Study of differentially expressed genes related to plant height and yield in two alfalfa cultivars based on RNA-seq

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Background. Alfalfa (*Medicago sativa* L.) is a kind of forage with high relative feeding value in farming and livestock breeding, and is of great significance to the development of animal husbandry. The rapid growth of the aboveground part of alfalfa is the main factors limiting crop yield. Clarifying the molecular mechanisms that regulate alfalfa vigorous-growing may contribute to the development of molecular breeding for alfalfa. This mechanism, however, has not been extensively studied for alfalfa.

Methods. In the present study, the phenotypes of five alfalfa cultivars were evaluated. We found that the rapid growth of stems significantly affected plant height and yield of tall-type alfalfa (WL 712) and low-type alfalfa (Aohan). The vigorous-growing WL712 and slow-growing Aohan exhibited significantly different plant heights, stem diameters and internode lengths. RNA-seq was performed on the stems of both cultivars. GO enrichment analysis was conducted on all differentially expressed genes (DEGs).

Result. In WL712 alfalfa cultivar, we found that seven DEG groups were found to be involved in the formation of water-conducting tissue in vascular plants, biosynthesis and degradation of lignin, formation of the primary or secondary cell wall, cell enlargement and plant growth, cell division and shoot initiation, stem growth and induced germination, and cell elongation. KEGG analysis showed that plant hormone signal transduction, photosynthesis, and phenylpropanoid biosynthesis regulated the rapid growth of stems. Members of the *WRKY* family related to plant growth and development. Members of the *NAC* and *MYB* gene families related to the synthesis of cellulose and hemicellulose, and the development of secondary cell wall fibres. Some *MYB* family members act as activators or inhibitors and are involved in plant growth regulation. Our research results not only enrich the transcriptome database of alfalfa, but also provide valuable information for explaining the molecular mechanism of fast-growing, and can provide reference for the actual production of alfalfa at the same latitude and similar soil in the world.

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Abstract

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Key words: Medicago sativa, RNA – seq, DEGs, Stem elongation, Vigorous-growing, Slow-growing

Introduction

The stem is an important vegetative organ between the root and leaf of a plant and transports nutrients and water (Ernest et al., 2020). The stems of alfalfa also play a role in photosynthesis, nutrient storage, and regeneration (Sena, 2014). In the process of stem growth and development, stem tips grow continuously, whereas branches, leaves, and lateral branches are produced successively, which together constitute a huge branch system (Yu et al., 2015; Jaykumar & Mahendra, 2016). The degree of stem development is closely related to the life cycle of plants (Sophia et al., 2021), especially the aboveground biomass of the plant (Kleyer et al., 2019). Alfalfa, with stems and branches as the main components of ~~grass~~ yield, is a typical representative crop.

Alfalfa is a feed crop with a high economic value (Kumar et al., 2018). In addition to its stress resistance properties, it has been the focus of research because of its perennial nature and high nutritional value (Wang et al., 2017; Diatta, Doohong & Jagadish, 2021). The stems and leaves of alfalfa have ~~the highest~~ nutrient content and are the main parts areas of animal forage (Sulc et al., 2021). Owing to the cross-pollination of alfalfa, most cultivars have a complex genetic background. Restricted by its genetic characteristics, growth performance and nutritional quality are uneven (Bambang et al., 2021). Alfalfa stalks are composed of nodes and internodes, which affect plant height and yield. The height and stem diameter of alfalfa are important factors that restrict its biomass (Monirifar, 2011). Therefore, increasing the number of alfalfa vegetative branches, vegetative growth time, and delaying the flowering time of plants are crucial for improving the nutritional quality and yield of forage grass (Aung et al., 2015), ~~which is also one of the hot spots that breeders pay attention to at this stage~~. Previous studies have reported significant differences in alfalfa plant height and hay yield (Ziliotto et al., 2010). The WL alfalfa series exhibited the best growth performance when compared to different alfalfa cultivars (Tetteh & Bonsu, 1997). Plant spacing and light significantly effect on alfalfa forage yield and weed inhibition in the field (Celebi et al., 2010). Compound fertilizers can increase the nutrient content of soil and improve the yield of alfalfa (Iryna, Rudra & Doohong, 2021; Na et al., 2021). Additionally, the growth and development periods of alfalfa are equally important for its yield (Martin et al., 2010). During the growth of alfalfa, the budding stage that has excellent nutritional quality and ~~grass~~ yield has always been a ~~hot~~ period of concern for breeders around the world (Fan et al., 2018). Currently, research on the growth performance of alfalfa mainly focuses on the physiological level. Few reports have revealed the molecular mechanism of alfalfa stem elongation and diameter enlargement and its effect on biomass at the gene level.

Owing to the lack of a complete reference genome sequence, previous studies on the stress-response genes of alfalfa have used nonparametric transcriptome analysis (Yuan et al., 2020; Wang et al., 2021; Gao et al., 2016; Arshad, Gruber & Hannoufa et al., 2018). Reference-free transcriptome refers to the sequencing of eukaryotic transcriptomes in the absence of a reference genome. After obtaining the original data for eukaryotic nonparametric transcriptome sequencing, the quality control splicing is first ~~performed into unigene, and then the unigene is used~~ as the

reference sequence for subsequent analysis. However, with the availability of whole-genome sequencing and annotation of alfalfa (Zhongmu 1), studying the alfalfa genome has become easier (Zhang et al., 2021). Transcriptome sequencing is the study of all mRNAs ~~transcribed by a specific tissue in a certain period~~, which is the basis for the study of gene function and is important for understanding the development of organisms (Wang, Gerstein & Snyder, 2009). With the advantages of high-throughput, high accuracy, and high sensitivity, RNA-seq can be used to study changes in the expression level of transcripts ~~in vivo~~ to understand or reveal the intrinsic relationship between gene expression and biological phenotypes (Guo et al., 2021). At present, RNA-seq technology has become a common method to study the growth and development of ~~*rice, Arabidopsis thaliana, upland cotton and wheat* (~~Chen et al., 2020; Kim et al., 2021; Zheng et al., 2021). Next-generation high-throughput sequencing technology can be used to comprehensively obtain the transcript information of alfalfa and screen out the significantly different genes related to stem elongation and diameter enlargement.

The fast-growing of alfalfa is a important factor of alfalfa that ~~can~~ affect plant height and yield (Yan et al., 2021). Exploring the molecular mechanisms ~~of vigorous-growing of alfalfa~~ may be helpful to improve the yield ~~of alfalfa cultivars~~. The application of gene editing technology may be more efficient than traditional techniques such as cross-breeding. We identified differentially expressed genes (DEGs) in the stem of alfalfa "WL712" (USA, FDC = 10.2) and "Aohan" (China, FDC = 2.0) using RNA-seq, further identified the key genes regulating vigorous-growing of alfalfa by bioinformatics analysis and predicted their functions. These results may be helpful in clarifying the molecular mechanism ~~of vigorous-growing of alfalfa~~, establishing a regulatory network of the growth and development of dominant cultivars, and laying a theoretical foundation for molecular breeding and the introduction of dominant cultivars.

Materials & Methods

Characterisation of phenotypic traits

Five cultivars of alfalfa (WL 712, Victoria, Kangsai, Knight 2, and Aohan) were planted at the experimental herbage station of Shihezi University, Xinjiang, China (N44 ° 20 ', E88 ° 30', altitude 420 m) (**Table S1a**). Its characteristic is temperate continental arid climate, with an average annual temperature of 8.1°C. Before planting, we adopted the “S” shaped sampling method, and nine soil samples were obtained. the nutrient status of the soil (20 cm) was as follows: available nitrogen 92.6 mg/kg, organic matter 12.4 g/kg, available potassium 168.5 mg/kg, available phosphorus 33.2 mg/kg, and pH 7.26 (**Table S1b**).

In June 2019 and 2020, alfalfa was planted in a 40 m^2 plot using a completely randomised design. To ensure consistency among the cultivars, thirty-six stems with well-growing single alfalfa were collected from each cultivar. Single-row planting method with sampling plant spacing of 40 cm and row spacing of 60 cm, with three biological replicates per cultivar. At the budding stage, agronomic traits of five randomly selected plants were determined from each of the three biological replicates. The absolute distance from the root to the top of the main stem was calculated as plant height using ruler. ~~This counting method calculates the~~ number of branches and nodes. The stem diameter and internode length were ~~calculated~~ using calipers. The leaf area was measured

using leaf area meter. Five plants in each row were randomly selected and weighed, and the average value was calculated as the total fresh weight per plant. By comparing and analyzing the growth indexes of different varieties, it was finally determined that WL712 represented a vigorous and fast-growing variety and Aohan represented a short and slow-growing variety (**Fig. S1**).

Cultivation of experimental materials and sample collection

Stems ~~with vigorous-growing~~ WL712 and ~~slow-growing~~ Aohan were collected and cut into 8 cm pieces, leaving an axillary bud. The ~~plant~~ were cultivated on cutting beds in the greenhouse (light/dark: 16 h / 8 h, Temp: 25 °C / 20 °C, humidity 70%) of the Beiyuan campus of Shihezi University for 20 days, and surviving plants were transplanted into plastic pots (diameter 32 cm, height 35 cm). Nutrient soil: vermiculite = 1: 1 (cultivation and management methods were consistent). More than 30 individual plants of both WL712 and Aohan survived in the greenhouse. Five plants of ~~fast-growing~~ WL712 and ~~slow-growing~~ Aohan alfalfa were randomly selected~~, respectively,~~ and the plant height, internode length, stem diameter, leaf area and yield were determined.

At the budding stage, stems (approximately 1.5 cm) of each cultivar were collected, quickly frozen in liquid nitrogen. Three biological replicates were used for per cultivar. WJ1, WJ2 and WJ3 represent samples from the WL712 cultivar. AJ1, AJ2 and AJ3 represent samples from the Aohan cultivar. Finally, six samples were used for RNA-seq.

Library construction and RNA-seq

Total RNA was isolated from stems using the RNeasy Plant Mini Kit (Qiagen, Germany). A total of 3 μg RNA per sample was used to build the library. Sequencing libraries were generated using a NEBNext® UltraTM RNA Library Prep Kit (NEB,USA). Messenger RNA was purified from each sample using magnetic beads and fragmented with divalent cations at elevated temperature. First-strand cDNA was obtained using segmented mRNA as template and random oligonucleotide as primer. Then, the second strand of cDNA was obtained in DNA polymerase I system. The double-stranded cDNA were purified using AMPure XP Beads (Beckman Coulter, Beverly, USA). The double-stranded cDNA was ligated to the sequencing adaptor after terminal repair and A tail, and 250-300 bp cDNA was obtained using AMPure XP beads. Finally, the PCR system was amplified, and the PCR products were purified again using AMPure XP beads to obtain the libraries.

Library quality was examined using the Agilent Bioanalyzer 2100 system. The effective concentration of the library (⩾2 nM) was quantified using qRT-PCR. After passing the inspection, the libraries were pooled and sequenced on the Illumina HiSeq X-10 (California, USA) platform by Beijing Novo Biotech Company, Ltd. Finally, each sample contained an average of 6.63 G of valid data, and 4.42×10^7 clean reads.

Quality control

To ensure the accuracy of data analysis, we filtered the original data and examined the sequencing error rate. Using in-house Perl scripts to process the raw reads of fastq format. Removing reads containing adapters, ploy-N sequences, and low-quality from the raw data to obtain clean reads. The Q20, Q30, and GC contents of the clean data were calculated. All

subsequent analyses dependd on clean data, high quality.

RNA-seq data analysis

The index of the reference genome was constructed using HISAT2 v2.2.1. The paired-end clean reads were obtained using HISAT2 v2.2.1 (https://cloud.biohpc.swmed.edu/index) aligned to the reference genome Zhongmu No. 1 (https://figshare.com/articles/dataset/genome fastq sequence and annotation files/12327602) to obtain mapped reads (Mortazavi, Williams & McCue, 2008). We also analysed of the proportion of mapped reads in the exons, introns, and intergenic regions of the genome.

The clean reads aligned to Zhongmu No. 1 were quantified using FeatureCounts v1.5.0-p3. Gene expression was tested by FPKM (fragments per kilobase of transcript per million fragments mapped), and differences between WL712 and Aohan FPKM values were compared using FeatureCounts v1.5.0-p3.

Differential expression analysis of the two comparison combinations was performed using the DESeq2 R package (1.16.1) (https://www.bioconductor.org/packages/release/bioc/html/DESeq2.html). DESeq2 determines the differential expression in digital gene expression data using a model based on a negative binomial distribution. The corrected P-values and $|\log_2 \text{foldchange}|$ are thresholds for significant differential expression. P-values were adjusted using the Benjamini & Hochberg method.

Gene Ontology (GO) (http://www.geneontology.org/) enrichment and KEGG (Kyoto Encyclopedia of Genes and Genome) (http://www.genome.jp/kegg/) statistical analysis of DEGs were performed using the clusterProfiler R package. A corrected P-value less than 0.05 was used as the threshold for significant enrichment of differentially expressed genes.

qRT-PCR

The accuracy of the RNA-seq was verified by qRT-PCR. Total RNA were isolated from stems, and cDNA was synthesised by using the PrimeScript™ RT reagent Kit (Takara, Tokyo, Japan). Alfalfa *β-Actin 2* was used as the internal gene. The primers in **Table S9** were used for qRT-PCR. qRT-PCR were completed using the LightCycler 96/LightCycler480 system. The solution of the 20 μL system contained 0.4 μL forward primer, 0.4 μL Reverse Primer, 10 μL TB Green Fast qPCR Mix (2X) (Takara, Tokyo, Japan) and 2 ng cDNA. The PCR procedure included 45 cycles, with 3 technical repetites for each reaction. According to Kenneth report, the relative expression of each gene was calculated (Livak & Schmittgen, 2001).

Statistical Analysis

All statistical analysis was using *SPSS* software (version 17; IBM Inc, USA). The data were compared using Student's t-test, and $P < 0.05$ was considered statistically significant. The power of our samples was calculated using RNASeqPower (https://bioconductor.org/Packages/release/bioc/html/RNASeqPower.html), and the RNASeqpower was 94.2%.

Result

Phenotypic analysis of five alfalfa varieties

To compare the differences in the growth patterns of the five cultivars (**Table S1a**), plant height, internode length and stem diameter of alfalfa at different growth stages were ~~continuously~~ measured in 2019 and 2020 (**Fig. 1, Table S10**). There were no significant differences in plant

height, internode length or stem diameter among cultivars at the seedling transplant stage. After the budding stage, plant height, internode length and stem diameter of different alfalfa varieties reached a plateau and remained relatively stable (**Fig. 1a-c**). In 2019 and 2020, WL712 and Aohan represented tall and short phenotypes, respectively, ~~in alfalfa~~ (**Fig. 1d**). Comparing the agronomic traits of alfalfa at the budding stage in 2019 and 2020, the plant height of WL 712 was approximately 1.78 and 1.91times those of Aohan, respectively, and the stem diameter of WL 712 was approximately 1.90 and 1.92 times those of Aohan (**Fig. 1d-e**). The internode length and number of lateral branches in WL712 were significantly larger than those in Aohan ($P < 0.01$), whereas the number of main branches in WL712 was significantly lower ($P < 0.05$) (**Fig. 1f, Fig. 2a-b**).

To identify the correlation between internode length and stem diameter and other traits, the fresh weight, leaf-stem ratio, and dry weight of the five cultivars were also determined. The results showed that the production performances of WL712 and Aohan were significantly different ($P < 0.05$) (**Fig. 2c-f**). Phenotypic correlation analysis based on 8 agronomic traits was done. We found that fresh and dry weight were positively and strongly correlated with the number of lateral branches, plant height, stem diameter, and internode length, and plant height was significantly positively correlated with internode length ($P < 0.01$). In addition, the number of main branches was negatively correlated with plant height, stem diameter, and internode length ($P < 0.01$) (**Table 1**).

From the screening of five alfalfa cultivars, WL712 and Aohan were identified as the cultivars with the most significant difference in growth performance. The growth trend of the two varieties in greenhouse is similar to that in field. The plant height, internode length, yield per plant, leaf area and stem diameter of WL712 alfalfa were significantly higher than those of Aohan alfalfa (**Table 2**).

Based on the above results, WL712 and Aohan were used as ~~high-type~~ vigorous-growing and ~~short-type~~ slow-growing experimental cultivars, while the stem base tissue was used for RNA-seq (**Fig. S1**).

RNA-seq analysis

Using RNA-seq ~~to~~ obtain 2.74×10^8 raw reads. The sequence error rate of a single base position was 0.03%, and the average GC content was 41.65%. After filtering from the raw data, 2.65×10^8 (96.94%) clean reads (39.76 G) were obtained. The Phred values were greater than 97% and 93% at Q_{20} and Q_{30}, respectively (**Table S1c**). The Pearson coefficient showed that the homology among the samples within the group was higher than 84.6% (**Fig. S2**).

We aligned the clean reads with the reference genome. The average proportions of exons, introns and intergenic regions in AJ samples were 72.72%, 3.61%, and 23.67%, respectively. Similarly, the WJ samples accounted for 74.14%, 2.96%, and 22.90%, respectively (**Table S2**). The reads aligned to the intron region may have been derived from the precursor mRNA. The reads aligned to the intergenic region may have been derived from ncRNAs.

Additionally, according to the comparison of RNA-seq data from WL 712 and Aohan, the RNASeqpower of our sample was 94.2%. The result may be beneficial to screen and explore the functional DEGs related to the vigorous-growing of alfalfa. These results demonstrated that the

experiments were reproducible and that the data were accurate.

Identification and functional annotation of DEGs in WL712 and Aohan

Generally, ~~FPKM is used to evaluate~~ the gene expression value of RNA-seq, which corrects the sequencing depth and gene length successively (**Fig. S3**). More than 90% of the clean reads were successfully mapped to the alfalfa genome. To clarify the function of the DEGs in WL712 and Aohan, we performed GO and KEGG enrichment analyses. In total, 954 remarkably enriched DEGs were assigned to 35 GO terms. Compared to Aohan, WL712 upregulated 578 genes and downregulated 376 genes. Among the molecular function, "*protein heterodimerization activity*" [GO:0046982] (114 DEGs, 11.95%) was the highest proportion, followed by "*UDP-glycosyltransferase activity*" [GO:0008194] (99 DEGs, 1.04%) and "*translation factor activity, RNA binding*" [GO:0008135] (86 DEGs, 9.01%). Among the cell components, "~~–~~*bounding membrane of organelle*~~–~~" [Go:0098588] (57 DEGs, 5.97%) represented the largest cluster, followed by "*whole membrane*" [Go:0098805] (49 DEGs, 5.13%) and "*peptidase complex*" [Go:1905368] (44 DEGs, 4.61%). Among the biological processes, "*translational elongation*" [GO:0006414] (41 DEGs, 4.30%) represented the largest cluster (**Table 3, Table S3 , Fig. 3a**).

Based on biological system network, the function of DEG was identified using KEGG classification. A total of 1324 genes were enriched in 110 KEGG pathways (**Fig. 3b**). "*Carbon metabolism*" [ath01200] (103 DEGs, 7.8%) and "*Ribosome*" [ath03010] (96 DEGs, 7.3%) were the most abundant pathways; followed by "*Biosynthesis of amino acids*" [ath01230] (81 DEGs, 6.1%), "*RNA transport*" [ath03013] (54 DEGs, 4.1%), "*Plant-pathogen interaction*" [ath04626] (52 DEGs, 3.9%), "*Protein processing in endoplasmic reticulum*" [ath04141] (52 DEGs, 3.9%) and "*Plant hormone signal transduction*" [ath04075] (44 DEGs, 3.2%) (**Table S4)**.

Expression and regulation of DEGs in WL712 and Aohan

KEGG analysis showed that DEGs related to stem elongation and diameter enlargement were widely involved in biological processes such as hormone signalling, photosynthesis and transcriptional regulation (**Table S5**).

Plant hormone signal ~~transformation~~ (Ath04075) involves many hormones that regulate the growth and development ~~of plants~~, such as auxins, cytokinines, gibberellins, brassinosteroids, jasmonic acid, and ethylene. Twelve DEGs were enriched in the auxin-mediated signalling pathway, including *auxin-responsive protein SAUR* (*SAUR*), *auxin-induced protein X10A* (new gene) and *auxin transporter-like protein* (*LAX*). Among these, *IAA9*, *IAA6*, *SAUR50*, *SAUR32* and *SAUR36* were significantly upregulated. In the cytokinin-mediated signalling pathway, four DEGs were enzyme genes, such as *adenylate isopentenyltransferase 5* (*IPT5*), *7-deoxyloganetin glucosyltransferase* (*UGT85A24*), *cytokinin dehydrogenase 6* (*CKX6*), and *cytokinin hydroxylase* (*CYP735A2*). *DELLA protein GAI* (*GAI*), *f-box protein GID2* (*GID2*), and *transcription factor PIF4* (*PIF4*) were enriched in the gibberellin-mediated signalling pathway. *Serine/threonine-protein kinase BSK8* (*BSK8*), *serine/threonine-protein kinase BSK1* (*BSK1*), and *Cyclin-D3-3 (CYCD3-3)* were enriched in the brassinosteroid-mediated signalling pathway. Five DEGs were enriched in the jasmonic mediated signalling pathway, including *Coronatine-insensitive protein homolog 1a* (*COII A*), *Protein TIFY 6 B* (*TIFY 6B*), *Protein TIFY 11 B* (*TIFY 6B*), *Protein TIFY 10 B* (*TIFY 10B*) and *Protein TIFY 3 B* (*TIFY 3B*). Four upregulated DEGs were enriched in the

ethylene-mediated signalling pathway, including *ethylene receptor* (*ETR 1*), *mitogen-activated protein kinase kinase 4* (*MKK 4*), *mitogen-activated protein kinase homolog MMK1* (*MMK 1*), and *protein ethylene insensitive 3* (*EIN 3*).

Fifteen DEGs were enriched in the photosynthetic (ath00195) pathway. Among them, *PPL1*, *PETC*, *PSBR*, *PSBS*, *PSAG*, *PSAO*, *PSB 27* and *PSB 28* were related to the photoreaction. *PLSN 2* was related to the activity of the chloroplast NAD(P)H dehydrogenase (NDH) complex. *ATPF 2* and *ATPC* are related to ATPase activity. Additionally, two oxygen-evolving enhancer proteins and ferredoxins have been identified. In the photosynthesis-antenna protein (ath00196) pathway, eleven DEGs were classified into *chlorophyll a-b binding proteins* and *chlorophyll a/b binding proteins*, which were expressed in chloroplasts. In the MAPK signalling (ath04016) pathway, twenty-two DEGs were mainly involved in biotic stress (pathogen infection), abiotic stress (cold/salt/drought/osmotic stress), and hormone synthesis during root growth and wounding responses.

Furthermore, the TCA cycle (ath00020), carbon fixation in photosynthetic organisms (ath00710), glycolysis/gluconeogenesis (ath00010), ribosome (ath03010), amino sugar and nucleotide sugar metabolism (ath00520), pyruvate metabolism (ath00620), and phenylpropanoid biosynthesis (ath00940) were also closely related to alfalfa growth (**Table S5**). In the TCA cycle pathway, 12 DEGs played a role in catalysis of the pyruvate dehydrogenase complex. In addition, *ATP-citrate synthase alpha chain protein 1* (*ACLA 1*) and *2 malate dehydrogenases* (*MDH*) were identified. *Pyrophosphate--fructose 6-phosphate 1-phosphotransferase subunit beta* (*PFP*) and *glycoaldehyde-3-phosphate dehydrogenase* (*GAPC1*) were highly expressed in the glycolysis/gluconeogenesis pathway. Seven *glyceraldehyde-3-phosphate dehydrogenases* (*GAPDH*) were enriched in carbon fixation in the photosynthetic organism pathway and were highly expressed in the cytoplasm or chloroplasts. Ribosomal proteins predominated in the ribosomal pathway and included 30 s (*RPS 1*, *RPS 13*, *RPSQ*, *RPS 16*), 40 s (*RP 24 a*, *RP 30 a*, *RP 15 d*, *RP 10 a*, *RP 20 a*), 50 s (*RPL 28*, *RPMJ*, *RPL 31*, *RPLX*) and 60 s (*RPP 3 a*, *RPL 21 e*, *RPL 37a*, *RPL 37 B*). *Dihydrolipoyllysine-residue acetyltransferase component 2 of the pyruvate dehydrogenase complex* (*At3g13930*) and *malate dehydrogenase* (*mMDH*) were highly expressed in the pyruvate metabolic pathway. *Beta-glucosidase 44* (*BGLU 44*), *beta-amylase 1* (*BAM 1*), *acid beta-fructofuranosidase* (*VCINV*), and *probable fructokinase-4* (*At3g59480*) were highly expressed in the starch and sucrose metabolism pathways. The genes with high expression in the phenylpropanoid biosynthesis pathway were *Probable cinnamyl alcohol dehydrogenase* (*CAD 2*), *beta-glucosidase 46* (*BGLU 46*), *trans-cinnamate 4-monooxygenase* (*CYP 73A3*), and *3 peroxidases* (*PER*).

DEGs enriched in a variety of biological processes

All DEGs were analysed using GO and KEGG analyses. We found that seven groups of DEGs related to stem elongation and diameter expansion, including formation of water-conducting tissue in vascular plants, cell division and shoot initiation, biosynthesis and degradation of lignin, cell enlargement and plant growth, formation of the primary or secondary cell wall, cell elongation, and stem growth and induced germination (**Table S6**). *Eukaryotic translation initiation factor 5A-1* (*EIF 5A*), *mitogen-activated protein kinase kinase kinase 3* (*ANP 3*), and *alpha, alpha-trehalose-*

phosphate synthase (*TPS 6*) were involved in the formation of water-conducting tissues. Fourteen DEGs were enriched in lignin biosynthesis and degradation. Peroxidases play an important role in this process. Additionally, *peroxidase 47* (*PER 47*) is a novel gene. Eleven DEGs were enriched in the formation of the primary or secondary cell wall. C*ellulose synthase A catalytic subunit* (*CESA*) plays an active role. Eighteen DEGs were enriched in cell enlargement and plant growth. AUXs, such as *auxin-responsive protein* (*IAA 9*), *auxin-induced protein* (*IAA 6*) and *auxin transporter-like protein* (*LAX 5*), play an active role. Additionally, *auxin-induced protein X10A* is a novel gene. Five DEGs were enriched in cell division and shoot initiation. Enzyme genes such as *7-deoxyloganetin glucosyltransferase* (*UGT 85A24*), *cytokinin hydroxylase* (*CYP 735A2*) and *cytokinin dehydrogenase 6* (*CKX 6*) play a dominant role. Two DEGs were enriched in stem growth and induced germination. Interestingly, *DELLA protein* (*GAI*) negatively regulated the gibberellin signalling pathway, whereas *F-Box protein* (*GID 2*) positively regulated the gibberellin signalling pathway. *Serine/threonine-protein kinase*（*BSK 1*）and *BSK 8* are related to cell elongation. Additionally, we identified genes that regulate senescence, including *protein ethylene insensitive 3* (*EIN 3*). Importantly, compared with Aohan, *cellulose synthase A catalytic subunit 8* (*CESA 8*), *beta-1,4-xylosyltransferase* (*IRX 9*), *probable beta-1,4-xylosyltransferase* (*IRX 14H*), *auxin-responsive protein* (*SAUR 36*), *peroxidase 16* (*PER 16*), and *peroxidase 51* (*PER 51*) were upregulated more than 8-fold in WL712, whereas *mitogen-activated protein kinase 3* (*MPK 3*), *pathogenesis-related protein* (*At2g14610*), *peroxidase 55* (*POD 55*), *beta-glucosidase 46* (*BGLU 46*), and *peroxidase 15* (*POD 15*) were downregulated more than 15-fold in WL712 (**Table S7**). All the genes related to stem growth and development were clustered together, as shown in **Fig. 4**.

~~TF~~s involved in alfalfa growth and development

TFs are essential in plant growth and development as protein molecules that regulate gene expression. In this study, 20 TFs were involved in the development of alfalfa (**Fig. 5a, Table S8**). Seven DEGs were upregulated, including *NAC domain-containing protein 73* (*NAC073*), *NAC domain-containing protein 10* (*NAC010*), *transcription factor MYB 46* (*MYB 46*), and *NAP-related protein 2* (*NRP2*). Additionally, *WRKY transcription factor 22* (*WRKY 22*), *transcription factor TGA1* (*TGA1*) and *Transcription factor MYB 86* (*MYB 86*) were novel genes. GO annotations revealed that *NAC073* and *NAC010* were involved in the synthesis of cellulose and hemicellulose and the development of secondary cell wall fibres. Thirteen DEGs were downregulated, and the *WRKY* and *MYB* family members played a dominant role. GO classification shows that *WRKY 51* was involved in the positive regulation of salicylic acid-mediated signal transduction and negative regulation of jasmonic acid-mediated signal transduction in the defense respons. *WRKY 54* is a negative regulator of plant growth and development. *MYB 46* is involved in secondary wall cellulose biosynthesis as a transcriptional activator. *MYB 86* is involved in lignin synthesis and accumulation. Additionally, *MYB 2* inhibited the expression of light-harvesting genes. All identified TFs were validated using qRT-PCR (**Fig. 5b**). The relative expression of *NAC 081* was significantly upregulated in WL 712 plant ($P < 0.001$). The relative expression levels of most TFs were similar to the the FPKM trend.

The reliability of RNA-seq was verified using qRT-PCR

To determine the accuracy and rationality of the data, we ~~randomly~~ selected 11 DEGs for

qRT-PCR validation. DEGs were mainly related to the formation of the primary or secondary cell wall, cell enlargement and plant growth, and biosynthesis and degradation of lignin. The changes in transcript abundance are shown in **Fig. 6a**. qRT-PCR revealed that *IRX 9*, *CESA 8*, *CESA 7*, *MKK 4*, *PER 16*, and *PER 51* were significantly upregulated in WL712 plant ($P < 0.05$). *MPK 3*, *At2g14610*, *BGLU 46*, and *POD 15* were significantly downregulated in WL 712 ($P < 0.05$) (**Fig. 6b**). However, the relative expression of *CAD 2* between the two varieties were not significantly different ($P > 0.05$) and were inconsistent according to RNA-seq transcript abundance. This may have been caused by RNA-seq errors in the acceptable range. Overall, the relative expression trend of the DEGs was similar to the RNA-seq.

Discussion

Alfalfa is an important component of feed, and the growth performance of its aboveground part affect the ~~grass~~ yield. The *FmS6K* plays an important role in regulating the development of plant stems (Sun et al., 2018). The yield of elephant grass has a strong positive correlation with internode length (Yan et al., 2021). However, the molecular regulatory mechanisms underlying the ~~vigorous-growing~~ of stems and branches in alfalfa remain unclear. In this study, the growth difference between ~~vigorous and~~ fast-growing variety WL712 and short and slow-growing variety Aohan ~~alfalfa varieties~~ was studied ~~by comprehensive method~~. ~~The phenotypes and RNA-seq~~ of ~~these two varieties were analyzed by using stems~~. The results of qRT-PCR showed that the expression trend of most DEGs was consistent with RNA-seq. The difference between qRT-PCR and RNA-seq of individual DEG may be caused by the error of RNA-seq within the acceptable range. Overall, the RNA-seq data could be used for subsequent analysis. All DEGs were ~~enriched~~ with GO; 954 significant DEGs were obtained, and seven DEG clusters were involved in ~~regulating the vigorous-growing of alfalfa~~ (**Fig. 4**). Additionally, KEGG revealed that hormone signal transduction, photosynthesis and phenylpropane biosynthesis ~~regulate the process of the vigorous-growing of alfalfa.~~ RNA-seq also identified several novel DEGs associated with the ~~vigorous-growing of alfalfa~~, including *PER47* and *TIFY10A*.

Plant organ growth is ~~regulated~~ by both developmental processes and environmental factors (Sun et al., 2018). In ~~most~~ cases, these changes are ~~mainly~~ due to hormone-mediated action (Verma, Ravindran & Kumar, 2016). In this study, auxin, cytokinin, gibberellin, ethylene, brassinosteroid, and jasmonic acid ~~affected the growth and development of alfalfa by regulating downstream DEGs,~~ such as *SAUR50*, *CKX6*, *GID2*, and *GAI*. These DEGs might play a role in ~~the vigorous-growing of~~ alfalfa. Previous studies have identified *SAURs* as a class of hormones that regulate plant growth and development and promote cell enlargement (Ren & Gray, 2015). Cytokinin ~~bio~~synthesis ~~was~~ required to activate shoot division in apple trees with the top removed (Tan et al., 2018). Relevant studies have shown that gibberellin regulates plant organ elongation and development (Nagel, 2020). *GAI* is an inhibitor of highly conserved gibberellin ~~(GA)~~ signalling in plants. The *SCF* (*GID2*) complex mediates degradation of DELLA proteins (*RLG2*, *RGA*, and *GAI*), and activates and positively regulates the gibberellin signalling pathway (Dill et al., 2004). In addition, in the plant hormone signal transduction pathway, the production of hormones that play a mediating role depends on the metabolism of amino acids or fatty acids. Tryptophan in plants is not only involved in the synthesis of ~~its own~~ proteins but also the precursor

of many ~~secondary~~ metabolites (such as auxin) (Manol & Nemoto, 2012). Jasmonic acid induces plants to prioritise defense over growth by interfering with the gibberellin signalling cascade, which is usually accompanied by significant growth inhibition (Yang et al., 2012). *TIFY*, which encodes jasmonic acid repressor, was significantly upregulated in Aohan (**Table S5**). This may explainwhy the low-dormancy alfalfa plant is a dwarf.

Photosynthesis is an essential metabolic process ~~that regulates the growth and yield of plant~~. Twenty-nine DEGs were related to photosynthesis. For example, *PIF1* and *PIF3* were significantly downregulated in WL712 (**Table S5**). These genes may play a regulatory role in the process of plant height and internode elongation. Plant height~~,~~ and leaf area of transgenic soybean ~~were~~ decreased by overexpressing *PIF4* (Arya, Singh & Bhalla, 2021). The deletion of *PIF1* and *PIF3* results in an increase in plant height, longer internodes, and late flowering (Hoang et al., 2021). The light-harvesting complex II (LHC II) functions as a light receptor and is related to the absorption of light (Gu et al., 2017; Sen et al., 2021). The up-regulation of these DEGs may enhance the photosynthesis of WL712 and promote the growth of plants. Additionally, circadian rhythm is also involved in the regulation of plant growth and development (Venkat & Muneer, 2022). Our research found that DEGs enriched in circadian rhythm pathway were mainly related to photoperiod flowering response. (**Table S5**).

Driven by differences in plant tissue growth, cells continue to divide, proliferate and differentiate, eventually forming various functional organs (Huang et al., 2018；Hilde & Nathalie，2020). RNA-seq analysis showed that 1531 DEGs related to rape stem growth (Yuan et al., 2019). Combined analysis of proteome and RNA–seq showed that DEGs and DEPs of Mikania micrantha stems were significantly enriched in photosynthesis, carbon sequestration and plant hormone signal transduction pathways (Can et al., 2021). We identified seven DEG clusters that were involved in stem elongation and enlargement. Fourteen DEGs were enriched in lignin ~~bio~~synthesis and degradation (**Fig. 4a**) ~~and mainly consisted of~~ peroxidase. Eleven DEGs were involved the formation of the primary or secondary cell wall (**Fig. 4b**), ~~and mainly consisted of~~ cellulose synthase. ~~These genes may regulate lignin biosynthesis and degradation in stems.~~ Previous studies reported that *CESA4* and *CESA8* were specifically enriched and expressed in the stem tissue during the fibre development stage (Guo et al., 2021). ~~The oxidation activity of peroxidases is limited to the lignified region during plant development (Hoffmann et al., 2020).~~ Eighteen DEGs were enriched in cell enlargement and plant growth (**Fig. 4c**) ~~and mainly consisted of~~ auxin. Five DEGs were involved in cell division and shoot initiation (**Fig. 4e**). ~~Interestingly, *TPS6* was also involved in the formation of water-conducting tissues (**Fig. 4h**)~~. Two DEGs were enriched in the ~~processes~~ of stem growth and induced germination (**Fig. 4f**), ~~which mainly affected plant growth and development by regulating the~~ gibberellin signalling pathway. Two DEGs were ~~found to be~~ involved in cell elongation (**Fig. 4g**). These DEGs might play a role in stem internode elongation, diameter enlargement and lateral branch formation. ~~Previous studies reported that *AtTPS6* completely compensates for the defects in reduced trichome and stem branching due to *csp-1* deficiency in Arabidopsis (Chary et al., 2008)~~. Deletion of *IAA17* in tomatos showed that the increase in fruit size ~~was~~ related to the higher ploidy level of peel cells (Su et al., 2015). Finally, the *TIFY* homologous related to ~~alfalfa~~ senescence were also identified (**Fig. 4d**). In addition, we

identified several members of *SPL* family, such as *SPL1*,*SPL6* and *SPL7*, which may be involved in the lateral branch development of alfalfa. Previous studies reported that *SPL13* regulates shoot branching in ~~*Medicago sativa*~~ (Gao et al., 2018). Overall, these DEGs may be involved in alfalfa growth and development.

TFs are essential in the regulation of development, morphogenesis and environmental stress~~in higher plants. Our~~ research found that most members of *NAC*, *WRKY* and *MYB* families ~~were~~ involved in the ~~bio~~synthesis of lignin, cellulose and hemicellulose (Wang et al., 2016). The *NAC*-mediated transcription network synergistically regulate~~d~~ biosynthesis of the plant secondary wall (Ryan, Zhong & Ye, 2011). *WRKY6* and *WRKY33* positively regulated ABA signal transduction during early development of Arabidopsis thaliana (Huang et al., 2016). *WRKY54* is a negative regulator of salicylic acid ~~(SA)~~ biosynthesis (Li, Zhong & Palva, 2017) and can significantly increase stem diameter, leaf area, and total dry weight of plants (Amin et al., 2013). Overexpression of *AtMYB44* in tomatoes resulted in slow growth (Shim et al., 2012). *MYB3R1* is a transcriptional repressor that regulates organ growth, and restricts plant growth and development by binding to target genes and promoters of specific genes (Wang et al., 2018). Under reduced light intensity, *MYB2* and *MYR1* acted as inhibitors of flowering and organ elongation, respectively (Zhao et al., 2011). In this study, excluding ~~for~~ *WRKY 22*, all *WRKY* members were significantly upregulated in dwarf alfalfa. Therefore, *WRKY 22* may positively regulate the growth and development of WL712. *NACs* are involved in the development of plant secondary cell walls. Among these, *NAC081* functions as a positive regulator. *MYB46* and *MYB86* positively regulate the synthesis of ~~plant~~ cellulose and lignin, and *MYB44*, *MYB3R1* and *MYB2* act as transcriptional repressors (**Table S8**).

Conclusion

Plant height is a important factor in determining forage biomass. The molecular characteristics of the DEGs ~~that regulate the vigorous-growing of alfalfa in WL 712 and Aohan alfalfa~~ were identified using RNA–seq. The trend of our qRT-PCR was largely consistent with those of RNA-seq, which indicated that the RNA-seq data could be used for subsequent analysis. All DEGs were analysed using GO ~~enrichment,~~ and 954 significant DEGs were identified. KEGG analysis indicated that hormone signal transduction, phenylpropane biosynthesis, and photosynthesis ~~are involved in the regulation of the vigorous-growing of alfalfa.~~ GO analysis ~~revealed that~~ seven clusters of DEGs ~~engaged in the~~ formation of water-conducting tissue ~~in vascular plants~~, cell division and shoot initiation, ~~bio~~synthesis and degradation of lignin, stem growth, formation of the primary or secondary cell wall, cell enlargement and plant growth, and induced germination and cell elongation. Additionally, the TFs ~~involved~~ in stem elongation and diameter expansion are mainly *WRKY*, *NAC*, and *MYB* family members. In summary, our research results not only enrich the transcriptome database of alfalfa, but also provide valuable information for explaining the molecular mechanism of ~~fast-growing~~, and can provide reference for the actual production of alfalfa at the same latitude and similar soil in the world.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

Qi Jiangjiao conceived and designed the experiments, performed the experiments, analyzed the data, prepared Fig.s and/or tables, and authored or reviewed drafts of the paper.

Yuxue, Wang Xuzhe and Zhang Fanfan performed the experiments.

Ma Chunhui conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Availability of data and materials

The data is available at the Sequence Read Archive (SRA) of NCBI: https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA807394.

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Reference

Aung B, Gruber M, Amyot L, Omari K, Bertrand A, Hannoufa A. 2015. Ectopic expression of LjmiR156 delays flowering, enhances shoot branching, and improves forage quality in alfalfa. *Plant Biotechnology Reports* 9(6):379-393

Arshad M, Gruber MY, Hannoufa A. 2018. Transcriptome analysis of microRNA156 overexpression alfalfa roots under drought stress. *Scientific Reports* 8:9363

Arya H, Singh MB, Bhalla PL. 2021. Overexpression of PIF4 affects plant morphology and accelerates reproductive phase transitions in soybean. *Food and Energy Security* 10(3):1

Amin AA, El-Kader AA Abd, Shalaby MAF, Gharib FAE, Rashad ESM, Teixeira da SJA. 2013. Physiological Effects of Salicylic Acid and Thiourea on Growth and Productivity of Maize Plants in Sandy Soil. *Communications in Soil Science & Plant Analysis* 44(7):1141-1155

Bambang S, Lukmana A, Nafiatul U, Bambang S. 2021. The performance and genetic variation of first and second generation tropical alfalfa (Medicago sativa). *Biodiversitas* 22(6)

Celebi SZ, Kaya L, Sahar AK, Yergin R. 2010. Effects of the Weed Density on Grass Yield of Alfalfa. In Different Row Spacing Applications. *African Journal of Biotechnology* 9(41):6867-6872

Chen L, Yang Y, K Mishina, Cui C, Zhao Z, Duan S, Chai Y, Su R, Chen F, Hu Y.G. 2020. RNA-seq analysis of the peduncle development of Rht12 dwarf plants and primary mapping of Rht12 in common wheat. *Cereal Research Communications* 48(2):139-14

Cui C, Wang Z, Su YJ, Wang T. 2021. New insight into the rapid growth of the Mikania micrantha stem based on DIA proteomic and RNA-seq analysis. *Journal of proteomics*: 104126

Chary SN, Hicks GR, Choi YG, Carter D, Raikhel NV. 2008. Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in Arabidopsis. *Plant physiology* 146(1):97-107

Diatta AA, Doohong M, Jagadish SVK. 2021. Chapter Two - Drought stress responses in non-transgenic and transgenic alfalfa--Current status and future research directions. *Advances in Agronomy*:35-100

Dill A, Thomas SG, Hu JH, Steber CM, Sun TP. 2004. The Arabidopsis F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. *Plant Cell* 16(6):1392-1405

Ernest BA, Alena PB, Mariam RS, Marian O, Edo G, Henk VA, Richard GFV, C Gerard VDL.

2020. Morphological and physiological responses of the potato stem transport tissues to dehydration stress. *Planta* 251(2):45

Etzold S, Sterck F, Bose AK, Braun S, Buchmann N, Eugster W, Gessler A, Kahmen A, Peters RL, Vitasse Y, Walthert L, Ziemińska Kasia, Zweifel R, Penuelas J. 2021. Number of growth days and not length of the growth period determines radial stem growth of temperate trees. *Ecology letters* 25:427– 43

Fan WQ, Ge GT, Liu YH, Wang W, Liu LY, Jia YS. 2018. Proteomics integrated with metabolomics: analysis of the internal causes of nutrient changes in alfalfa at different growth stages. *BMC Plant Biology* 18 (1):78

Gao RM, Austin RS, Amyot L, Hannoufa A. 2016. Comparative transcriptome investigation of global gene expression changes caused by miR156 overexpression in Medicago sativa. *BMC genomics* 17(1):658

Guo JD, Huang Z, Sun JL, Cui XM, Liu Y. 2021. Research Progress and Future Development Trends in Medicinal Plant Transcriptomics. *Frontiers in plant science*:691838

Gu JF, Zhou ZX, Li ZK, Chen Y, Wang ZQ, Zhang H, Yang JC. 2017. Photosynthetic Properties and Potentials for Improvement of Photosynthesis in Pale Green Leaf Rice under High Light Conditions. *Frontiers in plant science*:1082

Guo Y, ,Wen L, Chen JK, Pan G, Wu ZM, Li Z, Xiao QM, Wang YC, Qiu CS, Long SH, Zhao XL, Wang H, Wang YF. 2021. Comparative Transcriptomic Analysis Identifies Key Cellulose Synthase Genes (CESA) and Cellulose Synthase-like Genes (CSL) in Fast Growth Period of Flax Stem (Linum Usitatissimum L). *Journal of Natural Fibers*:1-16

Hoang QTN, Tripathi S, Cho JY, Choi DM, Shin AY, Kwon SY, Han YJ, Kim JI. 2021. Suppression of Phytochrome-Interacting Factors Enhances Photoresponses of Seedlings and Delays Flowering With Increased Plant Height in Brachypodium distachyon. *Frontiers in plant science*:756795

Huang CJ, Wang ZL, David Quinn, Subra Suresh, K Jimmy Hsia. 2018. Differential growth and shape formation in plant organs. *Proceedings of the National Academy of Sciences of the United States of America* 115 (49):12359-12364

Hilde N, Nathalie G. 2020. Understanding plant organ growth: a multidisciplinary field. *Journal of Experimental Botany* 71(1):7-10

Hoffmann N, Benske A, Betz H, Schuetz M, Samuels AL. 2020. Laccases and peroxidases co-localize in lignified secondary cell walls throughout stem development. *Plant physiology* 184(2):806-822

Huang Y, Feng CZ, Ye Q, Wu WH, Chen YF. 2016. Arabidopsis WRKY6 Transcription Factor Acts as a Positive Regulator of Abscisic Acid Signaling during Seed Germination and Early Seedling Development. *PLOS Genetics* 12(2):e1005833

Jaykumar JC, Mahendra LA. 2016. In Vitro Callus Induction and Plant Regeneration from Stem Explants of Ceropegia noorjahaniae, a Critically Endangered Medicinal Herb. *Methods in molecular biology*:347-355

Kleyer M, Trinogga J, Cebrin-Piqueras MA, Trenkamp A, Fljgaard C, Ejrns R, Bouma TJ, Minden V, Maier M, Mantilla CJ, Albach DC, Blasius B, Barua D. 2019. Trait correlation network

analysis identifies biomass allocation traits and stem specific length as hub traits in herbaceous perennial plants. *Journal of Ecology* 107(2):829-842

Kumar T, Bao AK, Bao Z, Wang F, Gao L, Wang SM. 2018. The progress of genetic improvement in alfalfa (*Medicago sativa L).* Czech Journal of Genetics and Plant Breeding 54(2):41-51

Kim S, Cho K, Lim SH, Goo TW, Lee JY. 2021. Transcriptome profiling of transgenic rice seeds lacking seed storage proteins (globulin, prolamin, and glutelin) by RNA-seq analysis. *Plant Biotechnology Reports* (1):77-93

Katyayini NU, Rinne PLH, Tarkowska D, Strnad M, Schoot C. 2020. Dual Role of Gibberellin in Perennial Shoot Branching: Inhibition and Activation. *Frontiers in plant science*:736

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25(4): 402-408

Li J, Zhong R, Palva ET. 2017. WRKY70 and its homolog WRKY54 negatively modulate the cell wall-associated defenses to necrotrophic pathogens in Arabidopsis. *Plos One* 12(8):1-22

Martin N, Brink G, Shewmaker G, Undersander D, Walgenbach R, Hall M. 2010. Changes in Alfalfa Yield and Nutritive Value within Individual Harvest Periods. *Agronomy Journal* 102(4):1274-1282

Monirifar H. 2011. Path Analysis of Yield and Quality Traits in Alfalfa. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca* 39(2):190-195

Mortazavi A, Williams BA, McCue K. 2008. Mapping and quantifying mammalian transcriptomes by RNA-seq. *Nature methods* 5(7):621-628

Manol Y, Nemoto K. 2012. The pathway of auxin biosynthesis in plant. *J Exp Bot* 63:2853-2872

Nagel R. 2020. Gibberellin Signaling in Plants: Entry of a new MicroRNA Player. *Plant physiology* 183(1):5-6

Ren H, Gray WM. 2015. SAUR Proteins as Effectors of Hormonal and Environmental Signals in Plant Growth. *Molecular Plant* 8(8):1153-1164

Ryan LM, Zhong RQ, Ye ZH. 2011. Secondary wall NAC binding element (SNBE), a key cis-acting element required for target gene activation by secondary wall NAC master switches. *Plant signaling & behavior* 6(9):1282-1285

Sulc RM, Arnold AM, Cassida KA, Albrecht KA, Hall MH, Min D, Xu X, Undersander DJ, Santen E. 2021. Changes in forage nutritive value of reduced-lignin alfalfa during regrowth. *Crop Science* 61(2):1478-1487

Sena G. Stem cells and regeneration in plants(Article). 2014. *Nephron – Experimental Nephrology* 126 (2):5-39

Sun H, Zhao XT, Liu Z, Yang K, Wang Y, Zhan YG. 2018. Bioinformatics of S6K Genes in Fraxinus mandshurica and Their Expression Analysis under Stress and Hormone. *Plant resear*ch 38(5):714-724

Sen S, Mascoli V, Liguori N, Croce R, Visscher L. 2021. Understanding the Relation between Structural and Spectral Properties of Light-Harvesting Complex II. *The journal of physical chemistry. A* 125(0): 4313-4322

Su LY, Audran C, Bouzayen M, Roustan JP, Chervin C. 2015. The Aux/IAA, Sl-IAA17 regulates quality parameters over tomato fruit development. *Plant Signaling & Behavior*

10(11):e1071001
Shim JS, Jung C, Lee S, Min K, Lee YW, Choi Y, Lee JS, Song JT, Kim JK, Choi YD. 2012. AtMYB44 regulates WRKY70 expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling. *The Plant Journal* 73 (3):483-495
Tetteh JP, Bonsu KO. 1997. Agronomic performance of seven cultivars of alfalfa (Medicago sativa L.) in the coastal savanna zone of Ghana. *Ghana Journal of Agricultural Science* 30(1):39-44
Tan M, Li GF, Qi SY, Liu XJ, Chen XL, Ma JJ, Zhang D, Han MY. 2018. Identification and expression analysis of the IPT and CKX gene families during axillary bud outgrowth in apple (Malus domestica Borkh). *Gene*:106-117
Verma V, Ravindran P, Kumar PP. 2016. Plant hormone-mediated regulation of stress responses. *BMC Plant Biology* 16:1–10
Venkat Ajila, Muneer Sowbiya. 2022. Role of Circadian Rhythms in Major Plant Metabolic and Signaling Pathways. Frontiers in plant science 13: 836244 DOI 10.3389/fpls.2022.836244
Wang J, Tang F, Gao CP, Gao X, Xu B, Shi FL. 2021. Comparative transcriptome between male fertile and male sterile alfalfa (Medicago varia). *Physiology and Molecular Biology of Plants* 27(7):1487-1498
Wang Z, Gerstein M, Snyder M. 2009. RNA-seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics* 10(1):57-63
Wang YJ, Li MC, Wu Wang, Wu HY, Xu YN. 2013. Cloning and Characterization of an AP2/EREBP Gene TmAP2-1 from Tetraena mongolica. *Bulletin of Botany* 48(1):23-33
Wang HZ, Yang JH, Chen F, Ivone Torres-Jerez, Tang YH, Wang MY, Du Q, Cheng XF, Wen JQ, Richard Dixon. 2016. Transcriptome analysis of secondary cell wall development in Medicago truncatula. *BMC genomics*:23
Wang WP, Sijacic P, Xu PB, Lian HL, Liu ZC. 2018. Arabidopsis TSO1 and MYB3R1 form a regulatory module to coordinate cell proliferation with differentiation in shoot and root. *Proceedings of the National Academy of Sciences of the Uniited States of Americal* 115(13):e3045-e3054
Yu L, Chen HW, Hong PP, Wang HL, Liu KF. 2015. Adventitious Bud Induction and Plant Regeneration from Stem Nodes of Salvia splendens‘Cailinghong’. *HORTSCIENCE*:869-872
Yuan JB, Sun XB, Guo T, Chao YH, Han LB. 2020. Global transcriptome analysis of alfalfa reveals six key biological processes of senescent leaves. *PeerJ* 8(1):e8426
Yu KMJ, McKinley B, Rooney WL, Mullet JE. 2021. High planting density induces the expression of GA3-oxidase in leaves and GA mediated stem elongation in bioenergy sorghum. *Scientific Reports* 11(1):46
Yan Q, Li J, Lu LY, Gao LJ, Lai DW, Yao N, Yi XF, Wu ZY, Lai ZQ, Zhang JY. 2021. Integrated analyses of phenotype, phytohormone, and transcriptome to elucidate the mechanism governing internode elongation in two contrasting elephant grass (Cenchrus purpureus) cultivars. Industrial Crops & Products (170): 113693
Yuan R, Zeng XH, Zhao SB, Wu G, Yan XH. 2019. Identification of Candidate Genes Related to Stem Development in Brassica napus Using RNA-seq. *Plant Molecular Biology Reporter* 37(4):347-364

Ziliotto U, Leinauer B, Lauriault LM, Rimi F, Macolino S. 2010. Alfalfa Yield and Morphology of Three Fall-Dormancy Categories Harvested at Two Phenological Stages in a Subtropical Climate. *Agronomy Journal* 102(6):1578-1585

Zhang H, Liu XQ, Wang XM, Sun M, Song R, Mao PS, Jia SG. 2021. Genome-Wide Identification of GRAS Gene Family and Their Responses to Abiotic Stress in Medicago sativa. *International journal of molecular sciences* 22(14):7729

Zheng XM, Chen YJ, Zhou YF, Shi KK, Hu X, Li DY, Ye HZ, Zhou Y, Wang K. 2021. Full-length annotation with multistrategy RNA-seq uncovers transcriptional regulation of lncRNAs in cotton. *Plant physiology* 185(1):179-195

Zhao CS, Atsushi H, Shinjiro Y, Kamiya Yuji, Beers EP. 2011. The Arabidopsis Myb genes MYR1 and MYR2 are redundant negative regulators of flowering time under decreased light intensity. *Plant J* 66(3):502-515

Figure 1(on next page)

Phenotypic evaluation of five alfalfa cultivars.

The dynamics of plant height (a), stem diameter (b) and internode length (c) development of five alfalfa cultivars at transplanting stage, b ranching stage , b-udding stage , e-arly flower stage and f-ull flower stage . Average plant height (d), stem diameter (e), internode length (f) of five alfalfa cultivars. The values are the average of fifteen biological replicates and error bars represent the standard deviation. Different letters indicate significant difference at $P < 0.05$ among the five cultivars as determined by Student's test.

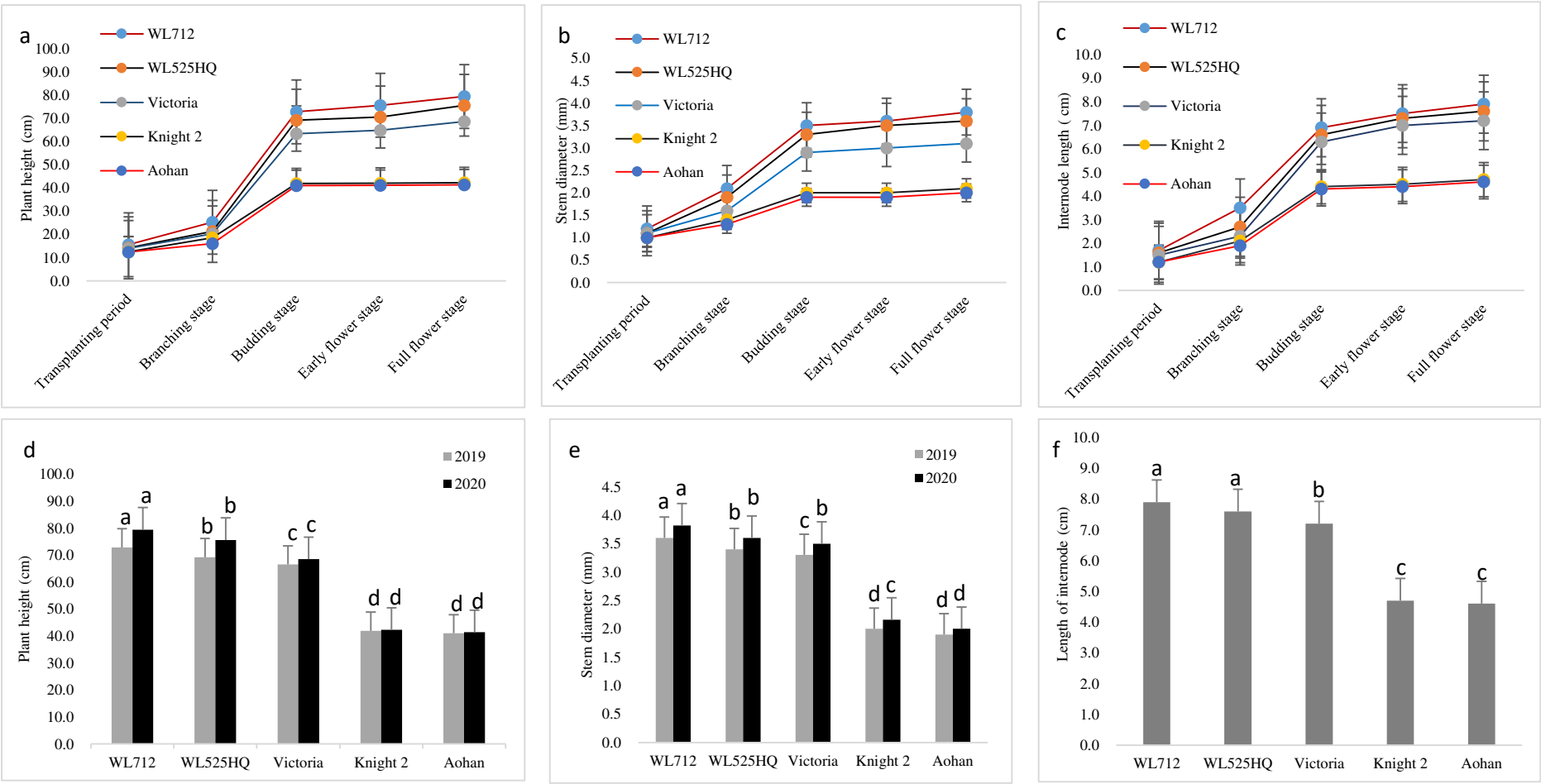


Figure 1 Phenotypic evaluation of five alfalfa cultivars. The dynamics of plant height (a), stem diameter (b) and internode length (c) development of five alfalfa cultivars at transplanting stage, branching stage, budding stage, early flower stage and full flower stage. Average plant height (d), stem diameter (e), internode length (f) of five alfalfa cultivars. The values are the average of fifteen biological replicates and error bars represent the standard deviation. Different letters indicate significant difference at $P < 0.05$ among the five cultivars as determined by Student's test.

Figure 2

Phenotypic evaluation of five alfalfa cultivars

Lateral branches number (a), branches number (b), leaf area (c), fresh weight (d), leaf to stem ratio (e) and dry weight (f) of five alfalfa cultivars. The values are the average of fifteen biological replicates and error bars represent the standard deviation. Different letters indicate significant difference at P < 0.05 among the five cultivars as determined by Student's test.

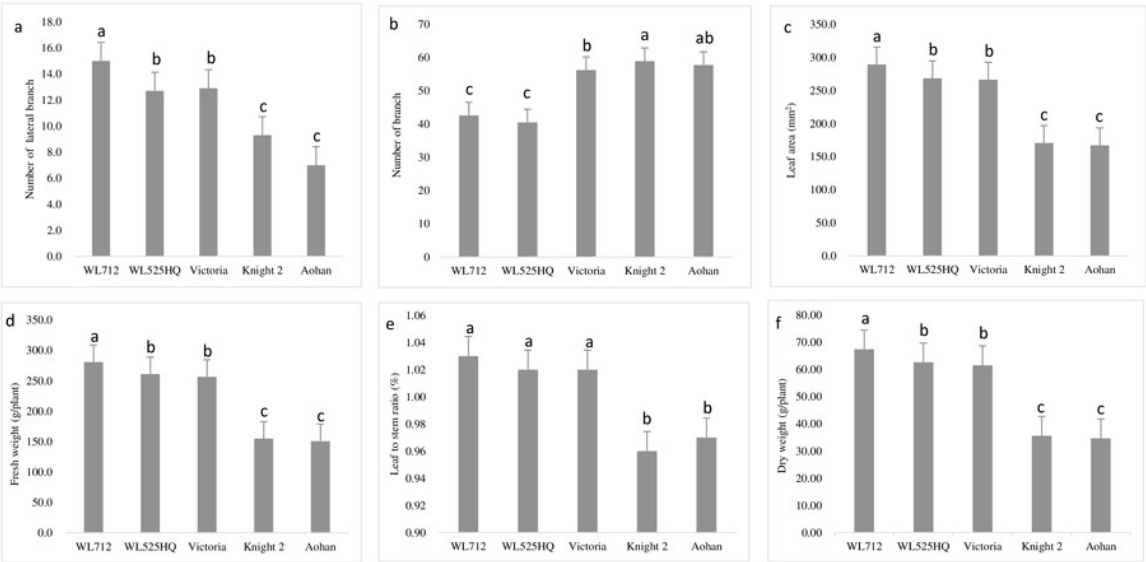


Figure 2 Phenotypic evaluation of five alfalfa cultivars. Lateral branches number (a), branches number (b), leaf area (c), fresh weight (d), leaf to stem ratio (e) and dry weight (f) of five alfalfa cultivars. The values are the average of fifteen biological replicates and error bars represent the standard deviation. Different letters indicate significant difference at P < 0.05 among the five cultivars as determined by Student's test.

Figure 3(on next page)

a.Scatter diagram of enriched GO functional. b.KEGG classification of DEGs

a. The “ GeneRatio ” shows the ratio of the number of ~~the~~ DEGs to the total number of differential genes ~~on the GO number~~. b. X axis is the number of gene annotations; Y axis is the type of KEGG pathway.

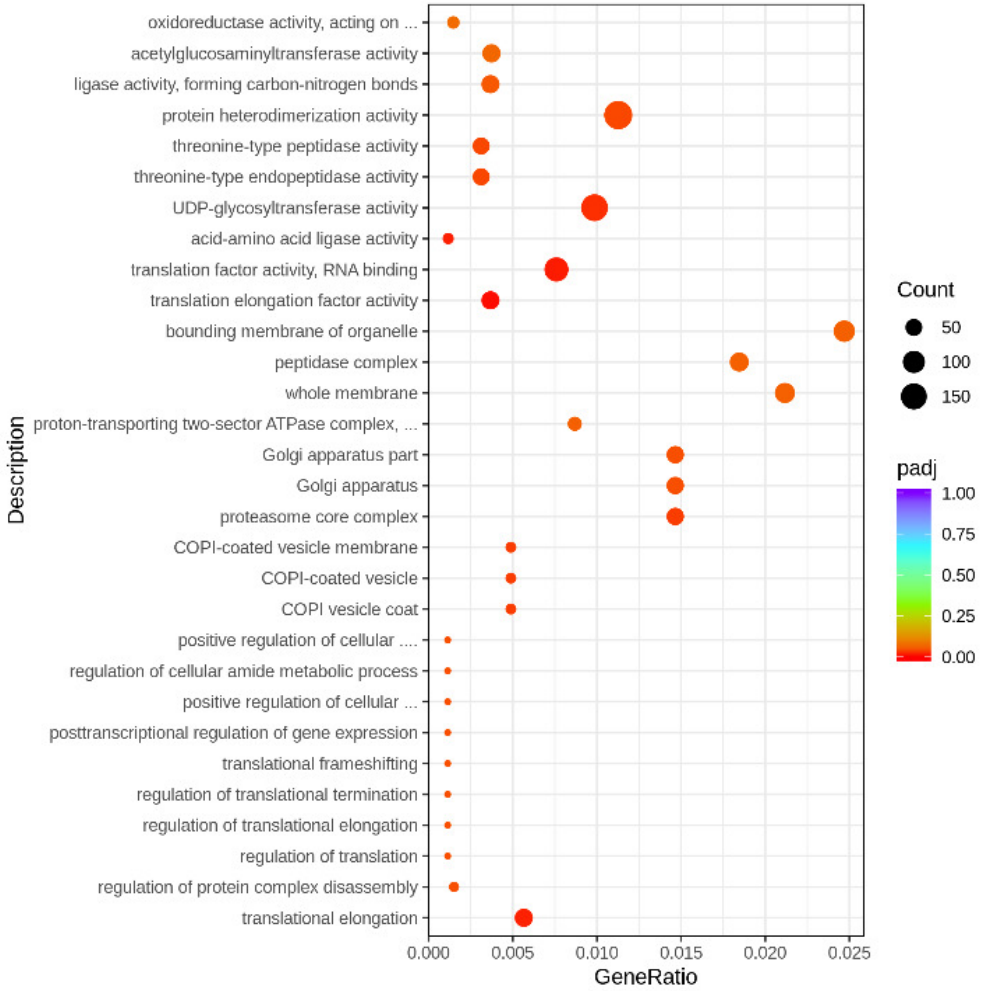


Figure 3a Scatter diagram of enriched GO functional. The "GeneRatio" shows the ratio of the number of the DEGs to the total number of differential genes on the GO number.

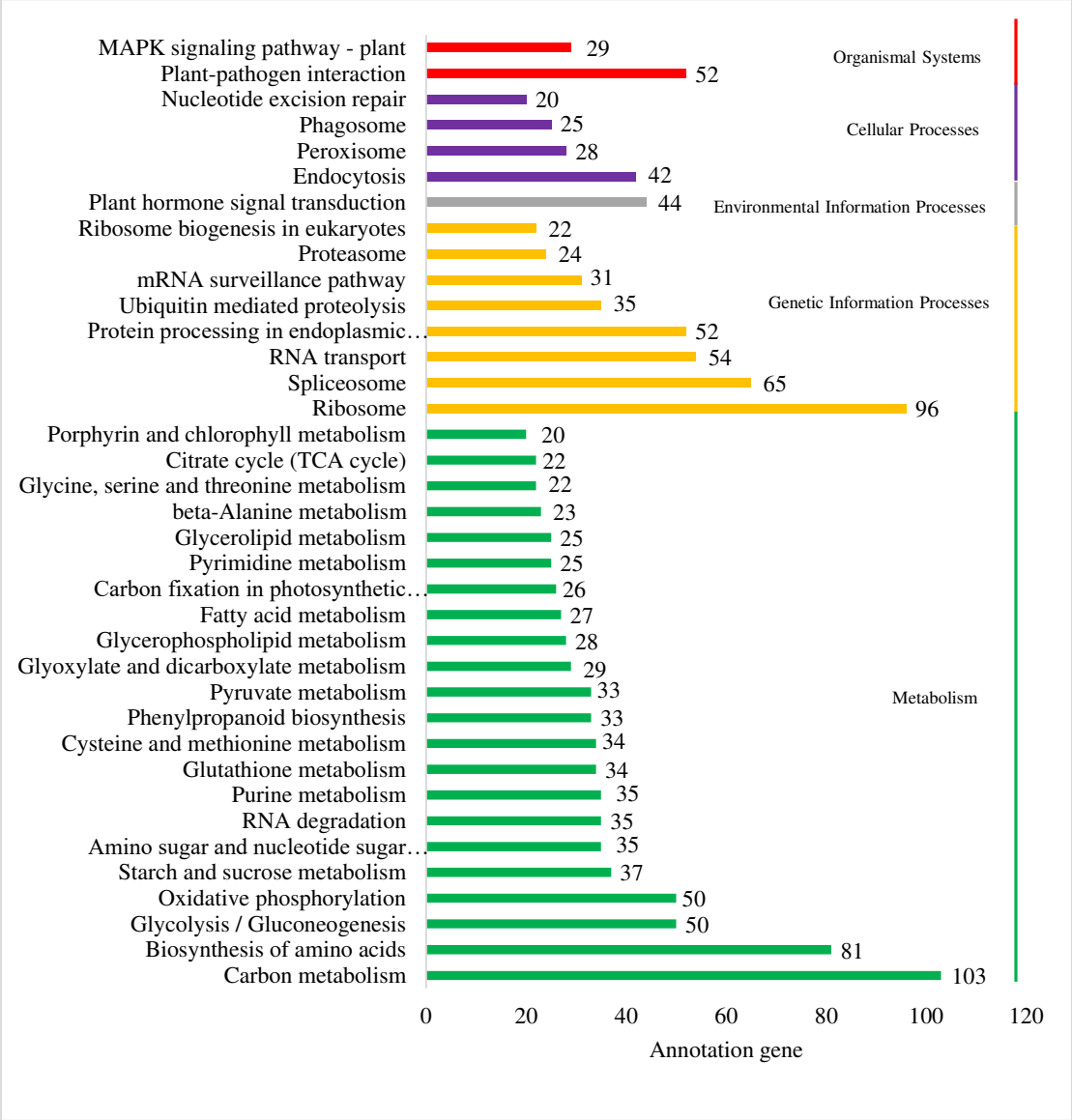
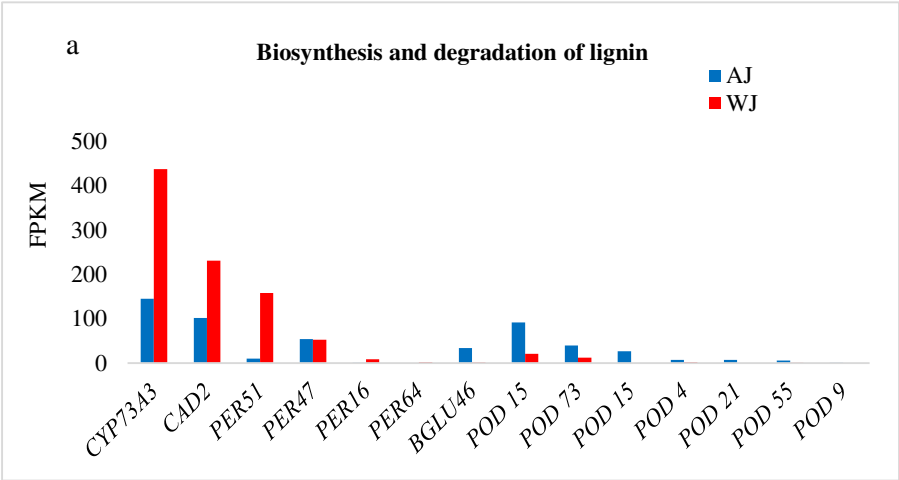


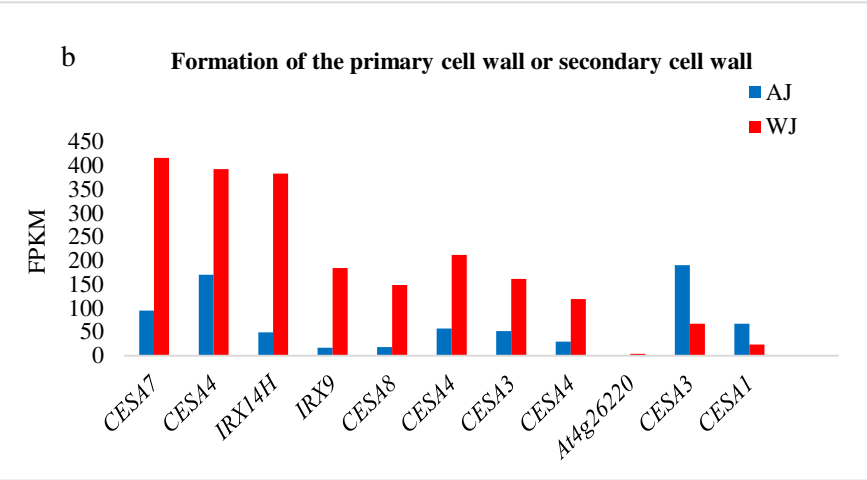
Figure 3b KEGG classification of DEGs; X axis is the number of gene annotations; Y axis is the type of KEGG pathway.

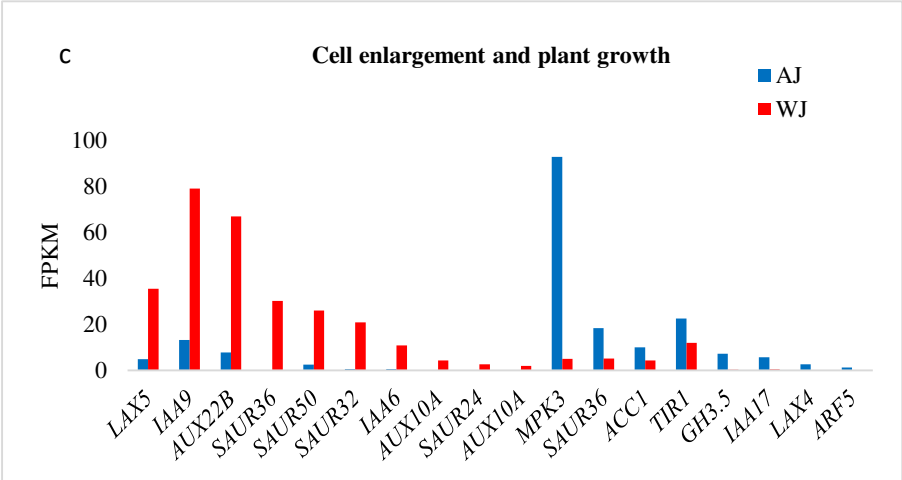
Figure 4(on next page)

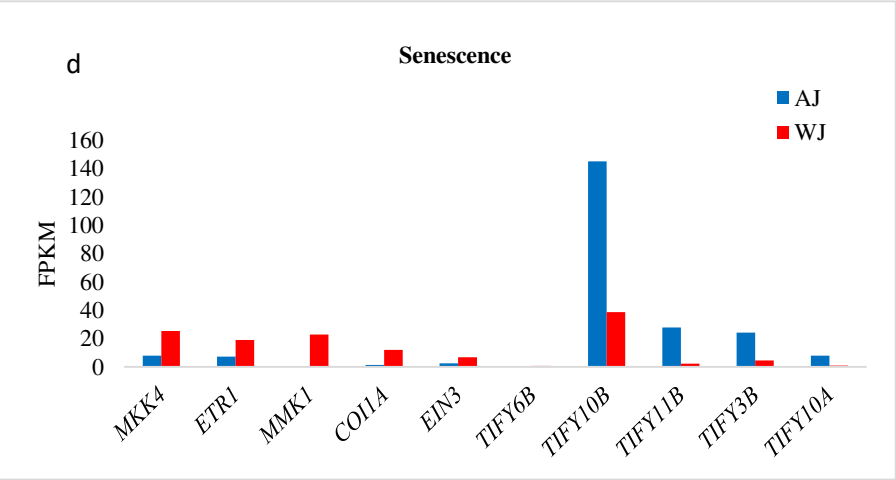
~~The bar~~ graphs showing F PKM of DEGs involved in eight biological processes by GO enrichment analysis.

(a) Biosynthesis and degradation of lignin; (b) Formation of the primary cell wall or secondary cell wall; (c) Cell enlargement and plant growth; (d) Senescence; (e) Cell division and shoot initiation; (f) Stem growth and induced germination; (g) Cell elongation; (h) Formation of water conducting tissues.


a
Biosynthesis and degradation of lignin
AJ
WJ
FPKM
500
400
300
200
100
0
CYP73A3
CAD2
PER51
PER47
PER16
PER64
BGLU46
POD 15
POD 73
POD 15
POD 4
POD 21
POD 55
POD 9


b
Formation of the primary cell wall or secondary cell wall
AJ
WJ
FPKM
450
400
350
300
250
200
150
100
50
0
CESA7
CESA4
IRX14H
IRX9
CESA8
CESA4
CESA3
CESA4
At4g26220
CESA3
CESA1


c
Cell enlargement and plant growth
AJ
WJ
FPKM
100
80
60
40
20
0
LAX5
IAA9
AUX22B
SAUR36
SAUR50
SAUR32
IAA6
AUX10A
SAUR24
AUX10A
MPK3
SAUR36
ACC1
TIR1
GH3.5
IAA17
LAX4
ARF5


d
Senescence
AJ
WJ
FPKM
160
140
120
100
80
60
40
20
0
MKK4
ETR1
MMK1
COI1A
EIN3
TIFY6B
TIFY10B
TIFY11B
TIFY3B
TIFY10A

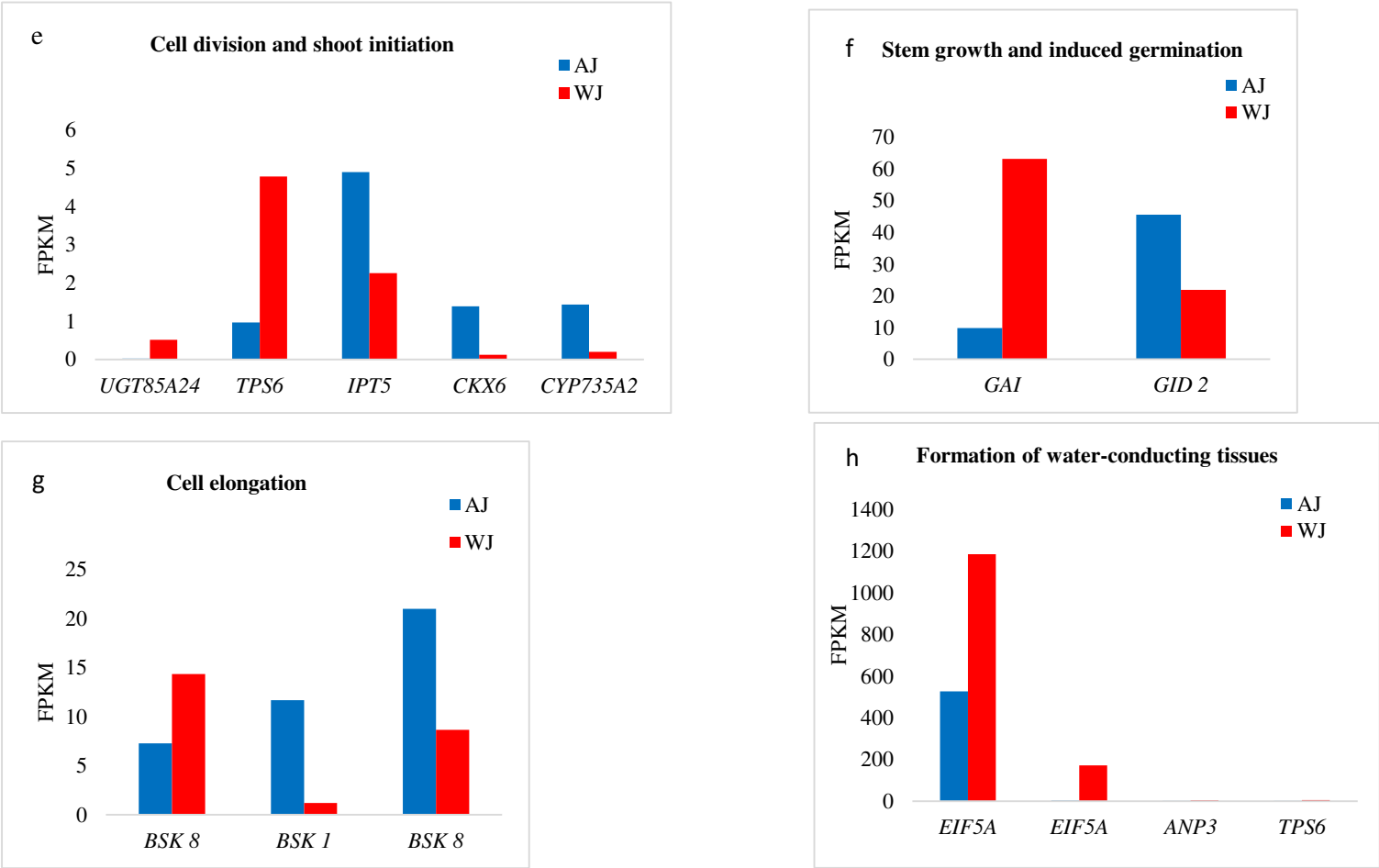


Figure 4 The bar graphs showing FPKM of DEGs involved in eight biological processes by GO enrichment analysis. (a) Biosynthesis and degradation of lignin; (b) Formation of the primary cell wall or secondary cell wall; (c) Cell enlargement and plant growth; (d) Senescence; (e) Cell division and shoot initiation; (f) Stem growth and induced germination; (g) Cell elongation; (h) Formation of water-conducting tissues.

Figure 5

~~TFs~~ involved in stem elongation and diameter enlargement in alfalfa

(a) Bar graphs shows the FPKM of nine DEGs ~~at different~~ transcriptome databases. Different colors represents different databases including Aohan (AJ) and WL712 (WJ). (b) ~~Histogram shows~~ the nine DEGs relative expression levels of AJ and WJ.

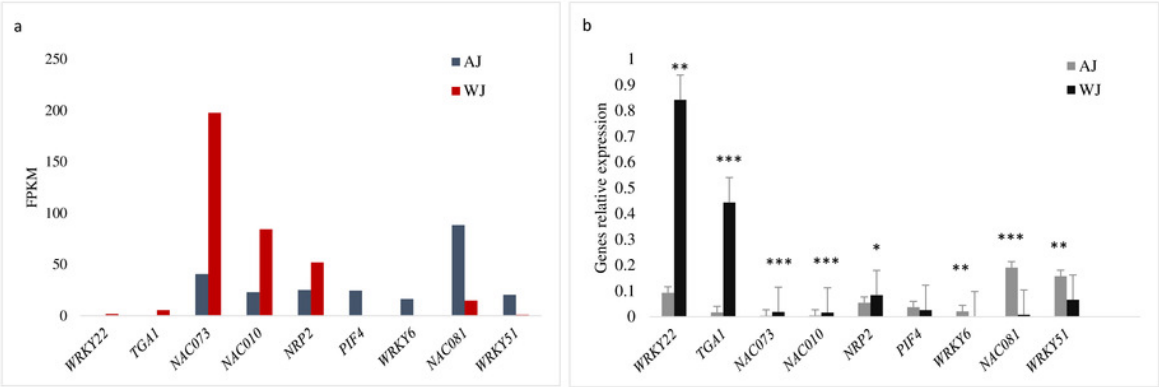


Figure 5 TFs involved in stem elongation and diameter enlargement in alfalfa. (a) Bar graphs shows the FPKM of nine DEGs at different transcriptome databases. Different colors represents different databases including Aohan (AJ) and WL712 (WJ). (b) Histogram shows the nine DEGs relative expression levels of AJ and WJ.

Figure 6

qRT-PCR validation of DEGs from the AJ and WJ RNA-seq databases in alfalfa.

(a) Histogram shows the Fpkm of eleven DEGs at different transcriptome databases. Different colors represents different databases including Aohan (AJ) and WL712 (WJ). (b) Histogram shows the eleven DEGs relative expression levels of AJ and WJ validated by qPCR.

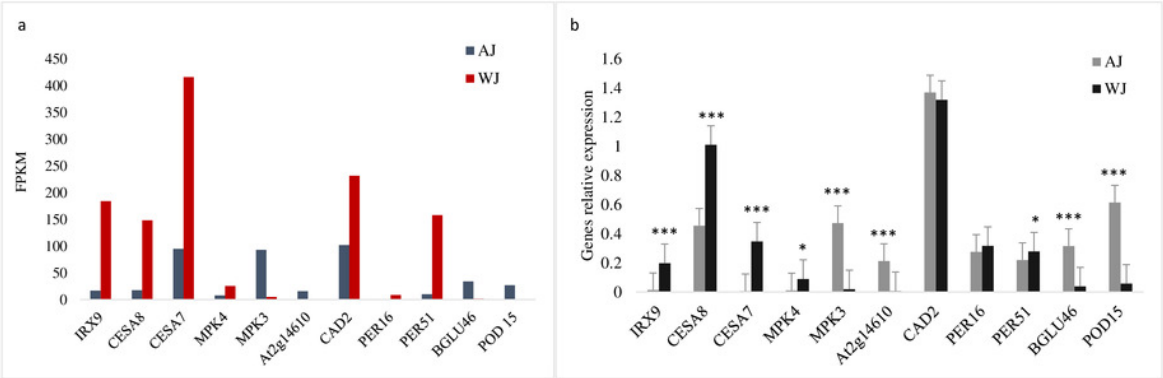


Figure 6 qRT-PCR validation of DEGs from the AJ and WJ RNA-seq databases in alfalfa. (a) Histogram shows the Fpkm of eleven DEGs at different transcriptome databases. Different colors represents different databases including Aohan (AJ) and WL712 (WJ). (b) Histogram shows the eleven DEGs relative expression levels of AJ and WJ validated by qPCR.

Table 1(on next page)

Phenotypic correlation coefficients between traits based on the five alfalfa cultivars

*, ** Significant at the 0.05, 0.01 ~~and 0.001~~ probability levels, respectively. PH, plant height; IL, internode length; SD, stem diameter; FW, fresh weight; LSR, leaf-to-stem ratio; DW, Dry weight; LBN, lateral branch number; MBN, main branch number.

Table 1. Phenotypic correlation coefficients between traits based on the five alfalfa cultivars

	PH	SD	IL	LBN	MBN	FW	LSR	DW
PH	1							
SD	0.98**	1						
IL	0.99**	0.98**	1					
LBN	0.89**	0.92**	0.90**	1				
MBN	- 0.84**	- 0.76**	- 0.79**	- 0.68**	1			
FW	0.98**	0.98**	0.98**	0.91**	-0.75**	1		
LSR	0.55**	0.54**	0.55**	0.51**	- 0.46*	0.60**	1	
DW	0.99**	0.99**	0.99**	0.82**	- 0.76**	1.00**	0.59**	1

*, ** Significant at the 0.05, 0.01 ~~and 0.001~~ probability levels, respectively.

PH, plant height; IL, internode length; SD, stem diameter; FW, fresh weight; LSR, leaf-to-stem ratio; DW, Dry weight; LBN, lateral branch number; MBN, main branch number.

Table 2 (on next page)

The growth index of the two varieties in greenhouse

Different letters indicate significant difference at $P < 0.05$ among the two ~~hormones~~ as determined by Student's test

Table 2 The growth index of the two varieties in greenhouse

	Plant Height (cm)	Lenght of Internodde (cm)	Stem Diameter (mm)	Leaf Areas (mm^2)	Plant Weight (g/plant)
WL 712	50.16 ± 0.9646 [a]	5.14 ± 0.0911 [a]	2.52 ± 0.0224 [a]	159.38 ± 0.6058 [a]	230.94 ± 2.4450 [a]
Aohan	28.73 ± 0.8027 [c]	2.94 ± 0.0695 [c]	1.19 ± 0.0274 [c]	127.36 ± 2.8085 [c]	141.06 ± 0.4037 [c]

Different letters indicate significant difference at $P < 0.05$ among the two hormones as determined by Student's test.

Table 3 (on next page)

Top 10 gene ontology function classification

Table 3 Top 10 gene ontology function classification

Category	Description	GO ID	Count	Percentage(%)
Biological process	translational elongation	GO:0006414	41	4.30
	regulation of protein complex disassembly	GO:0043244	8	0.84
	regulation of translation	GO:0006417	7	0.73
	regulation of translational elongation	GO:0006448	7	0.73
	regulation of translational termination	GO:0006449	7	0.73
	translational frameshifting	GO:0006452	7	0.73
	posttranscriptional regulation of gene expression	GO:0010608	7	0.73
	positive regulation of cellular protein metabolic process	GO:0032270	7	0.73
	regulation of cellular amide metabolic process	GO:0034248	7	0.73
	positive regulation of cellular amide metabolic process	GO:0034250	7	0.73
Cell component	bounding membrane of organelle	GO:0098588	57	5.97
	peptidase complex	GO:1905368	44	4.61
	whole membrane	GO:0098805	49	5.13
	proton−transporting two−sector ATPase complex	GO:0033177	20	2.10
	Golgi apparatus part	GO:0044431	36	3.77
	Golgi apparatus	GO:0005794	36	3.77
	proteasome core complex	GO:0005839	37	3.88
	COPI−coated vesicle membrane	GO:0030126	9	0.94
	COPI−coated vesicle	GO:0030137	9	0.94
	COPI vesicle coat	GO:0030126	9	0.94
Molecular function	translation elongation factor activity	GO:0003746	41	4.30
	translation factor activity, RNA binding	GO:0008135	86	9.01
	acid−amino acid ligase activity	GO:0016881	12	1.26
	UDP−glycosyltransferase activity	GO:0008194	99	1.04
	threonine−type endopeptidase activity	GO:0004298	37	3.88
	threonine−type peptidase activity	GO:0070003	37	3.88
	protein heterodimerization activity	GO:0046982	114	11.95
	ligase activity, forming carbon−nitrogen bonds	GO:0016879	35	3.67
	acetylglucosaminyltransferase activity	GO:0008375	35	3.67
	oxidoreductase activity	GO:0016638	12	1.26