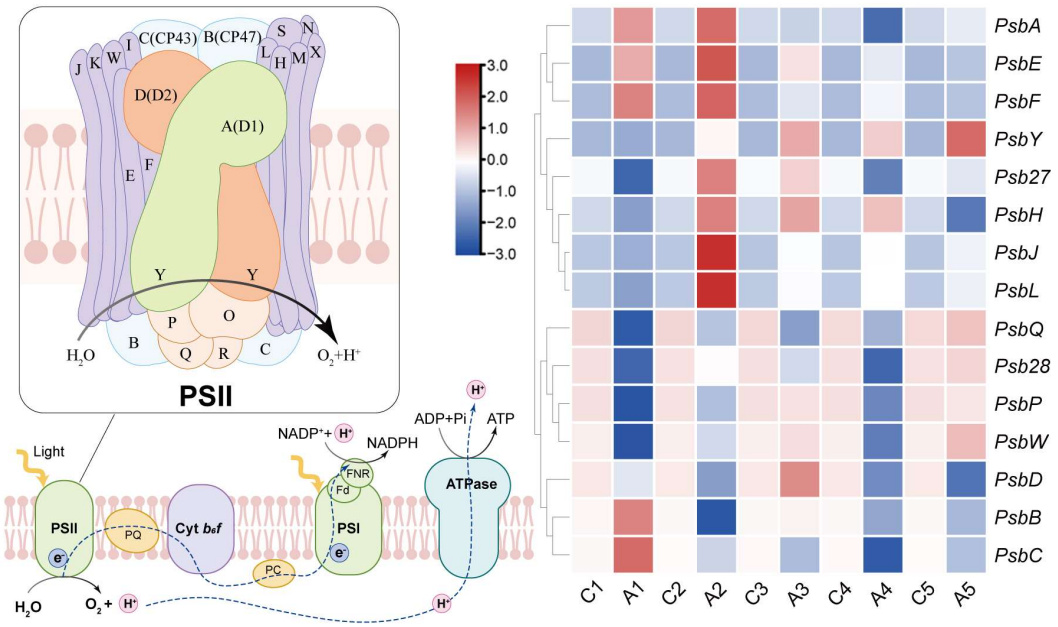

Figure 7. PSII reaction center structure and the effect of ALA treatment on the expressions of related genes in pear leaves. C1 to C5 and A1 to A5 represent the samples collected 1 to 5 weeks from the control and ALA foliar spraying for 1 to 5 weeks, respectively. The color gradient from blue to red corresponds to fold changes from low to high.

The above results indicate that ALA treatment may maintain the stability of the PSII reaction center and promote plant photosynthesis by promoting the expression of genes encoding PSII-RC core structural proteins and other functional small molecule proteins.

3.8. Effect of Exogenous ALA Treatment on Promoter Activity of PSII-Related Genes

To further explore the mechanism of ALA regulating the PSII reaction center protein, nine genes whose expressions were upregulated in the first two weeks after ALA treatment, including *PsbA*, *PsbB*, *PsbC*, *PsbE*, *PsbF*, *PsbH*, *PsbJ*, *PsbL*, and *Psb27*, were selected in this study. GUS staining was used to analyze the activation effect of ALA qualitatively and quantitatively on the promoters of these genes.

During the vector construction, only *PsbA*, *PsbB* and *PsbJ* were successfully constructed. The structural map of the pBI121 recombinant vectors carrying the promoters of the target genes is shown in Figure 8A. The Agrobacterium solution carrying the recombinant plasmid was injected into tobacco leaves, and some leaves were selected to be sprayed with 1 mg L^{-1} exogenous ALA solution. Then, the tobacco plants were cultured under dark conditions for 24 h and transferred to light conditions for 48 h. Leaves were collected and GUS staining was performed. The results (Figure 8B) show that the *GUS* staining degree of tobacco leaves injected with the *35S::GUS* empty vector bacterial solution was the highest, while that with ALA-treated bacterial solution was not significantly different from that of the control. Figure 8C shows that there was no significant difference in the relative expressions of the *GUS* gene promoted by *proPypPsbA::GUS*, *proPypPsbB::GUS* and *proPypPsbJ::GUS* in either the ALA treatment or the control. This indicates that exogenous ALA has no significant effect on the promoter activity of PSII reaction center protein genes of pear leaves.

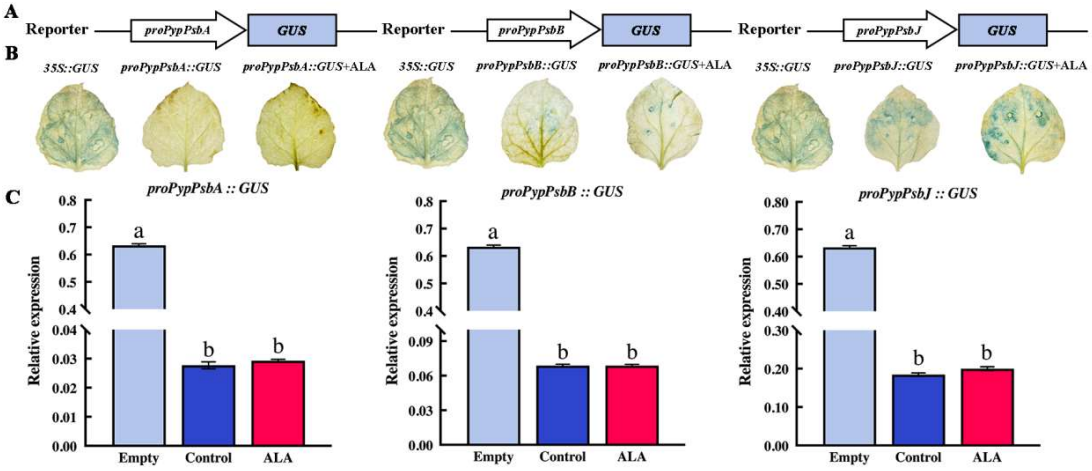

Figure 8. Effect of exogenous ALA on the promoter activities of genes associated with PSII. (**A**) Construction of gene vector atlas for *GUS* qualitative and quantitative analysis. (**B**) Analysis of *GUS* gene expression in tobacco leaves after injection with different agrobacterium liquids. (**C**) Analysis of *GUS* expression in tobacco leaves after injection of different Agrobacterium solutions. Empty represents the *GUS* expressions of tobacco injected with *35S::GUS* Agrobacterium solution, while ALA and control represent the *GUS* expressions in the tobacco injected with Agrobacterium liquids treated with or without ALA, respectively. Different lowercase letters in each histogram represent significant differences at $p = 0.05$.

4. Discussion

As early as 1997, Hotta et al. had proposed that exogenous ALA improves the photosynthetic efficiency of leaves of various plants [44]. Over the decades, the role of ALA in promoting photosynthesis has been confirmed in apple [11], grape [12], peach [15], melon [17], cucumber [24], pear [30], strawberry [45], and wheat [46]. At present, ALA's promotional effects on leaf photosynthesis have been widely utilized in various agricultural and forestry plant productions [47]. In peach, we have developed the "technical regulations

for the application of 5-aminolevulinic acid to peach trees" (TJAASS 88-2023 [36]), but whether this is suitable for pear production has not been proven. In this study, we applied ALA to the roots of pear trees before flowering, then sprayed the flowers at the late flowering stage, and sprayed the leaves at the young fruit development period, according to the regulation in peach. After that, we measured the photosynthetic gas exchange parameters on a weekly basis and the chlorophyll rapid fluorescence and gene expressions associated with chlorophyll synthesis and PSII-RC member proteins over the following five weeks, the results provide a theoretical basis for the elucidation of the mechanism of ALA in improving the photosynthetic efficiency in pear leaves.

Firstly, the P_n of pear leaves was significantly increased within five weeks after foliar spraying of ALA (Figure 1A). At the same time, the change trend of leaf G_{sw} was highly similar to that of P_n (Figure 1C). This is consistent with the results of Zhao et al. (2014) which state that ALA improves the photosynthetic capacity of tomato leaves under salt stress by maintaining the normal opening of stomata and reducing stomatal limitation [26]. In apple, ALA has been shown to upregulate *PP2AC* gene expression and PP2A protein phosphatase activity, promote SnRK2.6 dephosphorylation [28,29], downregulate Ca^{2+} and H_2O_2 content in guard cells [48], and upregulate flavonol content [49], thereby blocking ABA signaling and reversing ABA-induced stomatal closure [50]. ALA has a significant effect on the promotion of stomatal opening in pear leaves ($p < 0.05$), although the mechanism in pear leaves needs to be clarified further. In grape, exogenous ALA can alleviate ABA-induced stomatal closure when seedlings are subjected to drought stress [51]. However, compared with stomatal aperture, the promotion of ALA on the carboxylation efficiency of pear leaves may be more important. Within five weeks of ALA treatment, the instantaneous carboxylation efficiency of leaves remained at a high level, and the average P_n/C_i was 32.3% higher than that of the control, which was much higher than the average increase of G_{sw} (18.73%). In addition, ALA treatment significantly increased the water use efficiency of pear leaves (Figure 1F). These results suggest that the promotion of ALA on the photosynthetic efficiency of pear leaves has a variety of performances, and that this promotion is a comprehensive result of multiple factors.

Secondly, during the field experiment, the maximum temperatures were generally maintained at 30–34 °C. However, in the second week (May 22), the temperature soared to 39 °C. This may be an important reason for the decrease of P_n at this time point. From Figure 1, P_n, T_r and G_{sw} decreased significantly in the second week. However, the P_n and G_{sw} of leaves after ALA treatment were still significantly higher than those of the control, indicating that exogenous ALA improved the high temperature resistance of pear. This is similar to the observation shown in [20], where ALA was reported to increase leaf gas exchange parameters and photochemical efficiency during high temperature condition.

Thirdly, chlorophylls are the main pigment with which plant chloroplasts capture solar energy and drive electrons to the photosynthetic reaction center [52]. It has long been known that ALA is a key precursor of chlorophyll biosynthesis [53,54], and exogenous ALA treatment can promote plant chlorophyll synthesis [24,55]. Moreover, ALA can be used as a regulator for chlorophyll synthesis. Tanaka et al. (1993) have proposed that ALA promotes the conversion of chlorophyll a into chlorophyll b in cucumber leaves, which bind to the light-harvesting pigment protein complex II (LHCII) apoprotein to avoid the hydrolysis activity of chlorophyllase to Chl a, thereby increasing chlorophyll content [53]. The authors proposed that the Chl b/a ratio of leaves was increased after ALA treatment. However, we did not find a similar phenomenon in this study. Foliar application of ALA not only promoted the increase of Chl a, Chl b content and total chlorophyll content in pear leaves, but also significantly increased Chl a/b ratio. Therefore, different species may have different responses to ALA treatment in the conversion of chlorophylls. In addition, ALA promoted the increase of carotenoid content in pear leaves (Figure 4F). This is similar to tomato fruit [56], but opposite to tomato leaves [57]. Therefore, the relationship between ALA and carotenoids needs to be further studied.

Wu et al. (2018) have proposed that exogenous ALA upregulates gene expressions such as *HEMA* (encoding the key enzyme GluTR), *CHLH* (encoding chlorophyll chelatase), and *POR* (encoding protochlorophyllate oxidoreductase) in cucumber leaves under salt stress [24]. Wang et al. (2021) have also proposed that ALA promotes the expression of *HEMA1* and *HEMB* in pepper leaves under low temperature stress [58]. These indicate that exogenous ALA promotes endogenous ALA synthesis and metabolic decomposition. The results in the current study show that ALA not only promotes the expression of *HEMA*, *GSA* and *HEMB*, three key genes controlling ALA synthesis and metabolism (Figure 5), but also promote the synthesis and metabolism of endogenous ALA in pear leaves. Nevertheless, because exogenous ALA promotes the increase of endogenous ALA content, ALA promotion of the biosynthesis is greater than of the metabolism (Figure 3). The GUS staining has shown that ALA promotes the promoter activity of *PypHEMA* (Figure 6C). This not only validates the results of previous studies in cucumber [24] and pepper [58], but also proves that exogenous ALA itself induces biosynthesis. Thus, exogenous ALA treatment can exhibit these biological functions over a long period, and thus need not be applied too frequently. In 'Housui' pear, the increased photochemical efficiency induced by ALA application at the end of full blooming can be observed after 98 days [30]. The reason for this has never been clear. The finding in the current study, that exogenous ALA upregulates the gene promoter activity of endogenous ALA synthesis, can well explain the long-term effectiveness of ALA on leaf photosynthesis. However, the promoter of *GSA*, another key gene for endogenous ALA biosynthesis, was not regulated by ALA (Figure 6C). In addition, the mechanism of exogenous ALA activating *HEMA* promoter remains to be studied further.

Fourthly, chlorophyll synthesis is not only affected by ALA synthesis and metabolism, but also regulated by many other genes. In grape, Yang et al. (2023) have proposed that exogenous ALA upregulates the expressions of *DVR*, *POR*, *CHLG* and other genes, thereby promoting the increases of the leaf chlorophyll content [51]. In the current study, gene expressions related to chlorophyll synthesis were detected in pear leaves within five weeks after exogenous ALA treatment. The results show that the relative expressions of five genes, *HEMD*, *CHLM*, *DVR*, *CHLG* and *CAO,* were significantly lower than those of the control. The relative expressions of other genes, including *HEMF*, *HEMG*, *CHLH*, *CRD1* and *POR,* were significantly higher than those of the control (Figure 5). These show that exogenous ALA treatment can promote the expressions of chlorophyll synthesis genes in pear leaves to varying degrees. GUS staining showed that exogenous ALA stimulated the activity of the *PypCHLH* promoter (Figure 6C). *CHLH* encodes the H subunit of Mg-chelating enzyme, which catalyzes the chelation of Mg^{2+} into the porphyrin ring to form Mg-protoporphyrin IX. This is the first gene in the branch of chlorophyll synthesis [59]. The activation of ALA on the gene promoter may be an important mechanism for the upregulation of chlorophyll content in pear leaves. On the other hand, ALA treatment downregulates *CAO* expression in pear leaves. This may be the main reason for the increase of Chl a/b.

Fifthly, we detected the chlorophyll fast fluorescence curve and 820 nm modulated reflection fluorescence curve of pear leaves within five weeks of ALA treatment and analyzed the effects of ALA on the activity of PSII-RC and PSI-RC in pear leaves using fluorescence data. As shown in Figure 2 and Table 2, the promotion of ALA on PSII-RC activity in pear leaves was significantly higher than that of PSI-RC. V_{PSI} reflects the electron loss rate in the PSI-RC because of photo oxidization after strong light irradiation. We measured these for five weeks and, without exception, did not observe the effect of ALA. On the contrary, $V_{PSII\text{-}PSI}$ reflects the ability of electrons transferred from PSII-RC to reduce PSI-RC [40]. The $V_{PSII\text{-}PSI}$ of pear leaves after ALA treatment was always found to be higher than that of the control, and the difference was found to be significant in the second week ($p < 0.05$). Moreover, W_k is a parameter reflecting the inhibition of the PSII-RC donor side. φP_o reflects PSII-RC activity. M_o, φ_o and S_m reflect the characteristics of the receptor side of PSII-RC. We found that ALA treatment reduced W_k and M_o, but increased φP_o, φ_o and S_m. These effects were significant within at least three weeks of ALA treatment.

The quantum yield, φE_o, and the active reaction center density RC/CS for photosynthetic electron transport in pear leaves were significantly higher than those in the control, while the quantum yield for heat dissipation (φD_o) was lower than that in the control. The photosynthetic performance index, PI_{abs}, of ALA-treated leaves was significantly higher than that of the control. In addition, PI_{total}, including the two photosynthetic reaction centers of PSII and PSI, also increased significantly due to ALA treatment (Table 2). This indicates that ALA also has a promoting effect on the activity of the PSI reaction center. From Figure 2, one can see that the minimum value of MR_o/MR of 820 nm modulated reflectance fluorescence curve of pear leaves treated with ALA was lower than that of the control within 2–4 weeks, indicating that the ability of the PSI reaction center to reduce terminal electron acceptors was higher than that of the control [60]. In strawberry, Sun et al. (2011) have proposed that the promotion of ALA on photosynthetic efficiency is related to the activity of PSII-RC and PSI-RC [61]. In pear, Shen et al. (2011) have suggested that ALA has a significant promoting effect on the PSII reaction center of sand pear leaves [31]. Therefore, the regulation mechanism of ALA on PSII-RC activity in pear leaves is worthy of further study, and the effect of ALA on PSI-RC cannot be ignored.

Sixthly, in view of the important promoting effect of ALA on PSII-RC in pear leaves, the gene expressions of PSII-RC-related proteins to ALA treatment were determined in this study. The results show that the expressions of genes encoding the PSII-RC core protein D1 (*PsbA*), the inner light-harvesting pigment proteins CP43 (*PsbB*) and the CP47 (*PsbC*), cytochrome b_{559} (*PsbE* and *PsbF*) were significantly upregulated in pear leaves after spraying of ALA solution for one week. D1 is the core structure of PSII-RC [62] and the most vulnerable component of PSII [63]. ALA treatment significantly increased *PsbA* gene expression and D1 protein content in wheat under drought stress [46]. CP43 and CP47 connect PSII-RC and LHCII to transfer the energy generated by excitation to the reaction center [64]. As an electron transport branch, Cyt b_{559} has a light protective effect [65]. In addition, the genes encoding PSII-RC small molecule proteins, such as *PsbY*, *Psb27*, *PsbH*, *PsbJ* and *PsbL* were found to be significantly higher than those of the control at 2–4 weeks of ALA treatment (Figure 7). Therefore, it is suggested that the promoting effect of ALA on the photosynthesis of pear leaves may play a protective and promoting role by promoting the expression of PSII-RC core structural proteins and the coding genes of various small molecule proteins, and that this promoting effect has no obvious relationship with the regulation of gene transcription initiation. However, we did not find any direct regulation of ALA on the gene expressions, which needs further study.

5. Conclusions

In summary, the promotion of ALA on the photosynthesis of pear leaves can be maintained for at least three weeks, which means that ALA application is not frequently needed. Conversely, ALA should be applied at different specific stages. At the early stage of plant growth, rhizosphere application to prevent late spring coldness before fruit tree blooming, flower spraying to thin surplus flowers at the end of full blooming and foliar application to promote leaf development are all important for leaf photosynthesis and fruit quality. In the process of chlorophyll synthesis, exogenous ALA promotes its own synthesis and metabolism, promotes the branch of chlorophyll synthesis, and increases the content of photosynthetic pigments. In the process of photosynthetic electron transport, ALA promotes the expression of PSII-RC core protein genes in pear leaves with higher PSII-RC activity at the donor side and the accepting side, which facilitates the promotion of photosynthetic electron transport and energy conversion. Our published data suggest that the single fruit weight and the soluble sugar content of ALA treatment were, respectively, 19.7% and 14.5% higher than those of the control group when fruits were mature ($p < 0.05$), which may be the results of the increased photosynthesis. However, the regulatory mechanisms of ALA on photosynthetic dark reaction (such as RuBP carboxylase activity) of pear remains to be further studied.

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