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PhSPEAR1 Participates in Regulating the Branch Development of Petunia

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Abstract: *Petunia hybrida* is an economically important ornamental plant species. Branching in ornamental plants is closely associated with their ornamental traits, and branching is a significant agronomic trait in petunia, which shapes plant architecture and production cost. Although there are few studies regarding the involvement of the *SPEAR* genes in lateral branch development, they are known to regulate the development of plant organs. The *PhSPEAR1* gene in petunia, an ortholog of Arabidopsis *SPEAR1*, was isolated for study. According to the results of real-time quantitative PCR (qRT-PCR), *PhSPEAR1* was primarily expressed in the roots. The fluorescence signal indicated that PhSPEAR1 was localized in the nucleus and cytoplasm. An increase in *PhSPEAR1* expression was induced by cytokinin or decapitation. Overexpression of *PhSPEAR1* increased lateral branches in Arabidopsis. Based on our findings, *PhSPEAR1* participates significantly in the regulation of branch number in petunia.

Keywords: Arabidopsis; *Petunia hybrida*; branch development; regulation mechanism; *PhSPEAR1*

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1. Introduction

Branching is a crucial aspect of plant growth and development that influences plant morphogenesis, crop yield, and ornamental characteristics. The regulation of branch development involves pathways associated with strigolactone (SL), gibberellin (GA), auxin, and cytokinin (CK) [1–4]. Moreover, factors like sugar, temperature, nutrient availability, and light conditions can influence branch development [5–9]. The primary determinant of branching ability, however, is the genetic makeup of the plant itself. Thus, a key area of research for branch development is the study of gene function.

Over the last ten years, our knowledge of controlling shoot branching has become increasingly detailed. Auxin is regarded as the main trigger for apical dominance. The growing, immature leaves at the apex of the shoot produce auxin. Auxin influx and efflux carriers, which are polarly localized in plasma membranes, help to transport it basipetally [10]. However, lateral buds are not directly affected by auxin, which is derived from the shoot apex. It is therefore believed that the mechanism of auxin action is exerted through various methods, including canalization and secondary messengers. CK promotes bud outgrowth and is considered the second messenger of auxin. It is transported from the roots to the shoots [11], and its local synthesis in the stem is likely the primary means of regulating shoot branching [12]. Another class of factors implicated in regulating shoot branching in a variety of plant species is SLs [13]. SLs in the roots can be transported acropetally to shoots and inhibit shoot branching locally by inducing *BRC1/TB1* [14,15]. Research conducted on a diverse range of species revealed a negative correlation between bud activity and the abundance of ABA in buds [16]. In many species, including tomatoes

and *Ipomoea nil*, exogenous ABA hinders the growth of buds [17]. In a study by Arend et al. [18], it was observed that lowering ABA sensitivity led to greater lateral bud growth in *Populus* × *canescens*. These investigations point to ABA's role in regulating the development of branches.

In addition to the well-characterized transcriptional regulator *BRC1*, a number of genes also control branch processes, and their respective mechanisms of action have been elucidated. We still lack a thorough understanding of the control mechanisms underlying branching patterns, despite the identification and characterization of many of the genes that regulate them. SPL-like, EAR-containing (SPEAR) proteins are unique to embryophytes and not present in other organisms; therefore, it has been hypothesized that they are embryophyte-specific genes. They have the same general structure: an N-terminal NLS, which is followed by the SPL motif, and a C-terminal EAR motif (LxLxL) [19]. TCP interactors containing EAR motif protein 1 (TIE1) and TIE2, as members of SPEAR proteins, can interact with ARABIDOPSIS RESPONSE REGULATORs (ARRs) to modulate CK responses in roots [20,21]. TIE1 has also been found to be involved in the regulation of Arabidopsis branching. SPOROCYTELESS (SPL), another member of the SPEAR family, can interact with the TOPLESS (TPL) co-repressors in Arabidopsis and transcription factors of the TCP family to perform its transcription repressive function [19]. These existing studies indicate that SPEARs are involved in multiple developmental processes in plants. Although SPEARs have been studied in several species, they have not been reported in petunia yet.

Petunia hybrida Vilm. is an extremely important ornamental plant, and its branching characteristics directly affect its ornamental and commercial value. Therefore, the study of branch regulation in petunia has important practical application value. Secondly, petunia, as a model plant, can help to quickly and accurately explore the regulatory mechanisms of branch development. To investigate the function of *SPEAR1* in petunia, we cloned it and studied its expression characteristics, subcellular localization, and transgenic plant phenotype. This research will lay the groundwork for further understanding the regulatory mechanism of *SPEAR1*.

2. Materials and Methods

2.1. Plant Materials

The *Petunia* × *hybrida* cv. Mitchell Diploid, *Arabidopsis thaliana* Col-0, *Nicotiana benthamiana*, and the transgenic plants utilized in this research were all placed in the plant growth room of the School of Horticulture, Anhui Agricultural University. The growing conditions were adjusted to 25 ± 2 °C, with 3500 LX and a 16/8 h light/dark cycle.

2.2. Isolation of PhSPEAR1

We isolated total RNA from pooled leaves of 10 seedlings of *Petunia* × *hybrida* cv. Mitchell Diploid (40-day-old) utilizing the RNAprep Pure Plant Plus Kit (TIANGEN Biotech Co., Ltd., Beijing, China). The Prime-Script™ RT reagent kit with gDNA Eraser (TaKaRa Biotechnology Co., Ltd., Dalian, China) was then used to synthesize first-strand complementary deoxyribonucleic acid (cDNA) and remove DNA. Based on the genome sequence of petunia available on the Solanaceae Genome Network [22], primers F_{SPEAR1} (5′-3′): ATGGGCAGCAATTATTTTGGGGA and R_{SPEAR1} (5′-3′): TCAAAGGGAAAGTCTG AGCTCCA were used to amplify the *PhSPEAR1* with MegaFi^{TM} Fidelity 2 × PCR Master Mix (Toyobo Co., Ltd., Shanghai, China), and the amplified products were then purified and sequenced at General Biotechnology Co., Ltd. (Chuzhou, China). Sequencing was repeated three times to ultimately determine the correct sequence.

2.3. Bioinformatic Analysis

The protein physicochemical properties of PhSPEAR1 were predicted with the ExPASy-ProtParam tool (https://web.expasy.org/protparam/, accessed on 3 August 2023). MEGA software (version 7.0.14) was used to run the ClustalW program and perform multiple sequence alignment. Phylogenetic trees based on Arabidopsis SPEARs and PhSPEAR1

were generated using MEGA v7.0.14 software and the maximum-likelihood method with a bootstrapping value of 1000. Arabidopsis SPEARs sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 4 August 2023). The conserved domains were identified using the online software Pfam (http://www.sanger.ac.uk/Software/Pfam/, accessed on 5 August 2023).

2.4. Subcellular Localization Analysis

To generate the PhSPEAR1-enhanced green fluorescent protein (eGFP) fusion protein, the *PhSPEAR1* lacking the stop codon was amplified using primers SPEAR1-1300-F (5′-3′): GAGAACACGGGGACTCTAGAATGGGCAGCAATTATTTTG GGGA and SPEAR1-1300-R (5′-3′): GCCCTTGCTCACCATGGATCCTCAAAGGGAAA GTCTGAGCTCCA. The amplified PCR product was digested with *Hind III* (TaKaRa Biotechnology Co., Ltd., Dalian, China) and *Sal I* (TaKaRa Biotechnology Co., Ltd., Dalian, China). The recombinant reaction was performed according to the instructions of the NovoRec® plus One step PCR Cloning Kit (Novoprotein, Shanghai, China). The recombinant product was transformed into competent cells of *Escherichia coli* DH5α (Shanghai Tolo Biotechnology Co., Ltd., Shanghai, China). After screening for positive clones, the plasmid of pSuper1300-PhSPEAR1-eGFP was extracted and transformed into an *A. tumefaciens* GV3101 strain (Shanghai Tolo Biotechnology Co., Ltd., Shanghai, China) using the freeze–thaw method, and positive clones were selected for temporary infiltration into *N. benthamiana* leaves using a previously described procedure [23]. After 48 h of culture at 25 °C, the eGFP fluorescence was observed using a confocal microscope (Olympus FV1200, Tokyo, Japan).

2.5. qRT-PCR Analysis

Four plant parts—roots, stems, leaves, and buds—from 75-day-old plants of *Petunia* × *hybrida* cv. Mitchell Diploid were obtained to perform a tissue expression experiment. Notably, samples of the leaves, buds, and stems were taken from the shoot tip downward within the range of nodes 2 to 6. Furthermore, newly grown, tender roots were chosen.

In the treatment experiment, 40-day-old plants of *Petunia* × *hybrida* cv. Mitchell Diploid were split into three groups, including control, decapitated, and the third group that was treated with 10 µL of 50 µM 6-benzylaminopurine (6-BA) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) at the axillary buds at the top 4 th nodes. Buds at the first node (decapitation) or fourth node (6-BA treatment) were collected after six hours. For the control plants, the fourth bud from top to bottom was collected. Then, all the tissues were promptly submerged in liquid nitrogen and kept at −80 °C.

There were three biological and three technical replicates in every experiment, unless stipulated otherwise. For every technical replicate, 20–40 plants were pooled to provide tissue samples.

Two micrograms of DNase I (TaKaRa Biotechnology Co., Ltd., Dalian, China)-digested RNA from various samples were reverse-transcribed into cDNA. qRT-PCR was conducted with the Thermo PIKO REAL96 Real-Time PCR System employing a GoTaq qPCR Master Mix (Promega, Madison, WA, USA). Amplification was conducted by an initial denaturation at 95 °C for 30 s, then 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min) were performed. *P. hybrida Glyceraldehyde-3-phosphate dehydrogenase* (*PhGAPDH*) was employed as the reference gene with primers F_{GAPDH} (5′-3′): CAAGGCTGGAATTGCTTTGAG and R_{GAPDH} (5′-3′): CACCACTTTACTCCACTGATGCA. The $2^{-\Delta\Delta CT}$ method was utilized to calculate the relative expression [24]. In addition, all primers used in this study were designed and validated using Primer 5 software.

2.6. Construction and Transformation of 35S::PhSPEAR1

The primers SPEAR1-1300-F and SPEAR1-1300-R were synthesized by adding *Hind III* and *Sal I* restriction sites, and the target gene product was obtained by amplification with high-fidelity KOD DNA polymerase (Toyobo, Osaka, Japan). Furthermore, the pCAM-

BIA1300 vector was double-digested. The enzyme digestion vector and the target gene product were connected by homologous recombination. The recombinant product was transformed into *E. coli*-competent cells using the freeze–thaw method and cultured in LB medium containing kanamycin. After successful detection, the plasmid was extracted, and the 35S::PhSPEAR1 vector was obtained.

2.7. Arabidopsis Transformation

Using the *A. tumefaciens*-mediated flower-dipping method, the constructed pCAMBIA1300-PhSPEAR1-eGFP was introduced into Arabidopsis WT [25]. After the seeds of the infiltrated plants were harvested, they were transferred to a Murashige and Skoog (MS) medium (Solarbio Biotech Co., Ltd., Beijing, China) with 50 mg/L of kanamycin (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Kanamycin-resistant seedlings were cultivated in a growth chamber and transplanted into the soil after 15 days of germination.

2.8. Phenotype Analysis and Statistical Analysis

The number of rosette branches (bud length $\geq$ 10 mm) and the height of the main stems were measured for phenotypic analysis. All the data in this study was expressed as the mean value $\pm$ standard deviation.

2.9. Sample Preparation and Plant Hormone Extraction

The rosette sections of 25-day-old WT and transgenic plants were harvested, instantly frozen in liquid nitrogen, ground into powder, and kept at -80 °C until needed. About 50 mg of the plant sample was transferred into a 2 mL centrifuge tube kept in frozen liquid nitrogen and then dissolved in 1 mL of methanol/water/formic acid (15:4:1, $v/v/v$). To quantify the extraction solution as the internal standard, 10 µL of an internal standard solution (100 ng/mL) was added. After 10 min of vortexing, the mixture was centrifuged for 5 min at 6000$\times$ g and 4 °C. After centrifugation, the supernatant was transferred to clean plastic microtubes and dried before being dissolved in 100 µL of 80% methanol (v/v) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Following filtration through a 0.22 µm membrane filter (Beijing Heng Odd Instrument Co., Ltd., Beijing, China), LC-MS/MS culture analysis was conducted. MetWare (http://www.metware.cn/, accessed on 10 May 2024) was used to detect phytohormone contents utilizing the QTRAP 6500 LC-MS/MS platform (AB Sciex, Concord, ON, Canada).

2.10. Statistical Analysis

All the data were subjected to analysis of Student's *t*-tests using IBM SPSS Statistics software (version 27.0).

3. Results

3.1. Identification of SPEAR1 in Petunia

Five Arabidopsis SPEARs were used with the petunia SPEAR to generate a phylogenetic tree. Evolutionary analysis combined with sequence alignment suggested that the petunia SPEAR and Arabidopsis SPEAR1 were closely related (Figure 1), suggesting that the petunia *SPEAR* gene is a potential homolog of the Arabidopsis *SPEAR1* homolog, hence the name *PhSPEAR1*. Sequence alignment of the PhSPEAR1 and Arabidopsis SPEAR homologs was performed, and the results showed that the N-terminus of PhSPEAR1 protein contained an SPL motif (residues 33 and 49) and the NLS region (residues 16 and 25). A typical EAR motif sequence (DLELRL) is present in the C-terminal region (residues 185 and 192) (Figure 1B).

The full length of the *PhSPEAR1* gene was 579 bp, encoding a protein of 192 amino acids. Computationally, the molecular formula of the PhSPEAR1 protein was $C_{896}H_{1417}N_{273}O_{309}S_8$, the relative molecular weight was 21.2 KD, the isoelectric point was 8.54, and the instability parameter was 69.80. The highest content of PhSPEAR1 was Ser (19.8%, 38), and the lowest content was Trp (0.05%, 1) and Cys (1.0%, 2). Asp + Glu (total negatively charged residues)

had a total of 18, and Arg + Lys (total positively charged residues) had a total of 20. The theoretical half-life was 30 h.

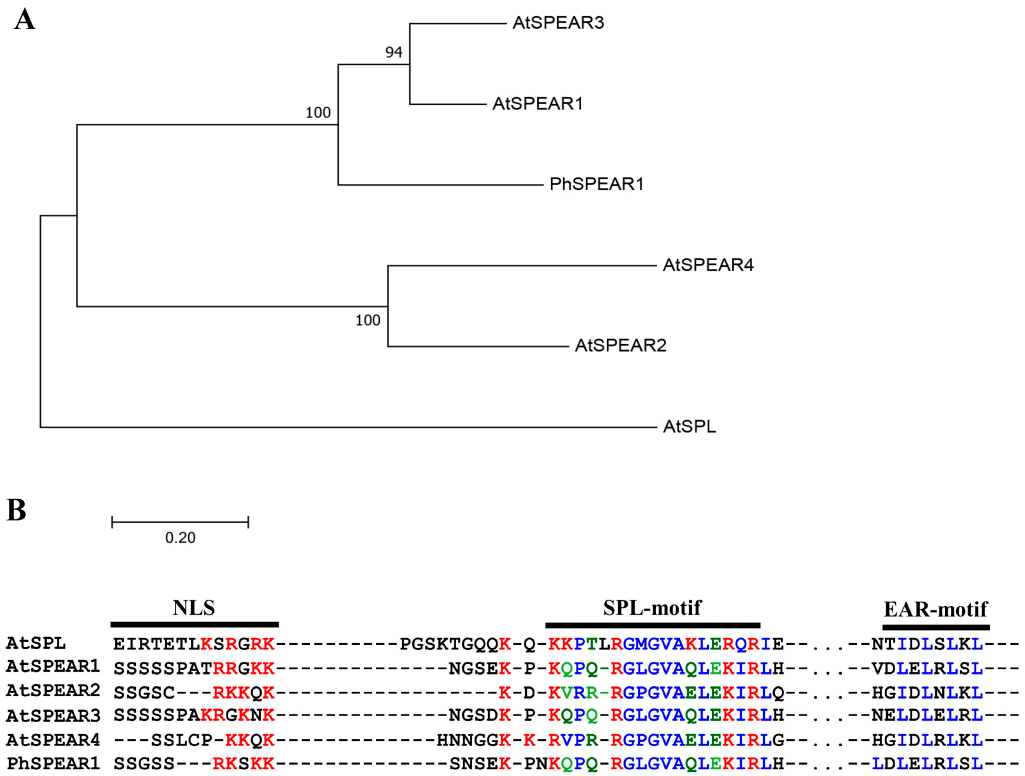

Figure 1. Comparison of SPEAR proteins. (**A**) Evolutionary maximum-likelihood tree analysis; (**B**) protein structure analysis of SPEARs. NLS, SPL motif, and EAR motif are all marked by black lines.

3.2. Tissue-Specific Expression Analysis of PhSPEAR1

The expression levels of the *PhSPEAR1* gene in the roots, stems, leaves, and axillary buds of petunia were determined by qRT-PCR to establish the expression level of the gene in various tissues. *PhSPEAR1* was expressed in all tested tissues. The *PhSPEAR1* gene was expressed highest in roots, followed by leaves, and was least expressed in buds. The expression level of *PhSPEAR1* in the buds was 1/13 of the expression in the roots (Figure 2).

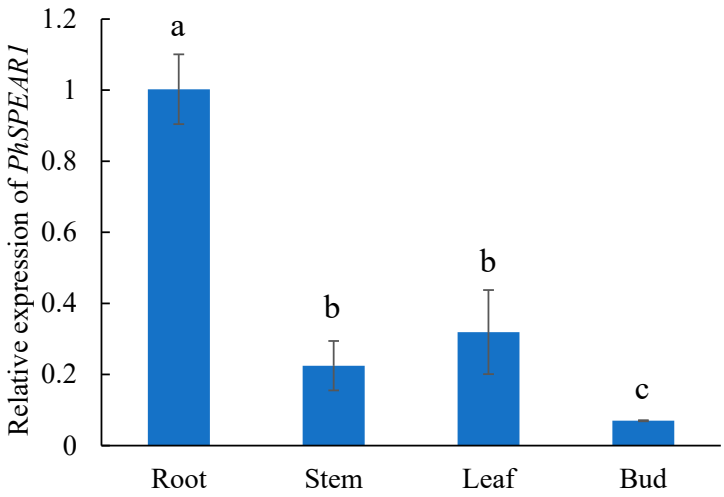

Figure 2. Expression analysis of *PhSPEAR1* in four tissues of *Petunia* × *hybrida* cv. Mitchell Diploid. Lowercase letters a, b and c indicate significant differences via Student's *t*-tests at $p < 0.05$.

3.3. Expression Analysis of PhSPEAR1 under Branch-Inducing Treatments

To investigate the response of the *PhSPEAR1* gene to decapitation and CK, 6-BA was applied to the axil of petunia seedlings. qRT-PCR was used to assess the expression levels of *PhSPEAR1*. Both 6-BA and decapitation both doubled the expression level of *PhSPEAR1* (Figure 3).

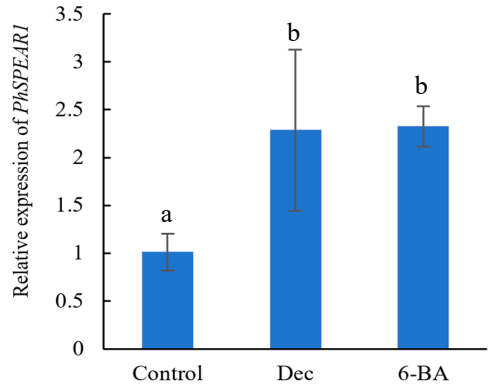

Figure 3. Expression analysis of *PhSPEAR1* under branch-inducing treatments. Dec: decapitation, 6-BA: 6-BA treatment. Lowercase letters a and b indicate significant differences via Student's *t*-tests at $p < 0.05$.

3.4. Subcellular Localization Analysis of PhSPEAR1

To examine PhSPEAR1's subcellular location, the 35S::PhSPEAR1:eGFP vector was constructed, transformed into Agrobacterium, and injected into tobacco leaves. The fluorescence was observed by confocal microscopy after 48 h. PhSPEAR1 fused with eGFP produced green fluorescence in the nucleus and cytoplasm, being that PhSPEAR1 was localized to the nucleus and cytoplasm (Figure 4).

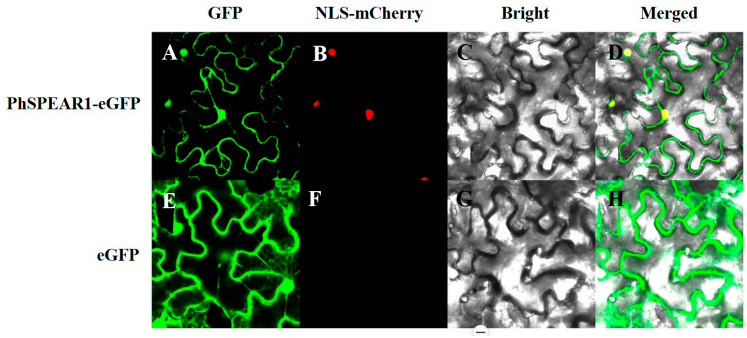

Figure 4. Subcellular localization of PhSPEAR1. (**A**,**E**) Green fluorescence images of PhSPEAR1-eGFP protein and eGFP (control); (**B**) red fluorescence images of NLS-mCherry; (**C**,**G**) bright field images; (**D**,**H**) the merged images. (**F**) Dark field.

3.5. Overexpression of PhSPEAR1 Increases Branch Number in Arabidopsis

To analyze the function of the *PhSPEAR1* gene, construct 35S::PhSPEAR1 was transformed into Arabidopsis, and the transgenic plants were screened. Our findings demonstrated that 35S::PhSPEAR1 plants produced more first-order rosette branches than the WT (Figure 5A–C). Compared with the WT, the average number of branches of transgenic plants increased from 1.8 to 3.5, 4.2, and 4.4, respectively (Figure 5A,C). WT plants displayed about four lateral branches after 60 d of growth, whereas 35S::PhSPEAR1 lines 1, 3, and 3 displayed about seven, eight, and eight lateral branches, respectively. RT-PCR revealed no *PhSPEAR1* expression in the leaves of 28-day-old WT plants, and in 35S::PhSPEAR1 transgenic plants, *PhSPEAR1* transcript abundance was detected (Figure 5D).

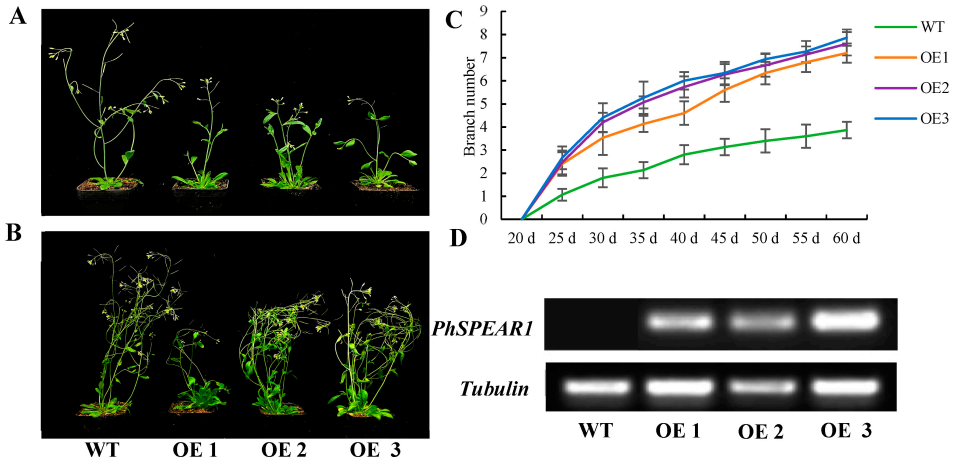

Figure 5. Phenotype analysis of WT and transgenic Arabidopsis plants overexpressing *PhSPEAR1*. (**A**) Comparison of phenotypes of WT and transgenic Arabidopsis plants after 30 d of growth; (**B**) comparison of phenotypes of WT and transgenic Arabidopsis plants after 60 d of growth; (**C**) branch number of WT and transgenic Arabidopsis plants at different times (n = 15); (**D**) expression analysis of *PhSPEAR1* in WT and transgenic Arabidopsis plants. OE represents transgenic plants overexpressing *PhSPEAR1*.

3.6. PhSPEAR1 Regulates the Synthesis of CKs, IAA, and ABA

To analyze the downstream regulatory pathway of *PhSPEAR1*, hormone levels were detected in the OE2 line plants of transgenic Arabidopsis overexpressing *PhSPEAR1*, which exhibited a more pronounced phenotype compared to other transgenic strains. As shown in Figure 6, overexpression of *PhSPEAR1* led to significant downregulation of IAA and upregulation of the important cytokinin types trans-zeatin (tZ) and trans-zeatin riboside (tZR). ABA levels were also significantly decreased. Based on the above conclusions, we speculate that *PhSPEAR1* affected branch development by promoting the synthesis of CK and inhibiting the synthesis of hormones such as IAA and ABA.

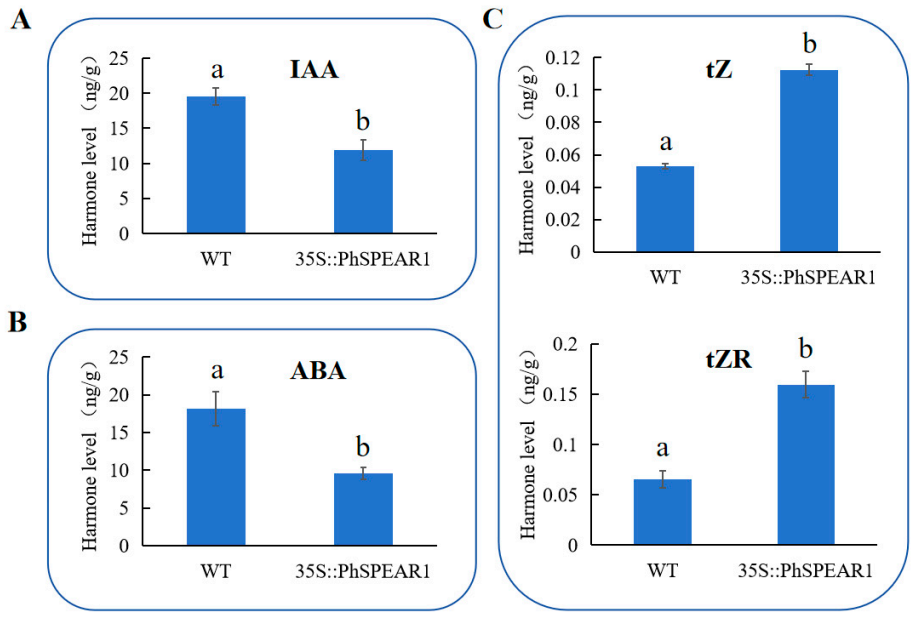

Figure 6. Hormone levels of transgenic Arabidopsis plants overexpressing *PhSPEAR1*. (**A**) Detection of IAA level; (**B**) detection of ABA level; (**C**) detection of tZ and tZR levels. Lowercase letters a and b indicate significant differences via Student's t-tests at $p < 0.05$.

4. Discussion

SPEAR refers to proteins with NLS, SPL motif, and EAR motif. SPL is the most extensively studied of the reported SPEAR proteins and is involved in multiple developmental processes in plants, such as sporocyte, ovule, fruit initiation, lateral organs, and stamen identity [26–30]. However, less research has been carried out on other SPEAR members, including AthSPEAR1 (AT2G20080), AthSPEAR2 (AT2G34010), AthSPEAR3 (AT4G28840), and AthSPEAR4 (AT1G29010). To enrich our understanding of the function of SPEAR proteins, we cloned a homologous gene of *SPEAR1* in petunia and analyzed its function.

We isolated one *SPEAR* gene in *Petunia* × *hybrida* cv. Mitchell Diploid, which contained three structural domains of SPEAR proteins. Phylogenetic tree and sequence alignment analyses show AtSPEAR1 was more closely related to PhSPEAR1, suggesting that this gene may be a homologous gene of AtSPEAR1.

Analysis of the expression characteristics of the *PhSPEAR1* gene in various tissues revealed that the gene was significantly expressed in the roots, followed by the leaves. These findings raise the possibility that *PhSPEAR1* plays a significant role in the development of roots and leaves in petunia.

Since there are reports showing that the *SPEAR* gene is involved in regulating plant branch development, in order to analyze whether *PhSPEAR1* is involved in branching regulation, we conducted branch-inducing treatments on it. Studies have shown that decapitation triggers axillary bud germination by activating multiple downstream pathways [31–33] and cytokinin functions by directly promoting axillary bud germination by regulating multiple genes, such as *BRC1* [2]. Our findings demonstrated that 6-BA and decapitation both upregulated the *PhSPEAR1* gene approximately twofold, suggesting that cytokinin and decapitation influence petunia bud outgrowth through upregulating *PhSPEAR1*. As a gene regulator, PhSPEAR1 is likely localized in the nucleus. To verify our hypothesis, we observed the fluorescence of PhSPEAR1-GFP. We found that it was localized to the nucleus and cytoplasm. Therefore, it likely interacts with genetic material.

While the candidate gene in petunia was divergent from Arabidopsis, key protein structures of the SPEAR family were maintained. Therefore, we tested the gene's function by overexpressing *PhSPEAR1* in Arabidopsis. The results showed that the overexpression of the *PhSPEAR1* gene resulted in a marked increase in the number of branches. The main stem of Arabidopsis WT was visible at 30 days old, with fewer rosette branches and shorter lengths. Transgenic plants, however, were relatively short in height overall and exhibited clear lateral branches growing at the base, along with weaker apical advantages. Transgenic plants typically had seven or eight branches when they reached 60 days of growth, compared to roughly 3.8 for wild types. It was evident that *PhSPEAR1* stimulated more dormant axillary buds, which caused them to germinate and produce more branches.

To determine whether *PhSPEAR1* controls the synthesis of hormones, we used transgenic Arabidopsis plants that overexpressed *PhSPEAR1* to perform hormone detection. The synthesis of CK was found to be significantly increased upon overexpression of *PhSPEAR1*, while the levels of IAA and ABA were found to be significantly decreased. These findings suggest that *PhSPEAR1* regulates branch development by influencing the synthesis of these three hormones. Considering that CKs can regulate the expression of *PhSPEAR1*, it is speculated that *PhSPEAR1* may have feedback regulation with CKs. The regulation of downstream hormone pathways or genes by *PhSPEAR1* at the molecular level will be analyzed through transcriptome sequencing in the future. This study established the biological role of *PhSPEAR1* in petunia, paving the way for additional analyses of its regulatory mechanism and the provision of genetic resources for the breeding of new petunia varieties.

Author Contributions: Conceptualization, L.D.; methodology, W.W. and C.L.; formal analysis, W.W., C.L. and Q.Z.; data curation, Y.W., X.L. and Q.Y.; writing—original draft preparation, W.W. and C.L.; writing—review and editing, L.D. All authors have read and agreed to the published version of the manuscript.

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