BMC Plant Biology

RESEARCH Open Access

Integrated physiological and transcriptomic analyses reveal that cell wall biosynthesis and expansion play an important role in the regulation of plant height in alfalfa*

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Abstract

Background Alfalfa (*Medicago sativa* L.) is a high-quality, high-protein forage, and the improvement and breeding of key traits are important for enhancing the productivity of alfalfa. Plant height is an important trait that affects crop yield, and its regulatory network mechanism has been widely reported in model plants, however, there are fewer studies on the developmental regulatory of plant height in alfalfa.

Results In this study, we screened tall (WL525HQ) and short (WL343HQ) alfalfa materials through field experiments and analyzed the regulatory mechanism of plant height based on the multidimensional joint analysis of phenotype, cell, physiology, and molecular biology. The results showed that internode length was an important factor determining plant height in alfalfa, and cell size affected the internode elongation to a certain extent, whereas cell size was limited by cell wall. Moreover, changes in cell wall components play an important role in cell wall expansion, especially lignin synthesis. Transcriptome analysis showed that the high expression of hydrolase activity in T1 (initiation growth period) facilitates the expansion of the cell wall, the significant enrichment of the cellular modification process in T3 (rapid growth period) increases the cell size, and the synthesis of cell wall structural constituents and plant-type cell wall organization in T5 (growth stabilization) further improves and modifies the cell wall structure. Differential genes involved in cell wall biosynthesis and expansion were mainly enriched in cellulose synthesis, pectin cleavage, lignin formation, expansion protein (EXP), and xyloglucan endotransglycosidase (XTH).

Conclusions These findings elucidated the plant height regulation mechanisms throughout the alfalfa plant and provided a theoretical basis for the generation of ideal alfalfa plant height germplasm.

Keywords Alfalfa, Growth periods, Plant height, Internode length, Cell size, Cell wall, Transcriptome

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Background

Plant height is a key factor in the processes of photosynthesis and nutrient allocation in plants, a reasonable plant height is the basis of a good plant shape [1–3]. The elongation of plant stalks is often accompanied by the expansion of meristematic tissues, and the corresponding increase in cell volume, degree of cell wall elongation, and the plant's control of cell growth determine the length of the internode and height of the plant [4]. The cell wall is a three-dimensional network composed of cellulose, hemicellulose, pectin, polysaccharides, and proteins [5]. Cellulose plays an important role in regulating the volume and determining the size of plant cells [6]. Furthermore, the altered binding of pectin and cellulose microfibrils may be a major factor in the regulation of internode elongation [7]. Cells reach their final size and shape after undergoing secondary cell wall development, and after differentiation, they begin lignification [8]. Appropriate levels of lignin deposition are essential for plant development because a decrease in lignin content results in plant dwarfism [9]. Most of the solar energy accumulated in plants is converted to chemical energy through photosynthesis and then locked in the cell wall polymers lignin, hemicellulose, and cellulose, which are important factors directly affecting cell elongation and plant height [10].

Plant cell wall loosening is a physiological process that is necessary for cell expansion and elongation throughout plant growth and development [11]. The cell wall provides rigid structural support for tissues and organs both by stretching to provide the elasticity required for cell expansion and by absorbing new polysaccharide polymers to maintain its thickness and toughness [12]. Expansion proteins (EXPs) are a class of proteins found within the plant cell wall and are mainly involved in cell growth, elongation, and cell wall modification, thereby promoting cell wall relaxation under acidic conditions [13, 14]. Xyloglucan endotransglucosylase (XTH) is a key enzyme that participates in plant cell wall remodeling, in which it relaxes and degrades the cell wall as well as facilitates cell wall strengthening and synthesis [15].

Alfalfa (*Medicago sativa* L.) has wide adaptability, high-stress tolerance, and high biological yield; its stems can be used as bioenergy materials; its leaves can be used for high-protein feed; and its root system can perform nitrogen fixation, making it a perennial legume crop with excellent development potential [16, 17]. The plant height of alfalfa is affected by its biological characteristics, and its growth pattern exhibits an "S" curve, i.e., the plant changes from slow growth to fast growth during the nutrient accumulation stage, and then the growth rate slows down or stops during the period from bud to flower [18]. Plant height is a complex agronomic trait that is regulated by genetic traits and environmental conditions. With the rapid development of plant sequencing and analysis technologies, such as transcriptomics, metabolomics, genomics, and genetics, plant height has been analyzed in rice (*Oryza sativa* L.) [19], maize (*Zea mays* L.) [20], tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) [21], Sugarcane (*Saccharum* L.) [22], and moso bamboo (*Phyllostachys heterocycla*) [23], among other crops, and the network mechanism of plant height regulation has been widely reported. However, only a few studies have explored the mechanisms regulating plant height development in alfalfa. Therefore, in this study, from 12 alfalfa varieties, we selected those with stable traits and substantial differences in their internode lengths as test materials and jointly analyzed the internode growth pattern of alfalfa based on phenotypic, cellular, physiological, and molecular biology perspectives. Furthermore, they were used to analyze the mechanisms of plant height development and elucidate the regulatory network of alfalfa plant height to provide a theoretical basis for future genetic improvement research on plant height traits of alfalfa and other leguminous forage grasses.

Results

Selection of alfalfa test material

To screen for tall- and short-stalked alfalfa materials with stable traits and substantial differences in internode length, we determined the average plant height, internode number, average internode length, and stem thickness of 12 alfalfa varieties in different crops in 2 consecutive years of field trials (Fig. 1). The plant heights of WL525HQ and Gannong No. 3 were not significantly different but were higher than those of other varieties for 2 consecutive years, whereas the plant heights of WL343HQ and WL354HQ were relatively low (Fig. 1A). There was some variation in the number of internodes in 2022 among the different alfalfa varieties, while the difference in the number of internodes in 2023 was not significant (Fig. 1B). The 2-year average internode lengths of WL525HQ and Gannong No. 3 were relatively high, while the average internode length of WL343HQ was the shortest for the 2 consecutive years (Fig. 1C). Through the determination of the phenotypic trait indexes, one tall- and short-stalked alfalfa materials with significant differences in their internode lengths were screened out as WL525HQ and WL343HQ, respectively. The average plant height, internode number, internode length, and stem thickness of WL525HQ in the 2 consecutive years were 27.20%, 7.40%, 27.24%, and 5.30% higher than those of WL343HQ, respectively (Fig. 1A-E).

Phenotypic traits of alfalfa materials

Changes in the phenotypic trait indexes of the test materials at 10-day intervals were analyzed using the short-stalked material WL343HQ as the control (Fig. 2). The

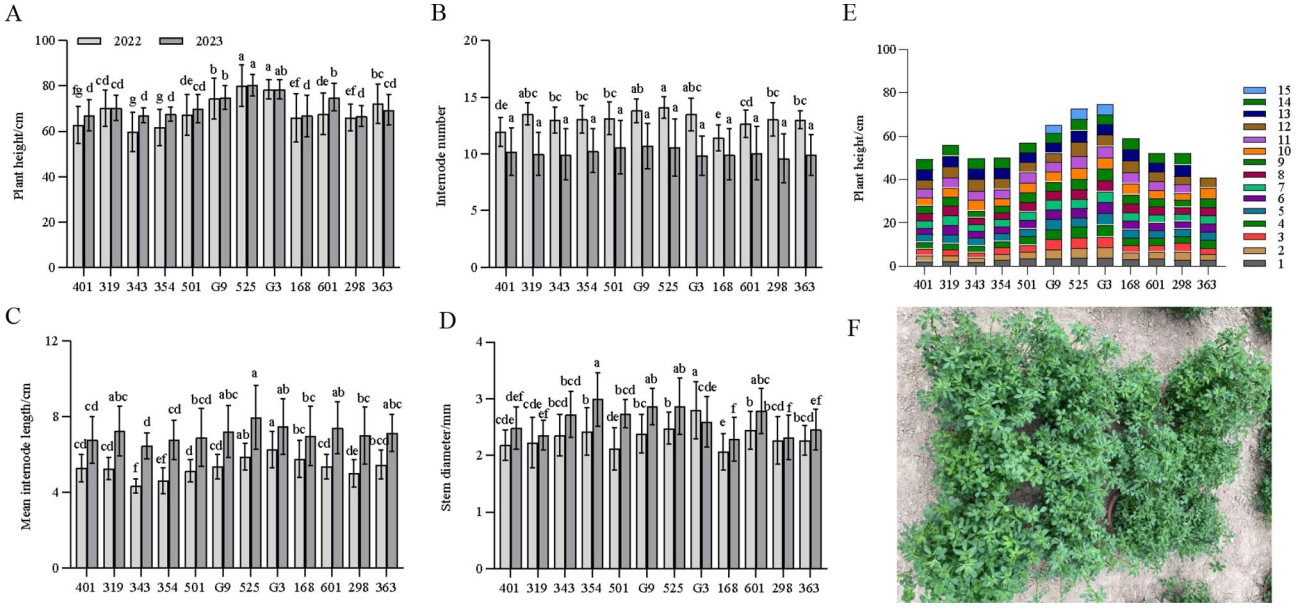

Fig. 1 Determination of phenotypic trait indexes in 12 alfalfa varieties. '401', '319', '343', '354', '501', 'G9', '525', 'G3', '168', '601', '298', and '363' represent respectively 'SG401', 'WL319HQ', 'WL343HQ', 'WL354HQ', 'SG501', 'Gannong No.9', 'WL525HQ', 'Gannong No.3', 'WL168HQ', 'SG601', 'WL298HQ', and 'WL363HQ'. Different lowercase letters indicate significant ($P < 0.05$) differences among varieties for the same index. (**A**) Plant height. (**B**) Internode number. (**C**) Mean internode length. (**D**) Stem thickness. (**E**) Plant height at first flowering. (**F**) Field growth charts of 12 alfalfa varieties

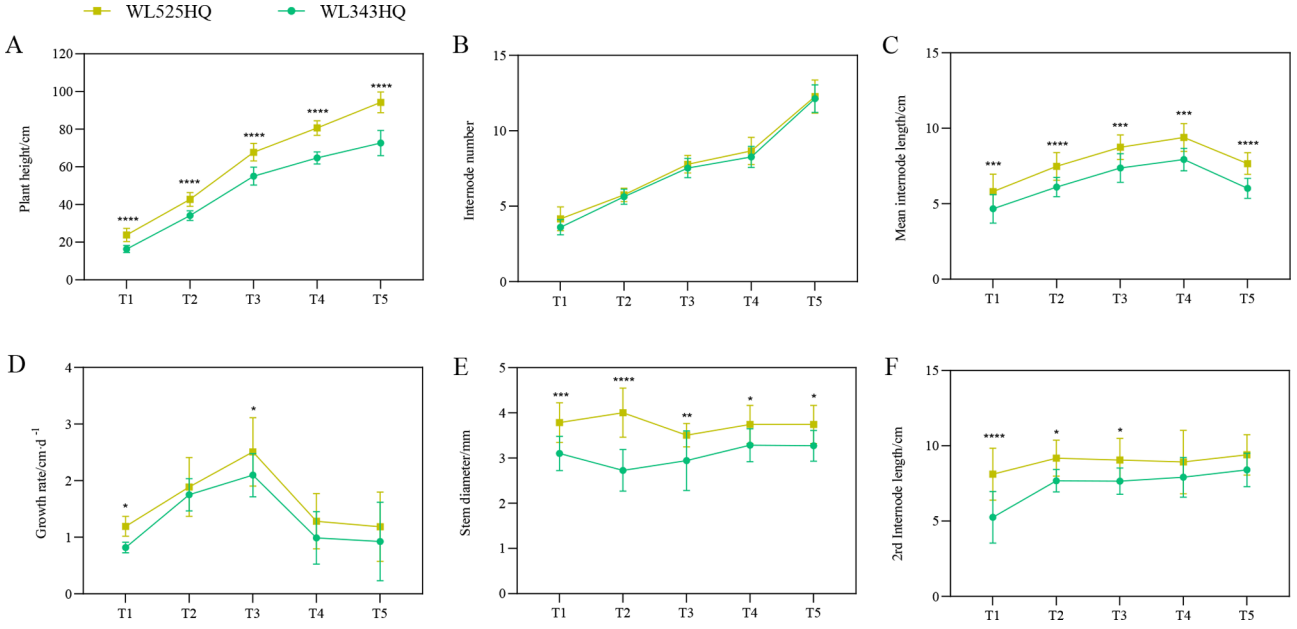

Fig. 2 Phenotypic trait indexes of tall-stalked alfalfa material WL525HQ and short-stalked alfalfa material WL343HQ at different periods. * indicates significant difference at the 0.05 level, ** indicates highly significant difference at the 0.01 level, *** indicates highly significant difference at the 0.001 level, and **** indicates highly significant difference at the 0.0001 level. T1-T5 represent seedlings sampled at 10-d intervals. (**A**) Plant height. (**B**) Number of internodes. (**C**) Mean internode length. (**D**) Growth rate. (**E**) Stem thickness. (**F**) Second internode length

number of internodes and internode length are two important indicators that affect the plant height of alfalfa, and the plant height (Fig. 2A) and number of internodes (Fig. 2B) of the two materials exhibited an increasing trend with the advancement of the reproductive period. Among them, the plant height of the tall-stalked alfalfa material WL525HQ was significantly higher than that of WL343HQ at different periods, and although the difference in the number of internodes between the two materials was not significant, it indicates that the internode length was the main factor affecting plant height in alfalfa. The average internode length (Fig. 2C) and

growth rate (Fig. 2D) of the two materials showed a tendency to increase and then decrease with plant growth and development.

The second internode tissues of the test materials were used in this study, in which the stem thickness of the second internode tissues of the taller materials was significantly higher than that of the control material at different periods (Fig. 2E). In addition, the difference in the length of the second internode of the two materials was the largest during the T1 period, which reached a significant level; this difference gradually decreased with the growth and development of the internodes, and the difference was not significant during the T4 and T5 periods (Fig. 2F). Finally, through the comprehensive analysis of the growth rate and length trends of the second internode at different periods, the T1, T3, and T5 periods were screened as important growth periods of alfalfa, which were named the initiation growth period (average growth rate of 1.01 cm/day), the rapid growth period (average growth rate of 2.30 cm/day), and the growth stabilization period (average growth rate of 1.06 cm/day).

Cytological analysis of alfalfa internodes

We observed paraffin section drawings of the second internode tissue at the same magnification during the important growth period of 2 alfalfa materials (Fig. 3A-B). It was found that the area of longitudinally cut cortical cells (Fig. 3C) of tall straw alfalfa material, WL525HQ, was highly significantly higher than that of short straw material, WL343HQ, while the area of transversely cut cortical cells (Fig. 3D) of short straw material, WL343HQ, was highly significantly higher than that of tall straw material at both T1 and T3 periods. With the growth and development of the plant, the cell area of the

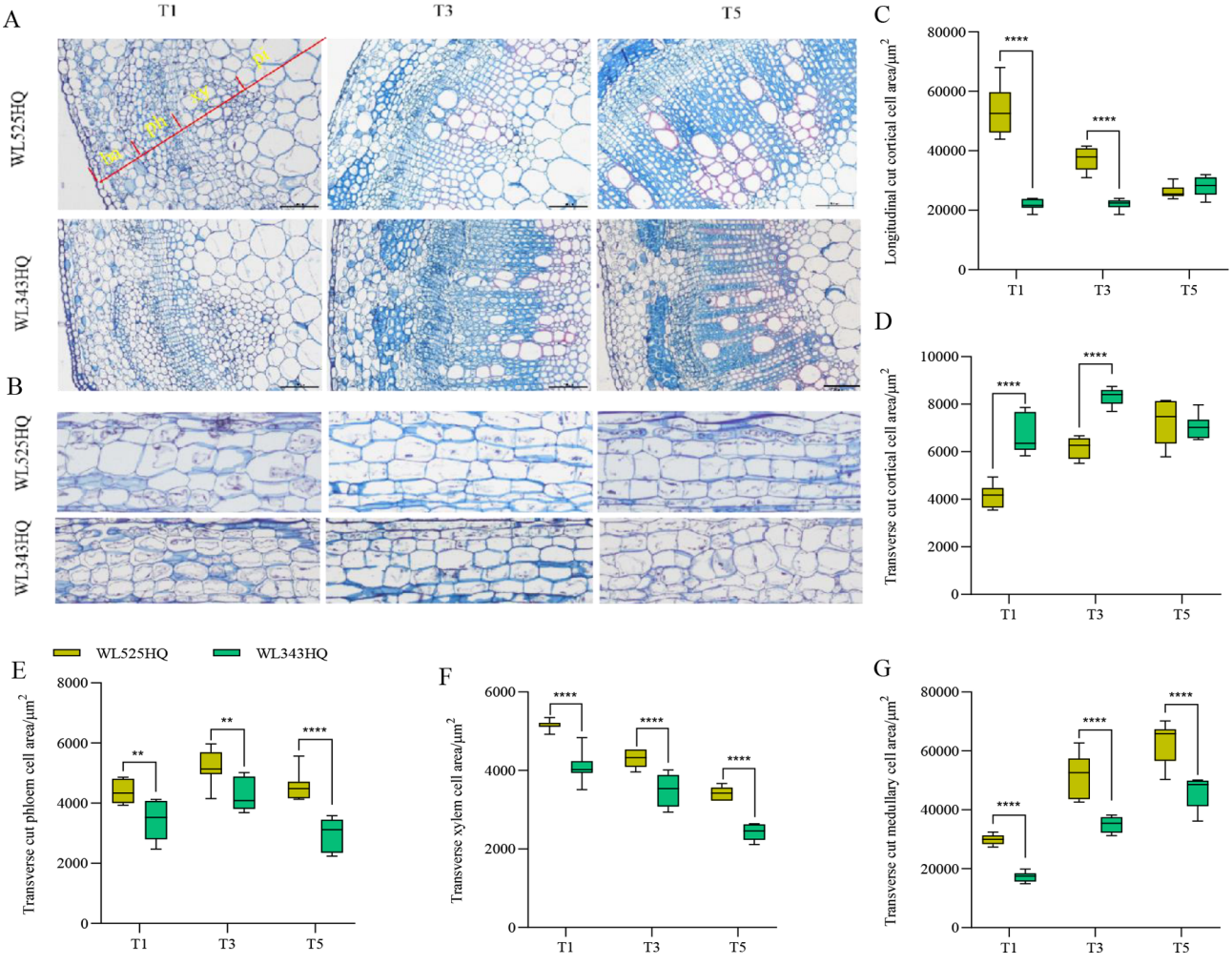

Fig. 3 Cytological analysis of tall-stalked alfalfa material WL525HQ (V1) and short-stalked alfalfa material WL343HQ (V2) at the T1, T3, and T5 periods. (**A**) Paraffin transverse view of the second internode. ba, bark; ph, phloem; xy, xylem; pi, pith. (**B**) Longitudinal paraffin view of the cortical section of the second internode. (**C**) Longitudinal cut cortical cell area. (**D**) Transverse cut cortical cell area. (**E**) Transverse cut phloem cell area. (**F**) Transverse cut xylem cell area. (**G**) Transverse cut pith cell area. ** indicates highly significant differences at the 0.01 level, and **** indicates highly significant differences at the 0.0001 level

two materials showed a tendency to increase and then decrease in the phloem (Fig. 3E), a tendency to decrease in the xylem (Fig. 3F), and a tendency to increase in the pith (Fig. 3G). In addition, the cell area of transverse sectioned phloem, xylem and pith of taller alfalfa material was highly significant higher than that of shorter material. By analyzing paraffin sections of internode tissues, it can be hypothesized that the size of cell area is an important factor influencing the length of alfalfa internodes, especially the longitudinal cell area.

Cell wall composition of alfalfa material

Some differences in the cell wall compositions (Fig. 4A-D) were observed between the tall- and short-stalked alfalfa materials during the three important growth periods; the lignin, cellulose, and protopectin contents exhibited an increasing trend with plant growth and development. The lignin and pectin contents of the tall-stalked material were greater than that of the short-stalked material at different periods, especially at T3. In contrast, the cellulose content of the tall-stalked material WL525HQ was significantly lower than that of the short-stalked material at T3, but the difference was not significant during the other periods. The hemicellulose content of the two materials first increased and then decreased, and it reached the maximum at T3. Notably, the cell wall compositions of the tall- and short-stalked alfalfa materials differed greatly during the rapid growth period (T3).

The soluble sugar content (Fig. 4E) and sucrose content (Fig. 4F) of the two materials did not have obvious patterns of change, and the soluble sugar content of the tall-stalked material WL525HQ was significantly higher than that of WL343HQ at T3 and T5, while the sucrose content of the short-stalked material WL343HQ was significantly higher than that of the tall-stalked material at the T3 period, and both differences were not significant at the T1 period. The starch content (Fig. 4G) of the two materials showed the same trend of increasing with plant growth and development, and the starch content of the tall-stalked material was highly significant higher than that of the short-stalked material at the T5 period.

Cell wall structure of alfalfa material

By staining the internodal tissues of alfalfa with resorcinol lignin (Fig. 5A-F), we found that the lignin of the two materials was mainly distributed in the xylem and both of them tended to increase with time. Among the three periods, the difference in the extent of lignin deposition was not significant at T1, but the thickness of lignin deposition over time was greater in the tall-stalked material than in the short-stalked material. The ultrastructure of the alfalfa internode tissues was analyzed via SEM (Fig. 5G-L), which revealed that the xylem area of the two materials increased with the development of internodes and the xylem cells were more tightly arranged. It was further observed that the xylem area was greater in the tall-stalked material than in the short-stalked material, whereas the xylem cells of the short-stalked material were more tightly arranged.

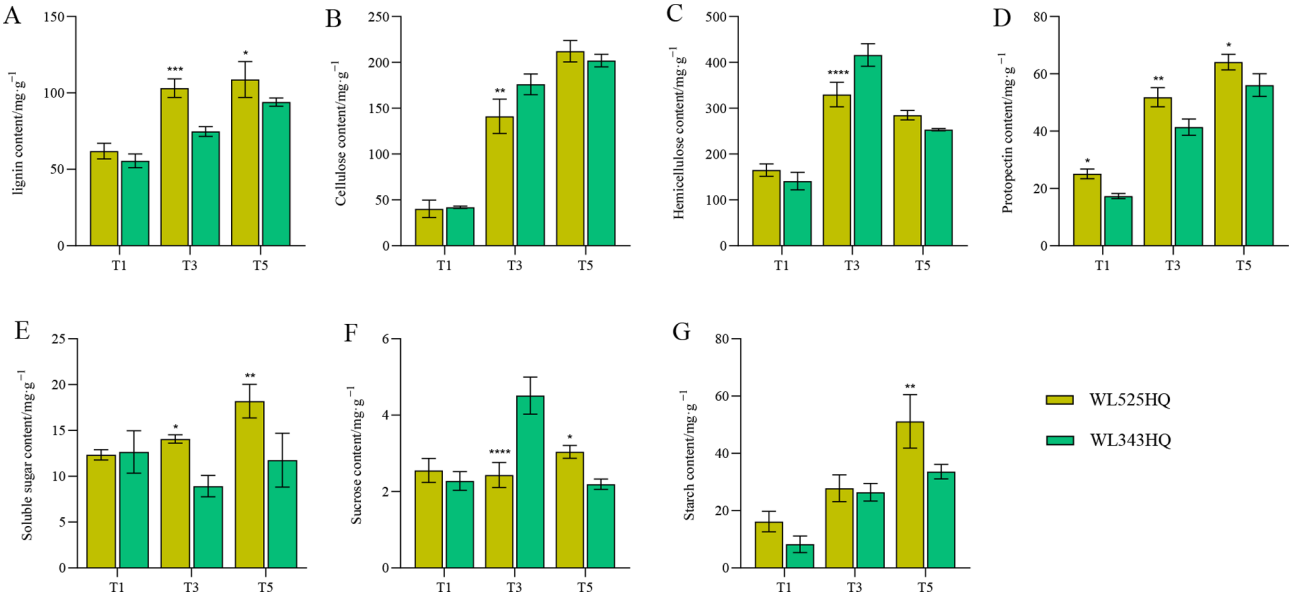

Fig. 4 Cell wall composition of tall-stalked alfalfa material WL525HQ (V1) and short-stalked alfalfa material WL343HQ (V2) at T1, T3 and T5 periods. * indicates significant difference at the 0.05 level, ** indicates highly significant difference at the 0.01 level, *** indicates highly significant difference at the 0.001 level, and **** indicates highly significant difference at the 0.0001 level. T1, T3, and T5 periods represent the time when the plants were sampled. (**A**) Lignin content. (**B**) Cellulose content. (**C**) Hemicellulose content. (**D**) Protopectin content. (**E**) Soluble sugar content. (**F**) Sucrose content. (**G**) Starch content

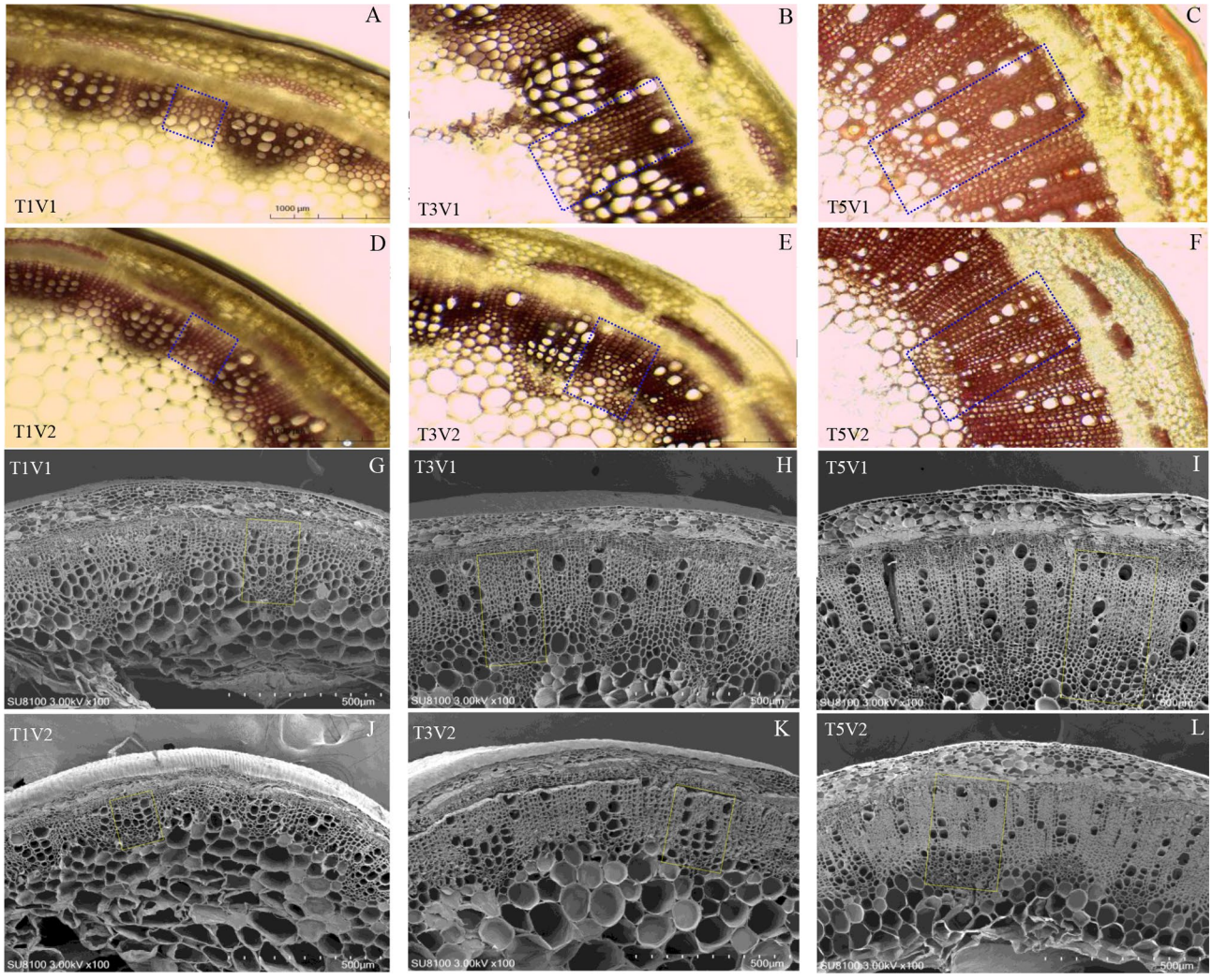

Fig. 5 Cell wall structure of tall-stalked alfalfa material WL525HQ (V1) and short-stalked alfalfa material WL343HQ (V2) at T1, T3 and T5 periods. (**A**–**C**) Plots of resorcinol lignin staining of WL525HQ. (**D**–**F**) Indicates resorcinol lignin staining of WL343HQ. (**G**–**I**) Indicates scanning electron micrographs of WL525HQ at 100x. (**J**–**L**) Indicates scanning electron micrographs of WL343HQ at 100x

Transcriptome analysis of alfalfa

To explore the molecular basis of alfalfa plant height differences, transcriptome analyses were performed during three important growth periods (Tables S1, S2). Using principal component analysis on the trial material, we found a good correlation between the biological replicates of all samples of the two alfalfa varieties in all three periods, which verifies the stability and reproducibility of the results (Fig. 6A). There were 4413 DEGs at the initiation growth period (T1), 3076 DEGs at the rapid growth period (T3), and 2086 DEGs at the growth stabilization period (T5). It was revealed that the total expression of the DEGs tended to decrease with the growth and development of the internodes (Fig. 6B). Using a Wayne diagram, we screened the DEGs that were common or unique among the comparison combinations, and the number of DEGs common to the two alfalfa materials was 721 between T1 and T3, 350 between T3 and T5, and 435 between T1 and T5, whereas the number of DEGs common to the two alfalfa materials was 209 between T1, T3, and T5 (Fig. 6C).

Through GO functional analysis, we found differences in the GO enrichment of genes in the two alfalfa materials during the three periods, with most being involved in BPs (Biological process) and MFs (Molecular function), and fewer were related to CCs (Cellular component) (Fig. 6D, Table S3). The GO enrichment analysis showed that the differentially expressed functions related to the CCs during the T1 period were mainly enriched in hydrolase activity that acts on glycosyl bonds, hydrolase activity that hydrolyzes O-glycosyl compounds, and cellular protein metabolic process. They were mainly enriched in cellular protein metabolic processes, cellular protein modification processes, and the regulation of the

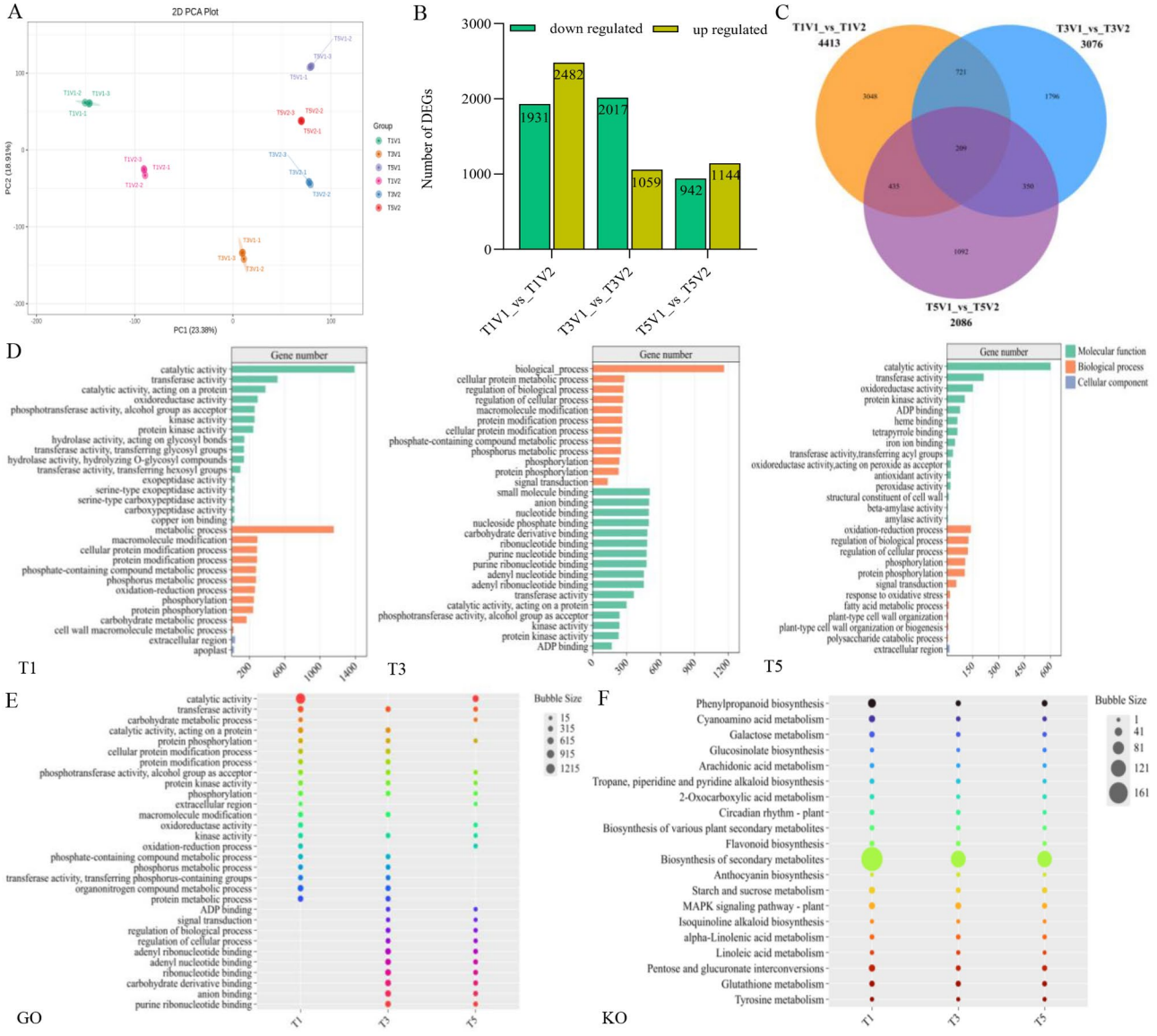

Fig. 6 Transcriptome analysis of tall-stalked alfalfa material WL525HQ (V1) and short-stalked alfalfa material WL343HQ (V2) at T1, T3 and T5 periods. (**A**) Principal component analysis (PCA) plot. (**B**) The number of up- and down-regulation of differential genes in different comparison combinations. (**C**) Differential gene comparison venn plot. (**D**) Histogram indicating GO enrichment of differential genes at T1, T3, and T5. (**E**) A bubble plot of GO function to which the top 50 GO terms of the 3 periods were co-enriched. (**F**) A bubble plot of pathways to which the top 50 KEGG pathways of the 3 periods were co-enriched

cellular processes in the T3 period; and in T5 they were mainly enriched in a structural constituent of the cell wall, the regulation of cellular processes, and plant-type cell wall organization. To an extent, this suggests that the high expression of hydrolase activity in T1 facilitates the expansion of the cell wall, the significant enrichment of the cellular modification process in T3 increases the cell size, and the synthesis of cell wall structural constituents and plant-type cell wall organization in T5 further improves and modifies the cell wall structure. The most differentially annotated functions coenriched in the three periods were the cellular and metabolic processes in the BP category, the binding and catalytic activity in the MF category, and the cellular anatomical entity in the CC category (Fig. 6E).

The KEGG pathway bubble diagram illustrates the top 50 metabolic pathways (Fig. 6F). We found that the pathways that were common to all 3 periods and had a high enrichment number of differentially expressed genes were Biosynthesis of secondary metabolites, Phenylpropanoid biosynthesis, Flavonoid biosynthesis, and Starch and sucrose. Notably, Phenylpropanoid biosynthesis is closely related to lignin biosynthesis, which is an important component of the cell wall.

Differences in the DEGs associated with the regulation of cell wall biosynthesis

The DEGs involved in cell wall growth during the important growth periods in tall- and short-stalked alfalfa material are presented in Fig. 7. Cellulose is synthesized at the plasma membrane by cellulose synthase subunits (CESs), and it was found that the number of DEGs encoding CESs was upregulated more in the T1 and T5 periods and less in the T3 period in the tall-stalked material compared to the short-stalked material. By evaluating the DEGs related to pectin lyase (PL) and pectin esterase (PM), we found that the expression of differentially upregulated genes of PL was higher in T1 than in the other periods and higher in the tall-stalked material than the short-stalked material, with a decreasing trend in the number of upregulated PL DEGs with the developmental modulation of internode growth. In addition, the number of DEGs related to PM was higher between the two enzymes, and there were more upregulated genes in the tall-stalked material than in the short-stalked material at the T1 period, and the number of DEGs decreased with the upregulation of PM regulated by the internode growth and development. The expression of the peroxidase, laccase, and PAL genes participated in lignin polymer formation. It was found that the number of upregulated DEGs encoding for peroxidase and laccase was higher at T1, and this upregulated expression was significantly higher in the tall-stalked material than in the short-stalked material, while the PAL DEGs were highly expressed at the T5 stage and were mainly enriched in the short-stalked material. These results further indicated that the DEGs associated with cell wall growth in the tall-stalked material were mainly formed during the T1 period, which is the critical period for cell wall growth in the tall-stalked material (Table S4).

Differences in the DEGs associated with the regulation of cell wall expansion

EXPs are nonhydrolyzed cell wall loosening proteins, and XTH has also been shown to be involved in cell expansion by loosening and rearranging the cell wall fibers in growing tissues. In this study, 11 members of the EXPs gene family and 13 members of the XTH gene family were found to be differentially expressed in the two alfalfa materials at different times. Specifically, five EXPs and eight XTH DEGs were expressed in high flux in the tall-stalked material at the T1 period, which indicates to some extent that the T1 period is a critical period for cell wall expansion and that these genes are essential for cell wall expansion and loosening. We evaluated other DEGs involved in cell wall modification and identified significant differences in the expression of 13 Aquaporin genes,

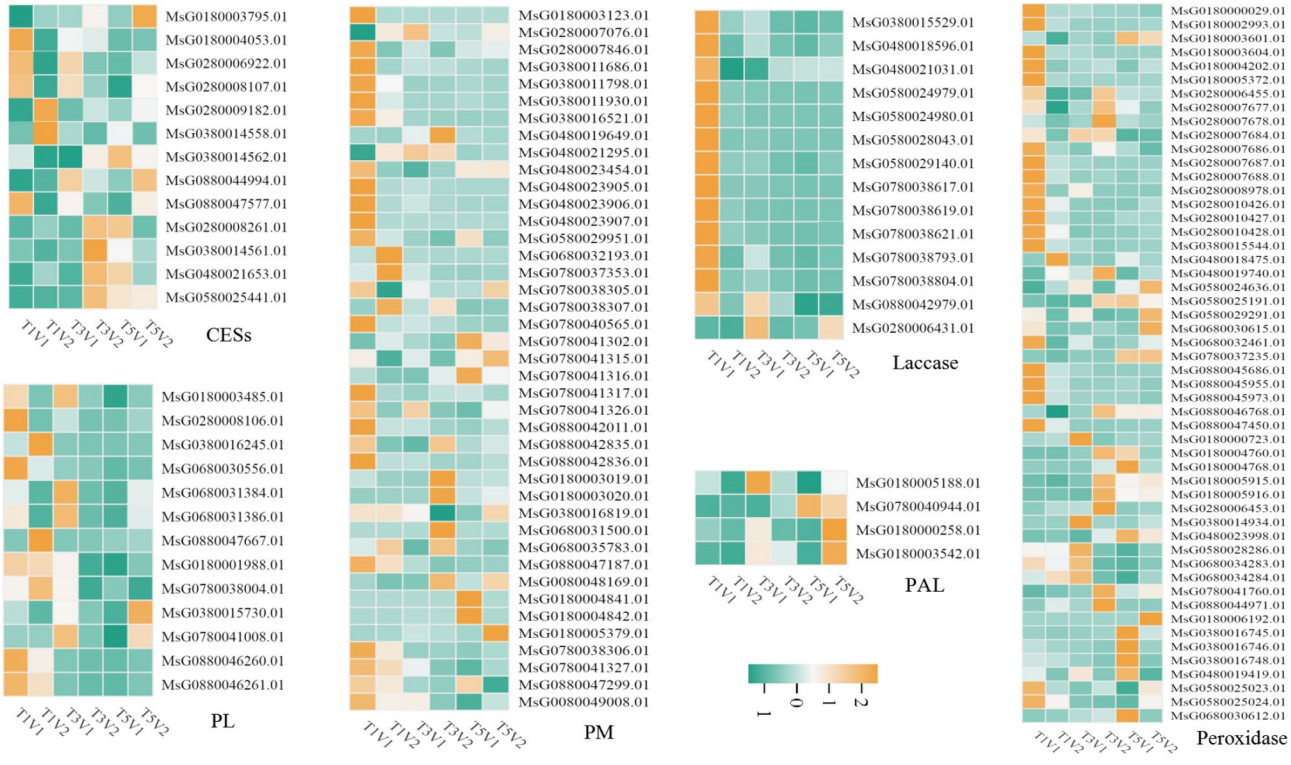

Fig. 7 Differential DEGs associated with plant cell wall biosynthesis. V1 represents the tall-stalked alfalfa material WL525HQ, and V2 represents the short-stalked alfalfa material WL343HQ. T1, T3, and T5 periods indicate the initiation growth period, rapid growth period, and growth stabilization period, respectively. CESs: Cellulose synthase; PL: Pectate lyase; PM: Pectinesterase; PAL: Phenylalanine ammonia-lyase

of which the upregulated expression of the DEGs was higher in the T1 period than in the other periods, and the expression was higher in the tall-stalked material than in the short-stalked material. In conclusion, these results revealed that the onset of growth in the T1 period is the critical period for cell wall growth and expansion in the tall-stalked material, and the differential expression of cell wall-related genes during this period determines the cell size (Table S4) (Fig. 8).

Quantitative real-time PCR (qRT-PCR) validation

To verify the relative expression pattern of single genes, we randomly selected nine differential genes related to the regulation of cell wall biosynthesis and expansion for qRT-PCR analysis, and determined the relative expression of the differential genes at each of the three periods (Fig. 9). The results showed that the relative expression of five differential genes in the tall-stalked material was highly significant higher than that of the short-stalked material at T1, namely Laccase, Expansins, CESs, XTH and PAL, while the relative expression of four differential genes was significantly or highly significantly lower than that of the short-stalked material at T5, and the difference in the expression of the other genes was not significant in these two periods. Among these nine genes, except for the PAL gene whose relative expression trend was slightly different from the transcriptome FPKM value, the relative expression of the other differential genes was basically the same as the FPKM value, which proved to a certain that the gene expression pattern of the transcriptome FPKM was supported by the results of qRT-PCR analysis.

Plant height regulatory networks in alfalfa

Finally, we proposed a hypothetical model for the regulatory network of plant height in alfalfa. In particular, the internode length is the main determinant of alfalfa plant height, and cell size influences the internode length to a certain extent, whereas the cell size is limited by the cell wall. The cell wall is a three-dimensional network formed by the interaction of lignin, cellulose, and pectin, and changes in the cell wall components play an important role in the regulation of cell wall expansion in alfalfa. The DEGs involved in the regulation of cell wall biosynthesis in alfalfa are related to cellulose synthesis, pectin cleavage, and lignin formation, and the DEGs involved in the regulation of cell wall expansion are related to EXPs and XTH, among others (Fig. 10).

Discussion

Alfalfa (*Medicago sativa* L.) can be used as a bioenergy material and high-quality, high-protein feed with high utilization value [16, 24]. Cultivating high-yielding and high-quality alfalfa germplasm is of great significance to improve alfalfa yield. Plant height, as an important indicator of alfalfa yield, is closely related to the ability of resistance to downfall, photosynthetic capacity, and harvest index [25, 26]. In this study, we screened tall- and short-stalked materials with significant differences in their internode length from 12 alfalfa varieties through a 2-year field trial. The average plant height, internode number, internode length, and stem thickness of the tall-stalked material WL525HQ for 2 consecutive years were 27.20%, 7.40%, 27.24%, and 5.30% higher than those of the short-stalked material WL343HQ, respectively. Alfalfa stem elongation is directly reflected by an increase in the number of internodes and the elongation of internodal tissues, which ultimately manifests as an increase in plant height [27]. Crops such as rice, wheat, and maize have been reported to have a similar property where the longer the internode length, the higher the plant height [28, 29]. In this study, we found that the difference in the

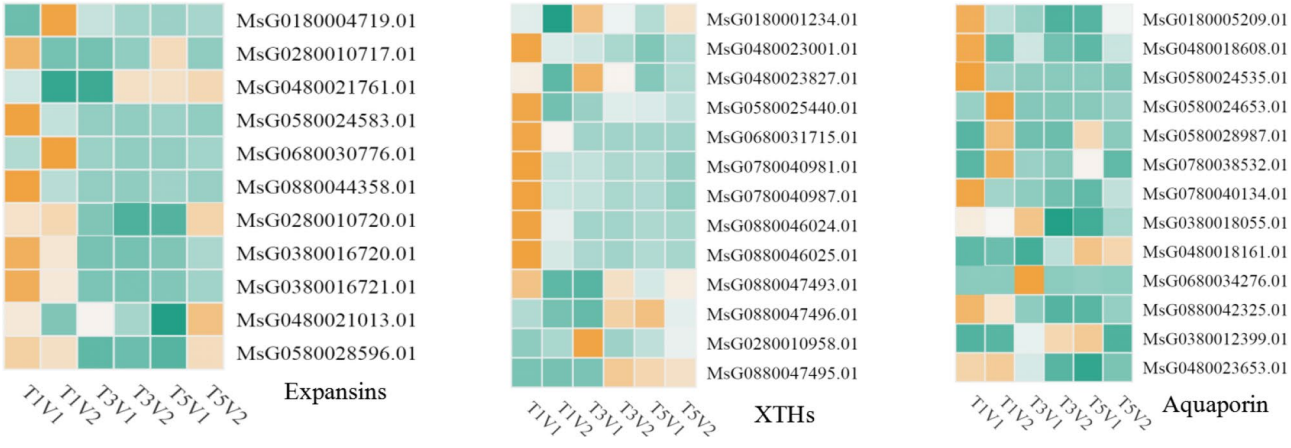

Fig. 8 Differential DEGs associated with plant cell wall expansion. V1 represents the tall-stalked alfalfa material WL525HQ, and V2 represents the short-stalked alfalfa material WL343HQ. T1, T3, and T5 periods indicate the initiation growth period, rapid growth period, and growth stabilization period, respectively. XTH: Xyloglucan endo-transglucosylase/hydrolase

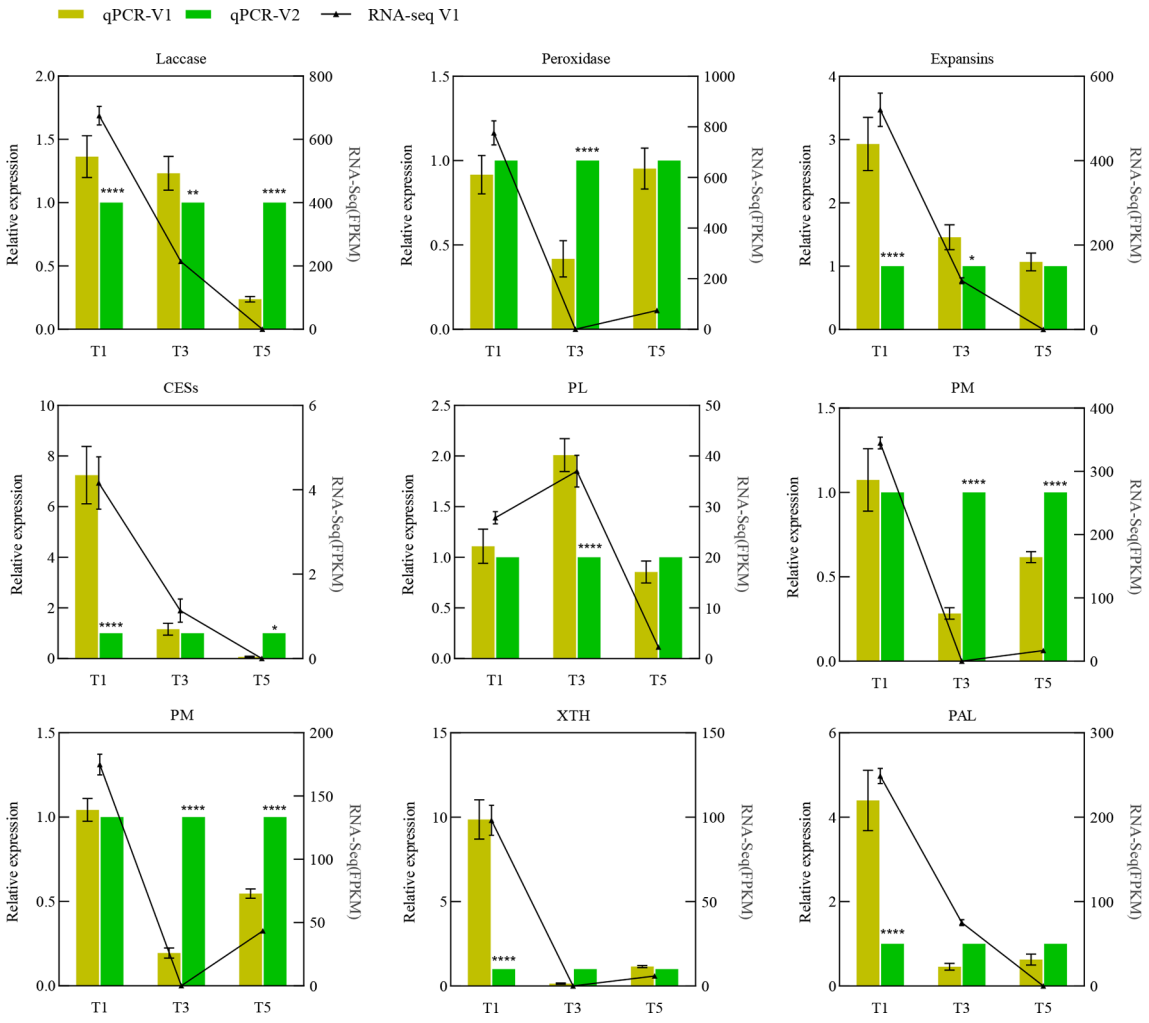

Fig. 9 Validation of the transcriptome data using qRT-PCR. CES: Cellulose synthase; PL: Pectate lyase; PM: Pectinesterase; XTH: Xyloglucan endo-transglucosylase/hydrolase; PAL: Phenylalanine ammonia-lyase

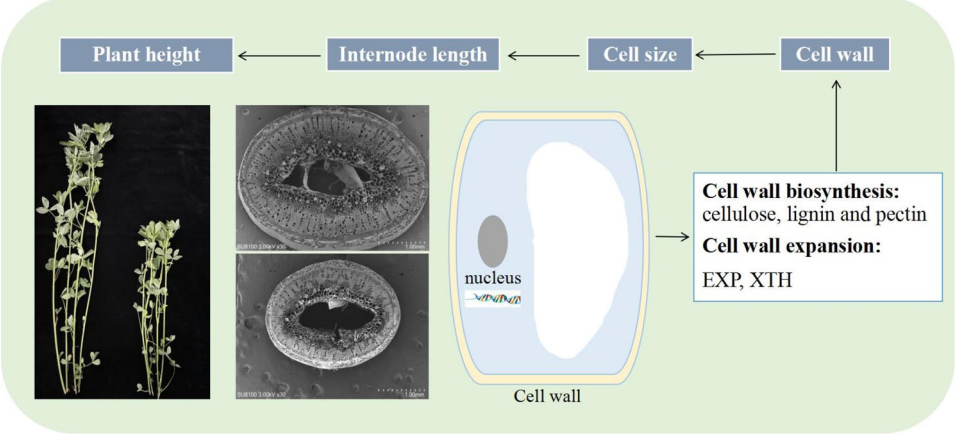

Fig. 10 Alfalfa plant height regulatory network. Where arrows indicate interactions. EXP: Expand protein, XTH: Xyloglucan endo-transglucosylase/hydrolase

number of internodes between the two alfalfa materials at different periods was not significant, although the difference in the average internode length was significant, which indicates that internode length is the main factor that affects plant height in alfalfa.

Internode elongation in plants is dependent on the longitudinal elongation of cells [30]. In this study, the difference in the internode length between the tall- and short-stalked alfalfa materials was greatest at the T1 period in the second basal node, which was significant, and this difference gradually decreased with the growth and development of the internodes. Cytological analysis revealed that the area of longitudinally cut cortical cells of tall-stalked alfalfa material was significantly higher than that of the short-stalked alfalfa material at the T1 and T3 periods, and this difference was greatest at the T1 period. Further experiments demonstrated that the cell size is an important factor that influences the internode length of alfalfa.

Plant growth and development require the regulation of cell size and morphology, which is limited by the cell wall, and plant cell wall loosening is an essential physiological process for cell expansion and elongation [31]. The plant cell wall is a three-dimensional network formed by the interaction of cellulose, pectin, and lignin [5]. During rapid cell growth, the formation of new cell walls depends on the deposition of large amounts of lignin, cellulose and hemicellulose, and the low content and weak deposition of cell wall components also counteracts to inhibit cell growth [32]. We found that the lignin, cellulose, and protopectin contents among the components that comprise the cell wall tended to increase with plant growth and development, and the lignin and pectin contents of the tall-stalked material were higher than those of the short-stalked material at different times. Further analysis by resorcinol lignin staining and SEM revealed that changes in cell wall components play an important role in cell wall expansion, especially lignin synthesis. It is widely believed that lignin deposition reduces the extensibility of the cell wall. However, on the other hand, silencing or mutation of relevant genes in the lignin synthesis pathway leads to a substantial reduction in lignin synthesis and defects in plant growth and development [33]. In Arabidopsis thaliana, suppression of the expression of genes in the lignin synthesis pathway leads to a 50% reduction in the lignin content of stem tissues in transgenic lines compared to the wild type, as well as severe dwarfing and male sterility, which affects their normal growth and development [34]. Either increase or decrease in lignin can lead to abnormal growth phenotypes such as plant dwarfism [35]. Next, we analyzed the factors that caused differences in the alfalfa plant height from the aspects of cell wall biosynthesis and expansion.

In higher plants, cellulose plays an important role in regulating cell volume and size as one essential cell wall component [5, 31]. The catalytic subunit of cellulose synthase (CESA) is responsible for the synthesis of cellulose in plant cell walls [36, 37]. There were 13 genes encoding CESA subunits in this study, of which the number of upregulated DEGs encoding CESA was less in the tall-stalked material than in the short-stalked material at the T3 period, which is a critical period for cell wall growth and expansion. This is consistent with findings by Wang, where more CESAs may be required for cell wall metabolism in dwarf mutant bamboo species compared to wild mosaic bamboo [38]. CESA mutants exhibit reduced cellulose and phenotypic growth defects [39]; furthermore, the overexpression of CESA genes affects the growth of transgenic plants [40]. These results suggest that CESA genes play an important role in cell wall biosynthesis and plant growth. In this study, GO enrichment analysis revealed that the highly expressed hydrolase activity during T1 promoted cell wall expansion, while the analysis of PL- and PM-related DEGs revealed that the expression of the upregulated genes was higher in T1 than in the other periods and was higher in the tall-stalked material than the short-stalked material. These results indicate that the expression of pectinase-related DEGs affected internode growth and development to a certain extent, and this effect was significant in the T1 period.

The primary function of lignin is to improve plant cell wall strength and stem bending resistance, and its monomer synthesis originates from the phenylpropane and lignin-specific pathways [41, 42]. In this study, lignin synthesis was found to play an important role in supporting cell wall expansion. In addition, KEGG analysis also revealed that the metabolic pathway was shared and there were significant differences in phenylpropanoid biosynthesis between the T1 and T5 periods, which is closely related to lignin biosynthesis. The main lignin monomer synthase genes are PAL, C4H, 4CL, COMT, CAD, and CCR, etc., and if the expression of these genes undergoes upregulation or downregulation, the lignin content and composition in plants change [43]. In this study, we found that peroxidase, laccase, and PAL participated in regulating the formation of lignin polymers in the tall- and short-stalked alfalfa through differential gene comparison.

Cell expansion is accompanied by irreversible cell wall extension under the control of a network structure comprising polysaccharides, such as cellulose and xylan [44, 45]. EXPs are nonhydrolyzed cell wall-loosening proteins that can reduce the network tension of the cell wall, thereby effectively relaxing the cell wall and promoting plant growth [46, 47]. Exogenous EXP can stimulate cell elongation, and the rate of cell elongation is positively correlated with EXP activity [48, 49]. In a previous study,

the increased expression of Nt EXPA5 contributed to the growth of tobacco stem and leaf cells and the overexpression of At EXPA10 and EXPA1 resulted in the enlargement of tobacco leaves [50]. In our study, 11 members of the EXP gene family were differentially expressed in two alfalfa materials at different periods; among these, five EXP DEGs were expressed in high flux in the tall-stalked material during the T1 period, suggesting that more EXP genes are involved in the cell wall expansion in the tall-stalked material than in the short-stalked material during T1. Moreover, T1 is a critical period when EXP genes cause plant height differences by relaxing the cell wall. It has also been shown that increasing the expression level of a particular EXP gene does not necessarily increase the plant height but may result in lateral cell expansion, thereby increasing the plant stem thickness [51, 52]. We also found that stem thickness, transverse xylem, phloem, and pith cell area were higher in the tall-stalked material than in the short-stalked material, which indicates to a certain extent that the expression of EXP genes not only causes longitudinal elongation of the cells but also lateral expansion of the cells.

XTH relaxes the cell wall and participates in cell wall synthesis during the growth of plant cells, but also functions in degrading the cell wall [53–55]. XTH9 is expressed in the apical meristematic tissue of *A. thaliana*, and its physiological role is related to stem elongation and growth, and a deletion mutation of this gene resulted in internode shortening of *A. thaliana* [56]. The overexpression of the Bc XTH1 gene promoted floral branch elongation and plant height increase in Arabidopsis [57]. In this study, we found that 13 members of the XTH gene family were differentially expressed in the two alfalfa materials at different periods, among which eight XTH DEGs were expressed in high flux in the tall-stalked material at the T1 period, which suggests that these eight XTH genes play an important role in cell wall expansion and loosening in the tall-stalked materials.

Conclusion

In this study, we screened tall- and short-stalked alfalfa materials (WL525HQ and WL343HQ, respectively) with significant differences in internode lengths in a 2-year field experiment and identified the three important periods of alfalfa plant height development: initiation of the growth period (T1), rapid growth period (T3), and stabilization of the growth period (T5). The DEGs involved in the regulation of cell wall growth in alfalfa were found to be related to cellulose synthesis, pectin cleavage, and lignin formation, and DEGs involved in the regulation of cell wall expansion were related to EXP and XTH.

Materials and methods

Experimental sites

The experiment was conducted at the forage training base of Gansu Agricultural University (E 105°41′, N 34°05′), which is located in the northwestern part of the Loess Plateau and the central part of Gansu Province. It has a mid-temperate continental monsoon climate, with a dry spring with little rainfall, a hot summer, a cool winter, and a large temperature difference between day and night. The average altitude is 1525 m above sea level, the average annual temperature is 10.3 °C, and the frost-free period is 180 d. The soil samples for testing were mixed by field soil samples and nutrient soil according to the mass ratio of 5:1. The field soil samples were taken from the 0 ~ 20 cm tillage soil samples of Gansu Agricultural University pasture training base, and the soil was yellow sheep soil with uniform fertility, and the nutrient soil was purchased from Gansu Sheng Hua Wei Trading Co.

Plant material

The 12 alfalfa varieties for testing have high yield and production performance, of which 'Gannong No.9' and 'Gannong No.3' were supplied by the College of Pratacultural Science, Gansu Agricultural University, and 'SG401', 'WL319HQ', 'WL343HQ', 'WL354HQ', 'SG501', 'WL525HQ', 'WL168HQ', 'SG601', 'WL298HQ' and WL363HQ' were purchased from Beijing Zhengdao Ecological Technology Co. The alfalfa varieties selected for this study were all commercial varieties, and the collection and use of plant materials in this study complied with relevant institutional, national and international guidelines and regulations.

Planting of test material

The test material was sown in August 2021 in pots of 12 varieties, 3 pots of each variety per plot, in a randomized block arrangement with 4 replications. Before sowing, soil samples were filled into plastic pots with a diameter opening of 24 cm and a height of 24 cm, and full-grown and uniformly sized alfalfa seeds were evenly sown, 30 seeds/pot. To maintain consistency with field conditions, alfalfa varieties were planted in test plots and watered regularly after planting. In 2022–2023, one pair of tall and short straw alfalfa materials with significant differences in internode length were screened out by measuring phenotypic indexes such as plant height, number of internodes, internode length, stem thickness, and other phenotypic indexes of 12 alfalfa varieties at the first flowering stage of different crops for 2 years. Phenotypic traits of tall and short-stalked alfalfa materials were measured every 10 d from seedling mowing on March 26, 2023, until the first flowering stage, for a total of five times. Meanwhile, the internode tissues affecting the important periods of growth and development of alfalfa

main stem were screened for cytological, metabolomic and transcriptomic measurements.

Measurement of plant height and related traits

A tape measure was used to measure the plant height of alfalfa, which was defined as the distance between the base and tip of the main stem. The number of internodes was based on a length of more than 1 cm, and the number of internodes was counted in two randomly selected alfalfa plants exhibiting good growth in each pot. The average internode length was the ratio between the height of the main stem and the number of internodes. The diameter of the second internode at the base of the main stem was measured as the stem thickness using vernier calipers. The plant height growth rate was determined using the following formula:

$$K_w\ (\mathrm{cm/day}) = (H_{n+1} - H_n) / (T_{n+1} - T_n)$$

where (H_{n+1}-H_n) is the growth of plant height at two consecutive sampling times, and (T_{n+1}-T_n) is the interval between the two consecutive sampling times. The plant measurements were counted from the bottom up for the first node, with the measurement of the diameter of the second internode determined from the base of the main stem upward.

Cytological observation of internodes

According to the results of the previous experiment, we divided the growth of the alfalfa main stem into three important growth periods. The intersegmental tissues in the middle of the second node of the main stem of tall- and short-stalked alfalfa materials during the three important growth stages were selected, and the thickness of 0.5 cm was cut and placed in FAA fixative (formalin: glacial acetic acid: 70% ethanol = 1:1:18), ensuring that the materials were fully immersed. The fixed samples were dehydrated using different alcohol gradients and gradually transitioned to pure xylene for transparency before being covered in wax for embedding. The embedded tissues were sliced into 8–10-μm-thick sections and then placed on slides, and slices were baked in an oven at 38 °C for 72 h. Transverse and longitudinal sections were deparaffinized, i.e., they were gradually transitioned from xylene to anhydrous ethanol. The slices were then removed and stained with a Senna staining solution, followed by a solid green staining solution. Neutral gum was used to seal the slices, which were then dried, processed, and photographed via observation with a Nikon-E200 light microscope (Jiangsu, China) to view the tissue cell morphology. The cell areas of the cortex, phloem, xylem, and pith in the transverse sections of the main stem were measured, and the cell areas of the cortex in the longitudinal sections were measured, among other indexes [58].

Determination of cell wall composition

The basal second internode tissue of the test material was randomly selected, dried at 80 °C until a constant weight, crushed, and sieved through a 40-mesh sieve for the determination of cell wall composition. The cellulose content was determined via anthrone colorimetry [59], hemicellulose content using the dinitrosalicylic acid (DNS) method [60], lignin content by the acetobromine method [61], and protopectin content using carbazole colorimetry [62]. The content of soluble sugars (SS) was determined by anthrone colorimetry [63], the content of sucrose (Suc) was determined by a kit (purchased from Beijing Solarbio Science &Technology), and the content of starch (Sta) was determined by perchloric acid hydrolysis-anthrone colorimetry [64].

Observation of cell wall structure

To observe the lignin in the cell wall of alfalfa at different times, a double-sided knife was used to make a transverse cut in the basal second internode tissue, and then the sections were placed on slides, stained with 0.2% resorcinol, diluted with a few drops of 25% hydrochloric acid for 1 min, and then placed under an orthotropic universal microscope OLYMPUS-BX61 (Tokyo, Japan) for observation and photographing [65]. To further observe the tissue structure, the collected internode tissues were fixed in 2.5% glutaraldehyde, and the fixed samples were then rinsed with 0.1 M phosphate buffer (PB) [pH 7.4]. The samples were then fixed with 1% osmium acid at 25 °C protected from light, dehydrated with different ethanol gradients, and then immersed in isoamyl acetate, after which the samples were transferred to the sample baskets for adventitious drying. The samples were then taped to the sample stage for coating, and they were examined using a scanning electron microscope (SEM, HITACHI Regulus 8100, Tokyo, Japan) for observation and photography [66].

cDNA library construction and transcriptome sequencing

The second internode tissue of the main stem from the three important growth stages of the tall- and short-stalked alfalfa materials was collected. Total RNA was extracted using TRIzol reagent (Tiangen, Beijing, China), mRNA was purified, and total RNA was measured and quantified. The mRNA was amplified via PCR to obtain a library. After the library was constructed, preliminary quantification was performed using a Qubit 2.0 fluorometer, and sequencing was performed on Illumina NovaSeq 6000 (Illumina, USA) according to the effective concentration of the library and the target downstream data volume. Then, the fragments per kilobase of transcript per million mapped reads (FPKM) values of each transcript in each sample were counted, and the value was used as the expression of the transcript. Differentially expressed

genes (DEGs) were screened using DESeq2 software (1.20) by setting the adjusted p-value (P-adj) ≤ 0.05 and fold change (|log2 (Fold change)| ≥ 1) as the screening thresholds. DEGs were analyzed using Cluster Profiler (3.8.1) for the Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway analysis. A threshold value of P-adj value ≤ 0.05 was used to identify the significantly enriched GO terms and KEGG pathways. The raw sequencing data have been uploaded to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the accession number PRJNA1173773.

Gene expression analysis using qRT-PCR

RNA was extracted from the second internode tissue of tall alfalfa WL525HQ and short alfalfa WL343HQ using RNA simple Total RNA Kit (TIANGEN BIOTECH, Co., Ltd, Beijing, China). RNA was reverse transcribed into cDNA using FastKing RT Kit (TIANGEN, Beijing, China), and primers specific for 10 differential genes were designed using Primer-BLAST at NCBI (Table S5). The designed primer sequences were synthesized by Shanghai Bioengineering, and alfalfa 18s RNA was selected as the internal reference gene. The qPCR amplification was performed on LightCycler 96 (Roche, Basel, Switzerland) according to the instructions of Servicebio's SYBR Green qPCR Master Mix. 3 biological replicates were set up, and 3 technical replicates were set up for each biological replicate [67]. The relative expression of each differential gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Data statistics and analysis methods

Data were organized and calculated in Microsoft Excel 2010 software. GraphPad 8.0.2 software was used for graphing. Differential gene analyses were performed using the website https://www.chiplot.online/.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06172-y.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Acknowledgements
We thank NES for its linguistic assistance during the preparation of this manuscript.

Author contributions
FJ, SLS and WJK conceived and designed the project. FJ performed most of the experiments. BW, BFL provided related culture methods. BW, BFL and JG provided laboratory assistance. FJ interpreted results and wrote the manuscript.

Funding
This work was funded by the Chief Scientist Project (No. 23ZDKA01-3) and the National Pastoral Industry Technology System of China (No. CARS-34).

Data availability
All data generated or analyzed in this study are included in this published article and its supplementary material. The raw sequencing data are available in the NCBI SRA repository, accession number PRJNA1173773 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1173773).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Clinical trial number
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 5 November 2024 / Accepted: 29 January 2025
Published online: 28 February 2025

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