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Exogenous Easily Extractable Glomalin-Related Soil Protein Induces Differential Response in Plant Growth of Tea Plants via Regulating Water Channel Protein Expression

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Abstract: Glomalin, a glycoprotein secreted by arbuscular mycorrhizal fungi (AMFs), exhibits multiple beneficial functions in regard to plant growth. However, the roles and regulatory mechanisms of exogenous easily extractable glomalin-related soil protein (EE-GRSP) in water and their effects on the quality of tea plants (*Camellia sinensis* (L.) O. Ktze.) remain unclear. The present study aimed to investigate the effects of a quarter-strength exogenous EE-GRSP solution (1/4 EE-GRSP), half-strength exogenous EE-GRSP solution (1/2 EE-GRSP), three-quarter-strength exogenous EE-GRSP solution (3/4 EE-GRSP), and full-strength exogenous EE-GRSP solution (full EE-GRSP) on plant growth, the root system architecture, leaf water status, and the tea quality of tea seedlings, along with examining the changes in the relative expression of water channel proteins (*AQPs*) in tea plants. The results indicated that exogenous EE-GRSP of different strengths had different effects on both the growth performance (height, leaf numbers, and biomass) and root architecture parameters of tea seedlings, and the best positive effects on plant growth and the root architecture appeared under the three-quarter-strength exogenous EE-GRSP treatment. Similarly, the exogenous EE-GRSP application also differently affected tea quality indicators, in which only the quarter- and half-strength exogenous EE-GRSP solutions significantly increased most of the indicators, including carbohydrates, tea polyphenols, total amino acids, catechins, and flavonoids. Moreover, the half- and three-quarter-strength exogenous EE-GRSP treatments significantly increased the leaf relative water content (LRWC), but all of the exogenous EE-GRSP treatments significantly decreased the leaf water potential (LWP). Furthermore, the expression of *AQP* genes in the root system of tea plants was related to the strength of the exogenous EE-GRSP treatments, and different genes were significantly up-regulated or down-regulated under the treatment of exogenous EE-GRSP at different strengths. Moreover, the correlation analysis showed that most of the relative expression of *AQPs* was significantly and positively correlated with tea plant growth, the root architecture, and the leaf relative water content, but negatively correlated with tea quality indicators; however, the expression of *CsNIPs* and *CsSIPs* was markedly and negatively correlated with plant growth performance. Therefore, we speculated that the application of exogenous EE-GRSP could facilitate plant growth and improve the quality indirectly by regulating the expression of root *AQPs*, thus ameliorating the water uptake and nutrient accumulation in tea plants.

Keywords: glomalin; aquaporins; tea quality; growth; root

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1. Introduction

Arbuscular mycorrhizal fungi (AMFs) are a kind of beneficial soil microorganism that form a mutually beneficial symbiotic relationship with approximately 80% of terrestrial plants [1]. In order to sustain their own growth and development, AMFs obtain about 20% of the photosynthetic carbohydrates from host plants, and in return, absorb water and

nutrients from the soil to provide favorable conditions for the growth and development of host plants [1]. In addition, AMFs play a positive role in enhancing plant stress resistance against drought, salt, heavy metals, etc. [2].

The contributions to the morphogenesis of plants' above-ground parts by AMFs are partly due to a globular protein known as glomalin, which is secreted by the hyphae and spores of AMFs during their coexistence with a host plant. The physicochemical properties of glomalin are similar to those of humic acid, which has the function of promoting plant growth and nutrient absorption [3]. Glomalin primarily exists within the AMFs' mycelium and spore wall structures, and it is released into the soil due to degradation [4]. Due to a non-specific method for glomalin extraction, Rilling [5] proposed the Bradford assay to characterize the presence of glomalin in the soil using glomalin-related soil protein (GRSP) [5,6]. There are two main fractions of GRSP, viz. easily extractable GRSP (EE-GRSP) and difficultly extractable GRSP (DE-GRSP), with their sum referred to as total extracted GRSP (T-GRSP) [7].

Previous studies have indicated that the application of exogenous EE-GRSP positively affects plant growth, development, metabolism, etc. In citrus rootstock seedlings, Wang et al. [8] first observed that the plant growth and development of citrus seedlings could be promoted by exogenous EE-GRSP application, and the strength of exogenous EE-GRSP was positively and curvilinearly correlated to their biomass. Subsequently, Chi et al. [9] showed that exogenous EE-GRSP significantly ameliorated leaf water status and photosynthetic parameters and markedly increased the concentrations of phytohormones in trifoliate orange seedlings, which was determined under drought stress. Another study on peanuts showed that the root fresh weight and photosynthetic parameters were positively correlated with the EE-GRSP content [10]. Interestingly, Liu et al. [11] found that plant growth was strongly promoted by exogenous EE-GRSP, but markedly inhibited by exogenous DE-GRSP, which may be an important clue supporting EE-GRSP functioning as a plant growth promoter, whereas some studies have revealed that GRSP can promote plant growth, which may be related to its ability to improve plant water conditions. On the one hand, GRSP can bind to soil particles due to its strong bonding effect, thus improving the size, proportion, and distribution of soil water-stable aggregates (WSAs), which helps to prevent soil water loss and ameliorate the water relationship between plants and soil [12]. On the other hand, it is likely that the effect of GRSP on plant growth is related to its regulation of the expression of water channel proteins (*AQPs*) in plants, and a previous study found a positive correlation between *AQP* expression and GRSP content [10].

Water channel proteins (*AQPs*) are a group of transmembrane proteins located in the biological membranes of plants [13], which have a wide range of functions, including the transmembrane transportation of water and a variety of small molecule solutes, such as ammonia, hydrogen peroxide, urea, metal elements, gases, and ions, and are also important in seed germination, plant growth, photosynthesis, cell elongation, reproduction, responding to stresses, etc. [14]. Based on the analysis by subcellular localization and sequence similarity, plant *AQPs* can be divided into seven subfamilies: plasma membrane intrinsic proteins (*PIPs*), tonoplast intrinsic proteins (*TIPs*), nodulin-26 like membrane intrinsic proteins (*NIPs*), small basic intrinsic proteins (*SIPs*), GlpF-like intrinsic proteins (*GIPs*), X intrinsic proteins (*XIPs*), and hybrid intrinsic proteins (*HIPs*) [15]. Of these, *PIPs* and *TIPs* are not only efficient water channels, but are also central to the perception and transduction of stress signals in plants. *PIPs* play a crucial role in water transport across the cell and plasma membrane, while *TIPs* are involved in cellular osmoregulation and show high expression levels in roots [14]. In contrast, *SIPs* only have moderate water transport activity, and *NIPs* primarily allow for the permeation of small mineral nutrients and organic solutes and exhibit low water transport activity [14]. Up to the present, there have been few studies on whether the expression of *AQPs* is involved in plant growth regulation by exogenous EE-GRSP, especially in tea plants.

As we all know, the economic value of tea, which is one of the world's top three non-alcoholic beverages, cannot be underestimated. Tea plants are very sensitive to soil

moisture levels, mainly due to their characteristics of liking humidity and being averse to waterlogging; therefore, water has been identified as a decisive factor affecting the growth and quality of tea trees [16]. The present study aimed to evaluate different strengths of exogenous EE-GRSP in response to plant growth, the root system architecture, water uptake, and tea quality, as well as determine the potential regulatory mechanisms in tea (*Camellia sinensis* (L.) O. Ktze) seedlings.

2. Materials and Methods

2.1. Experimental Materials

Camellia sinensis "FudingDabaicha" seedlings were used as the test material, and the tea seeds were provided by the Tea Research Institute of Guizhou Academy of Agricultural Sciences. Sterilization and germination were performed according to the method of Liu et al. [17].

Soil for extracting EE-GRSP was taken from a 13-year-old citrus orchard (Yangtze University, Jingzhou, Hubei Province, China) at a depth of 0–20 cm, which was air-dried and thoroughly mixed before debris was removed with a 4 mm sieve. The method of Koide and Peoples was followed to extract EE-GRSP [7]. In detail, a certain amount of the soil sample and a 20 mmol/L of citrate buffer (pH 7.0) were added in a ratio of 1:8, held for 30 min at 121 °C and 0.11 MPa, and centrifuged for 5 min at 10,000× *g*/min. The supernatant was collected as the exogenous full-strength EE-GRSP solution, and its concentration was determined to be 0.027 mg of protein/mL of citrate buffer according to Bradford's method [6].

2.2. Experimental Design

For this experiment, a completely randomized design was used and five treatments were included: (1) citrate buffer solution (20 mmol/L, pH 7.0), which was used as the control; (2) quarter-strength EE-GRSP solution (1/4 EE-GRSP); (3) half-strength EE-GRSP solution (1/2 EE-GRSP); (4) three-quarter-strength EE-GRSP solution (3/4 EE-GRSP); and (5) full-strength EE-GRSP solution (full EE-GRSP). According to our previous study on citrus plants [11,18], the solutions of EE-GRSP at 1/4, 1/2, and 3/4 strength were prepared by diluting the full-strength EE-GRSP solution with a certain proportion of the 20 mmol/L, pH 7.0, of citric acid buffer solution. Six replications of each treatment were performed. Two-leaf-old tea seedlings with uniform growth in autoclaved sands were transplanted into plastic pots that contained 1.5 Kg of autoclaved yellow soil (121 °C, 0.11 MPa, 2 h). One tea seedling was planted in each pot. For the first 4 weeks after transplanting, 50–100 mL of distilled water was poured into the soil every 3 days to maintain plant growth. In total, 80 mL of the exogenous EE-GRSP solution at different concentrations was applied once a week, resulting in four applications after four weeks of transplanting. The plants were harvested after 12 weeks.

2.3. Variable Determinations

The plant growth indexes, including plant height, stem diameter, and leaf numbers, were determined before being harvested by conventional methods. At harvest, seedlings were divided into above-ground and below-ground parts, and the fresh weight of the roots, stems, and leaves was weighed immediately. The intact fresh root systems of tea seedlings were scanned using the Epson Perfection V700 Photo Dual Lens System (J221A, Seiko Epson Corporation, Jakarta Selatan, Indonesia), and the root morphology was analyzed with WinRHIZO (Regent Instruments Inc., Montreal, QC, Canada). The whole root systems of the tea seedlings were laid flat on the experimental table, and the length of the main roots was measured with a tape measure, while the number of lateral roots at each level was manually counted and recorded.

The leaf relative water content (LRWC) and leaf water potential (LWP) were determined according to Guo et al. [18].

The glucose, sucrose, and fructose contents of the tea leaves were determined using the method of Wu et al. [19]. The Coomassie Brilliant Blue G-250 dye-binding method was used to assess the soluble protein content of the leaves, as described by Bradford [6]. The contents of tea polyphenols, free amino acids, and catechuic acids were determined according to the method of Cao et al. [20]. The leaf flavonoid content was determined using the method of Chu et al. [21].

The *AQP* family of genes was selected to analyze their relative expression. From the tea roots, total RNA was extracted and purified using the TaKaRa kit (TaKaRa Mini BEST Universal RNA Extraction Kit, Dalian, China). Then, an ultra-micro spectrophotometer (K5600C, Beijing Kaio Technology Development Co., Ltd., Beijing, China) was used to detect the concentration and purity of the RNA, while highly purified RNA was reverse transcribed using the TaKaRa kit (Prime Script™ RT Kit with gDNA Eraser). Three tubes of cDNA were reverse transcribed for each RNA sample. The *AQP* family of genes was screened with reference to the Tea Tree Genome Database (http://tpia.teaplants.cn/index.html) (accessed on 10 February 2019). The relevant gene sequences were obtained based on the NCBI database, and the specific primers were designed by Primer Premier 5.0 (Palo Alto, CA, USA) (Table 1) and synthesized by Shanghai SangonBio. Tech. Co. (Wuhan, China). The GADPH gene was used as an internal reference gene, and the relative expression of the *AQPs* was measured with the CFX96 Real Time PCR Detection System (BIO-RAD, Berkeley, CA, USA) on a real-time quantitative PCR instrument according to the Vazyme kit (ChamQ Universal SYBR qPCR Master Mix, Q711, Nanjing, China). The qRT-PCR reaction system consisted of 10 μL of AceQ qPCR SYBR Green Master Mix, 0.4 μL of forward primer, 0.4 μL of reverse primer, 2 μL of cDNA, and 7.2 μL of dd H_2O, which totaled 20 μL. The reaction program was carried out by preheating for 5 min at 95 °C, 10 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C for 40 cycles. The experiment was performed with three biological replicates and three technical replicates for each gene. Quantitative results were calculated with reference to the $2^{-\Delta\Delta Ct}$ method [22].

2.4. Statistical Analysis

One-way analysis of variance was performed using the SAS (8.1) software, and Duncan's method was used for multiple comparison analysis ($p < 0.05$). The Pearson correlation coefficient was calculated using the SAS software. Sigma Plot 15.0 and Origin 2023b were used for graphing.

Table 1. Gene-specific primer sequences used in this study.

Gene	Gene ID	Primer Sequence	Gene	Gene ID	Primer Sequence
CsPIP1;2	XM_028242073.1	F-AGATCGTCGGTACCTTTGTC R-GACTCCTTGCTGGGTTGATA	*CsTIP1;1*	XM_028239672.1	F-GTTTGTCACCAATGGCATGA R-ATAATTCCCAACTCGCCCTT
CsPIP1;3	XM_028197133.1	F-TGGTATCTCAGGAGGACACA R-AATCTCAGCACCAAGACCAT	*CsTIP1;2*	XM_028241002.1	F-TCGGTAGGGGCTAACATTTC R-CTCGAAAACTAGTGCGTTCC
CsPIP1;4	XM_028209956.1	F-TTGGTGCACTTAGCCACTAT R-TCACGACTTGGTGGTACAAA	*CsTIP1;3*	XM_028271063.1	F-ATAAGCTGACGGATAACGGG R-ATTGAACCCAAACACTGTGC
CsPIP2;1	XM_028250124.1	F-TCCAGAGCTCCTACTACGAC R-GGCTAGGTGAACCATGAACA	*CsTIP2*	XM_028212337.1	F-TCATTCAGTGTTGGGTCACT R-AGGTGACAGCTGGATTCAAA
CsPIP2;2	XM_028201387.1	F-ATTCTCAGCCAAGGACTACG R-TGCAGTATACGAGGACGAAG	*CsTIP2;1*	XM_028220182.1	F-TTGGTTTCATTGTTGGTGCC R-TTTGGATAGATGAGTCCGGC
CsPIP2;4	XM_028247087.1	F-GGTGGAATGATCTTCGTCCT R-GACGTAGTAGGCCTTTTGGA	*CsTIP3;1*	XM_028223148.1	F-CGCGCTTTTATTGGAGATCG R-GACCGAGCCAATAGATCCAG
CsPIP2;5	XM_028241134.1	F-TCTCAGCCAAGGATTACCAC R-GGGTCAATCTGGCTCTTGTA	*CsTIP4;1*	XM_028269043.1	F-GTCATCTTAATCCGGCGGTA R-GGGAGTAGTCCATTCCACTG
CsPIP2;7	XM_028229390.1	F-ACATTTGGGTTGTTCTTGGC R-AACAGTGTAAACGAGGACGA	*CsNIP1;1*	XM_028206307.1	F-TTAACCCTGCTGTCACCATT R-ACACGAAAGATTGCATGTCG
CsSIP1;2	XM_028198192.1	F-TAACCACCGTCCTTGTCTTC R-TGTTTTTGTACTGCATGGGC	*CsNIP1;2*	XM_028232417.1	F-TTAACCCTGCAGTCACCATT R-GAAAGACTGTGCGTCAGAAC
CsSIP2;1	XM_028214111.1	F-GGGGATTTCAGTCGATTCCT R-ATGTCAGAAATCCCTCCGTC	*CsNIP5;1*	XM_028230765.1	F-GCCTCTTAGATTTCCCTGCT R-GAGGGAGGGATTGAGATGTG
Cs-GADPH	XM_002263109	F-TTGGCATCGTTGAGGGTCT R-CAGTGGGAACACGGAAAGC			

3. Results and Analysis

3.1. Plant Growth Performance and Biomass

Compared with the citrate buffer control, all four different strengths of the exogenous EE-GRSP treatments improved plant growth performance in tea seedlings to varying degrees (Table 2). In detail, 1/4, 1/2, 3/4, and full EE-GRSP significantly increased the biomass of tea plants by 40.49~49.18%, 27.44~52.10%, and 20.76~42.28% in the leaves, shoots, and roots, respectively, and the leaf numbers were increased by 23.40~95.74% as well. In addition, both the 1/2 and 3/4 EE-GRSP applications markedly increased the plant height by 28.98% and 26.78%, and only the 1/2 EE-GRSP notably increased the stem diameter of the tea plants by 24.49%.

Table 2. Effects of different strengths of exogenous EE-GRSP on plant growth performance and biomass of tea (*Camellia sinensis*) plants.

Treatments	Plant Height (cm)	Stem Diameter (mm)	Leaf Number (#/Plant)	Biomass (g FW/Plant)		
				Leaf	Shoot	Root
Citrate buffer	15.18 ± 1.48 b	2.44 ± 0.30 b	7.83 ± 0.75 d	1.78 ± 0.13 b	0.58 ± 0.05 c	3.22 ± 0.11 c
1/4 EE-GRSP	16.52 ± 1.18 b	2.58 ± 0.25 b	9.67 ± 0.82 c	2.66 ± 0.18 a	0.74 ± 0.03 b	3.89 ± 0.22 b
1/2 EE-GRSP	19.58 ± 1.90 a	3.04 ± 0.29 a	12.67 ± 1.51 b	2.50 ± 0.22 a	0.89 ± 0.07 a	3.91 ± 0.15 b
3/4 EE-GRSP	19.25 ± 2.76 a	2.75 ± 0.20 ab	15.33 ± 1.86 a	2.66 ± 0.18 a	0.86 ± 0.03 a	4.59 ± 0.44 a
Full EE-GRSP	16.35 ± 1.47 b	2.63 ± 0.22 b	12.00 ± 1.41 b	2.55 ± 0.17 a	0.78 ± 0.06 b	4.55 ± 0.45 a

Data (means ± SE, $n = 6$) followed by different letters among treatments indicate significant differences at $p < 0.05$.

3.2. Root Morphology

The application of exogenous EE-GRSP at all strengths significantly increased the root volume, with the 3/4 EE-GRSP treatment showing the greatest magnitude of response (Table 3). The total root length and lateral root numbers of the first-order, second-order, and third-order were significantly increased by 23.33%, 65.16%, 53.79%, and 138.46% under 1/2 EE-GRSP treatments, by 37.29%, 120.97%, 93.23%, and 267.95% under 3/4 EE-GRSP treatments, and by 19.53%, 53.87%, 27.09%, and 72.44% under full EE-GRSP treatments, while the best results were obtained under the 3/4 EE-GRSP treatment. Furthermore, the projected area was prominently increased by 19.63% only under the full EE-GRSP treatment, while the taproot length was increased by 11.68% only under the 1/2 EE-GRSP treatment. The 1/2 and 3/4 EE-GRSP treatments significantly increased the surface area by 11.41% and 23.09%, respectively. However, all four different strengths of the exogenous EE-GRSP treatments did not alter the average diameter of the tea plants.

3.3. Tea Quality

Compared to the citrate buffer control, the 1/4 EE-GRSP treatment dramatically increased the contents of fructose, sucrose, tea polyphenols, catechins, and flavonoids in the tea leaves by 11.31%, 11.34%, 16%, 65.85%, and 10.32%, respectively, but it did not alter the glucose and total amino acid contents (Table 4). Under the 1/2 EE-GRSP treatment, the contents of glucose, tea polyphenols, total amino acids, catechins, and flavonoids were 15.94%, 21.73%, 30.12%, 53.48%, and 13.65% higher than that under the citrate buffer control, respectively (Table 4). The 3/4 EE-GRSP treatment only notably increased the contents of catechins and total soluble proteins by 35.59% and 36.65%, respectively, but significantly decreased the contents of glucose, total amino acids, and flavonoids by 9.71%, 26.56%, and 9.24%, respectively. The full EE-GRSP treatment only significantly increased the contents of fructose and catechin by 7.88% and 49.08%, but significantly decreased the contents of total amino acids and flavonoids by 43.50% and 7.38%, respectively (Table 4).

Table 3. Effects of different strengths of exogenous EE-GRSP on root system architecture of tea (*Camellia sinensis*) plants.

Treatments	Total Length (cm)	Projected Area (cm^2)	Surface Area (cm^2)	Average Diameter (mm)	Volume (cm^3)	Taproot Length (cm)	Lateral Root Numbers (#/Plant)		
							First-Order	Second-Order	Third-Order
Citrate Buffer	102.97 ± 8.49 c	10.13 ± 0.94 b	9.78 ± 0.23 c	0.75 ± 0.09 a	1.14 ± 0.11 d	10.70 ± 0.89 b	77.50 ± 4.65 c	389.50 ± 38.54 d	39.00 ± 2.00 d
1/4 EE-GRSP	116.38 ± 9.84 bc	10.91 ± 1.02 ab	10.39 ± 0.36 bc	0.73 ± 0.09 a	1.44 ± 0.07 c	10.68 ± 0.91 b	91.00 ± 7.30 c	411.50 ± 37.97 d	44.00 ± 4.08 d
1/2 EE-GRSP	126.99 ± 1.55 ab	11.61 ± 0.40 ab	10.90 ± 0.49 b	0.73 ± 0.05 a	1.63 ± 0.11 b	11.95 ± 0.67 a	128.00 ± 10.23 b	599.00 ± 55.78 b	93.00 ± 7.39 b
3/4 EE-GRSP	141.37 ± 5.74 a	11.71 ± 0.59 ab	12.04 ± 0.42 a	0.81 ± 0.07 a	2.02 ± 0.13 a	10.05 ± 0.42 b	171.25 ± 13.77 a	748.75 ± 24.92 a	143.50 ± 9.28 a
Full EE-GRSP	123.08 ± 10.40 b	12.12 ± 0.94 a	10.09 ± 0.70 bc	0.68 ± 0.04 a	1.67 ± 0.07 b	10.70 ± 0.73 b	119.25 ± 7.50 b	495.00 ± 11.34 c	67.25 ± 6.24 c

Data (means ± SE, $n = 6$) followed by different letters (a, b, c, d) among treatments indicate significant differences at $p < 0.05$.

Table 4. Effect of different strengths of exogenous EE-GRSP on carbohydrate content and the quality parameters of tea (*Camellia sinensis*) plants.

Treatments	Fructose (mg/g)	Sucrose (mg/g)	Glucose (mg/g)	Tea Polyphenols (mg/g)	Total Amino Acids (mg/g)	Catechins (mg/g)	Flavonoids (mg/g)	Total Soluble Protein (mg/g)
Citrate buffer	29.25 ± 1.22 c	49.49 ± 2.13 bc	14.65 ± 0.82 b	80.03 ± 1.10 c	30.26 ± 3.66 b	24.40 ± 2.34 c	83.50 ± 3.16 b	5.88 ± 0.88 b
1/4 EE-GRSP	32.56 ± 0.97 a	55.11 ± 2.98 a	14.76 ± 0.21 b	92.83 ± 3.57 ab	30.07 ± 1.59 b	40.47 ± 1.72 a	92.11 ± 0.87 a	6.52 ± 0.42 ab
1/2 EE-GRSP	31.49 ± 1.31 ab	53.01 ± 1.32 ab	16.99 ± 0.83 a	97.41 ± 2.98 a	39.37 ± 2.34 a	37.45 ± 4.34 ab	94.89 ± 2.45 a	7.15 ± 1.03 ab
3/4 EE-GRSP	29.56 ± 1.11 bc	47.84 ± 3.48 bc	13.23 ± 0.37 c	85.88 ± 4.89 bc	22.22 ± 5.91 c	33.09 ± 1.72 b	75.78 ± 0.27 c	8.04 ± 1.07 a
Full EE-GRSP	31.56 ± 1.24 ab	47.06 ± 3.58 c	13.61 ± 0.68 bc	86.92 ± 5.09 bc	17.10 ± 0.33 c	36.38 ± 4.14 ab	77.33 ± 2.22 c	7.65 ± 1.32 ab

Data (means ± SE, $n = 6$) followed by different letters (a, b, c) among treatments indicate significant differences at $p < 0.05$.

3.4. Leaf Water Status

The LRWC was notably increased by 5.34% under the 3/4 EE-GRSP treatment compared to the citrate buffer control, while the 1/4, 1/2, and full EE-GRSP treatments did not significantly affect the LRWC (Figure 1a). However, the 1/4, 1/2, 3/4, and full EE-GRSP treatments significantly reduced the LWP of tea seedlings by 31.03%, 20.69%, 55.17%, and 13.79%, respectively (Figure 1b).

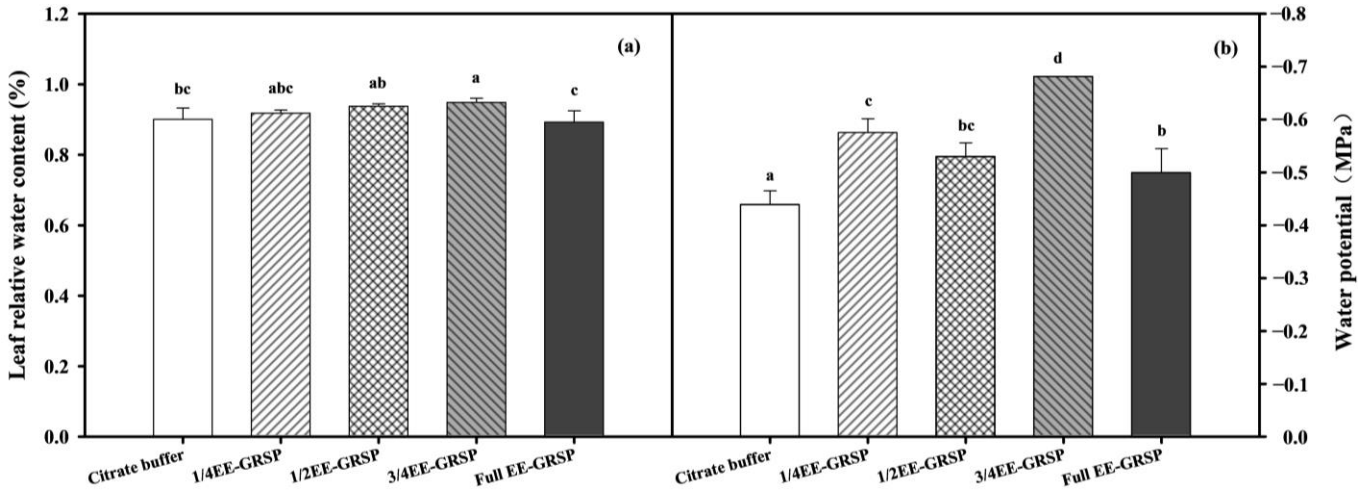

Figure 1. Effect of exogenous EE-GRSP on leaf relative water content (**a**) and leaf water potential (**b**) of tea (*Camellia sinensis*) plants. Different small letters (a, b, c, d) indicate significant difference within the same column at 0.05 level by LSD.

3.5. Relative Expression of AQP Genes in Roots

Compared to the citrate buffer control, the 1/4 EE-GRSP treatment significantly up-regulated the expression of *CsPIP2;1* by 1.49-fold, but significantly down-regulated the expression of the rest of the *PIPs* in the tea roots (Figure 2a). The 1/2 EE-GRSP treatment substantially up-regulated *CsPIP1;4*, *CsPIP2;1*, *CsPIP2;2*, *CsPIP2;4*, and *CsPIP2;7* expression by 1.61-fold, 1.12-fold, 1.36-fold, 2.40-fold, and 1.52-fold, respectively, which was coupled with down-regulation in the remaining genes' expression. The 3/4 EE-GRSP treatment notably up-regulated the expression of *CsPIP2;2* and *CsPIP2;7* by 1.57-fold and 2.82-fold, respectively, but significantly down-regulated the expression of the remaining genes except for *CsPIP1;4*. The full EE-GRSP treatment up-regulated *CsPIP1;2*, *CsPIP1;4*, and *CsPIP2;4* expression by 1.22-fold, 1.41-fold, and 1.11-fold, but significantly down-regulated the expression of *CsPIP1;3*, *CsPIP2;2*, and *CsPIP2;5* by 1.26-fold, 1.78-fold, and 1.75-fold (Figure 2a).

Among the family of *CsTIPs* in the tea roots, the 1/4 EE-GRSP treatment prominently up-regulated the expression of *CsTIP1;2*, *CsTIP1;3*, *CsTIP2;1*, *CsTIP3;1*, and *CsTIP4;1* by 2.15-fold, 1.25-fold, 2.15-fold, 14.00-fold, and 3.53-fold, respectively, but dramatically down-regulated the expression of *CsTIP1;1* and *CsTIP2* by 2.51-fold and 5.34-fold. The 1/2 EE-GRSP treatment significantly up-regulated *CsTIP1;2*, *CsTIP1*;3, and *CsTIP4;1* by 4.30-fold, 2.94-fold, and 34.71-fold, respectively, but markedly down-regulated the expression of *CsTIP1;1*, *CsTIP2;1*, and *CsTIP3;1* by 1.21-fold, 1.93-fold, and 5.08-fold. The 3/4 EE-GRSP treatment substantially up-regulated the expression of *CsTIP1;1*, *CsTIP1;2*, *CsTIP1;3*, and *CsTIP4;1* by 1.18-fold, 4.34-fold, 1.90-fold, and 37.06-fold, respectively, but significantly down-regulated the expression of *CsTIP2* and *CsTIP3;1* by 4.65-fold and 4.59-fold. The full EE-GRSP treatment significantly up-regulated the expression of *CsTIP1;2, CsTIP2*, *CsTIP2;1*, and *CsTIP4;1* by 3.28-fold, 1.47-fold, 1.56-fold, and 24.48-fold, respectively, but significantly down-regulated the expression of *CsTIP1;1* and *CsTIP1;3* by 1.35-fold and 1.98-fold (Figure 2b).

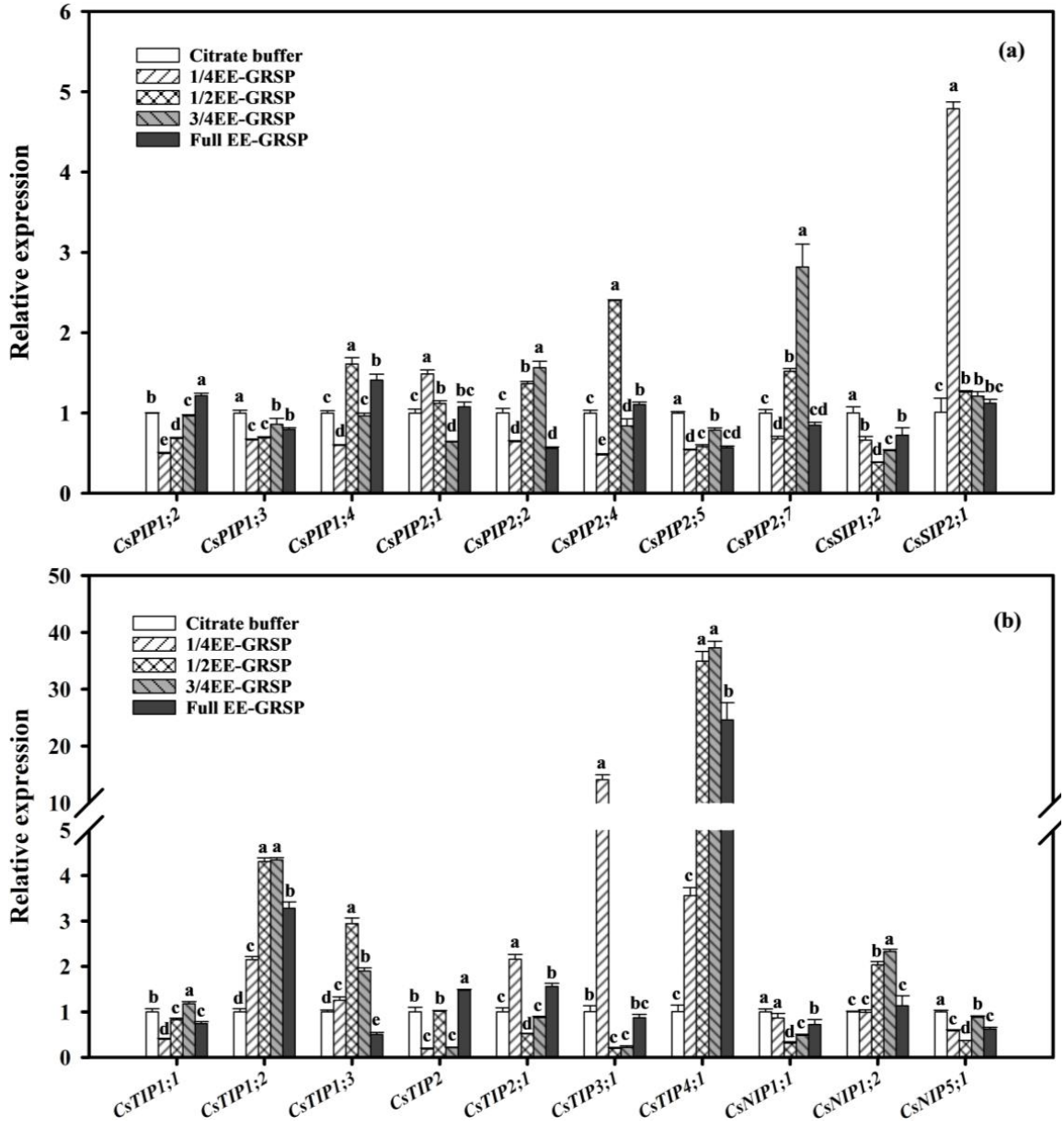

Figure 2. Effect of exogenous EE-GRSP on the relative expression of *AQPs* in the root system of tea (*Camellia sinensis*) plants. (**a**) *CsPIP* and *CsSIP* family of genes' relative expression; (**b**) *CsTIP* and *CsNIP* family of genes' relative expression. Different small letters (a, b, c, d) indicate significant difference within the same column at 0.05 level by LSD.

The application of the 1/2 and 3/4 EE-GRSP treatments markedly up-regulated the expression of *CsNIP1;2* by 2.03-fold and 2.33-fold, respectively. Nevertheless, the 1/4 EE-GRSP treatment significantly down-regulated the expression of *CsNIP5;1* by 1.72-fold. *CsNIP1;1* and *CsNIP5;1* expression in the tea roots was notably down-regulated under the 1/2, 3/4, and full EE-GRSP treatments (Figure 2b).

Application of the 1/4, 1/2, and 3/4 EE-GRSP treatments dramatically up-regulated the expression of *CsSIP2;1* by 4.75-fold, 1.25-fold, and 1.20-fold, respectively, which was coupled with the down-regulation of the expression of *CsSIP1;2* under the exogenous EE-GRSP treatments of all four different strengths (Figure 2a).

3.6. Correlation Analysis and Principal Component Analysis

CsNIP1;1 was significantly negatively correlated with plant height, stem diameter, and shoot fresh weight, while *CsNIP1;2* was significantly positively correlated with the plant height, lateral roots in different classes, root surface area, and leaf relative water content. Meanwhile, *CsNIP5;1* was markedly negatively correlated with the polyphenol content (Table 5). *CsPIP1;2* was dramatically negatively correlated with the sucrose content; *CsPIP1;3* was notably negatively correlated with the content of fructose, polyphenols, and catechins; *CsPIP2;5* was markedly negatively correlated with the content of fructose and catechins. Meanwhile, *CsPIP2;7* was dramatically positively correlated with lateral root numbers and the root surface area (Table 5). *CsTIP1;2* was notably positively correlated with the plant height, leaf number, shoot fresh weight, first-order and second-order lateral root numbers, total root lengths, root volume, and leaf soluble protein content. *CsTIP4;1* was remarkably positively correlated with the leaf number, shoot fresh weight, first-order and second-order lateral root numbers, total root lengths, and leaf soluble protein content. *CsTIP1;1* was evidently negatively correlated with the fructose content, and *CsSIP1;2* was observably negatively correlated with the plant height, stem diameter, and shoot fresh weight (Table 5).

Principal component analysis (PCA) was used to study the correlation between tea tree growth and tea quality based on major factors (exogenous EE-GRSP strength, root architecture, and root *AQP* genes) (Figure 3). The results showed that the accumulated contribution rate of the two principal components was 69.2% (39.2% and 23.7% for PC1 and PC2, respectively). The leaf relative water content, root architecture, and expression of some *AQP* genes (*CsPIP1;4*, *CsPIP2;2*, *CsPIP2;4*, *CsPIP2;7*, *CsTIP1;2*, *CsTIP4;1*, and *CsNIP4;1*) were positively correlated with tea plant growth indicators (plant height, stem diameter, leaf number, and biomass of each part) and the leaf soluble protein content, and these traits were significantly promoted under the 1/2 and 3/4 strength exogenous EE-GRSP treatments. In addition, the leaf relative water content, taproot length, and the expression of some *AQP* genes (*CsPIP2;1*, *CsPIP2;4*, *CsTIP1;3*, *CsTIP2;1*, *CsTIP3;1*, and *CsSIP2;1*) were positively correlated with tea quality (except soluble protein content), and the content of tea quality-related substances, with the exception being the soluble protein, was significantly promoted under the 1/4 and 1/2 strength of exogenous EE-GRSP treatments. Interestingly, the leaf water potential and the expression of some *AQP* genes (*CsNIP1;1*, *CsNIP5;1*, *CsPIP1;3*, *CsPIP2;5*, *CsTIP2*, and *CsSIP1;2*) were negatively correlated with tea plant growth and tea quality.

Table 5. Correlation coefficients between the relative expression of *AQP* genes in tea roots and various physiological indicators of tea (*Camellia sinensis*) plants under different concentrations of exogenous EE-GRSP treatment (n = 6).

	CsNIP1;1	***CsNIP1;2***	***CsNIP5;1***	***CsPIP1;2***	***CsPIP1;3***	***CsPIP2;5***	***CsPIP2;7***	***CsTIP1;1***	***CsTIP1;2***	***CsTIP4;1***	***CsSIP1;2***
Plant height	−0.964 **	0.943 *	−0.443	−0.249	−0.426	−0.317	0.743	0.321	0.917 *	0.877	−0.933 *
Stem diameter	−0.962 **	0.768	−0.708	−0.280	−0.561	−0.475	0.436	0.116	0.852	0.808	−0.926 *
Leaf number	−0.835	0.874	−0.207	0.165	−0.249	−0.297	0.811	0.429	0.947 *	0.942 *	−0.768
Shoot fresh weight	−0.934 *	0.783	−0.64	−0.179	−0.643	−0.635	0.541	0.057	0.971 **	0.889 *	−0.968 **
Number of first-order lateral roots	−0.792	0.898 *	−0.070	0.208	−0.122	−0.157	0.884 *	0.541	0.900 *	0.918 *	−0.697
Number of second-order lateral roots	−0.830	0.962 **	−0.054	0.133	−0.082	−0.065	0.934 *	0.610	0.886 *	0.914 *	−0.715
Number of third-order lateral roots	−0.792	0.949 *	0.006	0.154	−0.040	−0.026	0.949 *	0.632	0.859	0.890 *	−0.676
Total length of root system	−0.811	0.858	−0.226	0.059	−0.321	−0.346	0.801	0.351	0.931 *	0.899 *	−0.792
Root surface area	−0.726	0.915 *	−0.015	−0.123	−0.169	−0.076	0.927 *	0.477	0.783	0.757	−0.711
Root volume	−0.745	0.793	−0.176	0.159	−0.28	−0.348	0.767	0.343	0.901 *	0.876	−0.722
Leaf relative water content	−0.751	0.893 *	−0.171	−0.435	−0.289	−0.082	0.813	0.354	0.691	0.637	−0.774
Leaf soluble protein	−0.706	0.687	−0.214	0.336	−0.252	−0.397	0.644	0.314	0.886 *	0.882 *	−0.648
Sucrose	0.000	−0.166	−0.550	−0.966 **	−0.663	−0.415	−0.376	−0.692	−0.160	−0.344	−0.284
Fructose	−0.089	−0.294	−0.830	−0.526	−0.909 *	−0.928 *	−0.556	−0.917 *	0.125	−0.076	−0.385
Tea polyphenols	−0.630	0.305	−0.945 *	−0.681	−0.936 *	−0.820	−0.064	−0.526	0.542	0.371	−0.825
Catechins	−0.415	0.111	−0.827	−0.512	−0.969 **	−0.973 **	−0.139	−0.688	0.495	0.289	−0.692

* $p < 0.05$; ** $p < 0.01$.

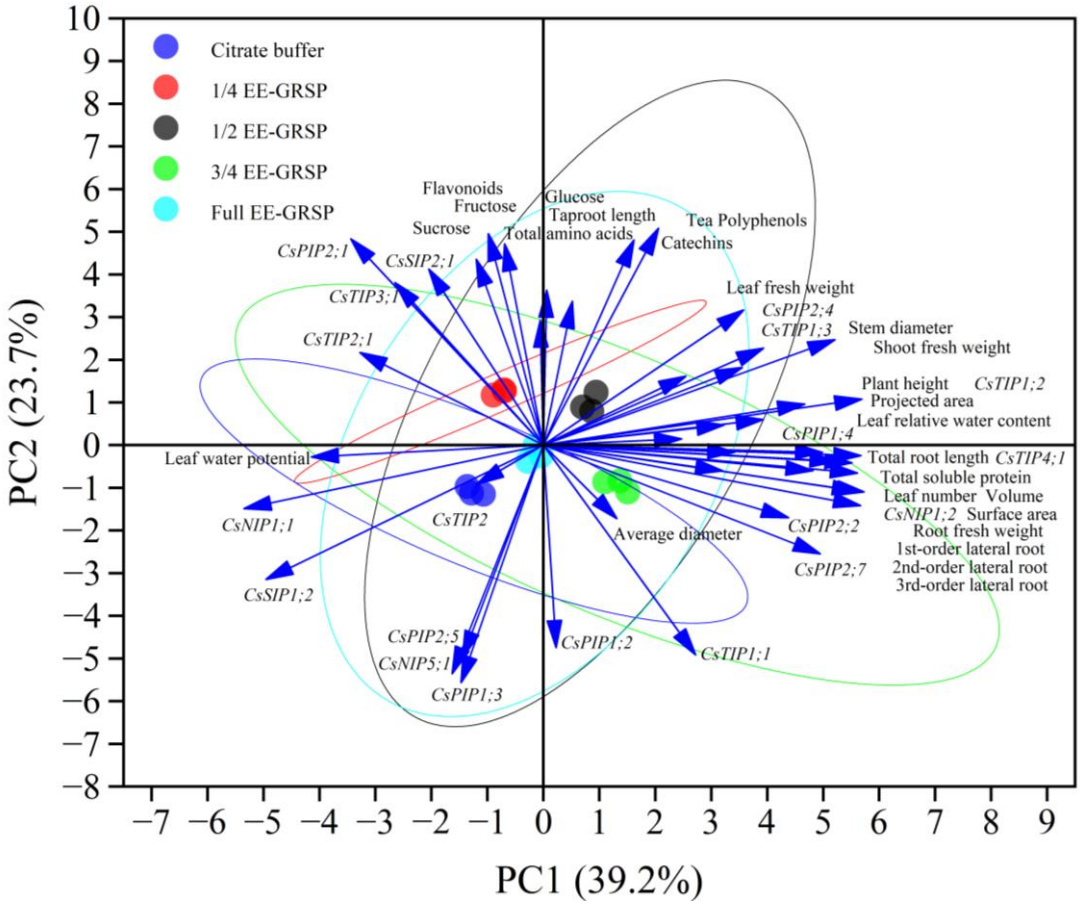

Figure 3. Unsupervised principal component analysis (PCA) of physiological and molecular parameters of tea (*Camellia sinensis*) plants under different strengths of exogenous EE-GRSP treatment. The circles in different colors represent the 95% confidence interval of the corresponding treatment.

4. Discussion

In this study, the application of exogenous EE-GRSP promoted the growth and increased the biomass of tea seedlings at different ranges; plant growth was optimal under the 1/2 EE-GRSP treatment, while the biomass was greatest under the 3/4 EE-GRSP treatment, which was consistent with the results from previous studies on trifoliate orange seedlings [11,18]. These results further confirmed that GRSP exerts a positive effect on plant growth. Additionally, the present study also showed a curvilinear relationship between plant growth performance in tea seedlings and exogenous EE-GRSP strength, which was consistent with the findings of Wang et al. [8], indicating that the promotion effects of exogenous EE-GRSP on plant growth were closely related to its strengths or concentrations.

Greater soil structure is an important factor in promoting root structure and development, and GRSP can significantly improve soil structure and promote plant growth by increasing the distribution and average weight diameter (MWD) of water-stable aggregates (WSAs) in the soil in citrus orchards [23–25]. In the present study, four different strengths of exogenous EE-GRSP all markedly regulated the root system architecture of tea seedlings to different degrees and increased the root system architecture parameters; these indexes were correlated with the strength of exogenous EE-GRSP in a positive curvilinear manner, which was in agreement with the results of Liu et al. [11] on trifoliate orange seedlings. However, the relative parameters of the root system architecture performed best under the 3/4 EE-GRSP treatment, which contradicts the previous research on citrus fruits in terms of lateral root development [18]. This is most likely because different plants have different genotypes, resulting in varying responses to exogenous EE-GRSP application, thus leading to different growth performance results. Moreover, the present study also indicated that,

although the full EE-GRSP treatment increased the root configuration parameters, its promoting effect was reduced or slowed down compared to the treatments of other strengths, which may be due to the deposited and sealed high-concentration GRSP components in the pores of these large aggregates, resulting in a slower rate of water infiltration into the WSAs [5].

In addition, the present study also reported that the lower-strength (1/4 and 1/2 strengths) exogenous EE-GRSP treatments exhibited a significant promoting effect that improved tea quality, which may be related to the material composition of GRSP. Firstly, GRSP contains substances similar to humic acids, which are prominent in the absorption and utilization of nutrients by plants [3,26]. In addition, EE-GRSP has also been proven to contain multiple essential nutrients, e.g., phosphorus (P), iron, etc., which could result in an increase of the sources required by plants after exogenous EE-GRSP application [27,28]. Meanwhile, high-strength (3/4 and full-strength) exogenous EE-GRSP treatments dramatically reduced the contents of glucose, total amino acids, and flavonoids in the tea leaves, which may be related to the eutrophication in the rhizosphere of tea plants caused by exogenous GRSP application. Generally, excessive organic or inorganic content in the soil may reduce the decomposition and release of proteases by the roots, thereby inhibiting the absorption and utilization of nutrients, such as nitrogen, by plant roots [29,30]. Therefore, we speculate that, in the present study, high-strength exogenous EE-GRSP application resulted in eutrophication of the tea rhizosphere soil, thereby decreasing nutrient absorption and utilization, as well as the transport efficiency of the tea roots. This also provides an explanation for the significant decrease in the contents of carbohydrates, total amino acids, and flavonoids under the high-strength exogenous EE-GRSP treatments.

A previous study revealed that the LRWC of citrus plants was notably increased under exogenous EE-GRSP treatment [25]. However, in this study, the LRWC was only significantly increased by 5.34% under the 3/4 EE-GRSP treatment, which may be related to the increase in soluble protein content in the leaves under the 3/4 EE-GRSP treatment. Soluble protein is an important osmoregulatory substance, which can improve the water retention capacity of cells as it accumulates [31]. Furthermore, the present study also revealed that the LWP in the tea leaves was dramatically reduced under all exogenous EE-GRSP treatments, and these findings were consistent with those of Guo et al. [18] on lemon seedlings, but contrary to those of Chi et al. [9] on trifoliate orange seedlings, which may be attributed to an increase in the solute concentration in tea leaves. Ultimately, exogenous EE-GRSP application accelerated nutrient accumulation in plant leaves, thereby increasing the cytoplasmic concentration and osmotic pressure [32], which may be the main reason for the decrease in the water potential.

PIPs are not only the most-important proteins that regulate water transport in plants, but they also affect the permeability of protoplasts, the leaf transpiration rate, and stomatal density [33], and they participate in water transport in the xylem and phloem [34], as they are mainly located on the cytoplasmic membrane [33,35]. Therefore, plants typically maintain water balance and stability by regulating the expression levels of different *PIPs*, which have different tissue specificities and functions. In this study, the *CsPIP* genes showed different response patterns under different strengths of the EE-GRSP treatments, and different *PIP* genes exhibited up-regulation or down-regulation under different strengths of the exogenous GRSP treatments; for example, *CsPIP2;1* was up-regulated under the 1/4 and 1/2 EE-GRSP treatments, but down-regulated under the 3/4 EE-GRSP treatment, indicating that different *CsPIPs* play a dominant role under different treatments, and the expression pattern of *CsPIPs* in the tea plants was regulated by the concentration or strength of the exogenous EE-GRSP. Additionally, correlation analysis showed that the expression level of *CsPIP2;7* was significantly and positively correlated with the lateral root numbers and root surface area of the tea seedlings, indicating that the *CsPIPs* were involved in the root development of tea plants due to their special role in taproot elongation and lateral root development [36]. Furthermore, the correlation analysis also revealed that *CsPIP1;2* was markedly and negatively correlated with sucrose, but *CsPIP1;3* and *CsPIP2;5* were

notably and negatively correlated with fructose and catechin contents, hinting that *CsPIPs* may indirectly affect tea quality by regulating water absorption in tea plants. However, this specific mechanism still needs to be researched further.

Within the tonoplast, *TIPs* are the most-abundant aquaporin proteins, and they are capable of regulating cellular water balance and responding to osmotic changes by facilitating water transport through the promotion of small-molecule transport [13]. The present study showed that *CsTIP1;2*, *CsTIP1;3*, and *CsTIP4* were significantly up-regulated under all four exogenous EE-GRSP treatments (except for the expression of *CsTIP1;3* genes under the full EE-GRSP treatment), and the expression levels of the above three genes were the most up-regulated under the 1/2 and 3/4 EE-GRSP treatments. These results indicated that *TIP* expression is regulated by EE-GRSP, which can enhance plant water status. Meanwhile, the results obtained in this study were slightly different from those of a previous study on lemon seedlings, which reported that the *TIP* expression was markedly up-regulated under the 1/4 EE-GRSP treatment [18]. This might be due to the different plant genotypes, and the expression of the *TIPs* was also influenced by the concentration of exogenous EE-GRSP [18]. In addition, correlation analysis revealed that *CsTIP1;2* and *CsTIP4;1* were significantly and positively correlated with the most plant growth indexes and root system parameters, indicating that *CsTIP1;2* and *CsTIP4;1* play a dominant role in regulating water uptake through EE-GRSP to promote plant growth. Furthermore, the involvement of *TIPs* in regulating plant growth may be related to the following two aspects: Firstly, *TIPs* have strong osmoregulation functions [37], which can promote water absorption and transport in tea seedlings. Secondly, a higher expression of *TIPs* is beneficial for the absorption of nitrogen in tea seedlings, as TIPs participate in the transport of NH_4^+ from the cytoplasm to vacuoles, and NH_4^+ is the nitrogen source that tea roots tend to utilize [38]. Moreover, a study by Reinhardt et al. [39] revealed that *TIPs* play a vital role in lateral root development, and a higher expression of *TIPs* can promote new lateral root primordium formation and development. The above result further confirmed that *TIPs* promote plant growth by regulating root development and water absorption.

NIPs have been found in the symbionts of leguminous plants that form root nodules, and they are also present in the plasma membrane and endoplasmic reticulum of plants [40]. Compared to *PIPs* and *TIPs*, *NIPs* have lower water transport activity, but higher permeability with organic small molecules and minerals, such as glycerol, boron, silicon, arsenic, ammonia (NH_3), and urea [41]. *SIPs* are associated with intracellular membranes, especially the endoplasmic reticulum, and play an irreplaceable role in facilitating cellular water transport [42]. The previous study on sugar beets showed that *SIP1;2* and *SIP2;1* were paired with *NIPs* in the transport of boron and nitrite through gene interaction analysis [43]. The present study showed that the four strengths of exogenous EE-GRSP treatments significantly down-regulated the relative expression of *CsNIP1;1*, *CsNIP5;1*, and *CsSIP1;2*; meanwhile, the relative expression of both *CsNIP1;1* and *CsSIP1;2* showed significant or highly significant and negative correlations with the plant growth indexes, including the plant height, stem diameter, and fresh weight of tea seedlings, suggesting a potential pairing relationship between these *SIPs* and *NIPs* for transporting small molecules and minerals. In addition, the relative expression of *CsNIP1;2* and *CsSIP2;1* was significantly up-regulated under both the 1/2 and 3/4 EE-GRSP treatments, while the expression of *CsSIP2;1* was prominently up-regulated by 4.75-fold under the 1/4 EE-GRSP treatment. Furthermore, the correlation analysis also displayed that *CsNIP1;2* was significantly and positively correlated with the LRWC, plant height, and most of the root architecture indexes, which indicates that exogenous EE-GRSP could adjust plant growth by regulating the expression of *NIPs* and *SIPs*, as these genes are highly involved in nutrient and water absorption.

5. Conclusions

Exogenous EE-GRSP regulates the expression of *AQPs*, especially *CsNIPs* and *CsTIPs*, in tea roots, improving water absorption and transport, thereby promoting root develop-

ment and improving tea quality. These findings open up the idea of employing EE-GRSP as a growth promoter for tea production in the future; however, more useful studies are required to clarify the physiological underpinnings of GRSP function and the growth response mechanism of GRSP.

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