

Article

Detecting the Different Responses of Roots and Shoots to Gravity in *Salix matsudana* (Koidz)

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Abstract: The study of the gravity response of roots and shoots is of great significance when exploring the polarity of plants and the development of the forest industry. In our study, normal and inverted cuts of *Salix matsudana* (Koidz) were cultured. The total RNAs of roots and shoots were extracted. Based on the comparative transcriptome, 412 and 668 genes were differentially expressed. The plasma membrane-, cell wall-, and extracellular region-related genes were up-regulated in the shoots, while the carbon metabolism and the nitrogen metabolism were up-regulated in the roots. Combining the alternative splicing genes, we found a potential gravity response network: in the shoots, LecRLKs were highly sensitive to gravity and further affected the alternative splicing of SNARE, as well as inducing an inhomogeneous distribution of auxin and a negative geotropism curve. In the roots, AP2/ERFs and STRKs were highly sensitive to gravity and regulated the expression level of STPKs and WAKs, finally resulting in a geotropism curve. Moreover, cell division was suppressed in both the roots and the shoots under inverted conditions with different mechanisms. Cell division inhibitors (KRPs) were up-regulated in the roots, while DNA helicase MCMs were down-regulated in the shoots. These results provide an important foundation for further studies of the molecular mechanisms and genetic regulation of plant responses to gravity and the plant polarity of forest trees.

Keywords: *Salix matsudana* (Koidz); gravity; roots; shoots; transcriptome

1. Introduction

Forests provide the most important natural raw material for industry and the environment [1]. Trees show obvious orientational growth in their stems and roots, and gravity is one of the most important environmental signals during this plant orientational growth [2]. Plants can reorient their growth after sensing gravitational stimuli to maintain the optimal angle of their organs. Although the first research of tropism was performed approximately 300 years ago, the fundamental aspects of the underlying mechanisms are still unclear [3].

Until now, researchers divided the response to gravity into four steps: (i) the sensing of gravity; (ii) the transduction of the gravity signal; (iii) the generating, maintaining, and transmitting of the auxin gradient; and (iv) the response of the organ [4,5]. The roots manifest positive gravitropism, while the shoots manifest negative gravitropism [3]. In the first step, statocytes, types of cells that are located in the root columella and the shoot endodermal, have been identified as being able to sense gravity [3,6]. Amyloplasts are enriched in these cells [7,8]. With amyloplast sedimentation, an auxin gradient is generated [9], which finally promotes the downward curvature of roots and the upward

curvature of shoots. Several genes have been verified as being able to generate an auxin gradient [10]. The most studied genes in this step are *PINs*, which encode auxin efflux carriers [11].

Previous studies have found that the localization pattern of PIN proteins is consistent with the direction of auxin transport, and their uneven polarity distribution on the plasma membrane is associated with a change in the auxin concentration gradient [12]. In *Arabidopsis*, a total of eight PIN proteins were detected, and at least five of them were found to be related to gravitropism. In the roots, PIN3 and PIN7 were identified in the lower side of the root cap columella cells, initiating the differential flow of auxin toward the lower flank of the root [13,14]. The AUXIN-BINDING PROTEIN 1 (ABP1) and the TRANSPORT-INHIBITOR-RESISTANT 1 (TIR1)/AUXIN SIGNALING F-BOX (AFB) receptors can bind to auxin and activate or suppress cell expansion on the upper and lower sides of the roots and shoots [15].

Previous studies have also identified that SNARES can affect the gravitropism of shoots [16]. SNARES are named after SNAP (soluble NSF attachment protein) receptors and are small proteins that mediate vesicle fusion [17,18]. However, the manner in which gravity signals transduce to auxin gradient-related genes is still unclear.

Salix matsudana (Koidz) is widely distributed across the world, especially in China [19]. Additionally, willows have the characteristics of easy rooting and germination. Thus, they are an ideal model system for studying the response to gravity. In this study, the responses of roots and shoots to gravity were compared, based on the transcriptome. Several functionally enriched items in the roots and shoots were identified. We also proposed a potential functional network for the curvature of roots and shoots. These results provide an important foundation for further studies on the molecular mechanisms and genetic regulation of plant responses to gravity and the plant polarity of forest trees.

2. Materials and Methods

2.1. Plant Materials and Tissue Collection

A cultivar of *Salix matsudana* (Koidz) (“9901”) was grown at the experimental forest farm in Nantong, Jiangsu, China, in 2015 [20]. The branches of “9901” were clipped to 10 cm in length and 1 cm in thickness. The cuts were hydroponically cultured in water under two conditions, with the morphology end of the cuts under water (i.e., the normal condition, marked as “CK”) and the cuts inverted, or with the morphology top of the cuts under water (i.e., the inverted condition, marked as “T”). The clipped branches were grown under each condition in three biological replications for RNA-seq at Nantong University in November 2020. After 20 days, the newly sprouted roots and shoots were collected from each replication. The excised roots and shoots were immediately frozen in liquid nitrogen and stored at -80°C until use.

2.2. RNA Sequencing and Library Construction

The excised roots and shoots were ground in liquid nitrogen. The Plant RNA Reagent kit (Tiangen, China) was used to extract total RNAs from three replications. The Nanodrop ND 2000 spectrophotometer (NanoDrop, Thermo, Waltham, MA, USA) was then used to quantify the RNAs. The RNAs were then stored at -80°C before performing the RNA sequencing. Finally, Illumina sequencing technology (Illumina, San Diego, CA, USA) was employed to perform RNA sequencing by Biomarker (Beijing, China).

2.3. Analysis of the Sequencing Data

To get clean full-length reads, low-quality and adapter reads were removed from the raw data [1]. Then, the clean reads were aligned onto the reference genome of *S. matsudana* (“Yanjiang”) [19], using HiSAT2 software with default parameters [21]. Then, StringTie was used to detect new transcripts [22]. To calculate the fragments per kilobase of transcriptome per million mapped reads (FPKM), RSEM was selected to normalize the length of the gene and the number of mapped reads.

The identified genes were annotated by using the BLASTx search in the NCBI non-redundant protein database to annotate the identified genes [23]. Then, Gene Ontology (GO) categorization, clusters of eukaryotic orthologous groups (KOG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using BMKCloud (www.biocloud.net, version 2.0, accessed on 10 November 2021). The data that supported the findings of this study were deposited into the CNGB Sequence Archive (CNSA) of the China National GeneBank Database (CNGBdb) with accession number CNP0002161.

2.4. Identification of Differentially Expressed Genes

To identify the differentially expressed genes (DEGs), DESeq2 was selected. Two standards were taken to detect the DEGs: (1) The fold change should be no less than 2 between different libraries, and (2) the adjusted false discovery rate (FDR) should be less than 0.05 [24].

2.5. Analysis of the Alternative Splicing Genes under Different Conditions

StringTie was used to splice the comparison results of Hisat2 [22], while alternative splice types and expression levels in each sample were obtained by ASProfile software [25]. Additionally, the alternative splicing events identified in all three biological replications were selected as alternative splicing genes. The alternative splice types of the alternative splicing genes were then analyzed.

2.6. Construction of a Protein Interaction Network

The DEGs in the roots and shoots were used to build protein–protein interaction (PPI) networks. The DEGs were first aligned to the *Populus trichocarpa* protein sequences to identify the homologous genes by using the BLAST tool with a threshold value no more than 10^{-10} [26]. Then, the data were submitted to the search tool for the retrieval of interacting genes database (<http://string-db.org/>, version 11.5, accessed on 10 November 2021) [27] and visualized using Cytoscape software (version 3.5.1) [28]. The top three ranked DEGs were treated as hub genes and selected for further analysis.

2.7. qRT-PCR Analysis of the Gravity-Related Genes

The total RNAs of each sample were extracted, and qRT-PCR analysis was performed. The One-Step SYBR Primer Script Plus RT-PCR kit (Takara, Beijing, China) was used to perform the qRT-PCR experiment. The *Actin* gene was used as the internal control for the candidate genes [20]. Finally, the relative expression levels (R.E.L.) were measured by using the $2^{-\Delta\Delta C_t}$ method. The primers are listed in the Supplementary Table S1.

3. Results

3.1. Overview of the RNA Sequencing Data

Gravity may affect many traits of the roots and shoots of plants. Twenty days after being hydroponically cultured, the roots showed positive gravitropism, while the shoots showed negative gravitropism (Figure 1a). Additionally, the shoots were much smaller and the roots much shorter under the inverted condition than under the normal condition. To characterize the role of the response of active genes to gravity in the roots and shoots, deep-sequencing libraries were generated using the total RNAs extracted from the roots and shoots under normal and inverted conditions. After trimming off the adapter sequences and removing the low-quality reads, we obtained 19,434,675–22,891,810 clean reads for the 12 libraries, with a single read length of 90 bp and a Q30 percentage (percentage of the sequences with sequencing error rates lower than 0.1%) over 94% (Supplementary Table S2). The clean reads were then mapped onto the reference genome of *S. matsudana* using HISAT2. In total, 35,585 (61.52% of the 57,841 gene models in the reference genome) genes were identified as being expressed in at least one library. Moreover, 5715 and 4525 genes were identified as only expressed in the shoots and roots, respectively. While considering the

effect of gravity, 1111 and 951 genes were specially expressed under the inverted condition in the roots and shoots, respectively.

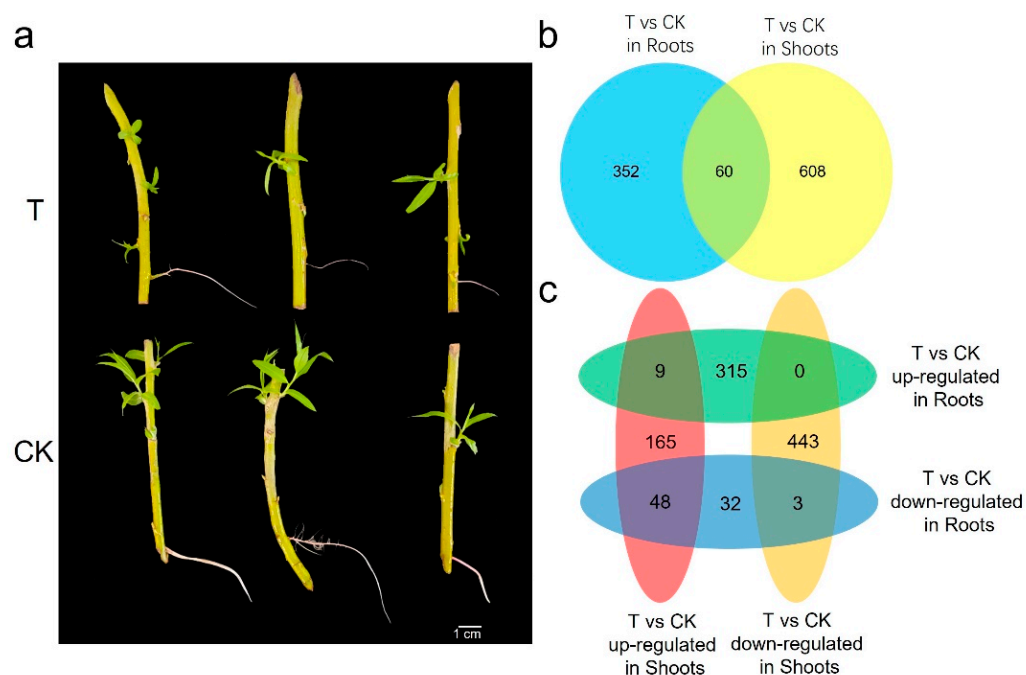


Figure 1. The roots and shoots showed different responses to gravity: (a) the phenotypes of the roots and shoots under normal and inverted conditions; (b) Venn analysis of the differentially expressed genes (DEGs) in the roots and shoots.; (c) Venn analysis of the up- and down-regulated DEGs in the roots and shoots.

3.2. The Differentially Expressed Genes between the Roots and Shoots under Normal and Inverted Conditions

The DEGs under normal and inverted conditions in the roots and shoots were then identified using a threshold $FDR \leq 0.05$ and the absolute value of $\log_2$ -fold change ≥ 1 , respectively. A total of 412 and 668 DEGs were identified in the roots and shoots, respectively (Figure 1b). Under normal conditions, most of the DEGs showed high expression levels in the roots (326/412) and low expression levels in the shoots (446/668) (Figure 1c). According to the Venn diagram (Figure 1c), nine and three DEGs were identified, respectively, as being up- and down-regulated in both the roots and shoots under the inverted condition. Moreover, 48 DEGs were identified as showing the opposite trend of expression in the shoots and roots. Interestingly, all of these 48 DEGs showed up-regulation in the shoots and down-regulation in the roots. Among the 412 and 668 DEGs, only 60 were identified in both the roots and the shoots. This result means that the mechanisms of the response to gravity in the roots and shoots may differ.

3.3. Construction of Gravity-Related Gene Expression Networks in the Roots and Shoots

To further understand the difference between the gravity response of the roots and shoots, enrichment analysis of their DEGs was performed. As shown in Figure 2a, most of the DEGs were enriched in transcription according to the eggNOG function classification. The DEGs were also selected for GO enrichment analysis. For molecular functioning, DNA binding, and transcription factor activity, sequence-specific DNA binding was enriched. The nucleus and the plasma membrane were enriched in the cellular component. For biological processes, oxidation reduction processes and the regulation of transcription were identified as being enriched.

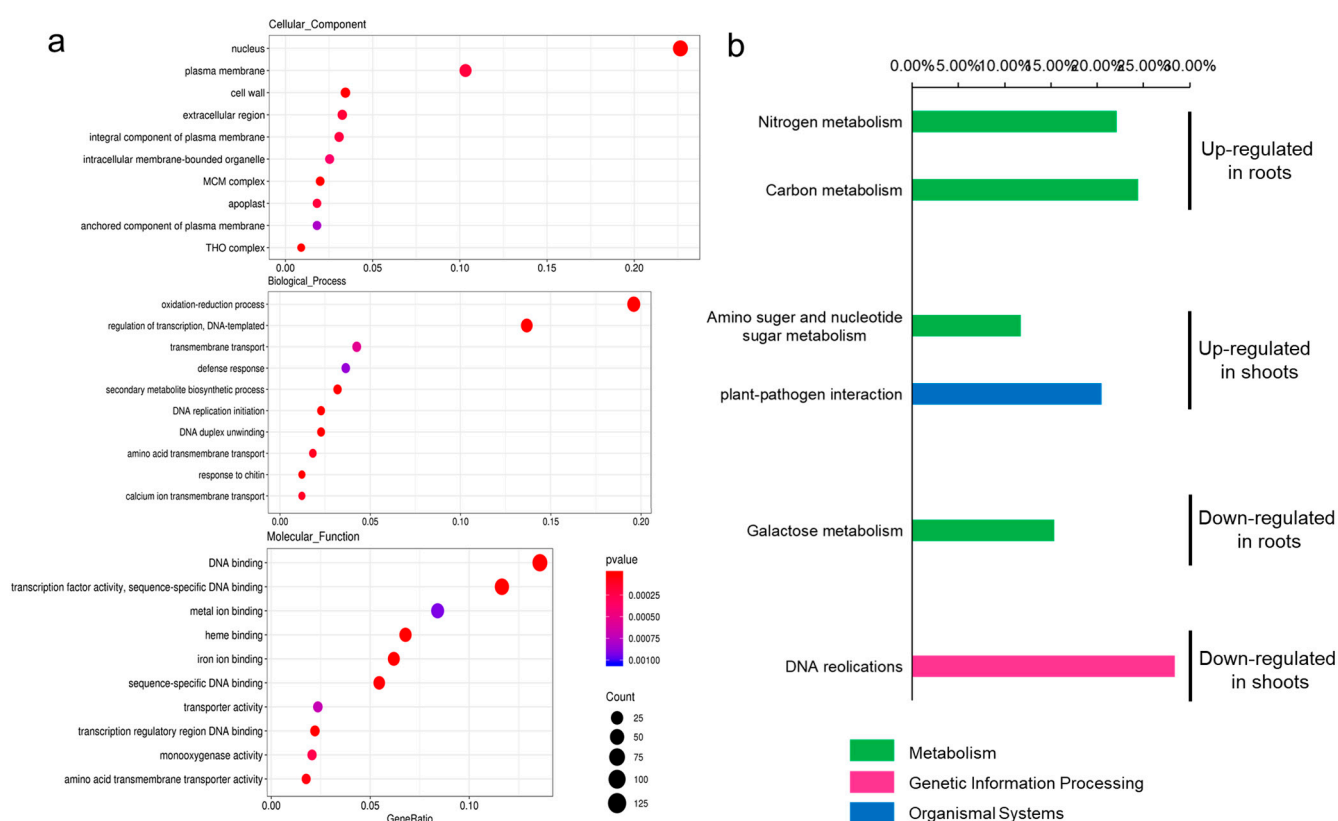


Figure 2. (a) GO and (b) KEGG analysis of the differentially expressed genes (DEGs).

We then compared the KEGG class of up- and down-regulated DEGs between the roots and shoots. The up-regulated genes were enriched in the carbon metabolism and nitrogen metabolism in the roots, while enriched in the amino sugar and nucleotide sugar metabolism in the shoots. Down-regulated genes were enriched in the galactose metabolism and DNA replication in the roots and shoots, respectively (Figure 2b). According to the GO enrichment analysis, the enriched items in the cellular component of the roots and shoots were detected. For the DEGs in the shoots, the up-regulated genes were mostly enriched in the plasma membrane, cell wall, and extracellular region, while the down-regulated genes were mostly enriched in the nucleus. In contrast, the up-regulated DEGs in roots were mostly enriched in the nucleus. Although the most enriched item of the down-regulated DEGs in roots was the nucleus, the plasma membrane was also significantly enriched (Figure 3a).

The KOG function classification analysis also showed that the signal transduction mechanism and transcription were enriched at high levels in both the shoots and the roots (Figure 3b). We then compared the DEGs in the signal transduction mechanism in the roots and shoots. Interestingly, all of these genes in the roots were up-regulated, annotated as serine/threonine protein kinases (STPKs) and Wall-associated receptor kinases (WAKs). In the shoots, the genes encoding the STPKs and MAPKs were down-regulated, while the genes encoding the lectin receptor-like kinases (LecRLKs) were up-regulated (Table S3). These results indicate that the roots' response to gravity by the STRKs regulated the network, while the shoots' response to gravity mainly through the LecRLKs regulated the network. Regarding transcription, opposite trends were also observed in the roots and shoots. Nearly all of the transcripts were up-regulated in the roots but down-regulated in the shoots, and more than half of the transcripts were AP2/ERFs, indicating that ethylene may be more important in the roots than in the shoots.

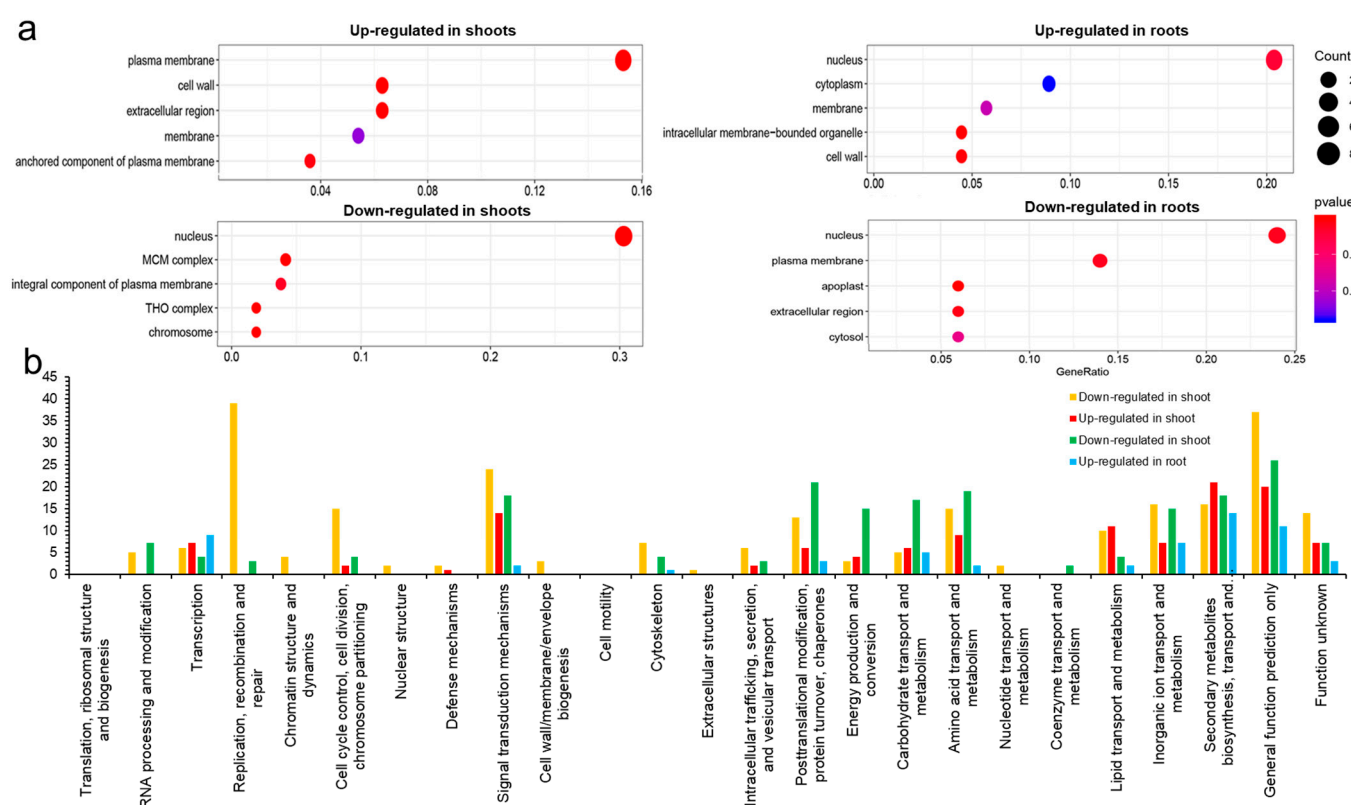


Figure 3. GO and KOG enrichment analysis of the up- and down-regulated differentially expressed genes (DEGs) in the roots and shoots: (a) cellular component enrichment of the up-regulated and down-regulated DEGs in the roots and shoots; (b) KOG enrichment analysis of the up- and down-regulated DEGs in the roots and shoots.

Protein interaction networks were constructed in the roots (Figure S1) and shoots (Figure S2). Furthermore, nine and 12 hub genes were detected in the roots and shoots, respectively. In the roots, all nine hub genes were up-regulated under the inverted condition. Moreover, four of the nine hub genes were annotated as Kip-related proteins (KRPs), which are cyclin-dependent kinase (CDK) inhibitors (CKIs) and can negatively regulate cell division (Figure S1) [29,30]. In the shoots, only two hub genes were up-regulated under the inverted condition, annotated as tyrosine kinases. Meanwhile, eight of the 10 down-regulated hub genes were annotated as being associated with DNA replication, recombination, and repair, such as minichromosomal maintenance (MCM) proteins and DNA polymerases (Figure S2). These results indicate that gravity may inhibit cell division in both roots and shoots in different ways. In the roots, cell division inhibitors (KRPs) were up-regulated, while DNA helicase MCMs were down-regulated in the shoots.

3.4. Analysis of the Gravity-Induced Alternative Splicing Genes in *S. matsudana*

We then analyzed the alternative splicing genes in the roots and shoots. As a result, a total of 185 and 114 genes were identified in the roots and shoots, respectively. Most of the gene alternative splicing events skipped exons, with only the genes in the roots being identified as mutually exclusive exons. Fourteen genes were identified as having alternative splicing events in both the roots and the shoots. Among these skipped exon genes, only 19 showed the existence of significant differences under the normal and inverted conditions. However, most of these 19 genes were annotated as uncharacterized proteins. According to the annotation, two genes were treated as gravity-related alternative splicing genes, encoding the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein and the APETALA 2/ethylene-responsive factor protein (AP2/ERF). SNARE was annotated as being related to the transport of auxin on the plasma membrane, while the AP2/ERF protein is known to transduce the signal of ethylene and regulate the

expression of downstream genes in the nucleus. These results indicate that auxin may play an important role in the negative geotropism of the shoots, while the geotropism of the roots may be mainly related to ethylene, which is consistent with the results above.

The protein sequences of these two genes were then analyzed. As shown in Figure 4a, the Snapin/Pallidin domain had changed in SNARE, and the first AP2 domain had changed in AP2/ERF. Under the inverted condition, SNARE and AP2 lost a domain in the roots and shoots, respectively. The alternative splicing of these two genes may result in a loss or change of their function.

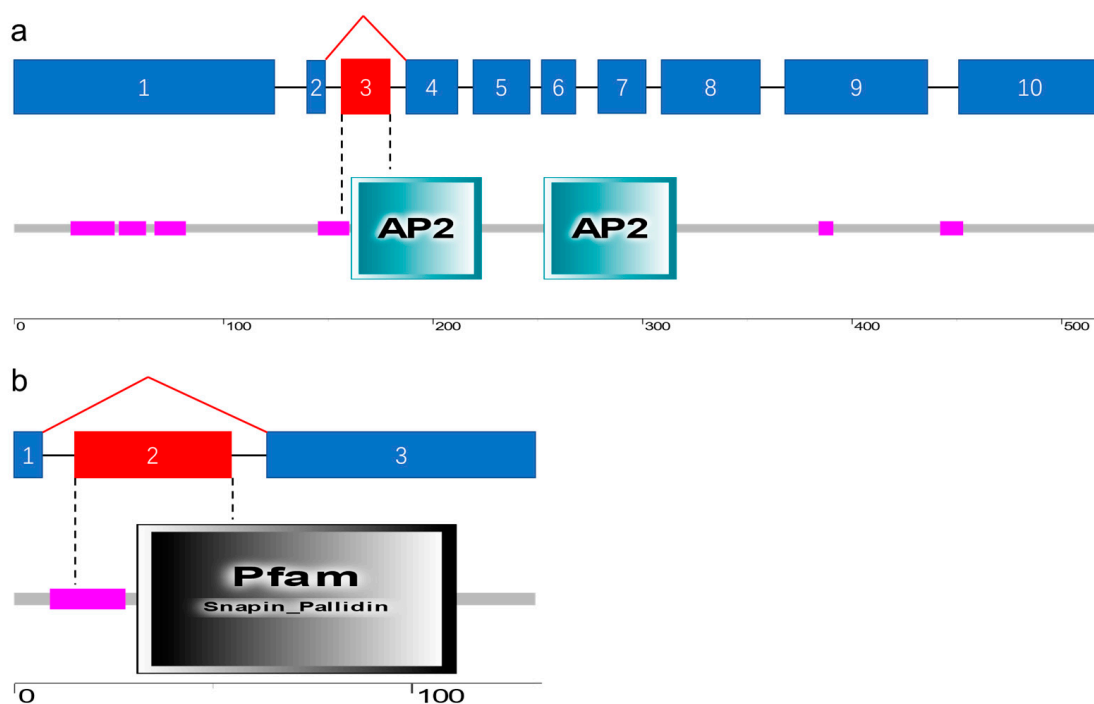


Figure 4. Alternative splicing-induced protein domain analysis: (a) alternative splicing caused the AP2 domain to change in AP2/ERF; (b) alternative splicing caused the loss of the Snapin/Pallidin domain in SNARE. The skipped exons are marked in red.

3.5. Expression Profiling of the Gravity-Related Genes

The relationships between these two genes and the DEGs were then analyzed, and the correlations between the alternative splicing genes and the DEGs were measured (Table S3). As expected, a total of 369 DEGs in the roots showed significant correlations with AP2/ERF. Moreover, AP2/ERFs were identified as showing opposite expression trends in the roots and shoots. In the shoots, the correlations between the expression levels of the DEGs and SNARE were not significant. The function of SNARE is to help transport auxin on the plasmalemma, which may not affect the expression level of the DEGs directly. According to the annotation enrichment analysis, correlation analysis, and the hub genes of the protein interaction networks, the *LecRLKs*, *SNAREs*, *AP2/ERFs*, and *MCMs* in the shoots and the *STPKs*, *WAKs*, *AP2/ERFs*, and *KRPs* in roots were further verified by qRT-PCR analysis (Figure 5). AP2/ERFs were significantly up-regulated in the roots and significantly down-regulated in the shoots (Figure 5a). The *LecRLKs*, which were highly expressed in the shoots and showed a much higher expression level under the inverted condition, were barely expressed in the roots (Figure 5b). In the roots, *STPK* and *WAK1* were highly expressed under the inverted condition (Figure 5c,d). We also examined the expression level of one SNARE gene, *SYP121*. As shown in Figure 5e, it was highly reduced in the shoots under the inverted condition. Two hub genes (*KRP* in the roots and *MCM* in the shoots) were also detected (Figure 5f,g), consistent with the transcriptome.

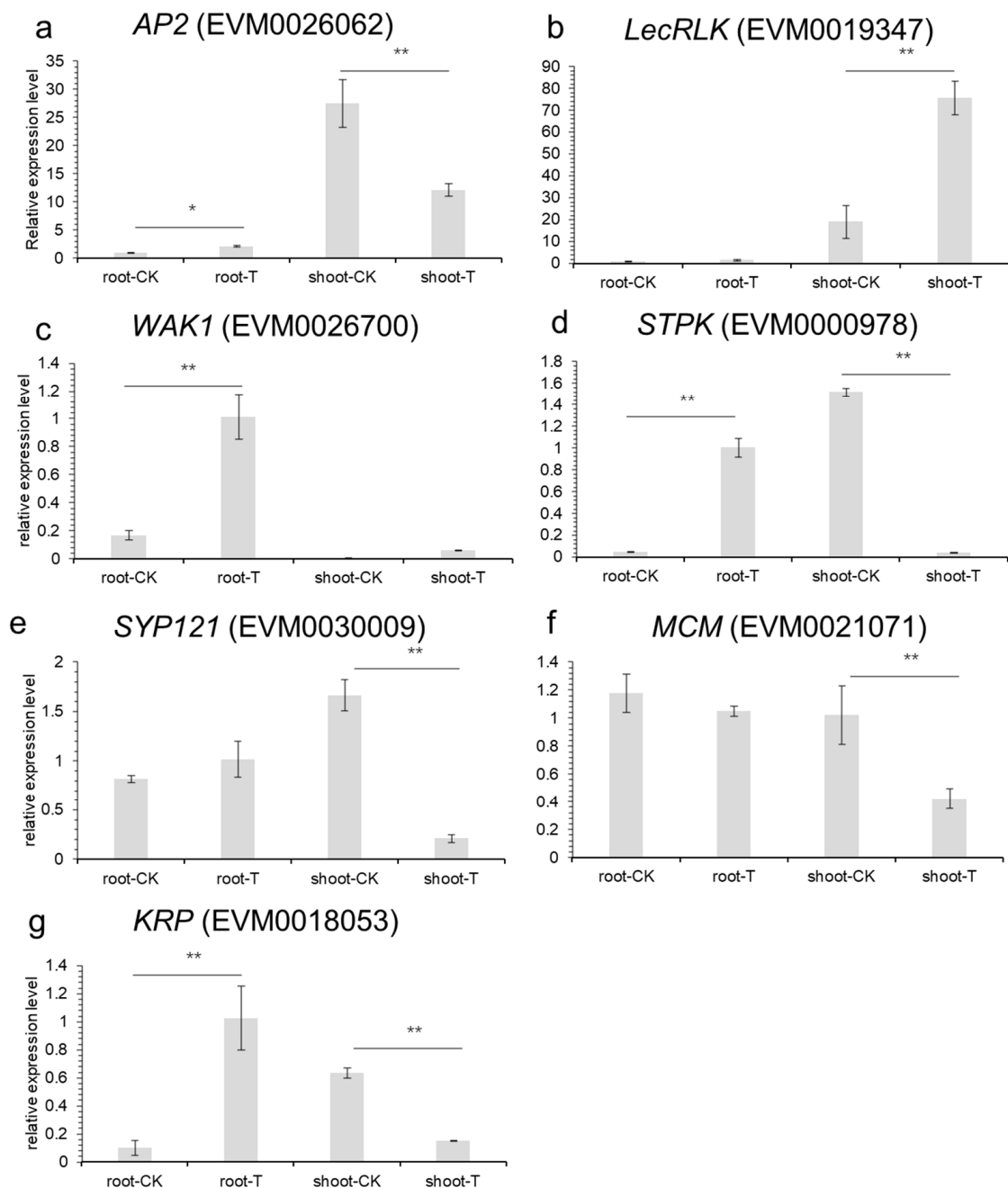


Figure 5. The expression trends of the gravity responses-related genes in the roots and shoots. The qRT-PCR analysis of the transcript levels of the candidate genes in the roots and shoots under the two conditions. The Y-axis represents the relative expression level, while the X-axis represents the different cultivars. The error bars denote the standard error (SE). The expression level was significantly different at * $p < 0.05$ and ** $p < 0.01$.

4. Discussion

4.1. The Difference in the Gravity Response between the Roots and Shoots of *S. matsudana*

The response to gravity could be divided into four steps: sense gravity, conduction of the gravity signal, asymmetrical distribution of auxin, and positive gravitropism of the roots and negative gravitropism of the shoots. Numerous research works have reported that the detection of gravity originates in statocytes [31], the tiny starch-accumulating amyloplasts acting as statolith sediment under gravity at the bottom of the statocytes [32]. Although the gravitropic signaling pathway has been studied since Darwin and Sachs

discovered plant tropisms, it is still not clear how physical signals convert into biochemical signals and produce the response of plant growth [33].

To further discover the different responses between the roots and the shoots, we decided to perform this study. In this study, a total of 412 and 668 DEGs were identified in the roots and the shoots, respectively. However, only 60 genes were differentially expressed in both roots and shoots, which indicates that the gravitropic signaling pathway in roots and shoots may differ. The KOG enrichment analyses showed that secondary metabolites, transport, and signal transduction may be the main reasons for the induction of the curvature of shoots and the down-regulation of nucleus-related items such as DNA replicating, recombination, and repair. These results mean that the gravitropic signaling pathway may affect the secondary growth and hormone transduction of cells. However, in the roots, secondary metabolites were down-regulated and the primary metabolism-related items were enriched, such as amino acid transport and metabolism, nucleotide transport and metabolism, and posttranslational modification. Furthermore, the GO enrichment analysis showed that the DEGs in the roots were enriched in the regulation of transcription, and numerous AP2/ERF transcription factor-regulated DEGs were identified. AP2/ERF could respond to ethylene, which has been reported to possibly affect plants in terms of losing their responses to gravity [34]. Moreover, two alternative splicing genes, *SNARE* and *AP2/ERF*, were identified as also potentially taking part in the gravity response in roots and shoots.

Protein interaction networks in the roots and shoots were also constructed. However, the hub genes in the roots and shoots did not show a direct relationship with curvature, but seemed associated with the size and length of the shoots and the roots.

4.2. A Potential Functional Network of the Genes Associated with Gravity

In this study, we put forward potential networks of gravity responses in roots and shoots. The up-regulated DEGs in the shoots were enriched in the plasma membrane, cell wall, and extracellular region, while the down-regulated DEGs were enriched in DNA replication. Meanwhile, in the roots, the up-regulated DEGs were enriched in the carbon metabolism and nitrogen metabolism, and the down-regulated DEGs were enriched in the galactose metabolism. According to the function classification analysis, we identified several signal transduction mechanism genes, i.e., *STPKs* and *WAKs* were up-regulated in the roots and down-regulated in the shoots. Similarly, the transcripts (*AP2/ERFs*) were also up-regulated in the roots but down-regulated in the shoots. Moreover, the *AP2/ERF* binding elements were enriched in the promoter regions of *STPKs*. *STRKs* and *WAKs* belong to receptor protein kinases (*RLKs*), which could affect plant growth, development, and stress response. *WAKs* have been reported to be located in the cell wall and can regulate cell growth, and a high expression level of *WAKs* could suppress the elongation of cells [35]. Previous studies have reported that *STPKs* can respond to plant disease resistance and light, salt, drought, and other signals [36]. In *Arabidopsis*, the over-expression of *ARK1*, an *STRK* gene, could result in the suppression of cell elongation and reduce the number of roots. In our study, we first identified that *STRKs* may also respond to gravity.

In the shoots, *LecRLKs* were identified as being up-regulated. *LecRLKs* also belong to *RLKs* and are located in the plasmalemma. Several *LecRLKs* have been reported to be regulated by hormones [37]. In *Arabidopsis*, the A4 subfamily of the *LecRLK* family participates in the ABA signal transduction pathway [38], with *AtLecRLK2* participating in the ethylene signal transduction pathway [39] and *LecRLK1* reducing the content of SA in tobacco [40]. We also detected alternative splicing genes in the roots and the shoots, a gene encoding the auxin-transporting protein *SNARE* in the roots, and a gene encoding *AP2/ERF* in the shoots. *SNARE* has been reported to regulate the fusion of secretory vesicles with the plasma membrane [41,42], which could affect plant polarity [43,44]. Combining the enrichment analysis of the transcriptome, we can put forward a potential functional network: in roots, gravity may affect the expression levels of *AP2/ERFs* and then regulate the expression levels of *STPKs* and *WAKs*, resulting in an alternative splicing

of SNARE and finally inducing an inhomogeneous distribution of auxin and geotropism curvature. In shoots, gravity may affect the alternative splicing and expression levels of *AP2/ERFs*, further down-regulating *STRKs* and up-regulating *LecRLKs*, thus resulting in a lower expression level of *SNARE* and finally inducing an inhomogeneous distribution of auxin and negative geotropism curvature.

Another effect of gravity is that shoots become much smaller and roots become much shorter under the inverted condition, compared to normal conditions. Based on the protein interaction networks in the roots and shoots, we could easily find that cell division was suppressed in both the roots and the shoots. However, the mechanisms for the roots and shoots were different. In the roots, cell division inhibitors (*KRPs*) were up-regulated, while DNA helicase *MCMs* were down-regulated in the shoots.

5. Conclusions

We analyzed the effect of gravity on the roots and shoots of *S. matsudana*. By comparing the gene expression levels of the roots and shoots under normal and inverted conditions, we identified several functionally enriched items in the roots and the shoots. Additionally, a potential functional network of the curvature of roots and shoots was proposed. We also analyzed why roots and shoots are much smaller under inverted conditions than under normal conditions. These results provide an important foundation for further studies of the molecular mechanisms and genetic regulation of plant responses to gravity and the plant polarity of forest trees.

Supplementary Materials: The following materials are available online at <https://www.mdpi.com/article/10.3390/f12121715/s1>: Figure S1, The protein interaction network of differentially expressed genes (DEGs) in the roots; Figure S2, The protein interaction network of differentially expressed genes (DEGs) in the shoots; Table S1, The primers used in this study; Table S2, Overview of the RNA sequencing data; and Table S3, Annotation of the DEGs in the roots and shoots.

Author Contributions: All the authors contributed to the study conception and design. Material preparation and data collection were performed by J.Z., G.L. and Y.L. (Yixin Li). Data analysis was performed by Z.F., J.G., H.Z., H.G., X.C. and Y.L. (Yaqi Li). The first draft of the manuscript was written by G.L. and J.Z., Y.C., C.Y., F.Z. and H.W. reviewed and edited the manuscript. All authors read final manuscript and agree to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data that supported the findings of this study were deposited into the CNGB Sequence Archive (CNSA) of the China National GeneBank Database (CNGBdb) with accession number CNP0002161.

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

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