forests

Article

Screening and Expression Characteristics of Plant Type Regulatory Genes in *Salix psammophila*

Kai Zhao [1], Rong He [2], Guosheng Zhang [2], Fucang Qin [1,3,*], Yongjie Yue [2], Long Li [1] and Xiaoyu Dong [1]

1 College of Desert Control Science and Engineering, Inner Mongolia Agricultural University, Hohhot 010010, China; zhaokmail@163.com (K.Z.)
2 College of Forestry, Inner Mongolia Agricultural University, Hohhot 010010, China
3 Inner Mongolia Forestry Research Institute, Hohhot 010011, China
* Correspondence: qinfucang@163.com; Tel.: +86-0471-3380181

Abstract: *Salix psammophila* is an important tree species adapted to sand-fixing afforestation in arid areas, and its different plant type characteristics can have different ecological functions. To identify the genes affecting the plant type of *S. psammophila*, this study used RT-qPCR and RNA-seq technology to establish a method for screening the candidate genes of the *S. psammophila* plant type based on the correlation coefficient of the crown–height ratio. We then screened out the gene combination that can best control the expression of the *S. psammophila* plant type. The results show the following: (1) The expression levels of the *FHY1* and *TAC2* genes were positively correlated with the crown–height ratio, whereas those of the *ATX1*, *RFK1*, *PYL1*, *ABF2*, *SPA2*, *TB1*, *ZFP4*, and *LAZY1b* genes were negatively correlated with the crown–height ratio. (2) The *ATX1* + *FHY1* gene combination had the greatest influence on the plant type traits of *S. psammophila*, and the correlation between the gene combination and the crown–height ratio reached 0.74. (3) The double- gene combination screening method improved the screening efficiency and accuracy, as well as the sensitivity and specificity, indicating certain universality. This strategy can be used for the determination of other plants or other traits, providing a theoretical basis for the directional breeding of forest trees.

Keywords: *Salix psammophila*; crown–height ratio; gene screening; gene expression; RT-qPCR; RNA-seq

Citation: Zhao, K.; He, R.; Zhang, G.; Qin, F.; Yue, Y.; Li, L.; Dong, X. Screening and Expression Characteristics of Plant Type Regulatory Genes in *Salix psammophila*. *Forests* **2024**, *15*, 103. https://doi.org/10.3390/f15010103

Academic Editor: John E. Carlson

Received: 7 December 2023
Revised: 26 December 2023
Accepted: 3 January 2024
Published: 4 January 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Plant traits are determined by gene expression and environmental factors, which reflect the morphology, function, and adaptability of plants [1]. Gene expression refers to the activity of genes at the transcriptional level, which determines the synthesis and regulation of proteins [2]. Different gene expression levels lead to different traits. For example, a higher content of genes controlling abscisic acid (ABA) synthesis corresponds to a slower a plant growth rate, and a higher content of genes controlling the tillering ability of *oryza sativa* corresponds to more panicles of *oryza sativa*. Changes in these traits directly affect the yield and quality of crops [3]. Plant type is an important form of plant morphological structure, and it is determined by the activities of meristem and lateral organs during plant growth and development [4]. Plant type is also a dynamic trait and is regulated by endogenous and exogenous factors [5]. Different plant species have different plant type characteristics, such as the erect type, evacuation type, open type, dwarf type and solitary type. The optimization of the plant type can improve the utilization efficiency of light energy and water, the resistance of plants to adversity, and the yield and quality of crops [6].

Salix psammophila is a shrub plant with the characteristics of wind–sand resistance, strong drought resistance, and easy reproduction. It is one of the most important tree species for sand fixation and afforestation. *S. psammophila* can also be used for pulping fiber, feed, and medicine and has a high economic value. The plant type of *S. psammophila* is the result of the interaction of genes and the environment. According to the different plant

types, *S. psammophila* can be divided into three types, namely, the erect, intermediate, and scattered types. Different plant types have different effects on the growth, development, and functional performance of *S. psammophila*. For example, the erect type is beneficial to improve the utilization rate of light energy and photosynthetic efficiency, as well as the ability of drought resistance and lodging resistance. It is suitable for growth in areas with strong wind– sand and less water. The intermediate plant type is beneficial to increase the number and length of branches, as well as improve the quality and yield of branches. It is suitable for growth in areas with weak wind– sand and sufficient water. The scattered plant type is beneficial to increase the crown area and coverage, as well as improve the effect of windbreak and sand fixation and soil retention ability. It is suitable for growing in areas with less wind and more water. Wang et al. [7] used the F2 population of willow to analyze the genetic characteristics of plant height and its components. They found that plant height is controlled by multiple genes, and significant epistatic effects and gene interactions exist. The correlation and genetic parameters between plant height and stem diameter, stem length, branch number, and other traits have been analyzed. The results have provided genetic information for *Salix matsudana* plant type breeding. Jia et al. [8] identified 28 *HD-ZIP* genes from the genome of *S. psammophila* using bioinformatics methods. The *HD-ZIP* gene family is a plant-specific transcription factor involved in plant development and the stress response. In this paper, these genes were divided into four subfamilies, and their gene structure, chromosome distribution, conserved motifs, and evolutionary relationships were analyzed. The expression patterns of these genes in different tissues and under different stress conditions were analyzed using RNA sequencing (RNA-Seq) data and real-time PCR (RT-qPCR) technology. Some genes that may be related to plant type development and stress resistance were found, such as *SpsHDZI-1*, *SpsHDZI-2*, *SpsHDZI-3*, *SpsHDZIII-1*, *SpsHDZIII-2*, and *SpsHDZIV-1*. This provides important basic data for further study on the function and regulation mechanism of *HD-ZIP* genes in *S. psammophila*.

To reveal the molecular mechanism and regulation method of *S. psammophila* plant type formation, this study used RT-qPCR and RNA-seq technology to establish a screening method for *S. psammophila* plant type candidate genes based on the crown–height ratio and correlation coefficient and to screen out the gene combinations that control the plant type of *S. psammophila*. The plant type characteristics of *S. psammophila* in the early stage of growth were evaluated. It was planted in afforestation land suitable for its ecological function to save on cultivation resources, shorten the cultivation cycle, and improve the adaptability and comprehensive benefits of *S. psammophila*. The method used in this study is also applicable for the determination of other plant traits, providing a theoretical basis for the directional breeding of the *S. psammophila* plant type or other plant traits, and it has certain practical significance.

2. Materials and Methods

2.1. Location and Materials

The experimental sites and materials in this study were from the clonal determination forest of *Salix* National Forest Germplasm Resource Library in Ordos City, Inner Mongolia Autonomous Region. The hydroponic site is located in the intelligent greenhouse of Inner Mongolia Agricultural University (average air humidity: 60%; average air temperature: 24 °C; average CO_2 concentration: 550 g/m^3). The instruments used in the experiment were as follows: PCR instrument; ultraviolet spectrophotometer (Nanodrop 2000C); RT-qPCR instrument Roche light Cycler 480II; high-speed centrifuge (Eppendorf Centrifuge 5430); micro-high-speed centrifuge; drying oven (PH070A type); and high-temperature, high-pressure sterilization pot (SX-500 type). The reagents used in the experiment were as follows: RT-qPCR enzyme (SYBR ® Premix Ex TaqTM II, Tli RNaseHPlus), RNA extraction kit (RNeasy Plant Mini Kit), and reverse transcription kit (FastKing RT Kit, KR116; Takara Company, Bejing, China).

2.2. Methods

2.2.1. Investigation of Plant Type

We numbered 200 clones in the clone determination forest, and then we investigated the plant height, crown width, ground diameter, number of sprouted branches, leaf characteristics, and other indicators for two consecutive years in 2021 and 2022. Then, the crown–height ratio was calculated. We found that the crown–height ratios of the two years showed the same trend and a normal distribution. Thus, the plant type traits of *S. psammophila* were stable, and its plant type control genes could be further studied (Figure 1).

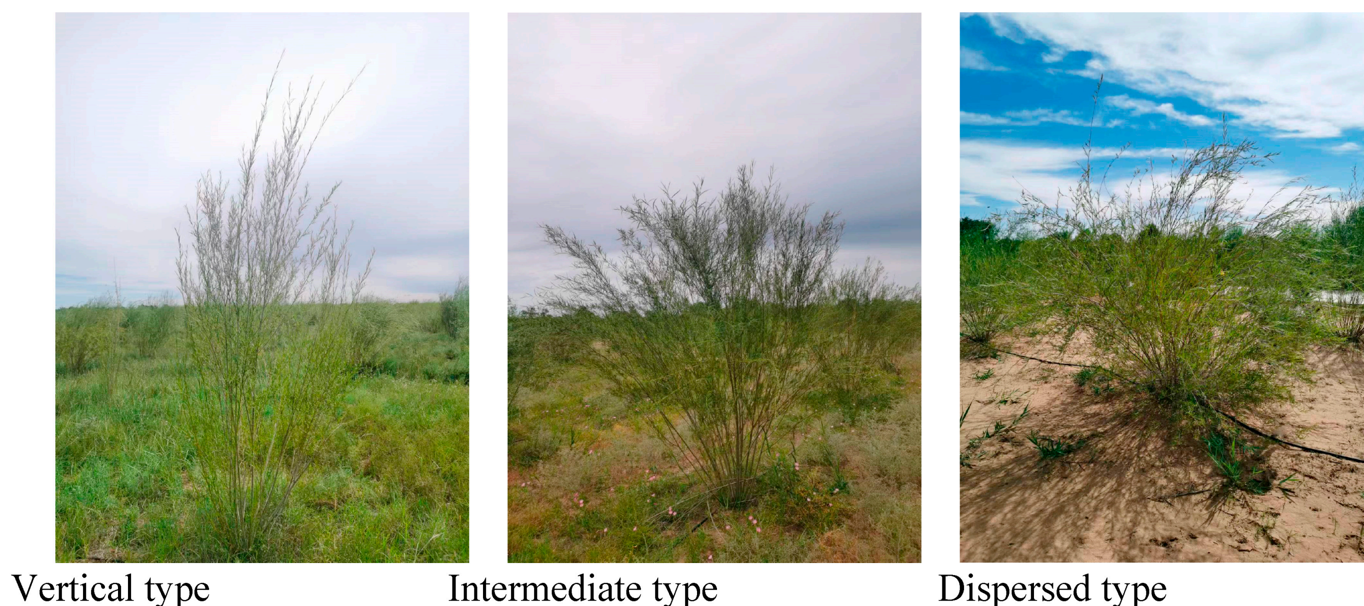

Figure 1. Three plant type characteristics of *S. psammophila*.

2.2.2. Construction of Hydroponic Prediction Group and RNA-seq

In May 2021, we selected nine *S. psammophila* clones with different crown–height ratios, which were upright, intermediate, and scattered, with three biological replicates for each type. We hydroponically treated the branches of these clones and placed them in a greenhouse as a hydroponic prediction group. After 2 months of hydroponic culture, we collected the tender stem samples of the hydroponic prediction group, extracted RNA, and performed an RNA-seq analysis. According to the reference genome of the RNA-seq, we classified the data obtained by sequencing according to known genes and unknown genes. The gene expression in the sequencing results was calculated using the FPKM algorithm.

2.2.3. Target Gene Selection

To select the target genes related to plant type regulation, we used the following strategies: First, we collected some known or predicted genes involved in plant type regulation from the literature and screened them according to their predicted expression levels in *S. psammophila*. Five candidate genes were obtained: *TB1* [9], *SPA2* [10], *ZFP4* [11], *PYL1* [12], and *ABF2* [13]. Second, we selected two genes related to branching that have been cloned and verified in *S. psammophila*: *TAC2* [14] and *LAZY1b* [15]. These two genes positively and negatively regulate the branching angle of plants, respectively. Significant differences existed in the expression levels of these two genes in different plant types of *S. psammophila*. Finally, we selected three genes with higher predictive values (correlation coefficients), namely, *ATX1* [16], *RFK1* [17], and *FHY1* [18]. Although these genes may be involved in plant type regulation, no exact experimental evidence is available to support this notion.

Next, we used SPSS software (v.22) for a rank-sum analysis to test the significance of the expression levels of the different genes and the crown–height ratio. Their mean,

standard deviation, and extreme value were calculated. We only retained genes that were statistically significant ($p < 0.05$) and performed a correlation analysis according to the double- gene combination. The genes were randomly combined according to C_n^m ($m \leq n$, where n is the number of genes with a significance level of $p < 0.05$, and 2 genes were randomly selected from n). Then, we used a multiple regression analysis to evaluate the correlation coefficient between gene expression and the crown–height ratio in each combination. After wards, we selected gene combinations that could significantly reflect the regulation effect of the plant type.

2.2.4. Construction of Field Test Group

We selected 78 clones with vigorous growth, few pests and diseases, and obvious plant type characteristics from the clonal determination forest of the germplasm resource bank. A field test group was then formed after marking. We then extracted RNA from the samples of the field test group. After reverse transcription, we used the cDNA of the test group as a template, with UBQ serving as an internal reference gene. A Roche lightCycler 480 II instrument was used to measure the expression of 10 target genes via RT-qPCR. The primer sequences are shown below (Table 1).

Table 1. The primers used in this study and their sequences.

Primer Name	Primer Sequences (5′ to 3′)
UBQ-F	AAGCCCAAGAAGATCAAGCA
UBQ-R	ACCACCAGCCTTCTGGTAAA
TB1-F	AAGCAAGCAAAACTATCGAGTG
TB1-R	GAAGAAACACTCTTGCTGTCAG
SPA2-F	CTTAGCCATTGTTGGTACATCG
SPA2-R	AAGGTATGACTACGGGAAATCC
Lazy1b-F	CACTGAAGGATTTTGCTATCGG
Lazy1b-R	CAGAAAACCATGGAATAGCTCG
TAC2-F	AAAGATGGGCTCGCTGGAAA
TAC2-R	GTGAATCCTCTACAGCGCGA
ATX1-F	TGGGGCTGTGAAAAGGGTTT
ATX1-R	GCATCTGGCTGCACATTTCC
FHY1-F	TGGGGATTTTTATGGTGAGGAA
FHY1-R	AAGTTTATGGATGCTTGCAGTG
RFK1-F	AAGGACAGACCAGCATCCAG
RFK1-R	GGAAGACGTGGAGGTGGATA
ABF2-F	CAAGAACTTCTCAAATGACCCG
ABF2-R	TGAAGCTCGTCAAAAGTTAACG
PYL1-F	GCAGGTCACGGGGTTTAGTA
PYL1-R	CCGTGTGTCTTCCTCGGTAT
ZFP4-F	AACCTGCATCACGTACCACA
ZFP4-R	AATGAGGATCCATGCAGAGG

2.2.5. RT-qPCR and RNA-seq of Field and Hydroponic Validation Groups

We grouped the field test groups according to the mean ± 0.5 standard deviation of the crown–height ratio. Six groups with different crown–height ratios were obtained. In each group, 4 clones were randomly selected for a total of 24 clones, which served as the field verification group. After collecting the tender stem samples of the field verification group, we immediately placed them in liquid nitrogen and stored them in a refrigerator at −80 °C. We then hydroponically treated the branch samples of the field validation group and placed them in a greenhouse, which served as the hydroponic validation group. On the 10th, 20th, 30th, 40th, and 50th days after the start of hydroponics, we collected tender stem samples from the hydroponic verification group and the field verification group for a total of 5 time points, with 48 samples at each time point. RNA was extracted from these samples and reverse- transcribed into cDNA. The cDNA of the validation group served as a template for RT-qPCR detection.

At each time point, we selected three samples from the hydroponic verification group and the field verification group (upright type, intermediate type, and scattered type), obtaining a total of 30 samples. An RNA-seq analysis was performed to verify the relationship between the FPKM value of the target gene and the crown–height ratio.

2.2.6. Bioinformatics Pre-Analysis

To sequence the transcriptome, we first extracted the total RNA from the sample, then removed the rRNA using a conventional kit, and enriched the mRNA. Next, we reverse-transcribed the enriched mRNA to generate double-stranded cDNA. After repairing the double ends of the cDNA, we added the adaptor and performed PCR amplification to construct a library for sequencing. We enriched the eukaryotic mRNA with polyA tail using magnetic beads with Oligo (dT), and we fragmented the mRNA with buffer. We synthesized the first strand of cDNA in an M-MuLV reverse transcriptase system, using the fragmented mRNA as a template and a random oligonucleotide as a primer. We then degraded the RNA strand using RNase H and synthesized the second strand of cDNA from dNTPs in the DNA polymerase I system. We purified the double-stranded cDNA and subjected it to end repair, A-tail, and the ligation of the sequencing adapter. We screened the cDNA of about 200 bp with AMPure XP beads, and we amplified and purified the PCR product with AMPure XP beads again. Finally, we obtained the library. We then carried out a library quality inspection. We analyzed the RNA integrity and DNA contamination of the samples via agarose gel electrophoresis. We detected the RNA purity (OD260/280 and OD260/230 ratio) using a NanoPhotometer spectrophotometer. We used a Qubit2.0 Fluorometer to accurately quantify the RNA concentration and an Agilent 2100 bioanalyzer to accurately detect RNA integrity.

We performed low-quality data filtering as follows: First, we applied fastp for the quality control of raw reads, filtered out low-quality data, and obtained clean reads. Then, we analyzed the composition and quality distribution of the bases to visually display the data quality. Next, we performed a sequence alignment analysis. We aligned clean reads with the ribosome database of the species using the short reads alignment tool bowtie2, removed the reads that matched the ribosome without allowing mismatches, and used the remaining unmapped reads for a subsequent transcriptome analysis. We conducted a comparative analysis based on reference genomes using HISAT2 software (Version 2.1.0). We calculated the distribution of reads in the reference genome based on the alignment results of all the reads (Total_Mapped reads) that could be mapped to the genome. We classified the regions aligned to the genome into exons, introns, and intergenic regions. We performed a gene analysis and displayed the expression level using the original read count and FPKM. The original read count represents the number of reads contained in the transcript, but it is not suitable for a comparison of differential genes between samples due to the influence of sequencing amount and gene length. To ensure the accuracy of the subsequent analysis, we first corrected the sequencing depth and then corrected the length of the gene or transcript to obtain the FPKM value of the gene for subsequent analysis [19].

3. Results

3.1. Gene Expression Analysis of Prediction Group and Test Group

RNA-seq was performed on the tender stems of nine clones in the prediction group. A total of 40,049 genes were detected, among which 37,865 were known genes and 2184 were unknown. To ensure the accuracy of the information analysis, the obtained raw data were filtered, and low-quality reads with connectors in the raw data were removed to obtain clean data. The Q20 value was above 96%, the Q30 value was above 91%, and the GC% was above 44%. The obtained clean reads were aligned using HISAT2 software. The reference genome was *Salix purpurea*. The proportion of reads successfully mapped to the genome ranged from 80.64% to 83.64%, and the proportion of only one matching point in the reference group ranged from 70.94% to 74.41%. In general, a lower proportion of known genes detected meant that the amount of sequencing data may be insufficient. Conversely,

a higher proportion of unknown genes meant that the integrity of the reference genome may not be sufficiently high.

We performed RNA-seq and RT-qPCR experiments on the samples of the prediction and test groups, measured the expressions of the target genes, and performed a correlation analysis with the crown–height ratio. The results show that the expression levels of the selected target genes showed the same trend in the prediction and test groups. A significant linear relationship existed with the crown–height ratio. The expression levels of the *FHY1* and *TAC2* genes were positively correlated with the crown–height ratio, indicating that these two genes may inhibit the upright growth of *S. psammophila*, whereas the expression levels of the *ATX1*, *ABF2*, *LAZY1b*, *PYL1*, *SPA2*, *TB1*, *ZFP4*, and *RFK1* genes were negatively correlated with the crown–height ratio. This finding indicates that these genes may promote the upright growth of *S. psammophila* or inhibit its dispersal growth. We also found that, except for a small amount of crossover between the *ATX1* and *PYL1* genes, the gene expression fitting lines of other genes in the hydroponic prediction group were higher than those in the field test group. Thus, the gene expression changes under hydroponic conditions were more significant (Figure 2).

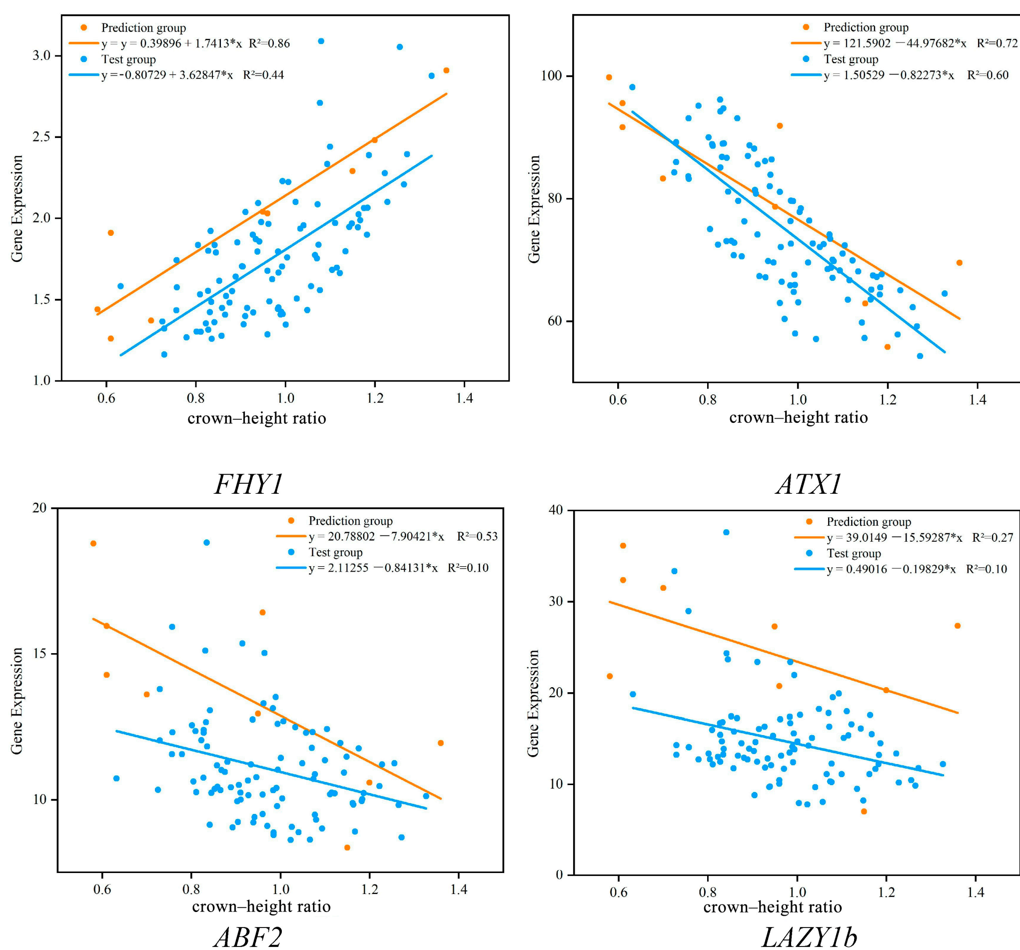

Figure 2. *Cont.*

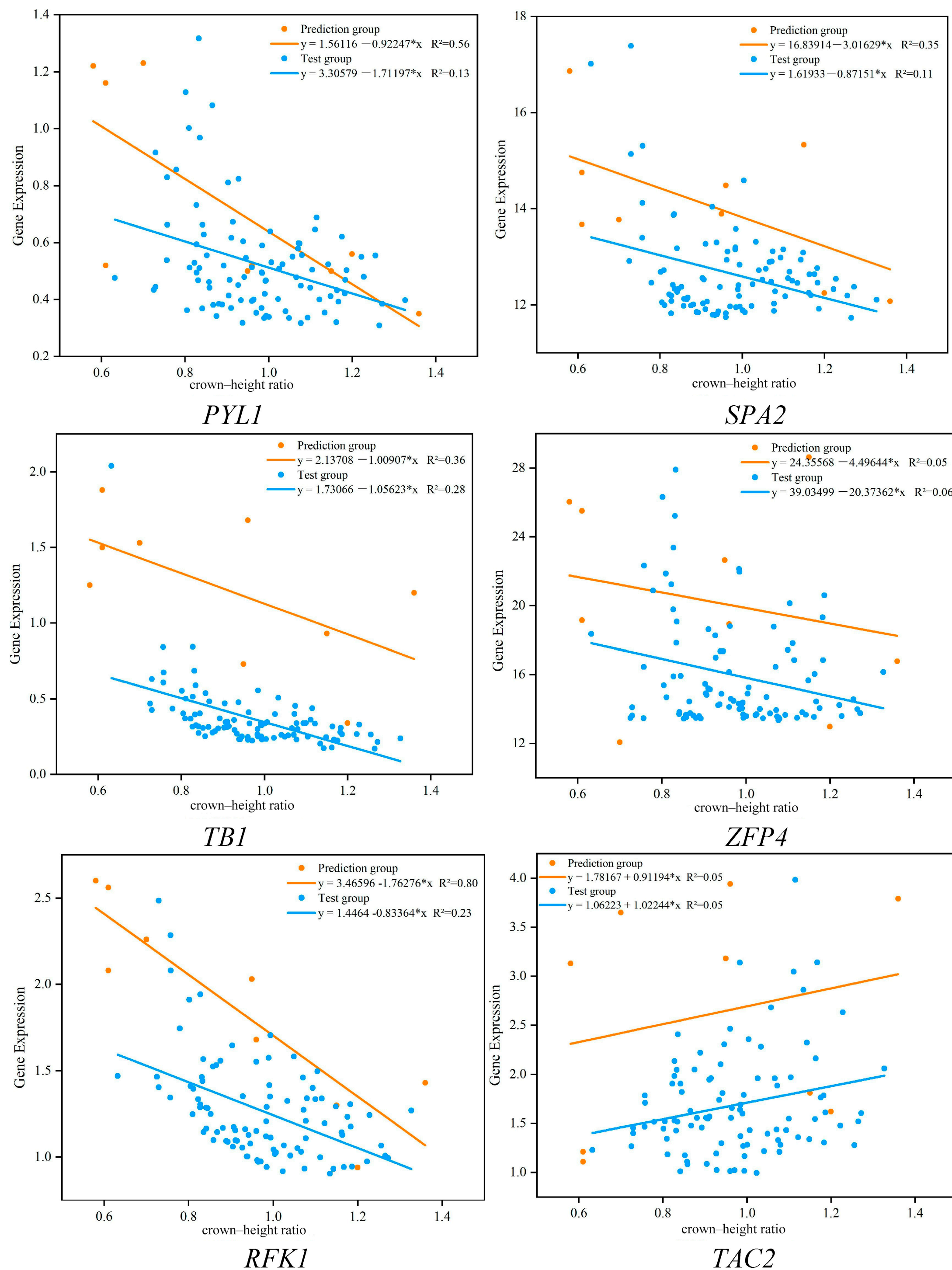

Figure 2. Trend of gene expression in the prediction and test groups.

3.2. Validation Group RT-qPCR and RNA-seq Analysis

To further prove the accuracy of the results, we compared the gene expression levels of the field verification group and the hydroponic verification group at different sampling times using RT-qPCR. The results show that the expression levels of the 10 genes had a consistent trend at different sampling times, and a certain linear relationship existed with the crown–height ratio. By observing the fitting curve, we found that the gene expression levels of most genes measured under hydroponic conditions were higher than those measured by direct sampling in the field, indicating that *S. psammophila* was less disturbed by environmental factors under hydroponic conditions. The addition of a nutrient

solution under hydroponic conditions can promptly supplement the nutrients required for *S. psammophila* so that its tender stems can grow better (Figure 3).

From the field and hydroponic validation groups, one strain of the erect type, intermediate type, and open type was selected, and RNA-seq was carried out at different times. The sequencing results showed that a total of 39,756 genes were detected, among which 37,865 were known genes and 1891 were unknown. The Q20 value was above 96%, the Q30 value was above 91%, and the GC% was above 44%. The obtained raw data were filtered to remove the low-quality reads with connectors in the raw data, and clean data were obtained. The obtained clean reads were aligned using HISAT2 software. The obtained clean reads were subjected to sequence alignment. The proportion of reads successfully mapped to the genome ranged from 83.82% to 87.39%, and the proportion of only one matching point in the reference group ranged from 73.40% to 77.31%.

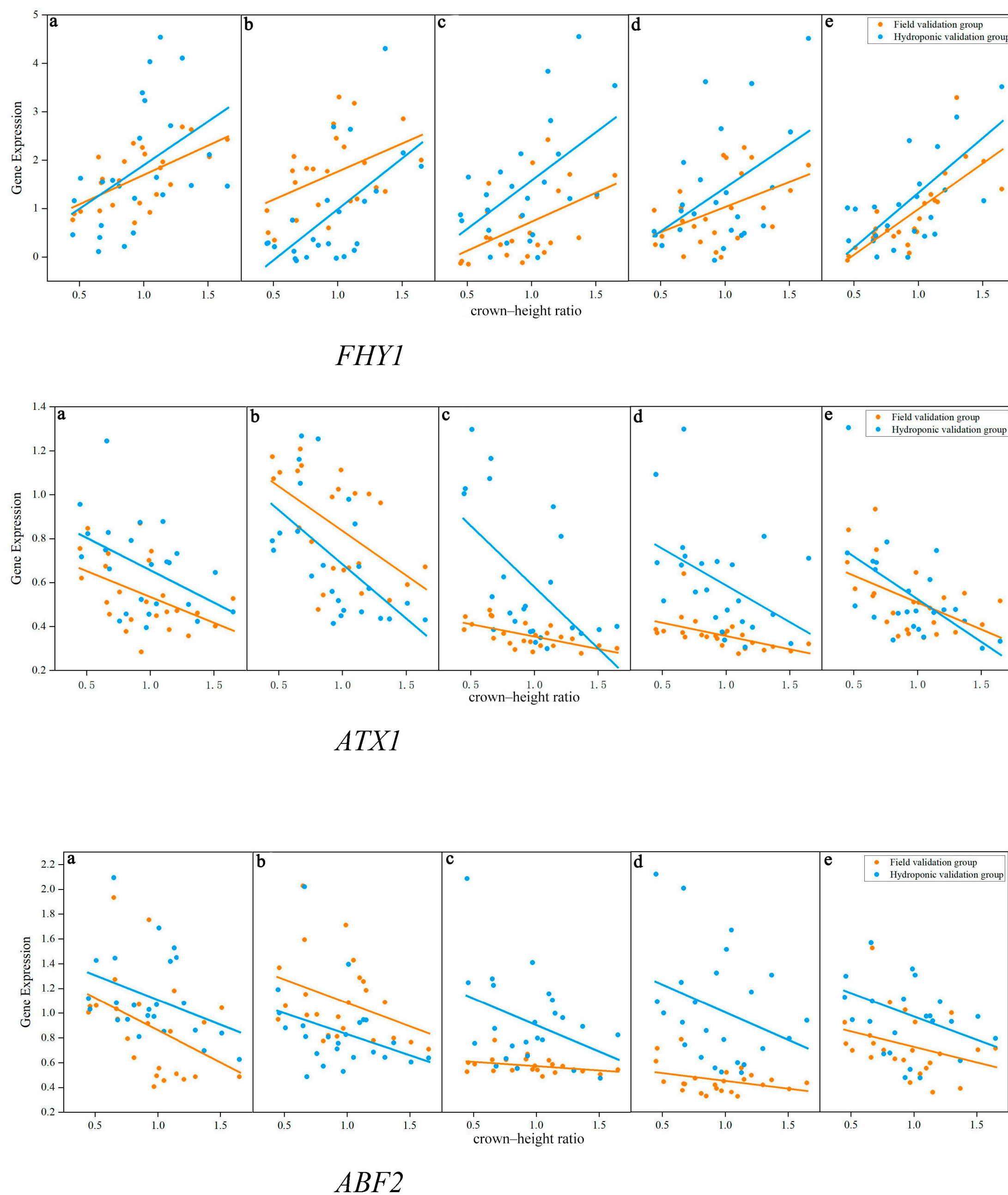

Figure 3. *Cont.*

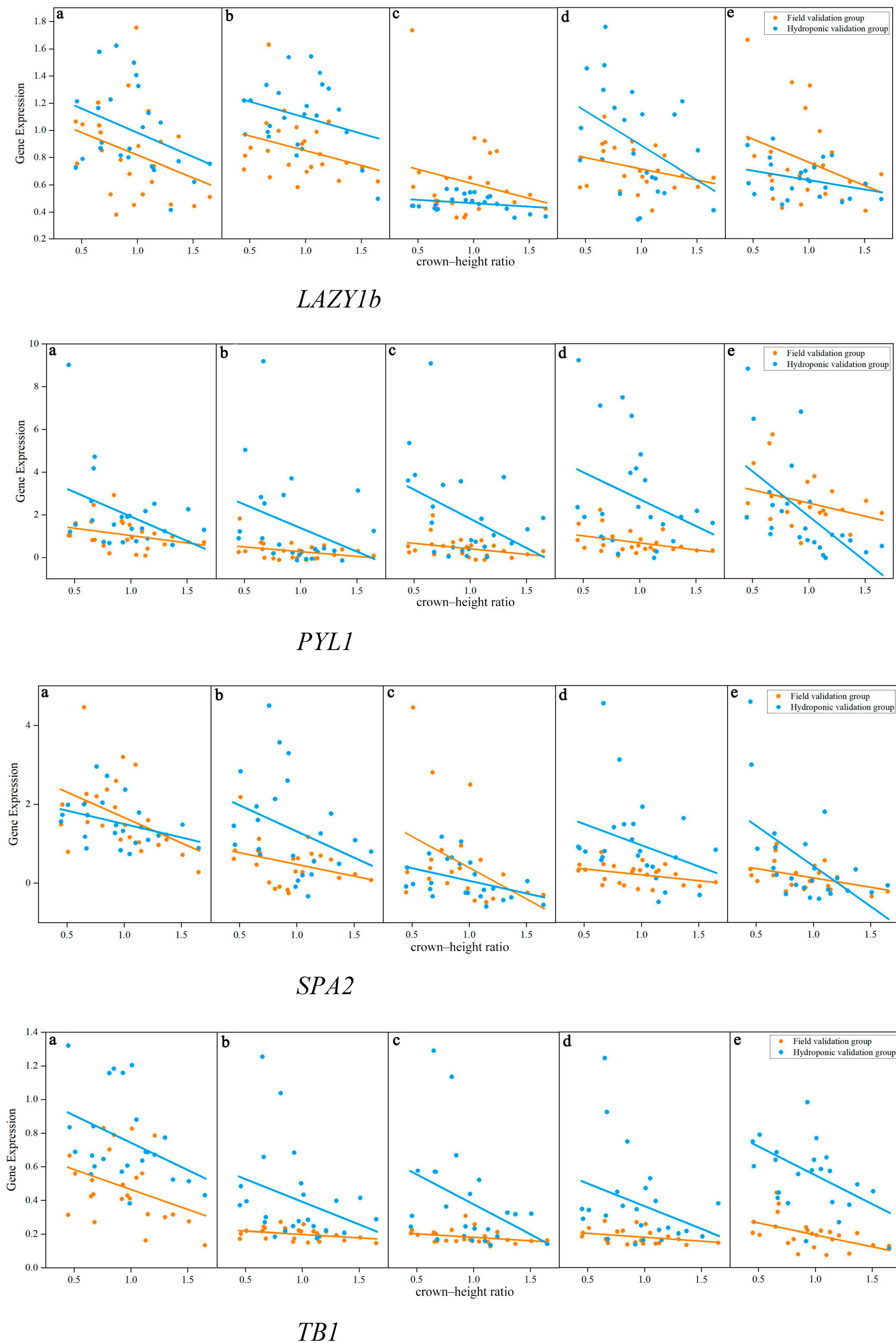

Figure 3. *Cont.*

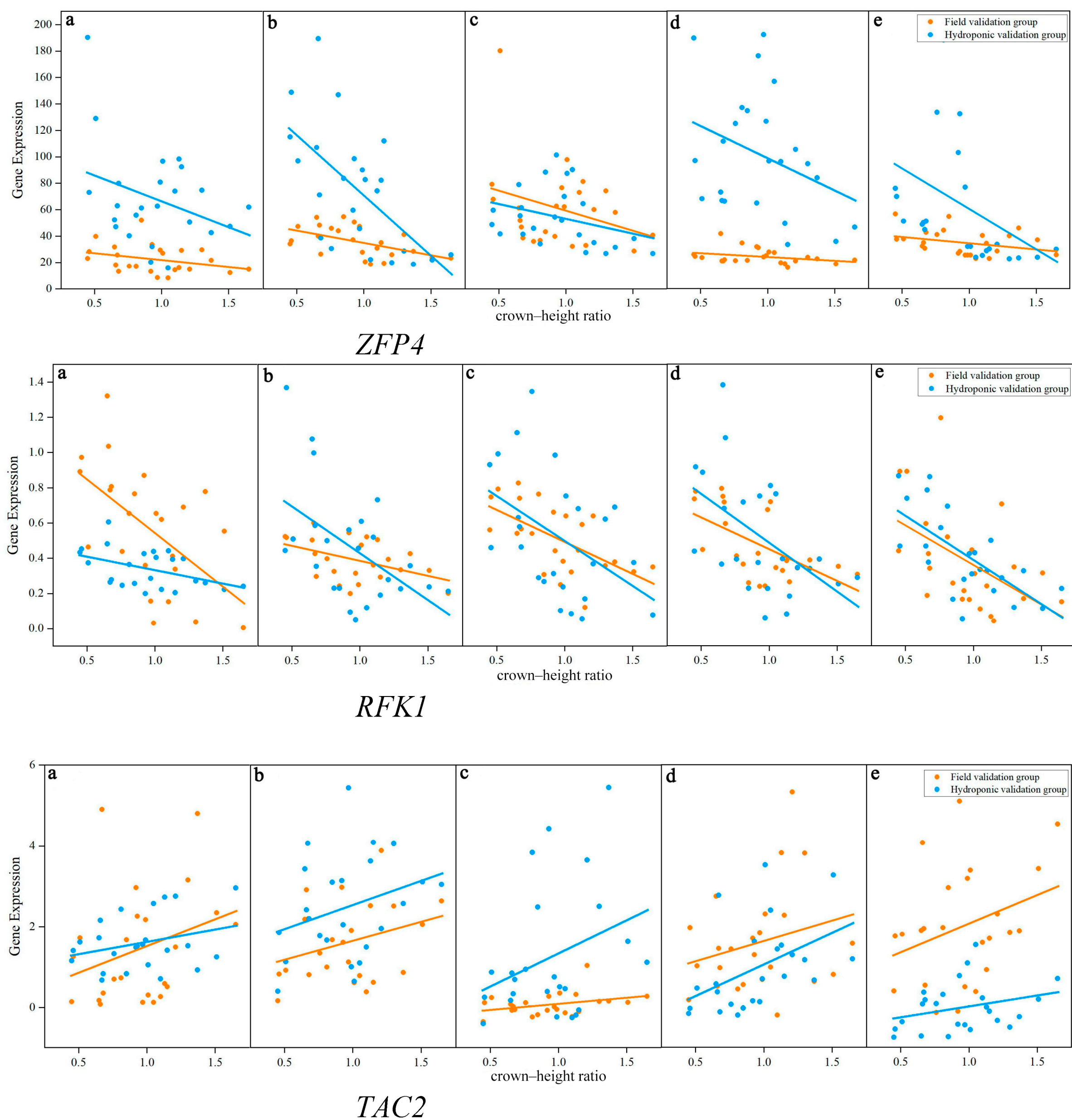

Figure 3. RT-qPCR and RNA-seq results of validation group. Note: (a–e) represent the sampling results of five time periods.

Finally, we analyzed the correlation between the FPKM value and the crown–height ratio. We found that the results were consistent with those of the prediction group. *FHY1* and *TAC2* were positively correlated with the crown–height ratio, whereas *ATX1*, *RFK1*, *PYL1*, *ABF2*, *SPA2*, *TB1*, *ZFP4*, and *LAZY1b* were negatively correlated with the crown–height ratio. Among them, *FHY1*, *ATX1*, and *RFK1* had a high degree of correlation in the hydroponic and field validation groups. Conversely, the correlation coefficients of *PYL1*, *ABF2*, *SPA2*, *TB1*, *ZFP4*, *LAZY1b*, and *TAC2* changed greatly, but the overall trend was consistent with the predicted value (Table 2).

We analyzed the gene expression levels in the prediction and validation groups using RNA-seq. The *ATX1* gene was highly expressed in both groups, whereas the *RFK1*, *PYL1*, and *TB1* genes were lowly expressed. These results are consistent with our previous ones using the FPKM value as a gene expression index, indicating that the FPKM value is a stable and reliable index. However, we also found that, owing to the differences in hydroponic and field cultivation conditions, as well as individual differences in the samples, the values

of gene expression differed between the two populations, resulting in a certain fluctuation in the FPKM values (Table 3). However, this fluctuation did not affect the overall trend and correlation of gene expression.

Table 2. Correlation coefficient between RNA-seq results of validation group and crown–height ratio.

Gene	Date / Cultivation	7.28	8.06	8.16	8.26	9.05
FHY1	Water	0.778	0.685	0.693	0.872	0.831
	Field	0.625	0.623	0.982	0.823	0.985
ATX1	Water	−0.962	−0.980	−0.872	−0.626	−0.996
	Field	−0.974	−0.834	−0.880	−0.881	−0.795
RFK1	Water	−0.729	−0.669	−0.736	−0.757	−0.998
	Field	−0.859	−0.974	−0.910	−0.775	−0.877
PYL1	Water	−0.931	−0.660	−0.899	−0.764	−0.507
	Field	−0.766	−0.775	−0.335	−0.813	−0.287
ABF2	Water	−0.875	−0.730	−0.700	−0.960	−0.255
	Field	−0.932	−0.892	−0.964	−0.357	−0.899
SPA2	Water	−0.668	−0.117	−0.466	−0.782	−0.891
	Field	−0.982	−0.789	−0.924	−0.643	−0.418
TB1	Water	−0.998	−0.994	−0.458	−0.964	−0.800
	Field	−0.813	−0.672	−0.992	−0.768	−0.689
ZFP4	Water	−0.440	−0.449	−0.498	−0.489	−0.529
	Field	−0.628	−0.465	−0.978	−0.761	−0.995
LAZY1b	Water	−0.123	−0.866	−0.360	−0.417	−0.829
	Field	−0.464	−0.230	−0.882	−0.280	−0.931
TAC2	Water	0.210	0.104	0.864	0.782	0.807
	Field	0.976	0.996	0.612	0.530	0.120

Table 3. Changes in FPKM values for the predicted and validated populations.

Groups / Gene	Prediction Group	Validation Groups	
		Field Validation Group	Hydroponic Validation Group
FHY1	1.0–3.0	7.0–17.5	7.0–27.6
ATX1	55.0–100.0	35.2−131.3	33.7–119.6
RFK1	0.9–3.0	0.1–8.3	0.0–6.6
PYL1	0.3–1.3	0.0–1.9	0.1–0.6
ABF2	8.0–19.0	9.6–33.5	11.1–80.0
SPA2	12.0–16.0	3.6–10.9	7.3–17.0
TB1	0.3–2.0	0.2–6.8	0.4–19.3
ZFP4	12.9–26.0	1.8–8.8	3.3–36.6
LAZY1b	5.0–30.0	0.8–3.1	0.2–1.6
TAC2	1.0–6.0	7.5–21.1	10.1–22.9

3.3. Gene Screening for Plant Type Regulation

To explore the correlation between the target genes, we performed a rank-sum analysis on these genes. The expression levels of *ATX1*, *TB1*, *ABF2*, *FHY1*, *RFK1*, and *PYL1* significantly differed in the clones with various crown–height ratios ($p \leq 0.05$), as shown in Table 4. Then, we combined these 6 genes in pairs to obtain 15 possible combinations. SPSS software was used to perform a multiple linear regression analysis on each combination and calculate their correlation coefficients to evaluate their influence on the crown–height ratio.

Table 4. Rank-sum analysis of target genes.

Gene Name	Mean	Standard Deviation	*p*	Min	Max
ATX1	0.726	0.309	0.000	0.130	1.410
LAZY1b	0.302	0.182	0.061	0.040	1.160
TAC2	2.345	1.847	0.207	0.340	8.810
TB1	0.730	0.579	0.000	0.200	5.190
ZFP4	19.732	23.706	0.099	0.110	109.230
ABF2	1.315	0.765	0.044	0.240	4.730
FHY1	2.631	1.596	0.000	0.170	8.090
RFK1	0.657	0.505	0.000	0.020	2.790
SPA2	0.794	0.760	0.078	0.070	4.520
PYL1	1.684	1.407	0.038	0.06	7.63

To further analyze the combined effect of each pair of genes on the crown–height ratio, we calculated the correlation coefficients of the 15 double- gene combinations. The results show that the correlation coefficient of the *ATX1* + *FHY1* combination was the highest, reaching 0.74, indicating that these two genes had the strongest correlation with the crown–height ratio. The correlation coefficient of the *ABF2* + *PYL1* combination was the lowest, only 0.184. Thus, the correlation between these two genes and the crown–height ratio was weak. The correlation coefficients of all double- gene combinations reached a significant level ($p < 0.05$), as shown in Table 5. We also found that the *ATX1* gene was the main factor influencing all the double- gene combinations, indicating that the *ATX1* gene is the most important factor in the regulation of the crown–height ratio.

Table 5. Two-gene combinations and their indices for regression analysis.

Gene Combinations	R^2	*F* Value	*p* Value	Models
ATX1 + *FHY1*	0.740	133.847	0.000	y = 1.159 − 0.566 × *ATX1* + 0.076 × *FHY1*
ATX1 + *TB1*	0.622	77.419	0.000	y = 1.477 − 0.634 × *ATX1* − 0.095 × *TB1*
ATX1 + *RFK1*	0.614	74.900	0.000	y = 1.482 − 0.656 × *ATX1* − 0.09 × *RFK1*
ATX1 + *ABF2*	0.602	71.035	0.000	y = 1.497 − 0.698 × *ATX1* − 0.033 × *ABF2*
ATX1 + *PYL1*	0.596	69.393	0.000	y = 1.472 − 0.741 × *ATX1* + 0.008 × *PYL1*
FHY1 + *TB1*	0.597	69.518	0.000	y = 0.823 − 0.206 × *TB1* + 0.012 × *FHY1*
FHY1 + *RFK1*	0.516	50.109	0.000	y = 0.79+0.103 × *FHY1* − 0.172 × *RFK1*
FHY1 + *ABF2*	0.460	39.988	0.000	y = 0.732+0.113 × *FHY1* − 0.061 × *ABF2*
FHY1 + *PYL1*	0.492	45.534	0.000	y = 0.737 − 0.050 × *PYL1* + 0.112 × *FHY1*
TB1 + *RFK1*	0.381	28.949	0.000	y = 1.226 − 0.208 × *TB1* − 0.194 × *RFK1*
TB1 + *ABF2*	0.323	22.441	0.000	y = 1.23 − 0.242 × *TB1* − 0.08 × *ABF2*
TB1 + *PYL1*	0.367	27.264	0.000	y = 1.232 − 0.249 × *TB1* − 0.061 × *PYL1*
RFK1 + *ABF2*	0.253	15.947	0.000	y = 1.188 − 0.240 × *RFK1* − 0.063 × *ABF2*
RFK1 + *PYL1*	0.274	17.709	0.000	y = 1.178 − 0.046 × *PYL1* − 0.234 × *RFK1*
ABF2 + *PYL1*	0.184	10.564	0.000	y = 1.174 − 0.061 × *PYL1* − 0.095 × *ABF2*

4. Discussion

4.1. Effects of Sampling Sites and Culture Methods on Gene Expression

The stem tip and tender stem of *S. psammophila* can affect the growth direction and morphology of the stem through cell division and differentiation. The stem tip is primarily responsible for the elongation growth of the stem, and the tender stem is primarily responsible for the branch growth of the stem. The growth direction of the shoot tip is regulated by plant hormones and environmental factors [20], whereas the branching growth of tender stems is regulated by genes and hormones. The growth direction of the stem tip has a relatively small effect on the plant type because the stem tip only determines the spindle direction of the stem, and the spindle direction of the stem does not necessarily determine the shape of the plant type [21,22]. The branch growth of tender stems has a relatively large effect on the plant type because tender stems determine the branch angle and branch

number of stems, and the branch angle and branch number of stems directly determine the shape of the plant type [23]. Therefore, the tender stem part of *S. psammophila* has a greater influence on its plant type than the stem tip.

Hydroponics is a kind of soilless culture technology with the advantages of saving resources and reducing the interference of exogenous factors [24]. In this work, hydroponic *S. psammophila* was used as a prediction group for RNA-seq to screen target genes. Through the RT-qPCR analysis of the test and verification groups, it was found that the results for the hydroponic prediction and verification groups had the same trend as the results obtained from the field sampling. This finding indicates that hydroponics had no significant effect on the expression trend of the *S. psammophila* plant type gene.

4.2. Analysis of the Relationship between Genes and Plant Types

LAZY1b and *TAC2* have been proven to be related to the branches of *S. psammophila*, but our experiment revealed a low correlation between their expression and the crown–height ratio. This result may be due to these two genes being non-major genes in the plant type of *S. psammophila*. They indirectly regulate plant morphology by regulating other genes. Plant morphology may also be regulated by endogenous hormones to affect the expression of functional genes and thus affect the plant type.

ZFP4, *TB1*, *SPA2*, *PYL1*, and *ABF2* are differentially expressed genes screened using GO and KEGG enrichment pathway analyses in the RNA-seq results. They are also genes that have been proven to be related to plant type development in other species. In *jatropha curcas*, gibberellin (GA) and cytokinin (CTK) synergistically inhibit *TB1* and promote the germination of lateral buds [25]. sPA is an important negative regulatory molecule in the red and far-red light signaling pathways of *Arabidopsis thaliana*. When light intensity, red light, and far-red light are weakened, its branches are inhibited [26]. Qin [27] found that the *ZFP4* gene in *Betula platyphylla* has multiple ABA -responsive cis-acting elements, which can regulate plant growth and development by regulating the ABA signaling pathway. *PYL1* and *ABF2* can affect plant growth by regulating ABA biosynthesis [28]. These genes indirectly affect the plant type by regulating downstream genes or other pathways. The experimental results of these target genes in the prediction, test, and verification groups had the same trend of change. They can preliminarily predict that these genes can also regulate the growth and development of plants in *S. psammophila*.

FHY1, *ATX1*, and *RFK1* are highly correlated with the plant type of *S. psammophila*. FHY transcription factors have been proven to be an important part of the far-red light signaling pathway. In *A. thaliana*, FHY transcription factors play important roles in regulating plant flowering and meristem formation [29]. *FHY1* can interact with phytochrome and regulate the plant type of *S. psammophila* through auxin (IAA) and GAs [30]. Copper is an important trace element required for plant growth and development, and *ATX1* is a copper chaperone protein. Its role is to supply the metal ions required for plant growth and discharge excessive metal ions to relieve heavy metal toxicity. *ATX1* may affect the growth and development of *S. psammophila* by transporting copper ions to regulate the copper concentration or by regulating the ethylene (ET) signal. The *RFK1* gene has not been proven to be related to plant type regulation in *S. psammophila*. In the present study, the *RFK1* gene in the tender stem part was highly correlated with the plant type of *S. psammophila*, which indicates that the *RFK1* gene can be used as a backup gene for the plant type of *S. psammophila*.

4.3. Plant Hormone Control Plant Type Analysis

ATX1 is a histone methyltransferase that catalyzes the trimethylation of the fourth lysine (H3K4) of histone H3, which is an epigenetic modification associated with transcriptional activation [31]. In *A. thaliana*, *Oryza sativa*, and *Gossypium hirsutum*, *ATX1* has been found to regulate the secondary cell wall synthesis of fiber cells and affect plant height, stem diameter, and stem strength [32]. *ATX1* primarily affects the synthesis of the secondary cell wall by activating the expression of NAC transcription factors in the secondary cell

wall, such as SND1 and NST1 [33]. NAC transcription factors are important plant hormone response elements, which can respond to the signals of GAs, ABA [34,35], jasmonic acid (JA), ET, and other hormones, thereby regulating plant growth and development and the stress response. Therefore, *ATX1* can affect the formation of plant architecture through epigenetic modification and hormone signal transduction.

FHY1 is a light-sensitive protein that binds to phytochrome A (phyA) and promotes the nuclear accumulation of phyA under far-red light irradiation, thereby affecting the phyA-mediated light response [36]. *FHY1* has been found to regulate plant height, branch angle, and inflorescence length in *A. thaliana*, *O. sativa*, and other plants, affecting plant type formation. *FHY1* primarily regulates the expression of transcription factors downstream of phyA, such as FHY3, FAR1, PIF3, PIF4, and PIF5, by affecting the nuclear localization of phyA [37]. These transcription factors can respond to the signals of hormones, such as GAs, ABA, and ET, and they can also regulate plant growth and development and stress responses [38]. Therefore, *FHY1* can affect plant type formation through phytochrome and hormone signal transduction.

In summary, *ATX1* and *FHY1* had the greatest influence on the plant type of *S. psammophila* because they can regulate the signals of plant hormones through different pathways, thereby affecting the cell division, differentiation, and elongation of plants. Ultimately, they influence the expression of the plant type, indicating that they were highly conserved and important.

4.4. Construction and Analysis of Prediction Group

Based on the RNA-seq results, traits and related genes were used to establish the screening of candidate genes for the *S. psammophila* plant type. The purpose of constructing the prediction group was to preliminarily determine the correlation between the plant type and gene expression and to subsequently predict the correlation between functional genes and traits through the basic principle of using fewer samples and obtaining more accurate data.

The different dispersion degrees of *S. psammophila* caused its plant types to vary. In this experiment, three biological replicates were selected for the RNA-seq of the *S. psammophila* upright, intermediate, and scattered types. The results show that the difference between the predicted values of the FPKM value and that of the crown–height ratio was very large, ranging from 0.9 to 0.0001. The predicted values of the target genes (LAZY1 b and *TAC2* genes were small; *FHY1*, *ATX1*, and *RFK1* genes were large; and *ZFP4*, *TB1*, *SPA2*, *ABF2*, and *PYL1* genes were in the middle) in the nine samples were consistent with the trend of the correlation coefficients in the clonal population. Similarly, the correlation coefficient with a large predicted value was large, and the correlation coefficient with a small predicted value was small. The correlation at different sampling time points was consistent with the predicted value, which also verified the accuracy of the RNA-seq results and proved that the selection of nine *S. psammophila* clones can be used to construct a prediction group.

4.5. Advantages of Double-Gene Combinations in Gene Selection

The trait performance of the organism was not the result of the action of a single gene but the function of multiple genes. When screening for the optimal gene of *S. psammophila* plant type regulation, the double-gene combination was used. The double-gene combination can simplify the screening process, reduce the calculation amount and experimental cost, and improve the screening efficiency and accuracy. If multiple genes are combined, it may increase the complexity and difficulty of screening, require more time and resources, and reduce the reliability and sensitivity of screening.

Moreover, the combination of two genes can avoid the interaction or interference between multiple genes, reduce complexity and uncertainty, and improve the reliability and stability of screening. If multiple genes are combined, it may lead to synergistic or antagonistic effects between the genes, thereby affecting gene expression and function.

The double-gene combination can also quickly determine the relationship between genes and plant types according to the size and direction of the correlation coefficient, enabling improved sensitivity and specificity of the screening. If multiple gene combinations are used, the distribution and change in the correlation coefficients may not be obvious, and distinguishing the correlation and causality between genes and plant types would be difficult.

Finally, the double-gene combination can reflect the main factors influencing genes and plant types and thus improve the importance and representativeness of screening. If multiple genes are combined, it may cause the influencing factors of genes and plant types to be too scattered and mixed. Determining the most critical and representative genes would also be difficult.

5. Conclusions

The candidate-gene mining technology of the *S. psammophila* plant type was discussed. The main conclusions are as follows: (1) the *FHY1*, *ATX1*, and *RFK1* genes were highly correlated in different time gradients whether in hydroponics or in field cultivation, and *FHY1* and *TAC2* were positively correlated with the crown–height ratio. *ATX1*, *RFK1*, *PYL1*, *ABF2*, *SPA2*, *TB1*, *ZFP4*, and LAZY1 b were negatively correlated with the crown–height ratio. (2) Through the correlation analysis of double-gene combinations, it was found that the *ATX1* + *FHY1* gene combination had the highest correlation with the crown–height ratio, which was 0.74. These two genes had the greatest effect on the expression of the *S. psammophila* plant type shape. The combination of two genes can improve the screening efficiency and accuracy, as well as the sensitivity and specificity, indicating certain representativeness. Additionally, nine clones were proven to be useful in constructing predictive populations.

Although two genes were selected as the optimal ones to control the expression of the *S. psammophila* plant type using this method, no transgenic verification was carried out. Accordingly, further research will be conducted in the future.

Author Contributions: Conceptualization, G.Z.; methodology, G.Z. and R.H.; software, K.Z.; validation, K.Z. and R.H.; formal analysis, K.Z. and R.H.; investigation, K.Z., R.H. and. X.D.; resources, G.Z., F.Q. and Y.Y.; data curation, R.H. and K.Z.; writing—original draft preparation, K.Z.; writing—review and editing, K.Z. and G.Z.; visualization, K.Z.; supervision, Y.Y. and L.L.; project administration, F.Q. and L.L.; funding acquisition, G.Z. and F.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by The Inner Mongolia Autonomous Region directly under the basic scientific research business costs of colleges and universities “Inner Mongolia Yellow River Basin sandy coarse sand area of forest and grass vegetation quality and efficiency of technological innovation team project” (BR22-13-10); The basic scientific research project of university “Study on the spatial and temporal variation characteristics of hydraulic erosion under different slope vegetation patterns in the coarse sand area of the Yellow River Basin” (BR220109); Project of Inner Mongolia Autonomous Region Science and Technology Office (2021GG0075); The Inner Mongolia Autonomous Region Natural Science Foundation Project (2018MS03013).

Data Availability Statement: The data presented in this study are available on request from the corresponding author or first author. The data are not publicly available due to the data are obtained from paid experiments.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gomez-Roldan, V.; Fermas, S.; Brewer, P.B.; Puech-Pagès, V.; Dun, E.A.; Pillot, J.-P.; Letisse, F.; Matusova, R.; Danoun, S.; Portais, J.-C.; et al. Strigolactone inhibition of shoot branching. *Nature* **2008**, *455*, 189–194. [CrossRef] [PubMed]
2. Umehara, M.; Hanada, A.; Yoshida, S.; Akiyama, K.; Arite, T.; Takeda-Kamiya, N.; Magome, H.; Kamiya, Y.; Shirasu, K.; Yoneyama, K.; et al. Inhibition of shoot branching by new terpenoid plant hormones. *Nature* **2008**, *455*, 195–200. [CrossRef] [PubMed]
3. Huang, J.; Zhou, Y.; Wenninger, J.; Ma, H.; Zhang, J.; Zhang, D. How water use of *Salix psammophila* bush depends on groundwater depth in a semi-desert area. *Environ. Earth Sci.* **2016**, *75*, 556. [CrossRef]

4. Vörösmarty, C.J.; Green, P.; Salisbury, J.; Lammers, R.B. Global Water Resources: Vulnerability from Climate Change and Population Growth. *Science* **2000**, *289*, 284–288. [CrossRef]
5. Cheng, D.; Duan, J.; Qian, K.; Qi, L.; Yang, H.; Chen, X. Groundwater evapotranspiration under psammophilous vegetation covers in the Mu Us Sandy Land, northern China. *J. Arid. Land* **2016**, *9*, 98–108. [CrossRef]
6. Priyana, Y.; Safriningsih, D. The Ready System of Clean Water for Population in Musuk District to Respon Dry Season. *Forum Geogr.* **2016**, *19*, 81. [CrossRef]
7. Wang, X.; Bi, C.; Wang, C.; Ye, Q.; Yin, T.; Ye, N. Genome-wide identification and characterization of WUSCHEL-related homeobox (WOX) genes in Salix suchowensis. *J. For. Res.* **2018**, *30*, 1811–1822. [CrossRef]
8. Jia, H.; Zhang, J.; Li, J.; Sun, P.; Zhang, Y.; Xin, X.; Lu, M.; Hu, J. Genome-wide transcriptomic analysis of a desert willow, *Salix psammophila*, reveals the function of hub genes SpMDP1 and SpWRKY33 in drought tolerance. *BMC Plant Biol.* **2019**, *19*, 356. [CrossRef]
9. Finlayson, S.A. Arabidopsis TEOSINTE BRANCHED1-LIKE 1 Regulates Axillary Bud Outgrowth and is Homologous to Monocot TEOSINTE BRANCHED1. *Plant Cell Physiol.* **2007**, *48*, 667–677. [CrossRef]
10. Ding, C.; Zhang, S.W. Overview of important regulatory genes in plant light signaling pathways. *Shanxi Agric. Sci.* **2015**, *43*, 3. (In Chinese)
11. Yin, M.; Wang, Y.; Zhang, L.; Li, J.; Quan, W.; Yang, L.; Wang, Q.; Chan, Z. The Arabidopsis Cys2/His2 zinc finger transcription factor ZAT18 is a positive regulator of plant tolerance to drought stress. *J. Exp. Bot.* **2017**, *68*, 2991–3005. [CrossRef] [PubMed]
12. Li, W.; Wang, L.; Sheng, X.; Yan, C.; Zhou, R.; Hang, J.; Yin, P.; Yan, N. Molecular basis for the selective and ABA-independent inhibition of PP2CA by PYL13. *Cell Res.* **2013**, *23*, 1369–1379. [CrossRef] [PubMed]
13. Lin, Y.Y.; Li, X.Y. Effect of overexpression of transcription factor AhAREB1 on growth hormone distribution in Arabidopsis thaliana. *J. South China Norm. Univ.* **2015**, *1*, 87–92. (In Chinese) [CrossRef]
14. Zhang, L.; Ye, Z.W. Cloning and bioinformatics analysis of salsify spslazy1a and spslazy1b genes. *J. Northwest For. Coll.* **2017**, *32*, 8. (In Chinese)
15. Yuan, M.R.; Yang, Y. Cloning, bioinformatics and tissue-specific expression analysis of the SpsTAC2 gene in *Salix salicifolia*. *Mol. Plant Breed.* **2019**, *7*, 2144–2151. (In Chinese) [CrossRef]
16. Zhang, Y.; Chen, K.; Zhao, F.-J.; Sun, C.; Jin, C.; Shi, Y.; Sun, Y.; Li, Y.; Yang, M.; Jing, X.; et al. OsATX1 Interacts with Heavy Metal P1B-Type ATPases and Affects Copper Transport and Distribution. *Plant Physiol.* **2018**, *178*, 329–344. [CrossRef]
17. Bohra, A.; Jha, U.C.; Adhimoolam, P.; Bisht, D.; Singh, N.P. Cytoplasmic male sterility (CMS) in hybrid breeding in field crops. *Plant Cell Rep.* **2016**, *35*, 967–993. [CrossRef]
18. Cheng, Y.; Deng, J. Genome-wide identification and expression analysis of the FAR1/FHY3 transcription factor family in tomato. *J. Plant Physiol.* **2021**, *10*, 1983–1995. [CrossRef]
19. *The cDNA/DNA/Small RNA Libraries Were Sequenced on the Illumina Sequencing Platform by Genedenovo Biotechnology Co., Ltd.*; Genedenovo Biotechnology Co., Ltd.: Guangzhou, China, 2023.
20. Vissenberg, K.; Claeijs, N.; Balcerowicz, D.; Schoenaers, S. Hormonal regulation of root hair growth and responses to the environment in Arabidopsis. *J. Exp. Bot.* **2020**, *71*, 2412–2427. [CrossRef]
21. Lu, D.Y.; Zhang, G.S. Research Progress of *Salix psammophila*. *Mol. Plant Breed.* **2020**, *18*, 6. (In Chinese) [CrossRef]
22. Yang, H.F.; Li, A.Y. Identification and anatomical structure of stem banding phenotype of *Salix psammophila*. *Desert China* **2021**, *41*, 45. (In Chinese)
23. Jiang, Y.; Zhu, Y.; Zhang, L.; Su, W.; Peng, J.; Yang, X.; Song, H.; Gao, Y.; Lin, S. EjTFL1 Genes Promote Growth but Inhibit Flower Bud Differentiation in Loquat. *Front. Plant Sci.* **2020**, *11*, 576. [CrossRef] [PubMed]
24. Li, Q.; Li, X.; Tang, B.; Gu, M. Growth Responses and Root Characteristics of Lettuce Grown in Aeroponics, Hydroponics, and Substrate Culture. *Horticulturae* **2018**, *4*, 35. [CrossRef]
25. Ni, J.; Gao, C.; Chen, M.-S.; Pan, B.-Z.; Ye, K.; Xu, Z.-F. Gibberellin Promotes Shoot Branching in the Perennial Woody Plant *Jatropha curcas*. *Plant Cell Physiol.* **2015**, *56*, 1655–1666. [CrossRef] [PubMed]
26. Yang, J.; Lin, R.; Hoecker, U.; Liu, B.; Xu, L.; Wang, H. Repression of light signaling by Arabidopsis SPA1 involves post-translational regulation of HFR1 protein accumulation. *Plant J.* **2005**, *43*, 131–141. [CrossRef] [PubMed]
27. Qin, L.L.; Zhang, X. Cloning of Birch BpZFP4 gene promoter and functional analysis of adversity response elements. *Plant Res.* **2019**, *6*, 917–926. (In Chinese)
28. Chen, N.Y.; Zhang, G.X. Role of Abf transcription factors in plant response to abiotic stress. *J. Plant Genet. Resour.* **2021**, *22*, 9. [CrossRef]
29. Wu, S.W. Molecular Mechanism of Arabidopsis thaliana SPA1 Interaction with Photosensitive Pigment B. Ph.D. Thesis, Chinese Academy of Agricultural Sciences, Beijing, China, 2009. (In Chinese).
30. Hou, Y.; Wang, X.; Zhu, Z.; Sun, M.; Li, M.; Hou, L. Expression Analysis of Genes Related to Auxin Metabolism at Different Growth Stages of Pak Choi. *Hortic. Plant J.* **2019**, *6*, 25–33. [CrossRef]
31. Wang, X.; Wang, D.; Xu, W.; Kong, L.; Ye, X.; Zhuang, Q.; Fan, D.; Luo, K. Histone methyltransferase ATX1 dynamically regulates fiber secondary cell wall biosynthesis in *Arabidopsis* inflorescence stem. *Nucleic Acids Res.* **2020**, *49*, 190–205. [CrossRef]
32. Liu, Y.; Sun, Y.; Yao, H.; Zheng, Y.; Cao, S.; Wang, H. Arabidopsis Circadian Clock Repress Phytochrome a Signaling. *Front. Plant Sci.* **2022**, *13*, 809563. [CrossRef]

33. Ding, Y.; Avramova, Z.; Fromm, M. Two Distinct Roles of ARABIDOPSIS HOMOLOG OF TRITHORAX1 (ATX1) at Promoters and within Transcribed Regions of ATX1-Regulated Genes. *Plant Cell* **2011**, *23*, 350–363. [CrossRef] [PubMed]
34. Hussain, S.; Zhang, N.; Wang, W.; Ahmed, S.; Cheng, Y.; Chen, S.; Wang, X.; Wang, Y.; Hu, X.; Wang, T.; et al. Involvement of ABA Responsive *SVB* Genes in the Regulation of Trichome Formation in Arabidopsis. *Int. J. Mol. Sci.* **2021**, *22*, 6790. [CrossRef]
35. Kim, H.; Lee, K.; Hwang, H.; Bhatnagar, N.; Kim, D.-Y.; Yoon, I.S.; Byun, M.-O.; Kim, S.T.; Jung, K.-H.; Kim, B.-G. Overexpression of *PYL5* in rice enhances drought tolerance, inhibits growth, and modulates gene expression. *J. Exp. Bot.* **2014**, *65*, 453–464. [CrossRef] [PubMed]
36. Hiltbrunner, A.; Tscheuschler, A.; Viczián, A.; Kunkel, T.; Kircher, S.; Schäfer, E. FHY1 and FHL Act Together to Mediate Nuclear Accumulation of the Phytochrome A Photoreceptor. *Plant Cell Physiol.* **2006**, *47*, 1023–1034. [CrossRef]
37. Liu, Y.; Wei, H.; Ma, M.; Li, Q.; Kong, D.; Sun, J.; Ma, X.; Wang, B.; Chen, C.; Xie, Y.; et al. Arabidopsis FHY3 and FAR1 Regulate the Balance between Growth and Defense Responses under Shade Conditions. *Plant Cell* **2019**, *31*, 2089–2106. [CrossRef]
38. Raza, A.; Salehi, H.; Rahman, A.; Zahid, Z.; Haghjou, M.M.; Najafi-Kakavand, S.; Charagh, S.; Osman, H.S.; Albaqami, M.; Zhuang, Y.; et al. Plant hormones and neurotransmitter interactions mediate antioxidant defenses under induced oxidative stress in plants. *Front. Plant Sci.* **2022**, *13*, 961872. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.