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Original Research

Study of the Function and Expression of the *AhRUVBL2* Promoter in *Arabidopsis*

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Abstract

Background: Cultivated peanut (*Arachis hypogaea* L.) is a major oil and economic crop. Pod size is one of the important agronomic traits of peanut variety, with a direct impact on peanut yield. **Methods**: In a previous study, *AhRUVBL2* was identified by map-based cloning technology as a candidate gene that regulates peanut pod size. **Results**: Overexpression of *AhRUVBL2* in transgenic *Arabidopsis* significantly increased plant height, branch number, leaf size, silique size, seed size, and thousand-seed weight. Further examination revealed an increase in the area of silique exocarp cells, and the number and area of endocarp lignified cells. A total of 337 differentially expressed genes, including *PRX* (*Periaxin*) , *SAUR* (*Small Auxin-up RNA*), and *PYL* (*Pyrabactin Resistance 1-like*), were identified by transcriptome analysis of transgenic *Arabidopsis* silique. *proAhRUVBL2-GUS* was found to be expressed explicitly in seeds, and the expression activity of *proAhRUVBL2-D893* was significantly greater than that of *proAhRUVBL2-79266*. Exogenous ABA (abscisic acid) and IAA (indole acetic acid) treatment of *proAhRUVBL2-GUS*-transformed tobacco leaves revealed that the *AhRUVBL2* promoter was hormone-responsive. **Conclusions**: This study sheds light on the function of *AhRUVBL2* in regulating plant growth and development. Moreover, characterization of the *AhRUVBL2* promoter provides a valuable genetic resource for enhancing peanut yield.

Keywords: peanut; *AhRUVBL2*; promoter; expression characteristics; pod size

1. Introduction

The cultivated peanut or groundnut (*Arachis hypogaea* L.) is one of the most important oil and economic crops, and is widely grown in semiarid tropical and subtropical regions. Global annual peanut production has risen rapidly, from 45.2 million tons in 2015 to 54.3 million tons in 2022 (http://www.fao.org/faostat, 2025). However, this still does not satisfy the demands of a growing global population, and increasing the peanut yield per unit area remains a major challenge for peanut breeders. Pod and seed size are direct determinants of peanut yield, with this harvest organ regulated by a variety of plant hormones [1]. Seed size is a typical quantitative trait regulated by many genes and significantly influenced by the environment [2–4]. Therefore, exploration of the function and mechanism of genes that regulate pod size is important for improving the breeding of peanuts.

In many crops, pod size is an economically important trait that affects the crop yield and quality. Researchers have made great progress in the study of genes related to crop improvement, as well as the various molecular mechanisms involved [5]. For example, by using 156 recombinant inbred lines constructed from ND_S and ND_L parents and 88 peanut germplasm resources, Zhao *et al*. [6] identified the gene *PSW1* that regulates peanut pod size. The different stages of pod growth and development are known to be associated with regulation of a variety of phytohormones, including auxin, abscisic acid, ethylene, cytokinin, and gibberellins [7–9]. Auxin is a key phytohormone that regulates plant pod development, and many of the genes involved in growth hormone signaling are involved in regulating pod size [10–12]. Previous studies have focused on the mechanism that regulates pod development [6,13]. With the progress of technology, it has been found that tissue-specific promoters exert a targeted expression and regulation effect on exogenous genes, thereby regulating gene expression in specific organs and tissues.

RUVBL (Recombinant human RuvB-like) proteins, named RuvA and RuvB, were first isolated from Escherichia coli and later shown to interact with TATA-binding proteins (TIP49a and TIP49b) in humans, rats, and yeast [14–16]. Both RUVBL2 and its homologous protein RUVBL1 are members of the AAA+ superfamily, which are evolutionarily highly conserved AAA+ ATPases involved in various cellular processes [17,18]. RUVBL2 and RUVBL1 share very similar structures and conserved structural domains, and both form a complex that plays a central role in regulating the cell cycle and mitotic progression [19–21]. Silencing of the RUVBL1 gene in *Arabidopsis* results in delayed plant development, dwarf plants, and abnormal leaf development [22]. RUVBL2 proteins are highly conserved ATPases involved in the regulation

of cell division and expansion, but less is known about these proteins in plants. A major QTL (Quantitative trait locus) related to pod size, *qAHPS07*, was fine mapped to a 36.46 kb interval on chromosome A07 using F_2, recombinant inbred line (RIL) and secondary F_2 populations derived from a cross between variety 79266 (as the female, small pod) and D893 (as the male, big pod). The results of forward and reverse genetics strongly indicated that *AhRUVBL2* (*Arahy.TSR8I7*) was the most likely candidate gene for *qAHPS07*. *AhRUVBL2* may regulate the proliferation of pod shell cells, which ultimately affects the pod size [23].

In this study, phenotypic and cytological analyses of *AhRUVBL2* transgenic *Arabidopsis* were carried out to study the regulatory mechanism of *AhRUVBL2* in plants. The *AhRUVBL2* promoter sequences of 79266 and D893 were cloned to allow construction of *proAhRUVBL2-GUS* expression vectors. These were transformed into *Arabidopsis* plants and tobacco leaves to analyse specific expression of the promoter in tissues. The regulatory mechanism of RUVBL2 protein expression in plants was clarified, thus providing a theoretical reference for transgenic breeding of the peanut.

2. Materials and Methods

2.1 Plant Materials

Arabidopsis (Col-0), tobacco, and peanut varieties D893 (large pod) utilized in this study were provided by the College of Agronomy, Shandong Agricultural University. The peanut varieties 79266 (small pod) was provided by the Shandong Peanut Research Institute. *AhRUVBL2* transgenic *Arabidopsis* was obtained through a previous investigation conducted by our research group [23]. All plant materials were cultivated inside an artificial climate chamber. Tobacco plants were grown in a mixture of nutrient soil and vermiculite at a 3:1 ratio and maintained at 22 °C with a 16-h light/8-h dark photoperiod, 70% relative humidity, and an illumination intensity of 2000 Lux. Each treatment was carried out with a minimum of three biological replicates.

2.2 Identification of Transgenic Arabidopsis

Genomic DNA from T_5 transgenic *Arabidopsis* seedling stage leaves was extracted using the CTAB (Hexadecyltrimethylammonium bromide) method [24]. PCR (Polymerase Chain Reaction) was performed using specific primers, and the expression of *AhRUVBL2* was evaluated by qRT-PCR (Quantitative reverse transcription polymerase chain reaction). cDNA sequence information for the *AhRUVBL2* gene was reported previously [23]. The specific primers for qRT-PCR were designed using Beacon Designer 8.0 software (Palo Alto, CA, USA). *Arabidopsis* UBQ5 (NP_191784, AT3G62250) was used as an internal control gene [25]. Three biological replicates and three technical replicates were performed for each sample. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [26].

2.3 Phenotyping and Cytological Observations of Arabidopsis

The phenotypic characteristics of wild-type and *AhRUVBL2* transgenic *Arabidopsis* lines grown under equivalent conditions were recorded, including plant height, rosette leaves, number of branches, silique and seeds. The results for bolting, flowering, and yellow fruiting stages of wild-type (WT) and transgenic *Arabidopsis* were assessed under the same growth conditions. WT and transgenic *Arabidopsis* silique were taken for cytological observations at 4 d, 6 d, 9 d, and 12 d after flowering [1]. Plant phenotypes and cytological features were photographed with a camera attached to microscope (Nikon D850, Tokyo, Japan), and ImageJ software (ImageJ 1.54, National Institutes of Health, Bethesda, MD, USA) was used for measurements.

2.4 Transcriptome Sequencing of AhRUVBL2 in Arabidopsis Silique

Silique from WT and overexpressing *AhRUVBL2* transgene *Arabidopsis* were used for transcriptome sequencing at 6 d after anthesis under normal growth conditions, with three biological replicates for each sample (Novogene Co., Ltd, Beijing, China). WT *Arabidopsis* silique was labelled WT-1, WT-2, and WT-3, while transgene *Arabidopsis* silique was labelled OE-1, OE-2, and OE-3 (OE, Overexpression). Total RNA was extracted using the FastPure Universal Plant Total RNA Isolation Kit (Vazyme, Nanjing, China), and RNA integrity was tested using the Agilent 2100 bioanalyzer. Samples that met the requirements for cDNA library construction were used in subsequent sequencing. Clean reads were compared for similarity to the *Arabidopsis* reference genome (TAIR10.1) using HISAT2 software (2.2.1, Johns Hopkins University, Baltimore, MD, USA). The expression levels of individual genes were normalised using Fragments Per Kilobase Million (FPKM). Differential gene analysis between WT and transgenic *Arabidopsis* was carried out using DESeq2 software (1.44.0, University of North Carolina, Chapel Hill, NC, USA), and differentially expressed genes (DEGs) were screened with the following parameters: adjusted $p \leq 0.05$ and $|\log2 \text{ (fold change)}| \geq 1$. Functional annotation and enrichment analysis of DEGs was carried out using the Gene Ontology (GO, http://geneontology.org/docs/download-ontology/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (https://www.kegg.jp/kegg/pathway.html), with the threshold for significant enrichment being $p < 0.05$.

2.5 Cloning and Expression Vector Construction of the AhRUVBL2 Promoter

Genomic DNA from peanut 79266 and D893 leaves was extracted using the CTAB method [24]. DNA concentration was estimated with a NanoDrop 2000 spectrophotometer, and DNA purity assessed by electrophoresis with 1% agarose gels. The sequence 2527 bp upstream of the

start codon (ATG) of *AhRUVBL2* (https://www.peanutbase.org/) was used as the promoter sequence. PCR primers for cloning of the *AhRUVBL2* promoter were designed using Primer 5 (5.0, PREMIER Biosoft International, Palo Alto, CA, USA) and SnapGene (6.3, GSL Biotech LLC, Chicago, IL, USA) (see **Supplementary Table 1**). Gel recovery of the PCR products was performed using the E.Z.N.A.® Gel & PCR Clean Up Kit (Omega, Norcross, GA, USA). Target fragments of *AhRUVBL2* promoter were cloned into the plasmid vector pUC18. The expression vectors *pBI121-proAhRUVBL2::GUS* were transformed into Agrobacterium tumefaciens GV3101 and used to infect *Arabidopsis* with the floral dip method [27].

2.6 Genetic Transformation of the Expression Vectors pBI121-proAhRUVBL2::GUS

Arabidopsis seeds were disinfected with 1 mL of 1% sodium hypochlorite for 5~10 min, uniformly spotted into 1/2 MS medium, and sealed with sealing film. The T_1 generation of *Arabidopsis* seeds harvested after infestation with *Agrobacterium tumefaciens* GV3101 (pBI121-*proAhRUVBL2::GUS*) were screened with a kanamycin plate. Green cotyledon plants were selected as the pure transgenic lines, and then continued to obtain the T_2 and T_3 generations of *Arabidopsis* [28]. Observations of the WT and transgenic *Arabidopsis* stems, leaves, flowers, fruit skin, and seeds were made following GUS (β-glucuronidase) staining [29].

2.7 Effect of Exogenous Hormones on Expression of the AhRUVBL2 Promoter in Tobacco

Healthy, non-flowering tobacco plants in good growth condition and with up to four leaves were selected for infestation using a syringe with the needle removed. The tobacco leaves were injected with equal amounts of a pBI121-*proAhRUVBL2 -79266::GUS* and pBI121-*proAhRUVBL2 -D893::GUS* recombinant plasmid-transformed *Agrobacterium tumefaciens* GV3101 resuspension. *pBI121-35S::GUS* was used as a positive control, and resuspension as a negative control. Tobacco leaves that had been transiently transformed for 48 h were sprayed with ddH_2O as the control and two phytohormones, ABA (abscisic acid, 100 µM) and IAA (indole acetic acid, 100 µM), and then incubated for 24 h. Three replicates were used for each treatment. Tobacco *Actin2* (NC_003074.8, AT3G18780.2) was used as an internal reference gene. The qRT-PCR-specific primer sequences for the GUS gene reference were previously reported by Anwar *et al.* [30].

2.8 Statistical Analysis

All experiments were conducted with three replicates. Excel 2016 (16.0.4266.1001, Microsoft Corporation, Redmond, WA, USA) and SPSS 26.0 software (26.0, IBM, Armonk, NY, USA) were used for statistical analysis of data, with one-way analysis of variance (Student's *t*-test). All values were calculated as the mean ± standard deviation (SD). Asterisks were used to indicate a significant difference as follows: * $p < 0.05$, ** $p < 0.01$. All images and photos were annotated using ImageJ software (ImageJ 1.54, National Institutes of Health, USA).

3. Results

3.1 Identification of AhRUVBL2 Transgenic Arabidopsis

PCR was performed on DNA from T_5 generation *Arabidopsis* strains using *AhRUVBL2* gene-specific primers. The target band of 1398 bp was amplified from the *AhRUVBL2* transgenic strain, but not the non-transgenic plants (**Supplementary Fig. 1**). Expression analysis performed using qRT-PCR showed the relative expression of *AhRUVBL2* in transgenic plants was significantly higher than that of WT *Arabidopsis* (**Supplementary Fig. 1**). The expression of *AhRUVBL2* in WT *Arabidopsis* was similar to that of the internal reference gene. The expression of *AhRUVBL2* in OE2 and OE3 transgenic *Arabidopsis* was 493-fold and 659-fold higher than that of the internal reference gene, respectively. These two transgenic lines, identified in the T_5 generation, were used in the subsequent experiments.

3.2 Phenotypic Analyses of T_5-Generation AhRUVBL2-Overexpressing Arabidopsis Lines

Phenotypic analyses were performed on plants from the T_5-generation *AhRUVBL2*-overexpressing *Arabidopsis* lines OE2 and OE3 grown normally for 4 weeks, with WT plants used as the control. *AhRUVBL2*-overexpressing plants were larger in size and had leaves of significantly greater length and width compared to the WT (Fig. 1A). The leaves from the OE2 and OE3 lines were 37.78% and 46.81% longer than the WT, respectively, and 25.69% and 42.21% wider, respectively (Fig. 2A,B). Overexpression of the *AhRUVBL2* gene accelerated the growth of *Arabidopsis* (Fig. 1B), with OE2 and OE3 plants being 46.18% and 49.95% taller, respectively, than WT plants during the growth period (Fig. 2C). Moreover, the number of branches per plant was increased by 0.74 and 0.47 in OE2 and OE3, respectively, compared to the WT (Fig. 2D).

Silique from WT, OE2 and OE3 were sampled at 4 d, 6 d, 9 d and 12 d after flowering, and their length and width measured. The silique length and width from OE2 and OE3 were significantly greater than those of WT at 4 d and 12 d after flowering (Fig. 1C). At 4 d after flowering, the silique length in OE2 and OE3 was 13.49% and 4.00% greater, and the silique width was 11.10% and 11.21% greater, respectively, compared to WT. At 12 d after flowering, the silique length was 3.71% and 6.18% greater, and the width 7.20% and 7.14% greater, respectively, compared to WT (Fig. 2E,F). Seeds from WT, OE2, and OE3 were harvested at maturity, dried at 37 °C, and photographed under a microscope (Fig. 1D). The length of WT, OE2, and OE3 seeds was 228.5 ± 7.5 µm, 270.9 ± 8.9 µm, and 276.1 ± 9.6 µm, respectively, and the width was 146.6 ± 5.8 µm, 167.8 ± 4.4 µm, and 161.6 ± 4.3 µm, respec-

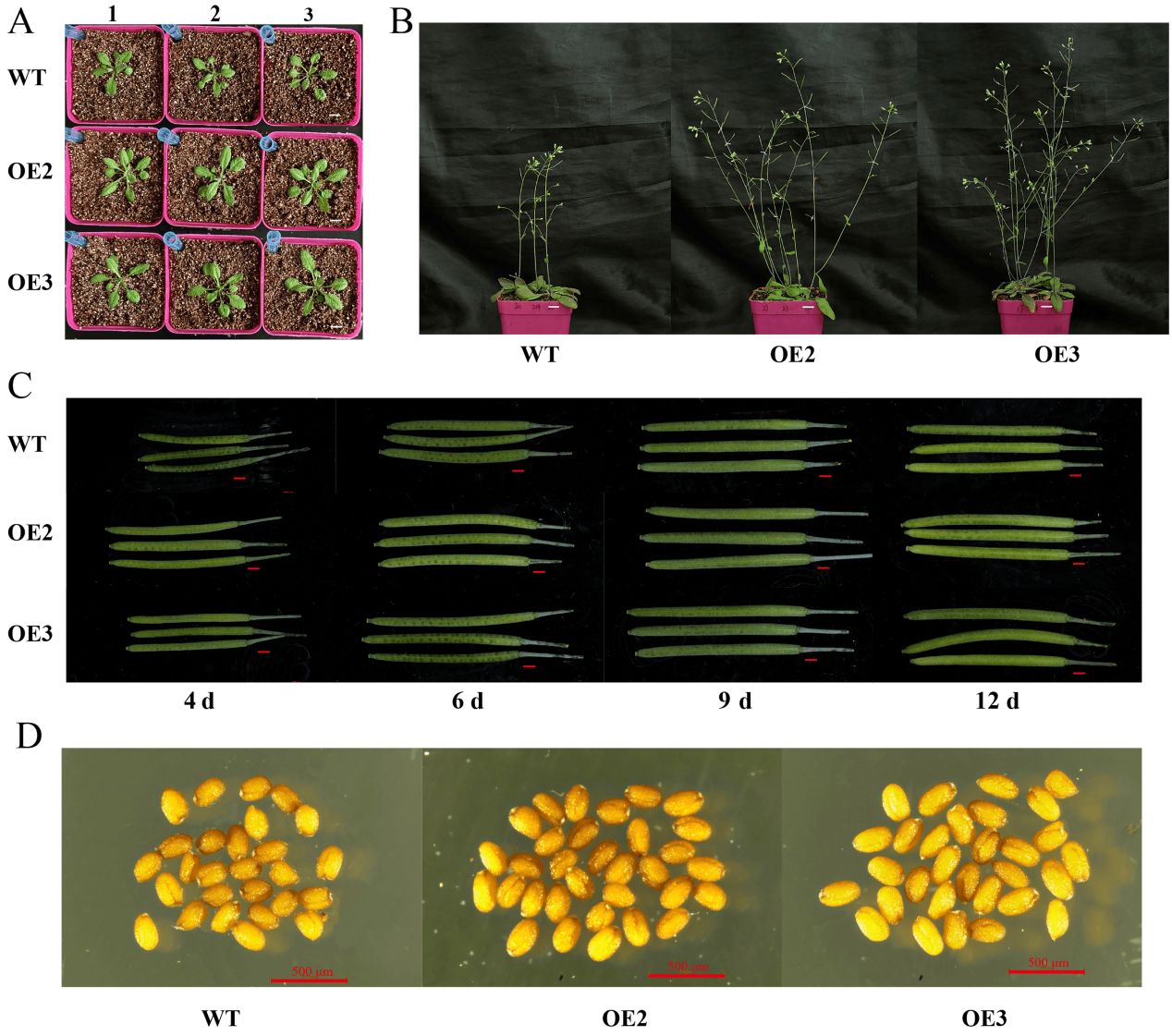

Fig. 1. Phenotype of wild-type and *AhRUVBL2*-overexpressing *Arabidopsis* lines. (A) Seedling stage phenotype of WT and *AhRUVBL2*-overexpressing *Arabidopsis*. 1 to 3, three replicates. Scale bar = 1 cm. (B) WT and *AhRUVBL2*-overexpressing *Arabidopsis* during the growth period. (C) The siliques of WT, OE2 and OE3 from 4 d to 12 d after flowering. Scale bar = 1 mm. (D) Phenotype of *Arabidopsis* seeds from WT, OE2 and OE3. Scale bar = 500 µm. WT, wild-type; OE2 and OE3, *AhRUVBL2*-overexpressing *Arabidopsis* lines.

tively (Fig. 2G,H). Therefore, the length and width of seeds from OE2 and OE3 were significantly greater than those from WT. The length of seeds from OE2 and OE3 was 18.54% and 20.84% greater, respectively, and their width was 14.44% and 10.19% greater, respectively, compared to those from WT. The thousand-seed weight for seeds from OE2 and OE3 was 45.97% and 66.02% heavier, respectively, than that of WT (Fig. 2I). In addition, OE2 and OE3 exhibited faster growth, entered the bolting and flowering stages earlier than WT, and exhibited early bolting and early flowering. The bolting stage for OE2 and OE3 was 2.5 d and 1 d shorter, respectively, than that of WT (Fig. 2J), while the flowering stage was 3 d and 1.7 d shorter, respectively (Fig. 2K). The time required to reach the yellow fruit stage in OE2 and OE3 was 0.3 d and 0.6 d longer, respectively, than that of WT (Fig. 2L).

3.3 Cytological Analysis of Silique Development in Transgenic and WT Arabidopsis

Paraffin-embedded tissue sections of silique from WT, OE2 and OE3 at 4 d, 6 d, 9 d and 12 d after flowering were prepared in order to observe the number and size of cells. OE2 and OE3 showed greater silique pericarp thickness at 4 d to 12 d after flowering compared to WT (Fig. 3A). At 6 d after flowering, the pericarp was 16.70% and 41.92% thicker in OE2 and OE3, respectively, than in WT. The thickest pericarp was found at 12 d after flowering in OE2, and at 9 d after flowering in OE3, both of which were signif-

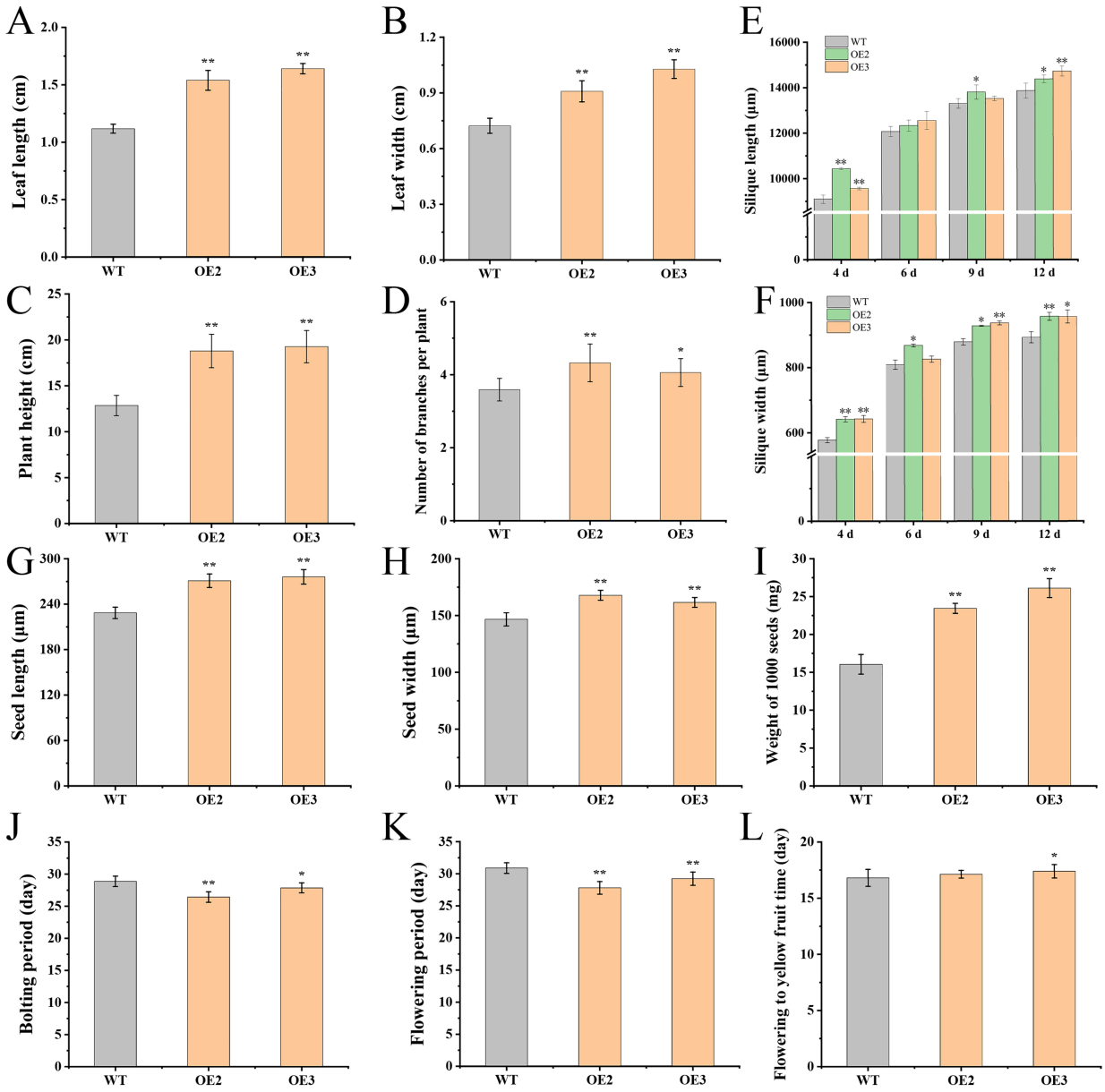

Fig. 2. Phenotypic analyses of wild type and *AhRUVBL2*-overexpressing *Arabidopsis* lines. (A,B) Seedling stage leaf phenotype (length and width) analysis. (C,D) Plant height and branches per plant. (E,F) Silique length and width analysis, from 4 d to 12 d after flowering. (G–I) Phenotype analysis of seed length, width, and thousand-seed weight. (J–L) Time for bolting stage, flowering stage, and yellow fruit stage. *, $p < 0.05$; **, $p < 0.01$. WT, wild-type; OE2 and OE3, *AhRUVBL2*-overexpressing *Arabidopsis* lines.

icantly greater than WT (Fig. 4A). The number and area of cells in a cross-section of silique were also evaluated from 4 d to 12 d after flowering (Fig. 3B). The number of exocarp cells did not differ significantly between the transgenic plants and WT. However, the exocarp cell areas in OE2 and OE3 from 4 d to 9 d after flowering were significantly larger than those of WT. The fastest growth in exocarp cell area was observed at 6 d after flowering in OE2 and OE3, with increases of 27.83 and 40.00%, respectively (Fig. 4B,C). The exocarp cell areas of OE2 and OE3 were still larger than those of WT at 12 d after flowering. The number of endocarp lignified cells at 6 d after flowering was 8.58 and 11.82% greater in OE2 and OE3, respectively, than in WT, while the area of endocarp lignified cells was 20.78 and 25.14% greater, respectively. The number of endocarp lignified cells at 4 d, 6 d, and 12 d after flowering was significantly higher in OE2 and OE3 than in WT. The fastest increase in the number of endocarp lignified cells was 10.76 and 13.16% in OE2 and OE3, respectively, at 4 d after flowering. The number of endocarp lignified cells increased by 8.58% in OE2 and 11.82% in OE3 at 6 d after flowering. Only a small change in the number of endocarp lignified cells was observed in OE2 and OE3 at 9 d to 12 d after flowering. Both OE2 and OE3 had significantly larger

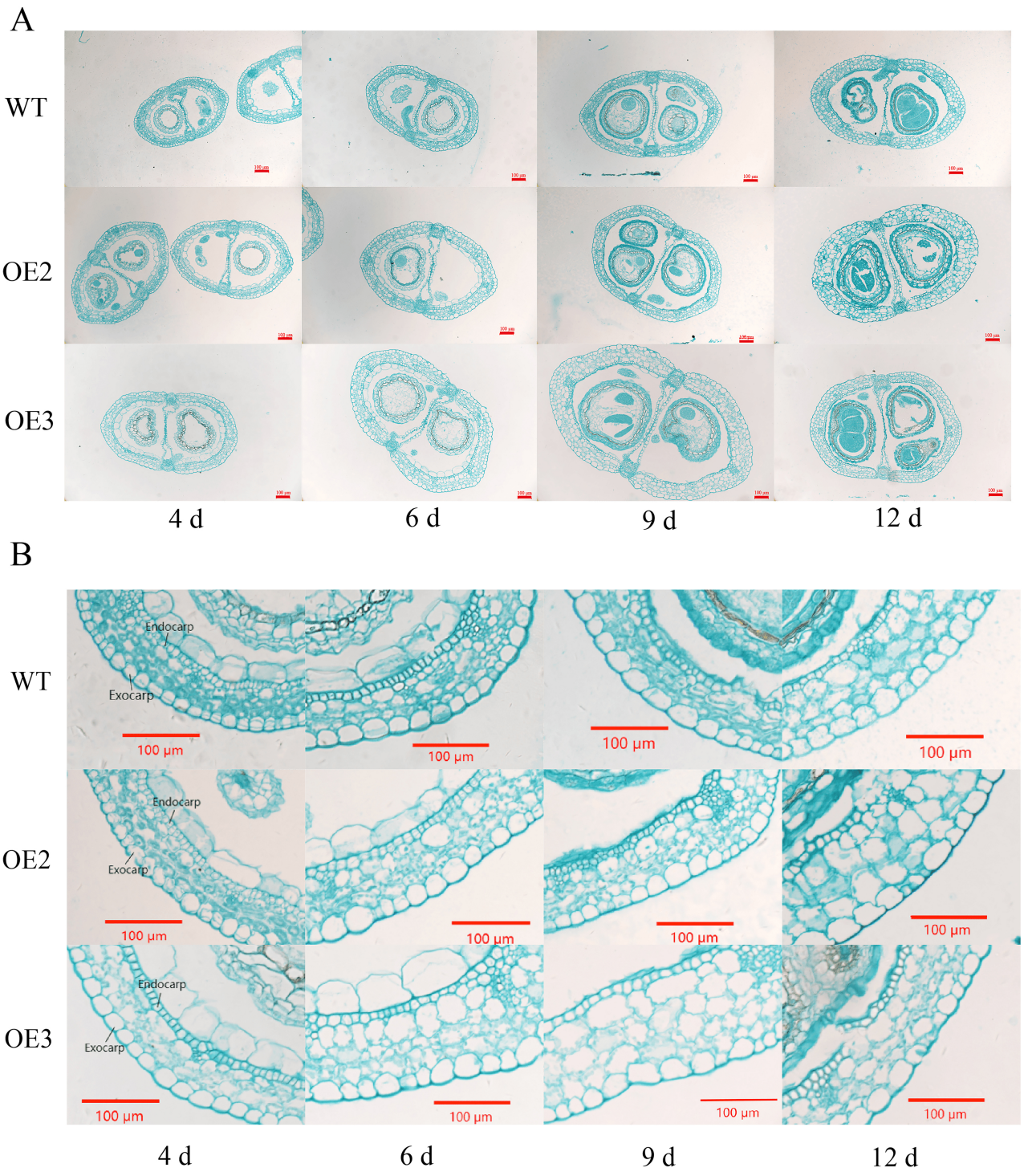

Fig. 3. Cytological analysis of *Arabidopsis* silique. (A) Cross section of pod shell from 4 d to 12 d after flowering. Scale bar = 100 μm. (B) Cross section of silique from 4 d to 12 d after flowering. Scale bar = 100 μm. WT, wild-type; OE2 and OE3, *AhRUVBL2*-overexpressing *Arabidopsis* lines.

endocarp lignified cell areas than WT at 6 d after flowering. The fastest growth of exocarp cell area was observed at 4 d after flowering in OE2 (31.15% increase) and at 9 d after flowering in OE3 (36.42%) (Fig. 4D,E).

3.4 Transcriptome Analysis of AhRUVBL2-Overexpressing Arabidopsis

WT and *AhRUVBL2*-overexpressing *Arabidopsis* silique at 6 d after anthesis and under normal growth conditions were selected for transcriptome sequencing. The clean bases for each sample were above 6.22 G, the

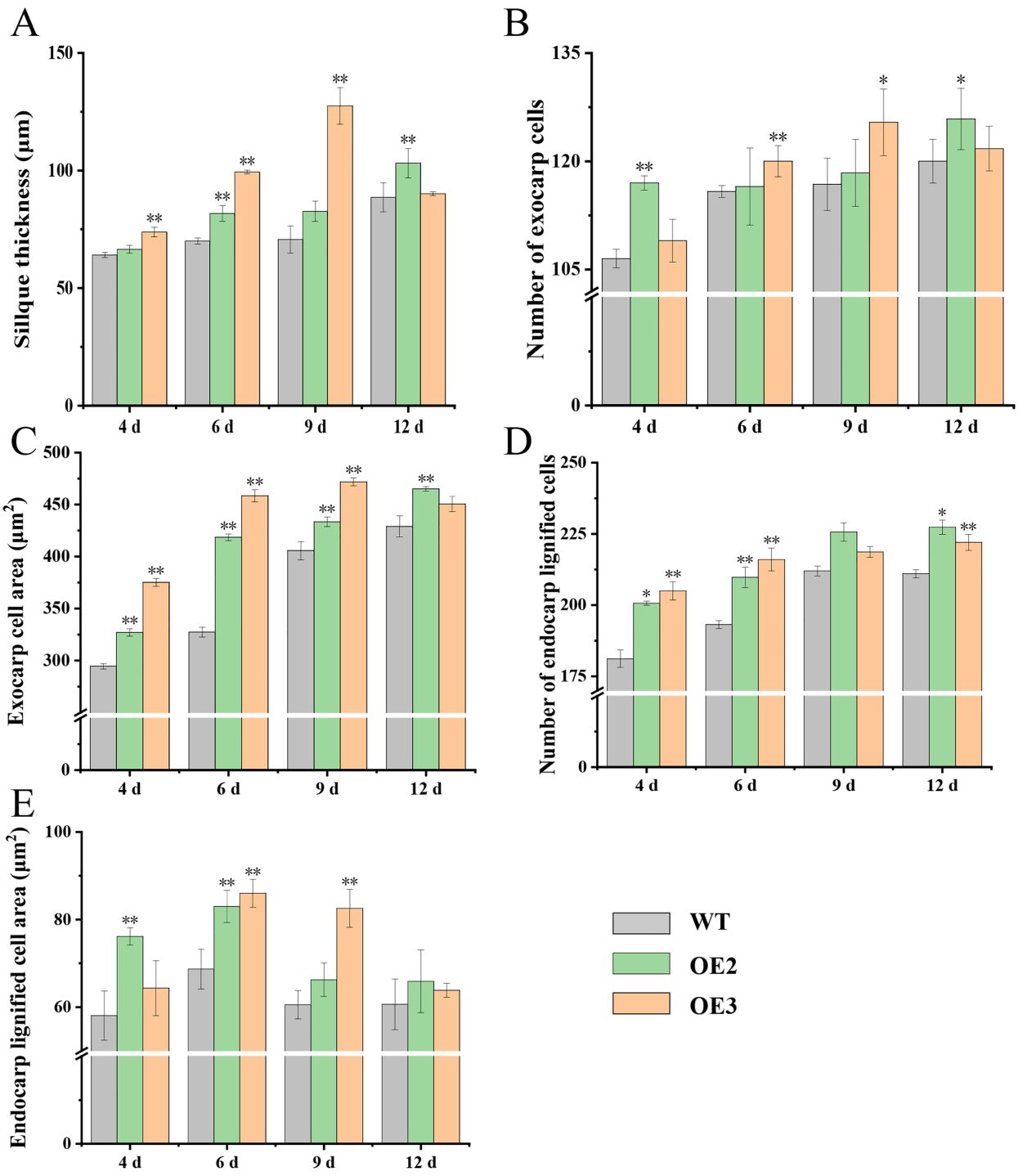

Fig. 4. Statistical analysis of *Arabidopsis* silique phenotypic. (A) Silique thickness from 4 d to 12 d after flowering. (B,C) The number and area of exocarp cells. (D,E) The number and area of endocarp lignification cells. *, $p < 0.05$; **, $p < 0.01$. WT, wild-type; OE2 and OE3, *AhRUVBL2*-overexpressing *Arabidopsis* lines.

Q30 content was 94.27–97.08%, and the comparison rate with the *Arabidopsis* reference genome was 96.05–97.76%. Hence, the data displayed a high degree of accuracy and satisfied the requirements for subsequent bioinformatics analysis (**Supplementary Table 2**). A total of 38,345 expressed genes were detected in the six sequenced samples, including 38,299 known genes and 46 new genes. There were 337 DEGs between the silique of WT and OE, including 127 up-regulated and 210 down-regulated genes. There are a total of 18,646 DEGs between wt and OE silique in the Venn diagrams (**Supplementary Fig. 2**). Of the 18,464 DEGs, 453 were expressed only in WT

Table 1. Key differentially expressed genes in the phenylpropane biosynthetic pathway.

Gene ID	Gene name	Definition	Locus
AT5G64120	*PRX71*	Peroxidase superfamily protein	*Chr5*: 25,659,257–25,661,007
AT5G51890	*PRX66*	Peroxidase superfamily protein	*Chr5*: 21,090,956–21,092,410
AT2G41480	*PRX25*	Peroxidase superfamily protein	*Chr2*: 17,296,894–17,298,725
AT1G14540	*PER4*	Peroxidase superfamily protein	*Chr1*: 4,974,021–4,975,705
AT1G24735	*ERF*	S-adenosyl-L-methionine-dependent methyltransferase superfamily protein	*Chr1*: 8,757,864–8,759,645
AT3G21560	*UGT84A2*	UDP-Glycosyltransferase superfamily protein	*Chr3*: 7,595,680–7,597,607

Table 2. Key differentially expressed genes in plant hormone signal transduction pathways.

Gene ID	Gene name	Definition	Locus
AT1G56150	*SAUR71*	SAUR-like auxin-responsive protein family	*Chr1*: 21,017,311–21,018,122
AT5G18060	*SAUR23*	SAUR-like auxin-responsive protein family	*Chr5*: 5,975,780–5,976,589
AT2G21200	*SAUR7*	SAUR-like auxin-responsive protein family	*Chr2*: 9,083,906–9,084,803
AT1G75590	*SAUR52*	SAUR-like auxin-responsive protein family	*Chr1*: 28,382,813–28,383,769
AT1G52830	*IAA6*	Indole-3-acetic acid 6	*Chr1*: 19,672,476–7,597,607
AT4G32280	*IAA29*	Indole-3-acetic acid inducible 29	*Chr4*: 15,583,332–15,584,903
AT4G01026	*PYL7*	PYR1-like 7	*Chr4*: 446,978–448,511
AT4G14560	*IAA1*	Indole-3-acetic acid inducible	*Chr4*: 8,360,996–8,362,033
AT4G14550	*IAA14*	Indole-3-acetic acid inducible 14	*Chr4*: 8,347,822–8,350,263

silique and 422 only in OE silique. In WT, 44 DEGs were down-regulated, while 35 DEGs were up-regulated in OE, all of which were associated with cellular metabolic processes.

3.5 GO and KEGG Enrichment Analysis of DEGs

GO and KEGG enrichment analyses were performed on the DEGs between WT, OE1 and OE2. GO enrichment analysis showed that DEGs were mainly enriched in cellular processes, metabolic processes and bioregulation of biological processes, binding and catalytic activities of molecular functions, and cellular components of cellular organisms and protein complexes. Among the top 30 metabolic pathways enriched with DEGs in GO analysis were stress response to hypoxia, transmembrane transporter protein activity, and glycosyltransferase activity (**Supplementary Fig. 3**). KEGG enrichment analysis showed that DEGs were mainly enriched in plant-pathogen interaction and phenylpropanoid biosynthesis, followed by MAPK (Mitogen-activated Protein Kinase) signaling pathway, amino sugar and nucleotide sugar metabolism, and ABC (ATP-binding Cassette) transporter protein pathways. Further KEGG analysis of DEGs expressed specifically in OE angiosperms showed enrichment in plant hormone signal transduction and plant-pathogen interaction pathways (**Supplementary Fig. 4**).

3.6 Identification of DEGs

Six DEGs related to the phenylpropane biosynthesis pathway were identified in silique: *PRX71* (AT5G64120), *PRX66* (AT5G51890) and *PRX25* (AT2G41480), *PER4* (AT1G14540) and *ERF* (AT1G24735) and *UGT84A2* (AT3G21560) (Table 1). *PRX71*, *PRX66* and *PRX25* are plant peroxidase genes with very specific functions in plant growth and development, and in stress conditions. These functions include cell elongation, cell wall metabolism, lignification, growth hormone and anthocyanin metabolism, oxidation, and biotic stress. They were significantly up-regulated in OE silique, with more than 2-fold higher expression than WT silique. PRX (Peri-axin) is also involved in cell proliferation, differentiation and apoptosis during cellular life processes. In contrast, the glycosyltransferase *UGT84A2* affects plant growth and development by delaying the onset of flowering. Nine phytohormone signaling pathway-related DEGs were identified: *PYL7* (AT4G01026), two IAA (AT4G14560 and AT4G14550), four *SAUR* (AT1G56150, AT5G18060, AT2G21200, AT1G75590) and two IAA (AT1G52830 and AT4G32280) (Table 2). A total of 203 differentially expressed transcription factors were identified amongst the DEGs of *AhRUVBL2*-overexpressing *Arabidopsis*, with 81 of them up-regulated in OE. The transcription factor families with the most up-regulated genes were *NAC* (9 genes), *MYB-related* (9), and *FAR1* (6) (Fig. 5).

3.7 Prediction of Proteins Interacting With RUVBL2 in Arabidopsis

The STRING database predicted 10 protein interactions with RUVBL2 in *Arabidopsis*. These included RUVBL2 protein (AT5G67630) interactions with *ARP5/ARP4* (actin), SWC2 (DNA-binding proteins), PIE1 (proteins of the deconjugating enzyme structural domains), EEN (subunits of the chromatin remodeling complex), TRA1A (phosphatidylinositol kinase family proteins), SWC4 (myb-like transcription factor family proteins), TRA1B (phosphotransferase), RIN1 (RuvB-like proteins), and SEF (HIT-

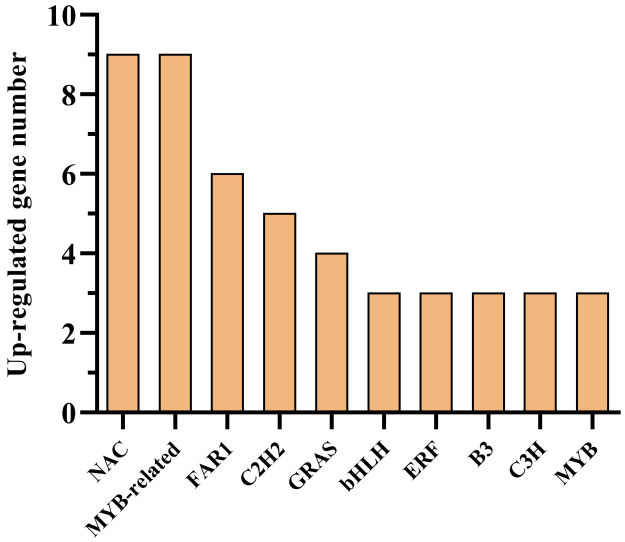

Fig. 5. Up-regulated transcription factor families in *AhRUVBL2*-overexpressing *Arabidopsis* silique. The x-axis indicates the transcription factor family, while the y-axis indicates the number of up-regulated genes in each family.

type zinc finger family proteins) (Fig. 6). Five interacting protein-related genes were predicted to be upregulated in OE silique, including *ARP4* (AT1G18450), *PIE1* (AT3G12810), *TRA1A* (AT2G17930), *SWC4* (AT2G47210) and *TRA1B* (AT4G36080), combined with transcriptome sequencing data for *AhRUVBL2*-overexpressing *Arabidopsis* (**Supplementary Table 3**).

3.8 Expression Characterization of the AhRUVBL2 Promoter

Positive plants were selected by kanamycin screening and transplanted into substrates for culture. *Arabidopsis* positive plants transformed with 35S, 79266, and D893 promoter-conjugated GUS expression vectors were named *pBI121-35S::GUS*, *pBI121-proAhRUVBL2-79266::GUS*, and *pBI121-proAhRUVBL2-D893::GUS*, respectively. Target fragments were amplified from both *pBI121-proAhRUVBL2-D893::GUS* and *pBI121-proAhRUVBL2-79266::GUS* positive plants (**Supplementary Fig. 5**). The T_1 generation of transgenic *Arabidopsis* at 6 d after flowering was selected for qPCR. GUS gene expression was detected in all specimens, with the activity of the 35S promoter found to be significantly higher than that of the *AhRUVBL2* 79266 and D893 promoters (**Supplementary Fig. 5**). Two *pBI121-35S::GUS* lines, three *pBI121-proAhRUVBL2-79266::GUS* pure lines, and four *pBI121-proAhRUVBL2-D893::GUS* pure lines were selected for further study.

Seedling and above-ground tissue samples from T_3 generation *pBI121-35S::GUS*, *pBI121-proAhRUVBL2-79266::GUS*, and *pBI121- proAhRUVBL2-D893::GUS* transgenic *Arabidopsis* plants underwent GUS staining (Fig. 7A). WT *Arabidopsis* showed no blue stain in all tissues. *Arabidopsis* transformed with *pBI121-35S::GUS* stained distinctly blue in the stems, leaves, flowers, pericarp, and seeds. *Arabidopsis* transformed with *pBI121-proAhRUVBL2-79266::GUS* or *pBI121-proAhRUVBL2-D893::GUS* displayed stems, leaves, flowers and pericarp that were devoid of blue staining, stamens that stained light blue, and significant blue staining in the seeds. PCR of WT and transgenic *Arabidopsis* cDNAs showed bright specific bands for both target and internal reference genes, with no primer dimers observed (**Supplementary Fig. 6**). The silique, stems, leaves, and flowers of T_3 generation trans *proAhRUVBL2::GUS Arabidopsis* plants were analyzed by qPCR. The *AhRUVBL2* promoter showed the highest expression in silique, with lower expression visible in the stems, leaves, and flowers (Fig. 7B).

3.9 Regulation of the AhRUVBL2 Promoter by Exogenous Hormones

The *proAhRUVBL2::GUS* vectors 893 and 79266 were injected into tobacco leaves by agrobacterium-mediated transformation. Injection of *pBI121-35S::GUS* empty vector was used as the positive control, and tobacco injected with buffer was used as the negative control. With the exception of the negative control, GUS activity was detectable as blue colour in all transgenic tobacco leaves. *pBI121-35S::GUS* displayed the strongest transcriptional activity, followed by *pBI121-proAhRUVBL2-D893::GUS*, and *pBI121-proAhRUVBL2-79266::GUS* showing weaker promoter activity (Fig. 8). Transiently transformed tobacco leaves were treated with IAA and ABA, and the expression of GUS genes detected by qPCR. The expression of *proAhRUVBL2-D893* was significantly down-regulated by ABA treatment, while that of *proAhRUVBL2-79266* was significantly up-regulated. IAA treatment had no significant effect on the expression of *proAhRUVBL2-D893*, but significantly down-regulated the expression of *proAhRUVBL2-79266* (Fig. 9).

4. Discussion

Pod size is the most important yield trait of the peanut, and one of the main traits studied in current research [31, 32]. The pod size is closely related to the number and area of cells [33,34]. A large number of QTLs have been identified, and many important genes have been cloned [1,35]. In *Arabidopsis*, silencing of the *RUVBL1* gene leads to retardation of plant growth, shorter plants, and deformity of leaf development [22]. Lu *et al.* [36] conducted genome-wide association studies (GWAS) on 28 agronomic traits across 390 peanut accessions. They identified the candidate gene *AhANT* associated with pod and seed weight, and within an 886.7 kb interval on chromosome B06. Functional validation in transgenic *Arabidopsis* revealed that *AhANT* negatively regulates cell number in organs. Given the limitations of peanut transgenic systems and the advantages of *Arabidopsis* as a model plant due to its short life cycle, re-

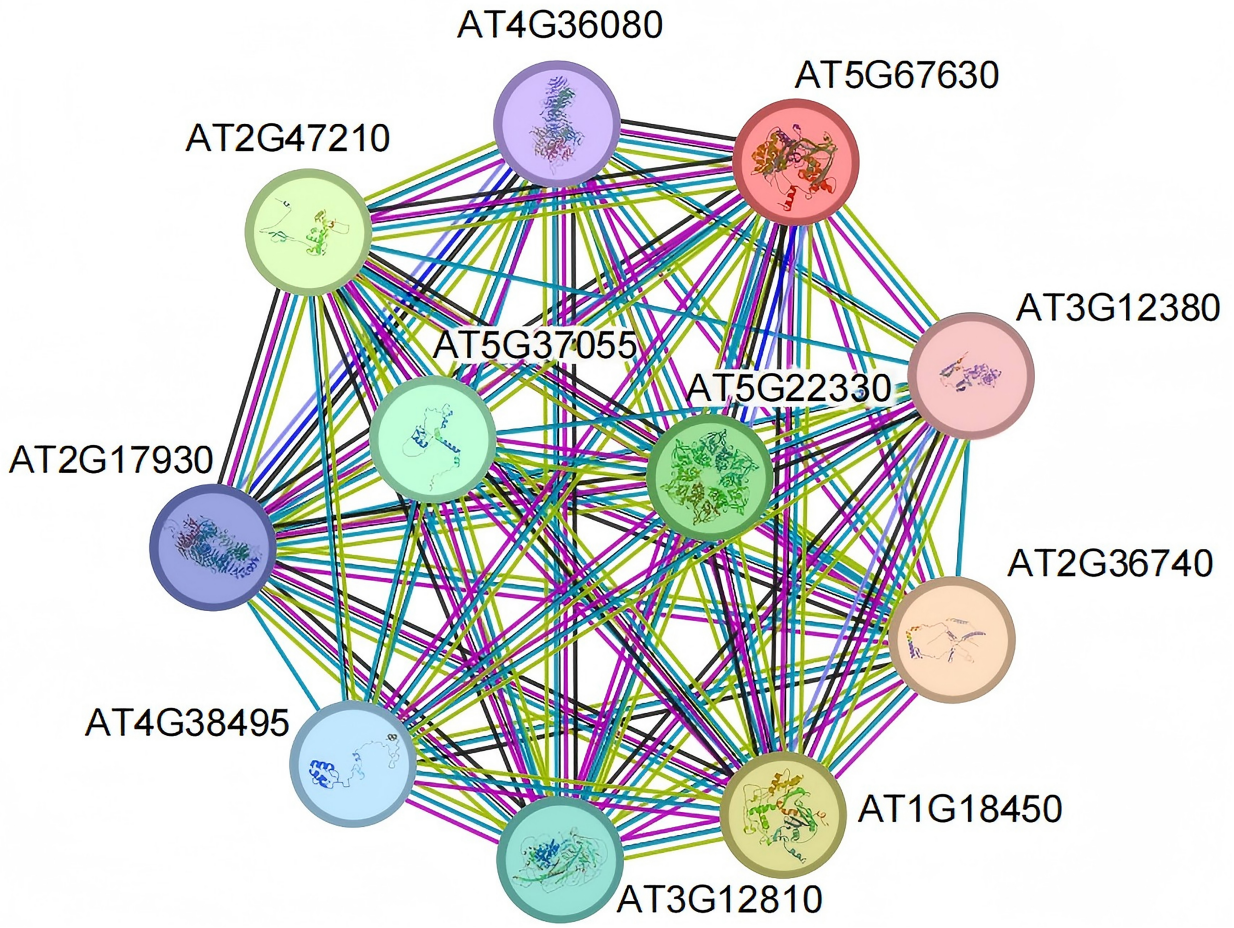

Fig. 6. Protein interactions with RUVBL2 in *Arabidopsis*.

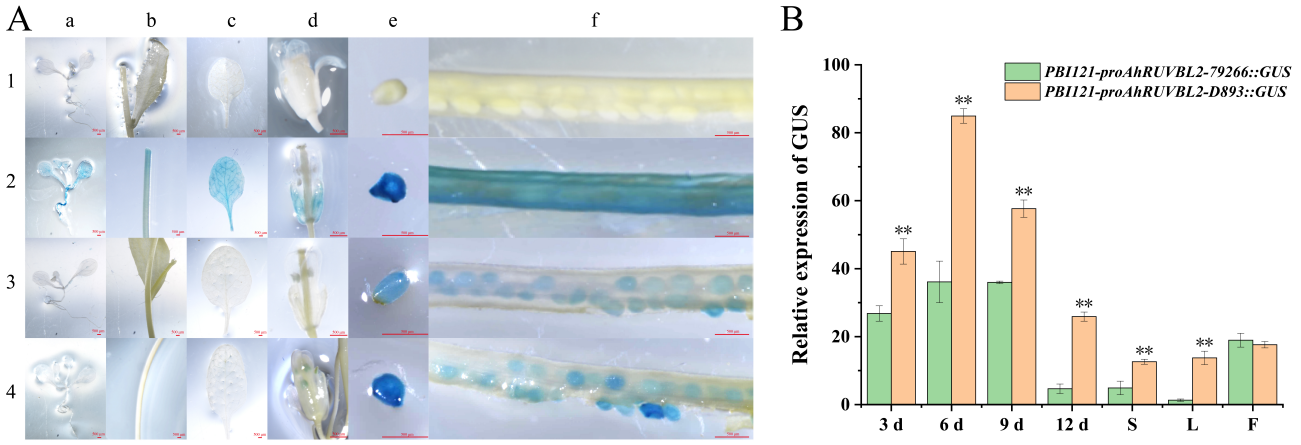

Fig. 7. GUS expression vectors constructed using different promoters in *Arabidopsis*. (A) GUS expression vectors constructed by different promoters in *Arabidopsis*. 1, Wild-type *Arabidopsis*; 2, *pBI121-35S::GUS* transgenic *Arabidopsis*; 3, *pBI121-proAhRUVBL2-79266::GUS* transgenic *Arabidopsis*; 4, *pBI121-proAhRUVBL2-D893::GUS* transgenic *Arabidopsis*; a, seedling plant; b, stem; c, leaf; d, flower; e, seed; f, silique. Scale bar = 500 μm. (B) Relative expression of the *AhRUVBL2* D893 and 79266 promoters in transgenic *Arabidopsis* tissues at days 3, 6, 9 and 12 of silique development after flowering; S, stem; L, leaves; F, flowers. **, $p < 0.01$.

searchers have increasingly utilized *Arabidopsis* for functional gene studies. Building on the prior work of Yang *et al.* [23] who identified the *AhRUVBL2* gene, the present study systematically analyzed phenotypic traits in *AhRUVBL2*-overexpressing *Arabidopsis* lines to investigate its biological roles. *AhRUVBL2* overexpression not only promoted the growth of *Arabidopsis* plants, but also significantly increased the area of exocarp cells in siliques, and the

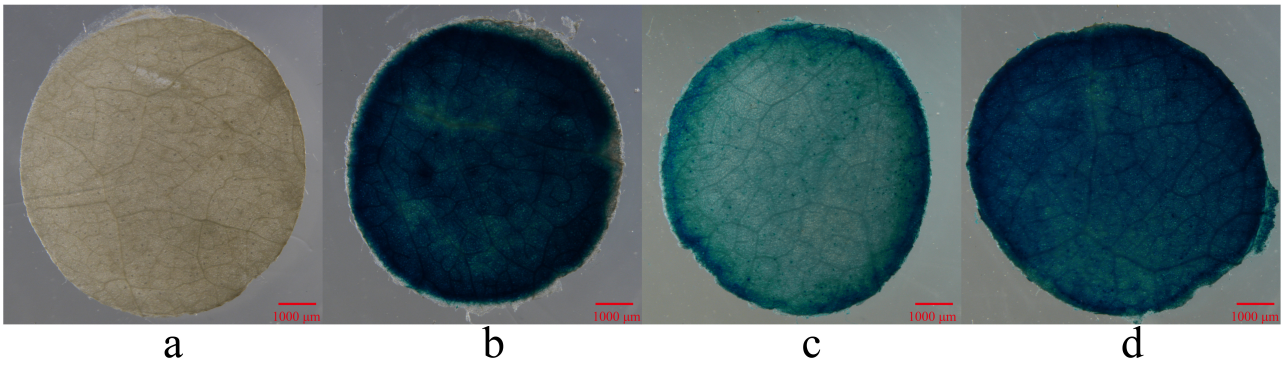

Fig. 8. Detection of GUS (β-glucuronidase) activity in tobacco leaves after transient promoter transformation. (a) Negative control. (b) Positive control. (c) *pBI121-proAhRUVBL2-79266::GUS*. (d) *pBI121-proAhRUVBL2-D893::GUS*. Scale bar = 1000 µm.

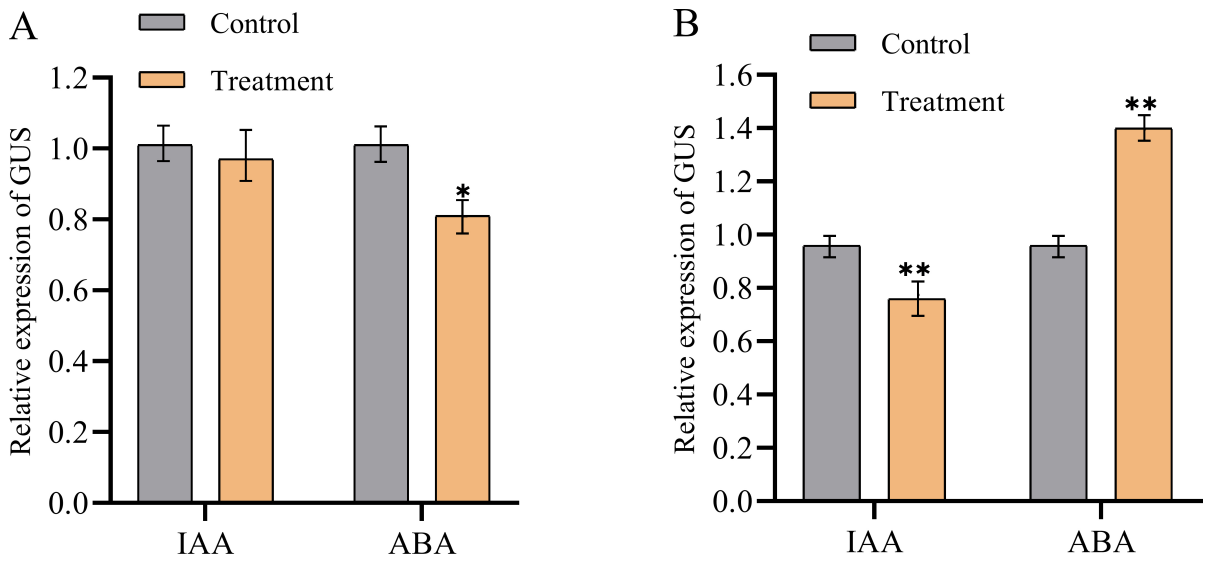

Fig. 9. GUS expression of *proAhRUVBL2* after hormone treatment (IAA, indole acetic acid and ABA, abscisic acid). (A) *proAhRUVBL2-D893*. (B) *proAhRUVBL2-79266*. *, $p < 0.05$; **, $p < 0.01$.

number and area of lignification cells in endocarp. In addition, *RUVBL2* and *RUVBL1* have very similar structures and conserved domains, with this complex playing a central role in regulating the cell cycle and mitosis [20]. Therefore, we speculated the *AhRUVBL2* gene regulates plant growth and development by controlling cell expansion and thus affecting pod size.

Plant promoters are critical regulatory elements that facilitate the binding of RNA polymerase and transcription factors, thereby playing a pivotal role in initiating and regulating gene transcription. Tissue-specific plant promoters are of particular interest due to their ability to drive the expression of exogenous genes in specific tissues or organs of transgenic plants. This targeted expression not only increases the efficiency of genetic transformation in desired plant parts, but it also has significant potential for improving crop quality through precise spatial and temporal regulation of transgene expression [37]. Seed-specific promoter is one of the tissue-specific promoters. Transcription factors may bind to conserved motifs in the promoter, thereby activating the specific expression of genes in seeds [38]. Seed-specific promoters generally have special regulatory elements, such as RY-motif, GCN4 (General Control Nonderepressible 4), AACA, CCAA and TATAA [39,40]. Analysis of the *AhRUVBL2* promoter sequence revealed the presence of seed-specific expression elements, namely AACC and CCAA motifs. Additionally, this promoter region contains conserved cis-acting elements such as the CAAT-box and G-box, which are commonly found in seed-specific promoters. Furthermore, the *AhRUVBL2* promoter exhibits characteristics of hormone-responsive promoters, which classifies it as an inducible promoter. Notably, ABA-inducible promoters typically harbor ABRE (abscisic acid-responsive element) cis-regulatory elements that are critical for mediating responses to abscisic acid signaling. In wheat and rice seeds, ABRE elements mainly regulate gene expression during the late stage of development [41]. Auxin-inducible promoters have a variety of auxin response elements, including AuxRE, TGA-box, As-1-box and Ocs-element. When the auxin concentration is high, this leads

to degradation of the transcription inhibitor Aux/IAA. The auxin response factor (ARF) can then bind to the AuxRE cis-acting element to promote the expression of auxin response genes [42].

In the current study, bioinformatics analysis of the promoter sequence located 2527 bp upstream of ATG in the *AhRUVBL2* gene revealed that it contained hormone responsive cis-elements, including two ABA response elements and one auxin response element. We first transferred GUS expression vectors containing the *AhRUVBL2* promoter sequences D893 and 79266 into Agrobacterium by transformation assay. WT *Arabidopsis* was then transformed by the floral dip method, followed by histochemical staining and real-time fluorescence quantification of GUS. The *AhRUVBL2* promoters D893 and 79266 were mainly expressed in the seeds of *Arabidopsis* siliques. By spraying exogenous hormones, ABA treatment was shown to significantly down-regulate the expression of *proAhRUVBL2-D893*, and significantly up-regulate the expression of *proAhRUVBL2-79266*. IAA treatment had no significant effect on the expression of *proAhRUVBL2-D893*, but significantly down-regulated the expression of *proAhRUVBL2-79266*. There is currently a dearth of information on the regulation of *AhRUVBL2* by plant hormones, and the role of auxin and ABA in the regulation of this gene in plants requires further study.

Plant hormones play important roles in regulating plant growth and development, as well as their adaptation to various abiotic and biotic stresses. The *SAUR* gene family is involved in plant-specific auxin responses and plays a vital role in seed development in many plants. In *Arabidopsis*, overexpression of *AtSAUR63*, a homologous gene of *SAURs*, leads to the elongation of hypocotyls, petals, stamen and filaments [43]. *PRX* is involved in regulating the hydrogen peroxide level during the cell cycle, which in turn is linked to signal transduction during cell proliferation, differentiation and apoptosis [44]. The plant peroxidase genes *PRX71* (AT5G64120), *PRX66* (AT5G51890) and *PRX25* (AT2G41480) are key genes in the phenylpropanoid biosynthesis pathway that regulate *Arabidopsis* siliques [45,46]. In the present study, *AhRUVBL2*-overexpression significantly promoted the growth of *Arabidopsis* and the development of silique cells. Transcriptome sequencing of the siliques of *AhRUVBL2*-overexpressing and WT *Arabidopsis* showed that *AhRUVBL2* can affect the growth and development of plants, and fruit ripening, by promoting the expression of genes associated with phenylpropanoid biosynthesis and plant hormone signal transduction. Previous transcriptome sequencing results [23] show that the expression pattern of *ARP4* (*Arahy.1T6SIZ*) in peanut is highly consistent with that of *AhRUVBL2*. Furthermore, *ARP4* was up-regulated in transgenic *Arabidopsis*, suggesting the protein encoded by this gene may interact with the AhRUVBL2 protein. In *Arabidopsis*, the loss of function of *AtARP4* leads to smaller leaves, demonstrating that ARP4 is involved in regulating plant growth [47]. Therefore, we speculate that AhRUVBL2 protein mainly interacts with ARP4 in the regulation of plant growth and development.

5. Conclusions

In this study, overexpression of *AhRUVBL2* in transgenic *Arabidopsis* was found to significantly increase plant height, branch number, leaf size, pod size, seed size, thousand-seed weight, and pericarp thickness, while significantly shortening the bolting and flowering stages. In addition, overexpression of *AhRUVBL2* increased the area of silique exocarp cells, and the number and area of lignified cells in endocarp. A total of 337 DEGs were identified in transgenic *Arabidopsis*. These included the key genes *PRX*, *SAUR* and *PYL* that regulate development of *Arabidopsis* siliques and are closely related to *AhRUVBL2* expression, as well as the ARP4 protein that interacts with AhRUVBL2. The *AhRUVBL2* promoter is a seed-specific and hormone-induced promoter, with the promoter activity of *AhRUVBL2* D893 being significantly greater than that of *AhRUVBL2* 79266. These results should prove useful for the breeding of high-yield peanut varieties and for the study of molecular mechanisms in peanut growth.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

FZL, LL and YSW conceived and designed the experiments. PHM, XLL, LL, KZ, HYY, YYL, HDL and HY performed the experiments. PHM, XLL and FZL analyzed the data and drafted the manuscript. FZL revised the manuscript. All authors discussed the results and commented on the article. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Arabidopsis (Col-0), tobacco, and peanut varieties D893 (large pod) utilized in this study were provided by the College of Agronomy, Shandong Agricultural University. The peanut varieties 79266 (small pod) was provided by the Shandong Peanut Research Institute. *AhRUVBL2* transgenic *Arabidopsis* was obtained through a previous investigation conducted by our research group.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL38940.

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