5-Aminolevulinic Acid Pretreatment Mitigates Drought and Salt Stresses in Poplar Plants

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Abstract: 5-aminolevulinic acid (ALA), a key precursor in the biosynthesis of porphyrins, can improve plant tolerance to various environmental stresses. However, it is unclear whether ALA can improve tolerance in poplar. Here, we investigated the effects of ALA on poplars under drought and salt stresses. ALA pretreatment exhibited less morphological damage, reduced leaf malonaldehyde content (MDA) and electrolyte leakage (EL), and increased leaf relative water content (RWC), proline (PRO), superoxide dismutase (SOD), and peroxidase (POD) content under stresses. Furthermore, exogenous ALA mitigated the decrease in photosynthetic capacity, and restored the chlorophyll content (Chl), net CO₂ assimilation rate, stomatal conductance (Gs), transpiration rate (Tr), maximal photochemical quantum yield of PSII (Fv/Fm), actual quantum yield of photosynthesis (YII), and electron transfer rate (ETR) of poplar under various stresses. qRT-PCR showed that ALA up-regulated the expression of antiporters and aquaporins genes, which are associated with Na⁺ exclusion in the leaf cells and the transport activity of aquaporins. In summary, ALA pretreatment significantly improved the stress tolerance of poplar, decreasing the degree of membrane lipid peroxidation and promoting the photosynthesis and antioxidant capacity of leaves. In addition, our results showed that ALA might mediate Na⁺ transporter and aquaporins activity, thereby increasing the salt tolerance of poplar.

Keywords: 5-aminolevulinic acid; drought stress; salt stress; poplar

1. Introduction

Poplar is an important economic and ecological tree species globally. Poplar has many advantages such as rapid growth, easy survival, and strong adaptability to soil [1–3]. Abiotic stresses such as drought and salt have a negative impact on plant growth. Water deficit has a negative effect on the production and accumulation of plant biomass. It also causes a series of damages to the plant, which are harmful to the growth of poplar [4–6]. In recent years, drought resistance and salt tolerance have become key areas of research regarding poplar resistance. With the development of biotechnology, genetic transformation technology has been widely used to improve the resistance of plants [7]. In addition, the application of exogenous substances can significantly increase plant stress resistance. For instance, the application of potassium fertilizer improved the photo-assimilation process and minimized the impact of drought stress in cotton [8]. Silicon enhanced the growth of wheat under short-term PEG-induced water deficit conditions by improving antioxidant defense [9]. CaCl₂ treatment can improve the drought tolerance of barley varieties [10].
Biochar-dependent bacterial inoculum and arbuscular mycorrhizal fungi can help host plants increase water relationships and promote plant growth under drought stress [11,12]. Exogenous application of putrescine can enhance the salt tolerance of guava and Luffa acutangula seedlings by significantly increasing antioxidant enzyme activity [13,14]. Exogenous melatonin improved the photosynthetic efficiency and antioxidant defense system of maize seedlings under salt stress [15]. Exogenous application of aminolevulinic acid and nitric oxide showed a significant increase in drought tolerance by up-regulating the oxidative defense system in canola plants [16].

5-aminolevulinic acid (ALA) is a plant growth regulator (PGR) that is present in all plants and has a variety of biological activities. ALA is a key precursor of porphyrin biosynthesis, such as chlorophyll, heme, and cytochrome [17,18]. Previous studies indicated that ALA was involved in several key physiological processes by promoting primary root elongation [19], including the promotion of seed germination [20], promoting plant biomass accumulation [21], improving photosynthesis [22,23], contributing to plastid-to-nucleus signaling [24], and inhibiting ABA-induced stomatal closure [25]. Notably, ALA is crucial for the response of plants to abiotic stress [26]. Previous studies have demonstrated that pre-spraying of ALA enhanced plant tolerance to various abiotic stresses, such as cold stress [27,28], chilling stress [29], waterlogging [17], heat stress [30], salinity [31] and water deficit stress [32]. For instance, ALA increased antioxidant enzyme activity and non-enzyme antioxidant levels in Kentucky bluegrass (Poa pratensis L.) by promoting drought tolerance [33]. ALA pretreatment significantly increased total chlorophyll and antioxidative activities in rice under NaCl stress [21]. Moreover, the application of ALA enhanced photosynthesis, antioxidant enzyme activity and accumulation of proline in tomato seedlings under NaCl stress [34]. ALA significantly increased salinity and drought tolerance in pakchoi (Brassica campestris) [19], cotton (Gossypium spp.) [35], potato (Solanum tuberosum) [36], date palm (Phoenix dactylifera) [37], oilseed rape (Brassica napus) [38,39], sunflower [40] and wheat [41].

The exogenous application of ALA is an effective means to resist various abiotic stresses in higher plants. However, there are minimal reports evaluating the effects of the application of ALA on poplar under drought and salt stresses. Therefore, in the current study, we evaluated the effects of exogenous ALA on the morphological, physiological and biochemical responses of poplar induced by drought and salt stresses. The results of these experiments are helpful for understanding the mechanism of ALA-induced stress tolerance in poplars. At the same time, they provide a new strategy for enhancing plant resistance. This study provides theoretical support for increasing synthesis/accumulation of ALA by genetic manipulation of plants to enhance stress resistance.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

One-year-old seedlings of NE-19 (Populus nigra × (Populus deltoides × Populus nigra)) were transplanted into individual pots (10 L) containing sandy soil (approximately 70% sand) and placed in a phytotron artificial climate chamber (photoperiod: 16 h/8 h, light/dark; temperature: 24 °C/20 °C light/dark; relative humidity: 60%) at Haidian, Beijing, China (40.000 N, 116.200 E; 49 m above sea level). Before treatment, the plants were irrigated with 1 L of Hoagland nutrient solution every 2 weeks for 2 months. ALA treatment was performed by spraying the leaves with 25 mg/L and 100 mg/L ALA solution with 1 g TWEEN 40 (Sigma-Aldrich, St. Louis, MO, USA). Meanwhile, the control plants were treated with 1 g TWEEN 40 in ultrapure water and sprayed on the leaves of NE19. ALA was sprayed on all the leaves of each seedling. When the back of the leaf began to drip, the spraying was stopped. Spraying was conducted after the light was turned off in the chamber. Plants were randomly divided into nine groups for subsequent treatments: (1) C, control without ALA; (2) C+A25, control of pretreatment with 25 mg/L ALA; (3) C+A100, control of pretreatment with 100 mg/L ALA; (4) D, drought treatment without ALA; (5) D+A25, drought treatment of pretreatment with 25 mg/L ALA; (6) D+A100, drought treat-
ment of pretreatment with 100 mg/L ALA; (7) S, 350 mM NaCl treatment without ALA; (8) S+A25, salt treatment of pretreatment with 25 mg/L ALA; and (9) S+A100, salt treatment of pretreatment with 100 mg/L ALA. ALA pretreatment was carried out by spraying all of the poplar leaves of each pot with two concentrations of ALA solution, and the same amount of water was sprayed into each pot of the control group. The drought and salt treatments were started on the fourth day after pre-spraying. The treatment concentrations were based on pre-experimental studies. Two concentration gradients, 25 mg/L and 100 mg/L ALA, were finally used. Leaves were sampled for analysis at 0, 4, 8, and 12 days after different treatments.

2.2. Short-Term Drought Experiments

The drought experiment was designed in a completely randomized block. The control group was watered normally. The treatment group was naturally arid. The water was stopped for 12 days after the spraying ALA for 4 days. The time of the last watering before treatment was recorded as the 0th day. Sampling and measuring occurred on 0th, 4th, 8th, and 12th days of the experiment.

2.3. Salt Treatment

After spraying ALA for 4 days, the control group was watered normally, while the treatment group was watered with NaCl solution (350 mM) by root watering. The 350 mM NaCl solution was given once and then the plants were incubated under normal conditions (photoperiod: 16 h/8 h, light/dark; temperature: 24 °C/20 °C, light/dark; relative humidity: 60%). Observation and sampling were the same as in the drought treatment.

2.4. Measurement of RWC in Poplar Leaves

The relative water contents (RWC) were determined. In brief, fresh leaves were weighed quickly and incubated in double distilled water for 24 h, then turgid weight was taken. The samples were placed at 105 °C for 15 min and then at 80 °C for 2 days until the dry weights were measured. The RWC was calculated using the formula RWC (%) = (fresh weight − dry weight)/(turgid weight − dry weight) × 100.

2.5. Malonaldehyde Concentration and Electrolyte Leakage

Malonaldehyde content was measured using a Plant Malondialdehyde (MDA) Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The poplar leaves (0.2 g) were cut into small pieces and added to 9 times the volume of reagent working extraction solution, according to a mass−volume ratio of 1:9; homogenate was made by an incision homogenizer in an icewater bath, spun at 8000 rpm 3 times (10 s for each time, interval of 30 s), and then the homogenate was transferred to a centrifuge tube and centrifuged at 3500 rpm for 10 min, creating supernatant for the assay. Then, reagents were added and mixed sufficiently by vortex, placed in a 95 °C water bath for 20 min, and cooled in tap water. Following this, we adjusted the enzyme-labeled instrument’s wavelength to 530 nm and measured OD values of the wells. The levels of MDA were expressed as (nmol/g).

Sampling was performed using a puncher. The leaf discs were thoroughly cleaned in distilled water and then the leaf discs were placed in 5 mL of double distilled water; the air was evacuated for 30 min using a vacuum pump. After 20 h at 20 °C, a lightning magnetic conductivity meter DDS-307 (Shanghai INESA Scientific Instrument Co., Ltd, Shanghai, China) was used to determine the initial conductance value, denoted as C0. Then, we diluted the leaves in boiling water for 20 min and then measured the final conductivity after cooling completely, denoted as C1; and the formula is EL = C0/C1 * 100% [42].
2.6. Chlorophyll

To determine total chlorophyll, a punch with a diameter of 6 mm was used to obtain fresh leaf samples. A UV/visible spectrophotometer Ultrospec™ 3100 pro (GE healthcare UK Ltd., Little Chalfont, England) was used to measure the absorbance of chlorophyll a at 663 nm and chlorophyll b at 645 nm. Chlorophyll a concentration was calculated using the formula \( C_a = 12.7A_{663} - 2.69A_{645} \). Chlorophyll b was calculated using the formula \( C_b = 22.9A_{645} - 4.68A_{663} \). Total chlorophyll concentration was defined as \( C_a + C_b \). The chlorophyll content per unit was calculated by the formula \( C = \frac{(C_a + C_b) \times V}{A \times 1000} \). (C: chlorophyll concentration (mg/L); V: total volume of extract (ml); A: sampling area (cm²)) [43,44].

2.7. Physiological Analysis

Net CO₂ assimilation, stomatal conductance and transpiration rate were measured by using the LI-6400 photosynthesis system (LI-COR 6400, Lincoln, NE, USA). The fluorescence parameters including \( Fv/Fm \), \( \Phi_Y \) (II), and ETR (II), were measured using DualPAM100 (Walz Heinz GmbH, Effeltrich, Germany) after dark and were adapted for 30 min.

2.8. Measurement of Proline Content and Antioxidant Enzyme Activities

The content of proline was obtained as described earlier [17]. For enzyme activity, the leaves (0.2 g) were ground in an ice-cold phosphate buffer solution (PBS, pH 7.8) before measurement. SOD activity was measured as described earlier [42]. The amount of enzyme required to obtain 50% SOD inhibition in 1 mL of reaction mixture was considered as one SOD activity unit (U). POD activity was measured using a Peroxidase Assay Kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer’s instructions. The levels of POD were expressed as (U/mgport).

2.9. Total RNA Isolation and Gene Expression Analysis

Total RNA was extracted by a plant RNA kit (OMEGA Bio-Tek, Inc., Norcross, GA, USA), and ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Kureha America Co., Ltd., Cincinnati, OH, USA) was used for reverse transcription; the final concentration was 100 ng/ul for subsequent real-time fluorescence quantification. Quantitative (qRT-PCR) was performed in 96-well plates, using Tli RNaseH plus ((Takara Biotechnology Co., Ltd, Dalian, China). The reaction was amplified for 45 cycles at 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 30 s using a qPCR Bio-Rad CFX Connect Real-Time System. Candidate genes were selected based on their known role in drought and salt stresses. Aquaporins (AQP) genes (\( NIP1, PIP2, TIP1, \) and \( TIP2 \)) had been confirmed to be involved in salt-induced water stress [45]. It had been confirmed that the \( NHX1 \) gene participated in the salt response process; the \( SOS1 \) gene is a critical gene in the salt overly sensitive (SOS) pathway (one of the signal transduction pathways that responds to salt stress in plants) [46]. Transcript levels of all candidate genes were determined using the \( 2^{-\Delta\DeltaCT} \) method. \( UBQ \) (ubiquitin) was used as an endogenous reference gene for data standardization [47]. Primers were designed using Primer6 (PRIMERE, Ivybridge, UK) software and are listed in Table 1.

Table 1. Primers used in this research.

| Gene   | Forward Primer (5’-3’)                  | Reverse Primer (5’-3’)                  |
|--------|----------------------------------------|----------------------------------------|
| UBQ    | AGACCTACACCAAGCCCAAGAAGAT              | CCAGCACCGCACTCAGCATATTAG               |
| NIP1   | GGCTGCTGTGATGTGTTCT                   | ATCCCGCTGATTGTTC                        |
| PIP2   | CACTGGATGTCGGCGCCTGA                 | TTGGTATGCTGACAGGCCTTCT                  |
| TIP1   | GAGCTTACAACACACACCCG                  | AGCAAGGACAGAGGAGGC                     |
| TIP2   | GCGACCTCTGTACCAGGCGGCCG              | ATGGGACGTGCGAAGCGT                     |
| NHX1   | CGGTGGATGAACAGAGGTCG                 | CCTATATCTAGGAGCCAGGAGTTC               |
| SOS1   | TCCCCGTGTGTAGGTTGGGA                 | GAAGTACGATCACCAGTGAAG                  |
2.10. Statistical Analysis

Statistical tests were performed with SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to compare the statistical difference in the mean among the plants under different treatments based on Duncan’s Multiple Range Test (DMRT) at a significance level of \( p \leq 0.05 \). Different lowercase letters indicate significant differences among mean values within the same (day of) treatment. Different capital letters indicate significant differences among the same type of plant while comparing different treatments.

3. Results and Discussion

3.1. ALA Alleviated Morphological Damage and Leaf RWC under Drought and Salt Stresses

Plant morphological analysis showed that the ALA alleviated morphological damage induced by drought and salt stresses. ALA had no significant influence on poplar morphology under normal conditions (Figure 1a). On the 12th day of drought treatment and the 8th day of salt treatment, leaves of the control poplars wilted, curled, and even fell off. ALA can slow leaf abscission under both normal and stress conditions. Compared with drought treatment and salt treatment groups, the pre-spraying ALA plants (D+ALA and S+ALA) had more green leaves (Figure 1b,c). Under salt treatment, the number of leaves in S+A25 was 32.87% higher than that of the S group (Figure 1d,e). This phenomenon showed that ALA had a better effect on plant growth under drought and salt stresses. ALA is a key precursor compound of tetrapyrrole and can be used as a plant growth regulator [38,48–50]. Previous studies have shown that ALA can effectively increase the tolerance in plants to various abiotic stresses including cold, drought and salt [51]. In this study, pretreatment with ALA effectively mitigated growth damage induced by drought and salt stresses (Figure 1). This is consistent with previous studies showing that foliar application of ALA mitigated the adverse impacts of drought and salt stresses in plants [52–54]. This study focused on ALA response to stresses. To our knowledge, this is the first report of ALA response to drought and salt stresses in poplar; thus, this study has practical reference significance.

The leaf RWC reflects the water status of plant tissues and is an important basis for the drought resistance of plants [55]. The leaf RWC was monitored during the whole experiment. Drought treatment for 12 days significantly reduced the RWC of poplar leaves. The pretreatment of ALA partially inhibited the decrease of RWC under drought conditions. On day 12, compared with D, C+A25 and C+A100 increased leaf RWC by 50.48% and 23.42%, respectively (Figure 1f). Similar results were obtained under salt stress (Figure 1g). Abiotic stress often leads to a decrease in RWC in plant leaves [17,56]. However, ALA pretreatment attenuated the decline in RWC in poplar leaves under drought and salt stresses. These results showed that ALA pretreatment significantly improved the water status and alleviated the damaging effects of drought and salt stresses on poplar plants.

3.2. ALA Reduced Leaf MDA and Electrolyte Leakage under Drought and Salt Stresses

To confirm the mitigation effect of ALA on the poplar under stresses, we determined the MDA content of the leaves. The content of MDA in leaves of control plants increased significantly under stresses, and up to 2.7-fold and 3.34-fold compared to control on the 8th day of drought stress and salt stresses, respectively. The result showed that pre-spraying ALA can reduce the content of MDA in poplar plants under drought and salt stresses (Figure 2a,b). The content of MDA in plants pretreated with 25 mg/L of ALA was similar to that in plants pretreated with 100 mg/L of ALA. These results indicated that the effect of ALA in buffering the increase in MDA content under drought and salt stress was not dose dependent. Furthermore, ALA pretreatment can inhibit the increase in MDA induced by drought and salt stresses. The electrolyte leakage of D and S group plants peaked at 12 days of the drought and salt treatments, at a level 2.2-fold higher than in the C group. However, the pretreatment ALA groups showed a significant reduction in electrolyte leakage (Figure 2c,d). MDA is an important product of membrane lipid peroxidation, which reflects the integrity of the cell membrane under stress [42,57,58].
Damage to the cell membrane can cause extravasation of plant electrolytes [59]. Our findings suggested that drought and salt stresses displayed a negative effect on membrane integrity, which may be the reason for the enhanced electrolyte leakage. Pretreatment with ALA decreased the electrolyte leakage in poplar leaves (Figure 2). These findings are in line with previous studies [18]. Abiotic stress can cause membrane damage in plants; the cell membrane changes from a normal fluid state to a restricted semi-crystalline state with low fluidity, and electrolyte leakage is therefore enhanced under stress [18,29,51]. Our current results showed that ALA pretreated plants have lower electrolyte leakage and higher RWC compared to the drought and salt treatment groups under drought and salt stresses (Figures 1 and 2). Generally, the content of MDA was used to evaluate the degree of oxidative damage to cell plasma membrane, and electrolyte leakage was a reflection of membrane stability [16,17]. Thus, exogenous ALA can reduce membrane oxidative damage and maintain cell membrane integrity under drought and salt stresses, thereby improving the ability of leaves to retain water.

Figure 1. Effect of ALA pretreatment on leaf morphology and RWC in poplar plants under drought and salt stresses. (a) Morphology of the poplar seedlings pretreated with or without ALA. Sprayed with 1 g Tween-40 deionized water for 4 days (control group) and with surfactant 25 mg/L ALA for 4 days (C+ALA). (b) Leaf morphology of the different treatment groups on the 12th day of drought. (c) Leaf morphology of the different treatment groups on the 8th day of salt. (d) Number of leaves on the 12th day of drought. (e) Number of leaves on the 8th day of salt. (f) RWC under drought stress. (g) RWC under salt stress. DMRT was carried out to determine the significance among different groups under different treatments. Means followed by different letters indicate significant differences at the $p \leq 0.05$ level. Different lowercase letters indicate significant differences among mean values within the same (day of) treatment. Different capital letters indicate significant differences among the same type of plant while comparing different treatments.
3.3. ALA Improved Chlorophyll Content in Poplar Leaves under Drought and Salt Stresses

Chlorophyll (Chl) plays a crucial role in photosynthesis. In particular, Chl is sensitive to abiotic stress and is easily degraded, resulting in reduced photosynthetic capacity [60,61]. We measured changes in chlorophyll content (Table 2). Chlorophyll content decreased significantly from the 4th day under drought and salt stresses, and these effects became more severe in the later stages of treatment. The results showed that the chlorophyll content of all poplars decreased after 12 days of drought and salt treatments. Compared with the D and S treatment groups, the chlorophyll content of C+A100 leaves increased 1.27-fold and 1.72-fold, respectively. These results suggested that the chlorophyll content of drought- and salt-stressed plants was lower than that of ALA pretreated plants, and ALA pretreatment reduced chlorophyll degradation under drought and salt stresses. Previous studies have found that ALA plays an important role in the rate of chlorophyll biosynthesis and photosynthesis [62,63]. In this study, consistent results were also obtained. Previous studies showed that, compared with control plants, plants pretreated with ALA improved the content of all types of pigments (Chl a, Chl b, total Chl and carotenoids) and increased the chloroplast length in both well-watered and salt stress conditions [31,34]; however, in the present study, the content of chlorophyll showed no significant change following ALA application at time d = 0. Microarray analysis indicated that ALA pretreatment of rice increased ALA-synthesizing capacity but did not result in a significant increase in chlorophyll content. The discrepancy may be due to the reduced levels of porphyrin intermediates or different growth conditions used in other studies [64]. Hence, the molecule mechanism of ALA involved in tetrapyrroles synthesis still requires further research.
Table 2. Chlorophyll content in poplar plants under different treatments. DMRT was carried out to determine the significance among different groups under different treatments. Means followed by different letters indicate significant differences at the $p \leq 0.05$ level. Different lowercase letters indicate significant differences among mean values within the same (day of) treatment. Different capital letters indicate significant differences among the same type of plant while comparing different treatments.

| Days | Treatments | Total Chlorophyll (mg/cm²) | Days | Treatments | Total Chlorophyll (mg/cm²) |
|------|------------|-----------------------------|------|------------|-----------------------------|
| 0    | C          | 0.033 ± 0.003 aA            | 0    | C          | 0.033 ± 0.003 bA            |
|      | C+A25      | 0.036 ± 0.000 aA            |      | C+A25      | 0.036 ± 0.000 bA            |
|      | C+A100     | 0.034 ± 0.002 aA            |      | C+A100     | 0.034 ± 0.002 bA            |
|      | D          | 0.033 ± 0.001 aA            |      | S          | 0.033 ± 0.001 bA            |
|      | D+A25      | 0.035 ± 0.004 aA            |      | S+A25      | 0.042 ± 0.003 aA            |
|      | D+A100     | 0.034 ± 0.001 aA            |      | S+A100     | 0.035 ± 0.001 bA            |
| 4    | C          | 0.032 ± 0.003 aA            | 4    | C          | 0.032 ± 0.003 abA           |
|      | C+A25      | 0.035 ± 0.002 aA            |      | C+A25      | 0.035 ± 0.002 bA            |
|      | C+A100     | 0.034 ± 0.002 aA            |      | C+A100     | 0.034 ± 0.002 bA            |
|      | D          | 0.026 ± 0.001 cB            |      | S          | 0.026 ± 0.002 cB            |
|      | D+A25      | 0.029 ± 0.002 bB            |      | S+A25      | 0.030 ± 0.000 abcB          |
|      | D+A100     | 0.027 ± 0.002 bCB           |      | S+A100     | 0.029 ± 0.002 bCB           |
| 8    | C          | 0.030 ± 0.002 abA           | 8    | C          | 0.030 ± 0.002 abA           |
|      | C+A25      | 0.031 ± 0.002 abA           |      | C+A25      | 0.031 ± 0.002 abA           |
|      | C+A100     | 0.032 ± 0.001 aA            |      | C+A100     | 0.032 ± 0.001 aA            |
|      | D          | 0.026 ± 0.002 cB            |      | S          | 0.020 ± 0.002 dC            |
|      | D+A25      | 0.029 ± 0.001 bB            |      | S+A25      | 0.028 ± 0.001 bCB           |
|      | D+A100     | 0.029 ± 0.002 bCB           |      | S+A100     | 0.027 ± 0.002 bCB           |
| 12   | C          | 0.032 ± 0.002 abA           | 12   | C          | 0.032 ± 0.002 aA            |
|      | C+A25      | 0.032 ± 0.002 abA           |      | C+A25      | 0.032 ± 0.002 abA           |
|      | C+A100     | 0.032 ± 0.000 aA            |      | C+A100     | 0.032 ± 0.000 aA            |
|      | D          | 0.020 ± 0.001 dC            |      | S          | 0.014 ± 0.001 dD            |
|      | D+A25      | 0.029 ± 0.000 eD            |      | S+A25      | 0.021 ± 0.001 eC            |
|      | D+A100     | 0.026 ± 0.002 bCB           |      | S+A100     | 0.024 ± 0.005 bCB           |

3.4. ALA Increased Photosynthetic Capacity under Drought and Salt Stresses

Many studies reported that biochemical changes in leaves led to a decrease in net photosynthetic rate under stress [65–67]. The net photosynthetic rate, stomatal conductance, and transpiration rate were monitored in vivo. Under drought and salt stresses, the pre-spraying of ALA on plants showed a higher net photosynthetic rate than that found in D and S group plants. On the 4th day, ALA pretreatment increased the stomatal conductance and transpiration rate of plants under drought and salt stresses. On the 8th day of drought and salt stresses, the net photosynthetic rates of the D and S groups were significantly lower than the C group by 99.05% and 83.25%, respectively (Tables 3 and 4). Thus, on the 8th day of salt treatment, the net photosynthetic rate was approximately 2.5-fold higher in S+A25 plants than in S treatment plants. After drought and salt treatments for 8 days, the pretreated ALA poplars showed higher photosynthetic efficiency than the C plants, which further verified that the pretreated ALA poplars are more tolerant to drought and salt stresses. Interestingly, the different response of net CO₂ assimilation rate to drought and salt stresses could be somehow connected to osmotic adjustments that characterize drought (rather than salt stress). Several studies showed that stress may inhibit the transfer of excitation energy from the light-catching chlorophyll-protein complex to PSII, causing interference in the ETR of PSII and leading to significant damage to photosynthetic biochemistry [34,67]. The genes involved in photosynthesis were up-regulated in ALA pretreated plants under osmotic stress [63,68]. Similarly, ALA significantly increased the photosynthetic rate in other species; this finding is supported by [22,69,70]. These results revealed that the application of ALA maintains the photosynthetic capacity in poplar seedlings under various stresses.
**Table 3.** Effect of ALA pretreatment on the net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (Tr) of poplar plants under drought stress. DMRT was carried out to determine the significance among different groups under different treatments. Means followed by different letters indicate significant differences at the $p \leq 0.05$ level. Different lowercase letters indicate significant differences among mean values within the same (day of) treatment. Different capital letters indicate significant differences among the same type of plant while comparing different treatments.

| Days | Treatments | Pn ($\mu$mol m$^{-2}$ s$^{-1}$) | Gs (mol m$^{-2}$ s$^{-1}$) | Tr (mol m$^{-2}$ s$^{-1}$) |
|------|------------|---------------------------------|----------------------------|-----------------------------|
| 0    | C          | 16.680 ± 0.751 aA               | 0.496 ± 0.069 aA           | 9.540 ± 1.233 bA            |
|      | C+A25      | 17.367 ± 0.535 bA               | 0.517 ± 0.101 aA           | 9.972 ± 1.297 bA            |
|      | C+A100     | 17.308 ± 1.435 bA               | 0.497 ± 0.031 aA           | 9.920 ± 0.974 bA            |
|      | D          | 18.063 ± 0.270 bC               | 0.483 ± 0.069 aA           | 9.210 ± 1.487 bA            |
|      | D+A25      | 18.083 ± 0.397 aA               | 0.487 ± 0.027 aA           | 10.797 ± 0.933 abA          |
|      | D+A100     | 18.08 ± 0.437 abA               | 0.487 ± 0.040 aA           | 11.067 ± 0.323 aA           |
| 4    | C          | 13.842 ± 0.33 dBb                | 0.510 ± 0.016 aA           | 6.451 ± 0.123 bB            |
|      | C+A25      | 13.992 ± 0.156 abb               | 0.524 ± 0.016 aA           | 6.469 ± 0.106 ab             |
|      | C+A100     | 14.167 ± 0.329 ab                | 0.517 ± 0.030 aA           | 6.033 ± 0.137 ab            |
|      | D          | 10.475 ± 0.845 cB                | 0.276 ± 0.044 dB           | 3.813 ± 0.181 cB            |
|      | D+A25      | 14.108 ± 0.131 ab                | 0.477 ± 0.043 bA           | 5.237 ± 0.356 bB            |
|      | D+A100     | 13.992 ± 0.808 aB                | 0.396 ± 0.034 cB           | 5.096 ± 0.235 bB            |
| 8    | C          | 13.667 ± 0.841 bb                | 0.465 ± 0.074 bA           | 9.363 ± 0.771 abA           |
|      | C+A25      | 13.933 ± 0.342 bb                | 0.523 ± 0.046 aA           | 9.599 ± 0.416 aA            |
|      | C+A100     | 14.575 ± 0.253 ab                | 0.461 ± 0.012 bB           | 9.077 ± 0.115 bA            |
|      | D          | 0.133 ± 0.040 dC                 | 0.010 ± 0.001 cC           | 0.419 ± 0.027 cc            |
|      | D+A25      | 0.768 ± 0.034 cc                 | 0.011 ± 0.001 cB           | 0.518 ± 0.086 cc            |
|      | D+A100     | 0.207 ± 0.024 dC                 | 0.009 ± 0.002 cC           | 0.417 ± 0.067 cC            |

**Table 4.** Effect of ALA pretreatment on the net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (Tr) of poplar plants under salt stress. DMRT was carried out to determine the significance among different groups under different treatments. Means followed by different letters indicate significant differences at the $p \leq 0.05$ level. Different lowercase letters indicate significant differences among mean values within the same (day of) treatment. Different capital letters indicate significant differences among the same type of plant while comparing different treatments.

| Days | Treatments | Pn ($\mu$mol m$^{-2}$ s$^{-1}$) | Gs (mol m$^{-2}$ s$^{-1}$) | Tr (mol m$^{-2}$ s$^{-1}$) |
|------|------------|---------------------------------|----------------------------|-----------------------------|
| 0    | C          | 16.68 ± 0.751 aA                | 0.496 ± 0.069 aA           | 9.540 ± 1.233 bA            |
|      | C+A25      | 17.367 ± 0.535 bA               | 0.517 ± 0.101 aA           | 9.972 ± 1.297 bA            |
|      | C+A100     | 17.308 ± 1.435 bA               | 0.497 ± 0.031 aA           | 9.920 ± 0.974 bA            |
|      | S          | 17.375 ± 0.357 bA               | 0.447 ± 0.011 bA           | 9.855 ± 1.044 aA            |
|      | S+A25      | 18.642 ± 0.458 aA               | 0.491 ± 0.070 aA           | 10.967 ± 0.689 abA          |
|      | S+A100     | 18.013 ± 1.298 bA               | 0.495 ± 0.020 aA           | 10.367 ± 0.065 aA           |
| 4    | C          | 13.842 ± 0.334 bb                | 0.510 ± 0.016 aA           | 6.451 ± 0.123 bB            |
|      | C+A25      | 13.992 ± 0.156 abb               | 0.524 ± 0.016 aA           | 6.469 ± 0.106 ab             |
|      | C+A100     | 14.167 ± 0.329 ab                | 0.517 ± 0.030 aA           | 6.033 ± 0.137 ab            |
|      | S          | 10.468 ± 1.824 cB                | 0.148 ± 0.050 cB           | 2.864 ± 0.742 cB            |
|      | S+A25      | 11.200 ± 0.432 cB                | 0.224 ± 0.066 bB           | 3.978 ± 0.854 bB            |
|      | S+A100     | 11.063 ± 1.725 cB                | 0.150 ± 0.061 bB           | 2.542 ± 0.989 cB            |
| 8    | C          | 13.667 ± 0.841 bb                | 0.465 ± 0.074 bA           | 9.363 ± 0.771 abA           |
|      | C+A25      | 13.933 ± 0.342 bb                | 0.523 ± 0.046 aA           | 9.599 ± 0.416 aA            |
|      | C+A100     | 14.575 ± 0.253 ab                | 0.461 ± 0.012 bB           | 9.077 ± 0.115 bA            |
|      | S          | 2.292 ± 0.885 dC                 | 0.021 ± 0.008 cC           | 0.759 ± 0.267 dc            |
|      | S+A25      | 5.647 ± 0.950 cc                 | 0.048 ± 0.012 cC           | 1.756 ± 0.374 cc            |
|      | S+A100     | 5.500 ± 0.365 cc                 | 0.046 ± 0.006 cC           | 1.753 ± 0.210 cc            |
3.5. ALA Increased Fluorescence Parameters under Drought and Salt Stresses

Stress poses a serious threat to photosystem II efficiency and the electron transport system [71,72]. Fluorescence parameters were monitored in vivo. Under drought and salt stresses for 8 days, the Fv/Fm of D+A25 and S+A25 groups was significantly higher than that of the D and S poplars, respectively (Tables 5 and 6). Higher Fv/Fm indicated that light-use efficiency of the pretreated ALA plants increased under stress conditions, and explained why the pretreated ALA plants had a higher photosynthetic rate than the drought and salt treatment plants. Under normal conditions, pre-spraying ALA significantly promoted PSII and ETR for photosynthesis [61]. Our data showed that ALA pretreatment increased PSII and ETRII under normal conditions (Tables 5 and 6). In combination with CO₂ assimilation/photosynthesis genes were significantly regulated by ALA [18,23,33]. We therefore concluded that ALA had a positive effect on photosynthesis (Tables 3 and 4). Under stress conditions, ALA treatment protected the photosynthetic apparatus and improved the ETR [21,73]. The PSII reaction center is the main site of stress injury, and osmotic stress had a negative impact on the electron transfer activities of the PSII reaction center, while ALA increased the expression of relative genes in the PSII reaction center core protein complex and repaired the PSII reaction center core proteins in strawberry leaves [74]. The levels of YII and ETRII were higher in pre-spraying ALA plants than in control plants in normal conditions, indicating that ALA pretreatment improved photosynthetic performance in PSII reaction centers. On the 4th day, the Fv/Fm of D group decreased to 0.786 under drought conditions, which was significantly lower than in D+A25. After 8 days of drought treatment, the YII and ETRII of C+A25 were 9.8% and 10.04% higher than in D plants, respectively. Under salt stress conditions, the YII and ETRII of C+A100 were 14.81% and 15.16% higher in S group plants, respectively. Although drought and salt stresses affect plant photosystem II electron transport and reduce chlorophyll content, the pretreated ALA poplars exhibited higher photosystem II efficiency than drought and salt treatment poplars did (Tables 5 and 6), leading to less injury under stress conditions. Our results showed that ALA pretreatment increased the ETR of S+A25 and S+A100 plants after 8 days salt treatment (Table 6); this explains the higher photosynthetic performances of these plants with respect to those not sprayed with ALA. Fv/Fm is widely used to assess the effect of stress on the photosynthetic efficiency of plants, and the increase in chlorophyll fluorescence is beneficial to photosynthesis [17,18,21]. However, ALA pretreatment did not improve the stomatal conductance under drought stress, but Fv/Fm increased and then slightly improved photosynthetic CO₂ assimilation of ALA pretreated plants (Tables 3 and 5). Overall, these results indicated that the photochemistry of photosynthesis was damaged by drought and salt stresses, and ALA pretreatment resulted in less damage to the photosynthetic system and improved the drought and salt tolerance of poplar seedlings.

3.6. ALA Increased PRO Content under Drought and Salt Stresses

Proline is usually accumulated in large amounts under stress, and many studies suggest it plays an important role in osmotic adjustment. However, whether the accumulation of proline under stress is related to osmotic tolerance or is merely a response to plant injury has been controversial [75–77]. The content of PRO showed an overall increasing trend while, in pretreatment, ALA poplars increased faster than those in D and S groups under drought and salt stresses. After 8 days of drought treatment, the contents of PRO in D+A25 were 26.73% and 17.55% higher than those of C and D poplars, respectively. Additionally, on the 8th day of salt treatment, the contents of PRO in D+A25 were 30% and 10.47% higher than those of C and D poplars, respectively. In this study, a significant increase in proline accumulation in all plants was observed under stresses (Figure 3a,b), as reported in leaves of Brassica napus L. [18]. Previous studies showed that exogenous ALA significantly increased the content of proline by regulating the expression of delta-1-pyrroline-5-carboxylate synthase (P5CS) and proline dehydrogenase (ProDH) in tomato seedlings under salt stress [34]. Our results showed that ALA might be involved in proline accumulation under drought and salt stresses. The accumulation of proline contributed to
the osmotic adjustment of plants under stress [16]. Therefore, plants pretreated with ALA were more tolerant to drought and salt stresses.

Table 5. Effect of ALA pretreatment on Fv/Fm, Y (II) and ETR (II) of poplar plants under drought stress. DMRT was carried out to determine the significance among different groups under different treatments. Means followed by different letters indicate significant differences at the $p \leq 0.05$ level. Different lowercase letters indicate significant differences among mean values within the same (day of) treatment. Different capital letters indicate significant differences among the same type of plant while comparing different treatments.

| Days | Treatments | Fv/Fm   | Y(II)   | ETR(II)   |
|------|------------|---------|---------|-----------|
| 0    | C          | 0.822 ± 0.003 aB | 0.636 ± 0.009 bA | 57.133 ± 0.802 bA |
|      | C+A25      | 0.828 ± 0.003 aA | 0.668 ± 0.003 aA | 59.500 ± 0.300 aA |
|      | C+A100     | 0.823 ± 0.002 aA | 0.677 ± 0.008 aA | 57.700 ± 1.000 aA |
|      | D          | 0.821 ± 0.004 aA | 0.637 ± 0.013 bA | 60.067 ± 0.252 bA |
|      | D+A25      | 0.822 ± 0.002 aB | 0.669 ± 0.023 aA | 59.800 ± 0.300 aA |
|      | D+A100     | 0.826 ± 0.007 aA | 0.672 ± 0.007 aA | 60.067 ± 0.252 bA |
| 4    | C          | 0.836 ± 0.007 abA | 0.662 ± 0.004 bA | 59.967 ± 0.651 baA |
|      | C+A25      | 0.846 ± 0.004 aA | 0.665 ± 0.004 aA | 60.067 ± 0.252 aA |
|      | C+A100     | 0.840 ± 0.010 abA | 0.668 ± 0.003 aA | 60.167 ± 0.551 aA |
|      | D          | 0.786 ± 0.012 cB | 0.614 ± 0.008 bB | 57.233 ± 1.102 bB |
|      | D+A25      | 0.834 ± 0.004 abA | 0.673 ± 0.005 aA | 55.100 ± 0.702 aA |
|      | D+A100     | 0.826 ± 0.008 ba | 0.665 ± 0.006 aA | 54.767 ± 0.702 aA |
| 8    | C          | 0.833 ± 0.003 aa | 0.642 ± 0.011 bA | 60.000 ± 1.967 bA |
|      | C+A25      | 0.838 ± 0.002 aa | 0.669 ± 0.003 aA | 60.500 ± 0.451 aA |
|      | C+A100     | 0.835 ± 0.015 aa | 0.670 ± 0.006 aA | 60.267 ± 0.451 aA |
|      | D          | 0.785 ± 0.027 bB | 0.610 ± 0.008 bB | 60.400 ± 0.656 cB |
|      | D+A25      | 0.818 ± 0.006 ab | 0.670 ± 0.005 aA | 59.800 ± 0.500 aA |
|      | D+A100     | 0.817 ± 0.026 aA | 0.639 ± 0.033 bA | 57.933 ± 2.268 bA |

Table 6. Effect of ALA pretreatment on Fv/Fm, Y (II) and ETR (II) of poplar plants under salt stress. DMRT was carried out to determine the significance among different groups under different treatments. Means followed by different letters indicate significant differences at the $p \leq 0.05$ level. Different lowercase letters indicate significant differences among mean values within the same (day of) treatment. Different capital letters indicate significant differences among the same type of plant while comparing different treatments.

| Days | Treatments | Fv/Fm   | Y(II)   | ETR(II)   |
|------|------------|---------|---------|-----------|
| 0    | C          | 0.822 ± 0.003 aB | 0.636 ± 0.009 bA | 57.133 ± 0.802 bA |
|      | C+A25      | 0.828 ± 0.003 aA | 0.668 ± 0.003 aA | 59.500 ± 0.300 aA |
|      | C+A100     | 0.823 ± 0.002 aA | 0.677 ± 0.008 aA | 57.700 ± 1.000 aA |
|      | S          | 0.822 ± 0.002 aA | 0.669 ± 0.008 aA | 59.967 ± 0.651 baA |
|      | S+A25      | 0.831 ± 0.006 ab | 0.630 ± 0.003 aA | 61.500 ± 0.300 aA |
|      | S+A100     | 0.827 ± 0.004 ab | 0.668 ± 0.008 ba | 60.033 ± 0.702 bA |
| 4    | C          | 0.836 ± 0.007 abA | 0.662 ± 0.004 bA | 59.500 ± 0.300 bA |
|      | C+A25      | 0.846 ± 0.000 aa | 0.665 ± 0.004 aA | 59.800 ± 0.300 aA |
|      | C+A100     | 0.840 ± 0.010 abA | 0.668 ± 0.003 aA | 60.067 ± 0.252 aA |
|      | S          | 0.812 ± 0.011 bAB | 0.539 ± 0.007 cB | 53.633 ± 0.351 cB |
|      | S+A25      | 0.815 ± 0.001 bB | 0.544 ± 0.013 cB | 50.550 ± 1.150 bB |
|      | S+A100     | 0.812 ± 0.006 bB | 0.615 ± 0.006 bB | 55.950 ± 0.212 bB |
| 8    | C          | 0.833 ± 0.003 aA | 0.642 ± 0.011 bA | 57.700 ± 1.000 bA |
|      | C+A25      | 0.838 ± 0.002 aa | 0.668 ± 0.003 aA | 60.067 ± 0.252 aA |
|      | C+A100     | 0.835 ± 0.015 aa | 0.670 ± 0.006 aA | 60.167 ± 0.551 aA |
|      | S          | 0.773 ± 0.035 bB | 0.536 ± 0.005 eB | 48.433 ± 0.651 cC |
|      | S+A25      | 0.823 ± 0.020 aAB | 0.556 ± 0.013 dB | 49.967 ± 1.150 dB |
|      | S+A100     | 0.786 ± 0.011 bc | 0.620 ± 0.004 eB | 55.767 ± 0.351 cB |
3.7. ALA Enhanced SOD and POD Activities under Drought and Salt Stresses

Drought and salt stresses lead to excessive reactive oxygen species (ROS) production in plant cells, causing oxidative stress and impairing plant growth and development [78]. Studies have reported that ALA improved the antioxidant capacity of various plants under stress, and protected plants from oxidative stress [30,32,68]. In the present study, under normal conditions, there was no difference in leaf SOD activity between the control group and the pre-spraying ALA group. On the 8th day of drought and salt stresses, the SOD activity of all poplars increased, and the ALA pretreatment groups were higher than those in the D and S groups. The activities of SOD in D+A100 and S+A100 were 17.48% and 17.34% higher than in the D group, respectively (Figure 4a,b). POD activity showed the same trend, which was significantly increased on the 4th day, and this effect persisted on the 8th day. On day 4, the activities of POD in D+A25 and S+A25 were 16.86% and 14.36% higher than in S treatment plants, respectively (Figure 4c,d). These results indicated that pre-spraying ALA on the leaf’s surface had little effect on the enzyme activity of plants grown under normal conditions, but significantly increased the SOD and POD activities of leaves under stress. These results are in accordance with those of previous studies, such as those examining cucumber [79], pepper [29], melon seedlings [22], and maize [78] under different stresses. Plants producing more antioxidative enzymes show better resistance to oxidative damage from ROS [21]; this is in accordance with the current study, where the activity of SOD and POD was significantly enhanced in drought and salt stresses. The reason why exogenous ALA enhanced the activity of antioxidant enzymes may be that ALA is an indispensable precursor of heme synthesis, so it can improve the activity of heme molecules such as superoxide dismutase [32]. This indicates that ALA can improve plant growth and enhance plant stress resistance by increasing proline accumulation and the protective enzyme system in poplar plants (Figures 3 and 4).
3.8. ALA Regulated the Expression of Aquaporins and Na\(^+\)/H\(^+\) Antiporter Proteins under Salt Stresses

Previous studies indicated that salt stress caused osmotic and ion toxicity (Na\(^+\) stress) effects on cells [4,80]. AQPs are channel proteins that facilitate the transcellular membrane movement of intercellular water and play an important role in plant response to salt stress [57,81,82]. The expression of AQP genes was regulated under salt stress [83]. Na\(^+\)/H\(^+\) antiporter proteins play an important role in salt stress resistance, which can greatly reduce the osmotic potential of the vacuole and reduce the damage of Na\(^+\) to the cytoplasm [46]. Therefore, further research is needed to study the expression pattern of the aquaporin gene to determine the relationship between ALA and water transport. As can be seen from the figures, exogenous ALA treatment can significantly induce the expression of aquaporins (NIP1, PIP2, TIP1 and TIP2) and Na\(^+\)/H\(^+\) antiporters (NHX1 and SOS1) in poplar leaves under salt stress (Figure 5a–f). The expression trend of different aquaporins genes has similar trends. After 8 days of salt treatment, the expression of aquaporin genes...
in the C+A25 group was significantly up-regulated compared to the C group by up to 1860 times (Figure 5a–d). The relative expression of aquaporin genes showed that the TIP1 and TIP2 genes in the tonoplast were significantly increased by ALA compared with NIP1 and PIP2 in the plasma membrane. Previous studies showed that the PIP and TIP families can activate water channels in *Xenopus oocytes* [83]. Moreover, NIPs also play an important role in maintaining the water balance of plants in response to drought and salt stresses [45]. The expression of AQPs in S+A25 plants was up-regulated under salt treatment, which can activate the water channel regulation to increase water permeability and maintain the water status of poplars. To explore the mechanism behind ALA-induced Na\(^{+}\) metabolism in leaves under salt stress, the expressions of SOS1 and NHX1 were analyzed by qRT-PCR. On the 4th day of salt stress, the expression of NHX1 in the vacuolar membrane increased, and then decreased with the time of salt treatment. Until day 8, the expression of the NHX1 gene in the S+A25 group dropped to a normal level (Figure 5e). On the 8th day of salt stress, SOS1 located in the plasma membrane was significantly induced in the S+A25 group, and the relative expression of the gene was 4.76 times higher than that of the S group (Figure 5f). Vacuoles play an important role in maintaining the turgor pressure of plant cells. Vacuolar partitioning of Na\(^{+}\) is a main adaptive strategy for reducing cytoplasmic Na\(^{+}\) toxicity in plants under salt stress [46,84]. These results suggest that ALA may limit the loss of water in the cells by partitioning Na\(^{+}\) into the vacuole at the early stage of stress. As stress increases, the plant cells will efflux excess Na\(^{+}\) from the cytoplasm through the SOS pathway, greatly reducing the osmotic stress of the vacuole and reducing the damage of Na\(^{+}\) to the cytoplasm. At the same time, ALA can also enhance the water absorption capacity of plants and improve the tolerance of poplar to salt stress. In the present study, we explored the expression of aquaporin genes under salt stress in leaves of poplars. However, the expression patterns of AQPs family members under ALA treatment and drought stress remain unclear. Further studies are needed to elucidate the molecular mechanism of ALA alleviating drought stress in poplars.

### 3.9. Model for the Mechanisms of ALA-Induced Stress Resistance

Overall, ALA pretreatment can alleviate the damage induced by drought and salt stresses in poplars. The results of the present study revealed that ALA alleviated the damage of drought and salt stresses by maintaining leaf water content, enhancing photosynthetic efficiency, and improving the oxidative defense system. In addition, the alleviated effect of ALA pretreated poplars under salt stress was attributed to the intracellular ion homeostasis regulated by the SOS pathway. Hence, we concluded that ALA pretreatment can improve poplar tolerance to drought and salt stresses (Figure 6).
Figure 5. Effect of ALA pretreatment on the relative expression of aquaporins and Na⁺/H⁺ antiporter proteins under salt stress: (a) expression levels of NIP1; (b) expression levels of PIP2; (c) expression levels of TIP1; (d) expression levels of TIP2; (e) expression levels of NHX1; (f) expression levels of SOS1. DMRT was carried out to determine the significance among different groups under different treatments. Means followed by different letters indicate significant differences at the $p \leq 0.05$ level. Different lowercase letters indicate significant differences among mean values within the same (day of) treatment. Different capital letters indicate significant differences among the same type of plant while comparing different treatments.
4. Conclusions

In conclusion, this study provides preliminary evidence that the exogenous application of 5-ALA enhanced drought and salt tolerance in poplars. Our study also provides insights into the physiological mechanisms induced by ALA in plants under drought and salt stresses. However, further studies are required to determine the regulatory mechanism of ALA in its alleviation of stress in poplars at the molecular level.

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