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Improved forage quality and biomass yield of alfalfa (*Medicago sativa* L.) by Arabidopsis *QQS* orphan gene

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ABSTRACT

Improving the forage quality of alfalfa in terms of digestibility and crude content is essential for any alfalfa quality breeding programs. *Arabidopsis thaliana* orphan gene *QQS* (*Qua-Quine Starch*) has been shown to improve protein content and alter carbohydrate composition in different food crops. However, there are significant differences in agronomic traits and nutritional conditions between alfalfa and other food crops. To explore the biological function and molecular mechanisms of *QQS* in alfalfa, we generated *QQS* transgenic plants and their segregated population (T1 generation), and evaluated their performance under normal- and nitrogen-deficient conditions. Our findings indicate that *QQS* can significantly enhance the total nitrogen and crude protein content of alfalfa and increase nodule weight under low-nitrogen conditions. Furthermore, *QQS* transgenic lines also showed reduced levels of neutral detergent fiber (NDF) and lignin, improving forage digestibility. By RNA sequencing and RT-qPCR analysis, we found that *QQS* affected the expression of genes involved in carbon and nitrogen metabolism, lignin biosynthesis and amino acid biosynthesis and degradation pathways in alfalfa. In addition, *QQS* also improved alfalfa biomass yield by increasing branch number and plant height in both greenhouse and field conditions. Our results demonstrate that *QQS* as a useful molecular tool can improve alfalfa biomass yield and overall forage quality and could have significant implications for the alfalfa breeding industry in satisfying the constant demands for high-quality and high-yielding forage.

1. Introduction

Alfalfa (*Medicago sativa* L.) is a valuable forage crop due to its high biomass yield and good forage quality, provided by its high digestibility and crude protein content. Increasing crude protein content is particularly important as it positively correlates with the price of alfalfa hay [17]. However, the high lignin content in alfalfa impedes the digestion of cellulose, resulting in an unbalanced nitrogen-to-carbohydrate ratio in the rumen [20], making simultaneous improvement of forage digestibility and crude protein content a challenge.

Genetic modification of alfalfa through transgenic methods has been attempted to improve the forage quality. Overexpressing bacterial aspartate kinase and adenylyl sulfate reductase genes has increased sulfur-containing amino acids content in alfalfa [35,37], while down-regulating lignin biosynthesis genes has enhanced forage digestibility [4]. However, an effective way for simultaneous improvement of the forage digestibility and crude protein content of alfalfa is still unknown.

Plants utilize nitrogen metabolism to produce components like proteins, nucleic acids, chlorophyll, and hormones essential for growth and

Abbreviations: *QQS*, Qua-Quine Starch; TGs, transgenic plants; WT, wild type; PCR, polymerase chain reaction; RT-qPCR, reverse transcription quantitative real-time PCR; *SPS*, sucrose phosphate synthase; *GS*, glutamine synthetase; *F5H*, ferulic acid-5-hydroxylase/coniferaldehyde-5-hydroxylase gene; *PAL*, phenylalanine ammonia-lyase gene; *4CL*, 4-coumarate-CoA-ligase gene; *CCR*, cinnamoyl-CoA reductase gene; *PDH*, proline dehydrogenase gene; *GDH*, glycine dehydrogenase gene; *PK*, pyruvate kinase gene; *PT*, plastid transketolase gene; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; Ls, glufosinate resistant seedlings; *GS1*, glutamine synthetase *1*; *PDH*, proline dehydrogenase; *GDH*, glycine dehydrogenase; *PK*, pyruvate kinase; *PT*, plastid transketolase; *F5H*, ferulic acid-5-hydroxylase/coniferaldehyde-5-hydroxylas; NDFD, NDF digestibility; ADFD, ADF digestibility; PEPC, phosphoenolpyruvate carboxykinase.

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development, while carbon metabolism fulfills organism's energy requirements and provides structural support and mechanical resistance [13]. Studies have found associations among carbon/nitrogen metabolism, plant cell wall composition, and crude protein content [1,15]. Kaur et al. [15] reported that overexpressing genes of key enzymes like sucrose phosphate synthase (SPS) involved in sucrose synthesis in leaves and glutamine synthetase (GS) involved in ammonia assimilation in root nodules, increased forage digestibility, crude protein content, and biomass yield of alfalfa. Therefore, regulating the carbon/nitrogen metabolism could be a promising strategy for enhancing forage quality.

QQS (*Qua-Quine Starch*; At3g30720) is an orphan gene unique (lacking any similar sequence of protein-coding genes in any other species) in *A. thaliana*, which has been shown reported to regulate carbon and nitrogen partitioning by binding to the conserved transcription factor nuclear factor Y, subunit C4 (NF-YC4) in various species [3,18,19,21,31,32,38]. Up-regulation of *QQS* has been reported in Arabidopsis carbohydrate mutants such as *Atss3* (lacking starch synthase III), *Atmex1,* and *Atsis8* [18]. In Arabidopsis, overexpression of *QQS* led to increased protein content and decreased starch content in leaves, while its down-regulation had the opposite effect [19]. Ectopic expression of *QQS* in corn, rice, and soybean also enhanced the protein content in grains and leaves without affecting plant morphology [18,19,21]. However, the effect of *QQS* on alfalfa forage quality is yet to be investigated. Unlike annual food crops, alfalfa is a perennial plant that can form symbiotic relationships with rhizobia to fix nitrogen from the atmosphere, providing a nutritional advantage to the plant. Therefore, previous reports on *QQS* in annual food crops may not predict its actual effect on alfalfa forage quality.

This study aimed to evaluate the impact of introducing *QQS* into alfalfa, under normal and nitrogen-deficient conditions to determine *QQS* effect on plant's performance and forage quality. To investigate potential mechanisms, we also conducted RNA-seq and RT-qPCR analysis to detect differential gene expression related to forage quality. Similar to food crops, *QQS* has shown conservative functions such as increasing crude protein content and changing carbohydrate structure, as well as specific functions such as improving biomass output, soluble sugar content, and nodule weight in alfalfa. Our results support using *QQS* as a molecular tool for high-yield and high-quality alfalfa breeding.

2. Materials and methods

2.1. Vector construction and alfalfa transformation

The T-DNA region of the *QQS* overexpression vector used, shown in Fig. 1A is based on a previous report [19]. The *QQS* gene expression was driven by the CaMV 35S promoter, and the *Bar* (phosphinotricin acetyltransferase) gene was used as a selectable marker (Fig. 1A) [19]. The vector was then transformed into *Agrobacterium tumefaciens* strain EHA105 and used to transform the alfalfa cultivar 'Baoding' [40]. Transgenic (TG) plants and wild-type (WT) plants were cultured in flowerpots filled with soil in a greenhouse with natural light and 25 ℃ of temperature [24].

2.2. Transgenic plants identification and quantitative real-time PCR tests

Genomic DNA was extracted from TG and WT plants using the CTAB method for PCR [24]. A 249-bp fragment that spans the CaMV 35S promoter and *QQS* gene was amplified using primer pair 35S-F2 and QQS-R1 (Table S1) to verify the TG plants. Total RNA was extracted from young leaves using Trizol reagent (Thermo Fisher Scientific, San Jose, CA, USA). One microgram of RNA was digested with DNase enzyme and employed to generate the first strand cDNA for RT-PCR and quantitative real-time PCR (RT-qPCR) analysis (Takara RR047 kit, Dalian, China). A 107-bp fragment specific to *QQS* was amplified using primer pair RT-QQSF and RT-QQSR to determine the expression of *QQS* in transgenic plants (Table S1).

The TB Green Premix Ex Taq™ (Takara RR420 kit, Dalian, China) with primer pair qRT-QQS-F and qRT-QQS-R (Table S1) was used for RT-qPCR that was carried out on an Eco Real-Time PCR System (Illumina, EC-100-1001, CA, USA). The relative expression levels of genes were calculated by the $2^{-\Delta\Delta Ct}$ method [25] using alfalfa *β-actin* gene (JQ028730.1) as an internal reference [22]. Three biological replicates were used for statistical analysis for this experiment.

2.3. Plant culture and phenotype analysis

WT and TG plants were propagated by the stem-cutting method and cultured in a greenhouse. The T1 generation seeds were harvested from each transgenic line planted in the field (Beijing, China, N40.09, E116.22). The seeds of different lines were planted in a greenhouse and identified by PCR and RT-PCR. The PCR-positive T1 seedlings from the same transgenic line were treated as a half-sib pool (L) for subsequent research, and PCR-negative (non-transgenic) seedlings were used as controls (CK). T1 generation plants were cultured in a greenhouse and cut every other month. Before the fourth cutting, the T1 plants and WT control plants were used for plant phenotype analysis. The absolute height of the plant was measured by a random selection of fifteen branches. The leaf and stem weight of each plant was measured separately to calculate the leaf-to-stem ratio. The above-ground biomass was harvested for forage quality and amino acid analysis. Three plants from each pool were used for phenotypic and forage quality analysis as biological repeats.

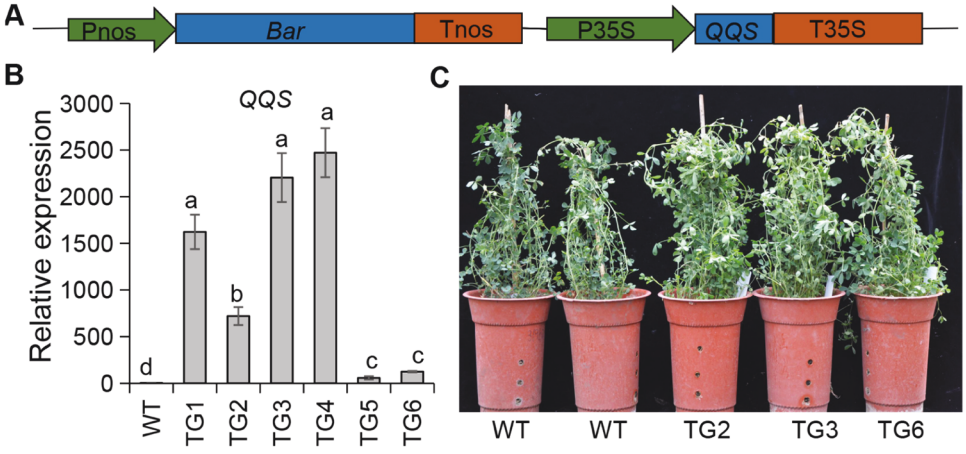

Fig. 1. Generation and morphological features of *QQS* transgenic alfalfa plants (TGs). (A) The schematic diagram of the T-DNA region of the vector used for *QQS* overexpression. (B) The relative expression of *QQS* in both WT and TGs through quantitative real-time PCR tests. The data are shown as mean ± SD ($n = 3$). Different letters indicate a significant difference ($P < 0.05$). (C) Compares the visual phenotype and morphological features of WT and TGs, specifically in their above-ground growth.

2.4. Forage quality analysis

Plant materials were dried at 65 ℃ for 48 h in an oven and then passed through a 100-mesh sieve to measure neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) by Van Soest herbage analysis using an ANKOM A2000i semi-automatic fiber analyzer (ANKOM, Macedon, NY, USA) [36]. Total nitrogen was measured with the Kjeldahl method by using Kjeltec 2300 machine (FOSS, Hillerød, Denmark) as previously reported [16] and used to calculate the crude protein content. Soluble sugar was determined by anthrone colorimetry [10], and starch was stained by KI/I_2 solution [18]. The hand-sliced stem was stained with a phloroglucinol solution to observe lignin deposition. For each line, three biological replicates (each containing three technical repetitions) were used for statistical analysis.

2.5. Amino acids analysis

The amino acid content was determined by high-performance liquid chromatography (HPLC, Alliance e2695, Waters, USA) [6]. A 0.05 g sample per replicate was used to perform HPLC. Sample treatment, analysis, and calculation methods were performed according to the previous study [7]. Three biological replicates were used for each line.

2.6. Low-nitrogen facilitated rhizobia inoculation

T_1 generation seeds were germinated and grown on sterilized filter paper moistened with sterile water containing 10 mg/L PPT for three days. The germinated and non-germinated seeds were separated and cultured with sterile water for four days. Seven-day-old seedlings were transplanted into test tubes (diameter=1.5 cm, height=30 cm) with a piece of folded sterile filter paper or planted in flowerpots with vermiculite. The test tube was wrapped with foil and contained 3-mL low-nitrogen (0.36 mM nitrogen) nutrient solution. The formula of the low-nitrogen nutrient solution was reported previously [9,29]. Seedlings in test tubes were cultured in an incubator with sixteen hours of natural light and eight hours of darkness at 25 ℃. Seven-day-old seedlings in flowerpots were treated with non-nitrogen or normal nitrogen (5 mM nitrogen) solution. Three *QQS* T1 plants (containing L2, L3, and L6) and one CK were cultured in one flowerpot. Fifteen repeats were performed for non-nitrogen and normal nitrogen. Seedlings were inoculated with the rhizobia (*Sinorhizobium meliloti* 1021, sm1021) cultured in TY liquid medium (5 g/L Tryptone, 3 g/L Yeast extract) up to OD_{600} = 0.8 [8]. 20 mL of low-, non-, or normal-nitrogen nutrient solution and 20 mL of rhizobia resuspended in low-nitrogen nutrient solution were added to every tube or flowerpot. Plants were watered twice with water and followed by once with nutrient liquid. Phenotype and nitrogenase activity [23] were analyzed after growing for 28 days. Following a PCR test, twenty T1 transgenic seedlings were chosen for analysis, where non-transgenic seedlings were used as CK. Refer to [8] for information on the non-nitrogen nutritional liquid composition. Non-nitrogen nutrient liquid with additional 0.36 mM and 5 mM KNO_3 were the low- and normal- nitrogen nutrients, respectively.

2.7. In-vitro digestibility

About 0.5 g of sample was put into fiber bags that had been treated with acetone before being sealed with a sealing machine. Fiber bags were put into a Dasiy^{II} Incubator digestion tank (ANKOM, NY, USA) containing 266 mL of buffer A, 1600 mL of buffer B, and 60 mL of rumen fluid and treated for 48 h at 39 ℃. One liter of buffer A has 10 g KH_2PO_4, 0.5 g $MgSO_4•7H_2O$, 0.5 g NaCl, 0.1 g $CaCl_2•2H_2O$, and 0.5 g urea, and one liter of buffer B has 15 g Na_2CO_3, 1 g $Na_2SO_4•9H_2O$ [28,34]. Fiber bags were rinsed with cold water after 48 h of digestion, dried at 65 ℃ for 48 h, and weighed. Digestibility was calculated according to a previous report [28,34] with three biological replicates.

2.8. RNA sequencing and data analysis

In the isolated T1 generation population, three transgenic plants with comparable QQS expression levels from L2, L3 and L6 and three non-transgenic plants were used to characterize the global gene expression profiles. The third mature leaves from top-down on a healthy branches were collected as samples for total RNA extracted. RNA sequencing and analysis were performed as the previously reported method [42]. Ten genes were selected randomly for measurement the relative expression level by qRT-PCR to validation the results of RNA-Seq analysis. The primers were listed in Table S2.

Key genes in carbon and nitrogen metabolism, lignin synthesis and amino acid metabolism were also selected for RT-qPCR analysis (Table S4). Relative expression of genes determined by RT-qPCR includes cinnamyl alcohol dehydrogenase gene (*CAD*); ferulic acid-5-hydroxylase/coniferaldehyde-5-hydroxylase gene (*F5H*); phenylalanine ammonia-lyase gene (*PAL*); 4-coumarate-CoA-ligase gene (*4CL*); cinnamate-4-hydroxylase gene (*C4H*); cinnamoyl-CoA reductase gene (*CCR*); glutamine synthetase gene (*GS*); proline dehydrogenase gene (*PDH*); glycine dehydrogenase gene (*GDH*); pyruvate kinase gene (*PK*); plastid transketolase gene (*PT*); phosphoenolpyruvate carboxykinase gene (*PEPC*); transcription factor nuclear factor Y, subunit C4 (NF-YC4) and subunit B5 (NF-YB5); acetyl-CoA c-acetyltransferase (AACT) and 2-oxoglutarate dehydrogenase E1 component (2-OGDH).

2.9. Statistical analyses

The data collected from biological duplicates were subjected to a one-way ANOVA with SPSS 20.0. Different letters or asterisks represent a significant difference ($P < 0.05$).

3. Results

3.1. QQS improved biomass yield in alfalfa

To investigate the function of *QQS* in alfalfa, we introduced the gene into alfalfa cultivar 'Baoding'. PCR and RT-PCR analysis confirmed the generation of six independent transgenic lines (TG1–6) (Fig. S1A and B). The relative expression of *QQS* in the TG lines was significantly higher than in the WT plants, as confirmed by RT-qPCR (Fig. 1B). Among TG plants, TG2, TG3, and TG6 displayed more vigorous growth of above-ground parts than the WT plants in both greenhouse and field conditions, and were therefore selected for further study (Fig. 1C; Fig. S2). The T1 generation seeds of each individual transgenic line were collected, and the T1 generation seedlings were used for verification using PCR and RT-PCR (Fig. S1C and D). The transgenic T1 plants showed significantly higher expression of *QQS* than the WT plants (Fig. 2A). After growing the T1 plants for one month, we observed that *QQS* overexpression led to increased branches and height in half-sib plants (Ls) compared to control plants (CK) (Fig. 2B-E), which resulted in a significant increase in biomass yield (Fig. 2F). Our results demonstrate that overexpression of exogenous *QQS* gene significantly improved biomass yield of transgenic alfalfa plants.

3.2. QQS enhances crude protein content in alfalfa

Previous studies have shown that overexpression of *QQS* can increase the protein content in soybean and rice seeds and leaves. In our study, we observed that the above-ground of parts of *QQS*-T1-Ls had significantly higher total nitrogen content than the CK (Fig. 3A). Moreover, the crude protein content in Ls was increased from 19.42 % in CK to 20.25–21.27 % (Fig. 3B). Additional testing revealed no appreciable difference between CK and Ls in terms of crude protein digestibility and leaf-to-stem ratio (Fig. 3C and D).

We conducted HPLC analysis to measure the content of fourteen essential amino acids, including aspartic acid, glutamic acid, serine,

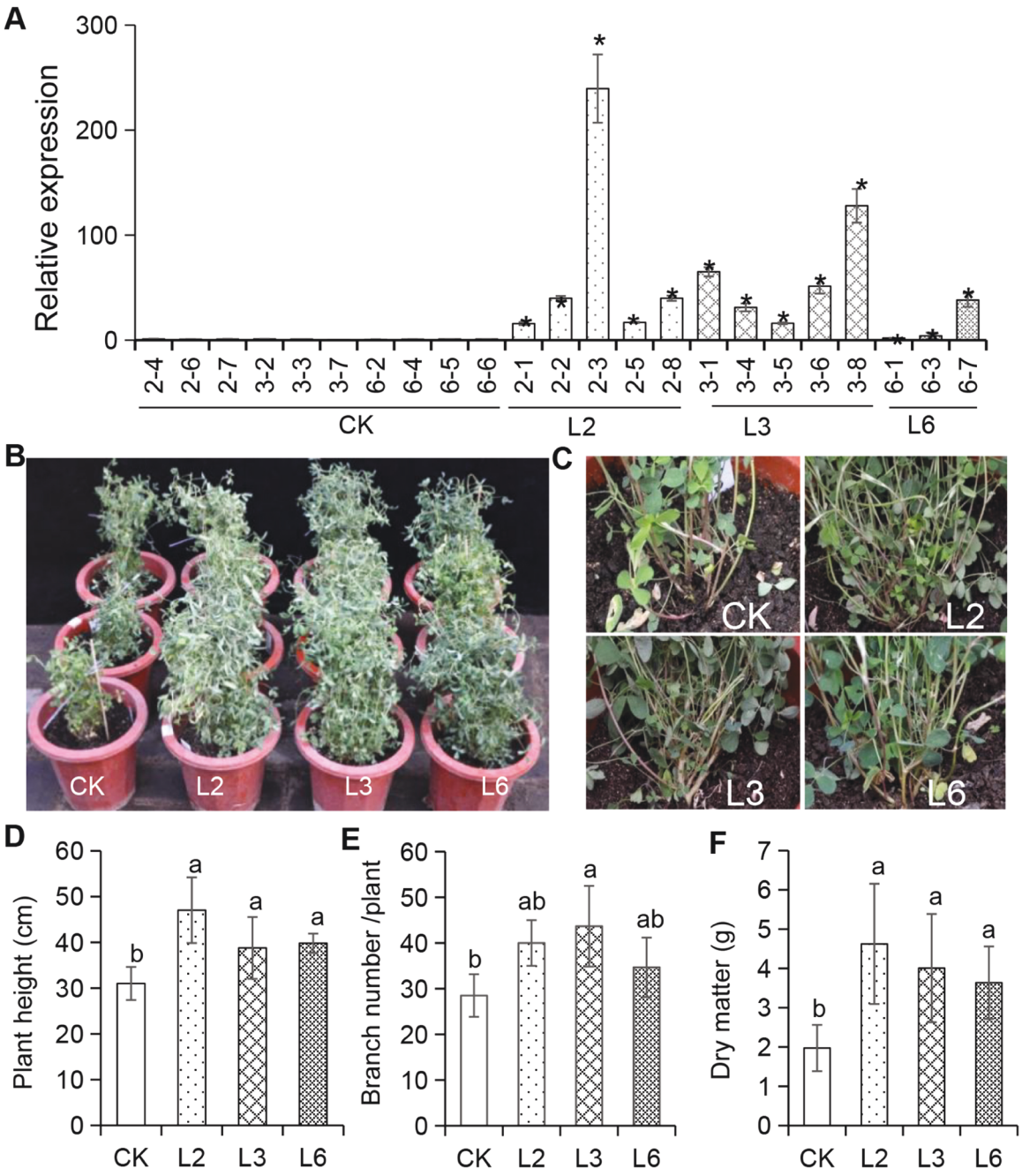

Fig. 2. Verification and phenotype analysis of the T1 generation of *QQS* transgenic alfalfa plants (TGs).(A) The relative expression of *QQS* in the T1 generation of TGs. L2, L3, and L6 represent the transgenic half-sib pools harvested from TG2, TG3, and TG6 plants, respectively. CK, non-transgenic plants. (B) Visual phenotype and morphological features of CK and Ls plants one month after the third cutting. (C) Close-up view of the branch of CK and Ls plants. (D-F) Comparison of plant height (D), branch number (E), and dry matter yield (F) of CK and Ls one month after the third cutting. Values are presented as mean ± SD ($n = 3$). Different letters and an asterisk denote a significant difference ($P < 0.05$).

histidine, glycine, threonine, arginine, alanine, tyrosine, valine, phenylalanine, isoleucine, leucine, and lysine. Result showed that, except for lysine, the content of other amino acids was significantly higher in Ls compared to WT (Fig. 3E). The relative change rates of different amino acids in Ls showed a similar tendency (Fig. 3E). These findings indicate that *QQS* can enhance the crude protein content of alfalfa, suggesting its potential use in breeding high-quality alfalfa.

3.3. *QQS increased nodules weight per plant to increase nitrogen fixation*

Symbiotic nitrogen fixation is crucial for alfalfa growth and development. To investigate the increased total nitrogen content in Ls, we examined the symbiotic nitrogen fixation of CK and Ls. During seed germination, we separated the glufosinate-resistant seedlings (Ls) and sensitive seedlings (CK) by culturing with water containing 10 mg/L PPT for three days, which did not effect the seedling post-germinated growing (Fig. S3A and B). Seven-old seedlings inoculated them with *Rhizobium* bacterium (Fig. S3C). After 28 days of growth cultured in a test tube, the above-ground and subterranean parts of TG plants were larger than WT plants (Fig. 4A and Fig. S3C). The number of nodules per unit root length did not differ significantly between CK and Ls (Fig. 4B). However, the nodules of transgenic lines were predominantly clustered, while those of WT plants mainly grew individually (Fig. 4C). The ratio of nodules with more than five bifurcations was significantly higher in Ls

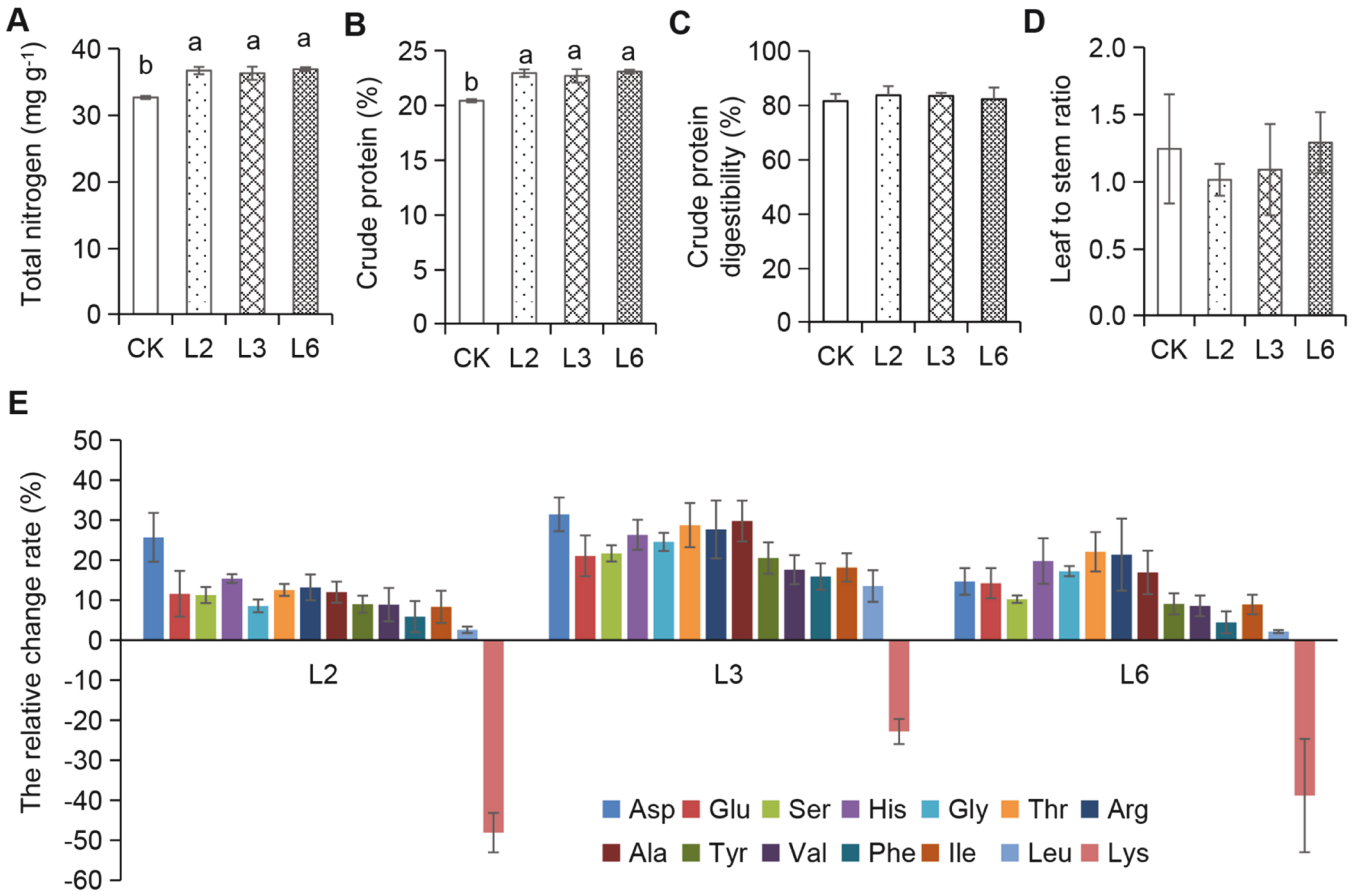

Fig. 3. *QQS* overexpression improved alfalfa's crude protein content. (A-D) Comparison of total nitrogen (A), crude protein (B), crude protein digestibility (C), and leaf-to-stem ratio (D) of CK and Ls. (E) The relative change rate of amino acids in Ls compared to CK. Values are presented as mean ± SD ($n = 3$). Different letters in the same row represent a significant difference ($P < 0.05$).

than CK (Fig. 4D). These changes in nodule phenotype in Ls led to a significantly higher single nodule weight (Fig. 4E), although the nitrogenase activity of nodules remained unchanged (Fig. 4F). Our findings indicate that overexpression of *QQS* improves the nodule weight per plant, leading to increased nitrogen fixation.

3.4. Improved growth and nitrogen fixation of QQS transgenic plants under nitrogen-deficient conditions

To evaluate the performance of CK and Ls under low-nitrogen growth conditions, we cultured the plants under non-nitrogen (-N) and normal (+N) conditions and inoculated with *Rhizobium* bacterium (Fig. S4A and B). After 28 days of culture under -N condition, the leaves of CK plants showed obvious nitrogen-deficient symptoms (Fig. S4C), and the plant height was significantly shorter compared to Ls (Fig. S4E). Ls had a higher tiller number, as well as significantly higher dry matter and crude protein content than CK after -N treatment. However, under the +N treatment, the phenotype differences between WT and Ls were not significant (Fig. S4D and F).

Under the +N and -N treatments, Ls did not show any significant difference in terms of branch number, plant dry weight, crude protein content, and nodule fresh weight, except for the increase in crude protein content in L2 (Fig. 5A-D). In contrast, the WT plants showed significant improvements in these parameters under the +N treatment compared to the -N treatment. These results demonstrate that overexpression of *QQS* enhances alfalfa plant growth and nitrogen fixation under low-nitrogen stress.

3.5. QQS alters alfalfa carbohydrate composition and improves forage quality

Our study investigated the effect of overexpressing *QQS* on alfalfa, and found that it improved both carbohydrate composition and forage quality. Specifically, *QQS* overexpression improved alfalfa symbiotic nitrogen fixation by enhancing nodule growth, while photosynthesis rates were similar between CK and Ls plants, as well as net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate (Fig. S5A-D). Additionally, we performed leaf KI/I_2 staining and measured starch content, which showed no significant difference between CK and Ls (Fig. 6A and B). However, the soluble sugar content of L3 and L6 was significantly higher than CK (Fig. 6C).

We also examined the forage quality using phloroglucinol staining to detect lignin deposition in the stem. Our results indicated that *QQS* overexpression reduced the lignin content of alfalfa cell wall, as evidenced by the lighter red color in hand-sliced Ls stems compared to the CK (Fig. 6D). Furthermore, we found that NDF and ADF content were significantly lower in Ls plants than in WT plants, except for the NDF content of L2 (Fig. 6E and F). Similarly, ADL content was also significantly lower in Ls than CK, except for L6 (Fig. 6G). Notably, the NDF digestibility (NDFD) of L2, L3, and L6 was 43.5 %, 43.7 %, and 51.2 %, respectively, which was significantly higher than WT (34.1 %) (Fig. 6H). Additionally, the ADF digestibility (ADFD) of Ls was 39.4 %, 40.1 %, and 48.0 %, respectively, which was also significantly higher than WT (29.1 %) (Fig. 6I). Overall, our findings suggest that *QQS* overexpression significantly improved alfalfa forage quality by reducing cell wall lignin and cellulose content.

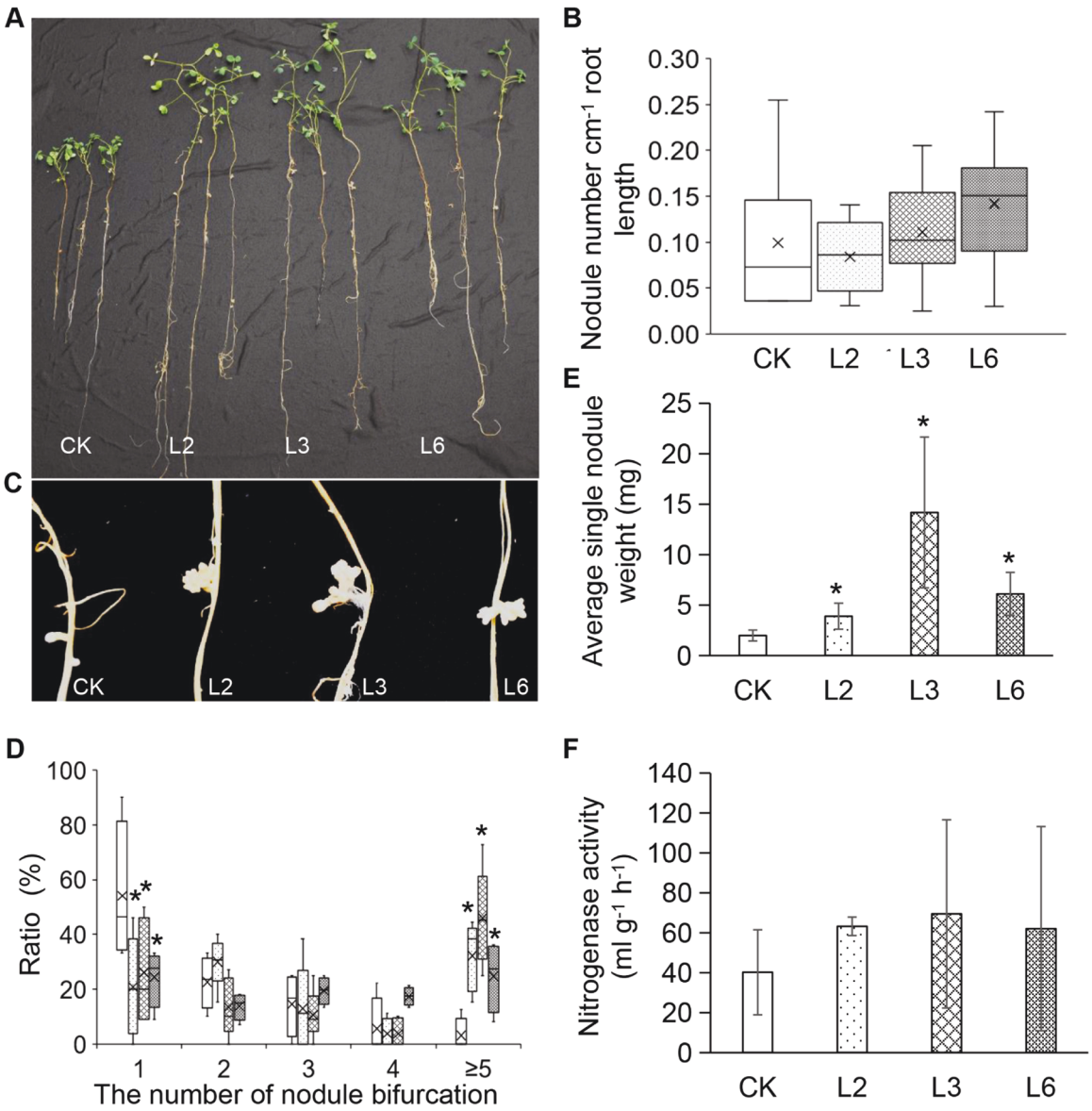

Fig. 4. *QQS* transgene enhances nodulation of transgenic alfalfa under low-nitrogen conditions. (A) Comparison of visual phenotype of CK and Ls under low-nitrogen (0.36 mM nitrogen) conditions. (B) Statistical analysis of nodule number per centimeter root. The data is presented as box plots with minimum to maximum plots, where the center lines represent the medians, "×" represents the mean value, and the down and up whiskers extend to the minimum and maximum values. At least 20 plants were used for measurement. (C) Comparison of nodules growing on CK and Ls. (D) Statistical analysis of the number of different nodule bifurcations accounts for the total nodule number per plant. (E) Statistical analysis of the average single nodule weight of CK and Ls. (F) Statistical analysis of the nitrogenase activity of CK and Ls. Values are presented as mean ± SD ($n = 20$) in D-F. The asterisk represents a significant difference ($P < 0.05$) by one-way ANOVA analysis.

3.6. QQS modulates the expression of genes involved in carbon and nitrogen metabolism as well as lignin biosynthesis

To uncover the potential molecular mechanism in improvement of protein content and forage quality by *QQS*, we characterized the global gene expression profiles between Ls and CK. The results showed that there were 548 up-regulated and 316 down-regulated expressed genes were detected in Ls plants compared to CK (Fig. S6). We performed qRT-PCR tests of ten randomly selected genes to validate the reliability of the RNA-seq data. The relative expression level of them was consistent with the results of RNA-Seq analysis (Fig. S7). The DEGs between Ls and CK enrich into 114 KEGG pathways, especially for protein processing in endoplasmic reticulum, starch and sucrose metabolism, biosynthesis of amino acid, and degradation of lysine, valine, leucine and isoleucine (Fig. S8 and Table S3). By examining the relative expression level of the genes involved in carbon and nitrogen metabolism, we found that the genes related to nitrogen metabolism, including *GS*, *PDH*, *GDH*, *PK*, *PT*, and *PEPC*, had significantly higher expression levels in Ls lines compared to WT (Fig. 7A). On the other hand, the expression of lignin biosynthesis-related genes, *F5H* and *PAL*, was significantly decreased in transgenic lines compared to WT (Fig. 7B). In contrast, the expression of *4CL* was significantly increased in transgenic lines (Fig. 7B). The relative expression of *CAD* was increased in L2, decreased in L6, and there was no significant difference in L3 (Fig. 7B). Moreover, the relative expression of *C4H* was increased in L2 and L6 plants, while the expression of CCR did not differ between transgenic plants and WT (Fig. 7B). Several DEGs associated amino acid biosynthesis and degradation were listed in Table S4. Among them, two genes associated with lysine degradation, AACT and 2-OGDH E1 subunit, were significant upregulated in Ls than in CK. Above that, we also found the nuclear factor Y, subunit C4, which reported bind with QQS, and subunit B5 significant upregulated in Ls compared them in CK respectively (Fig. 7C). These results suggest that

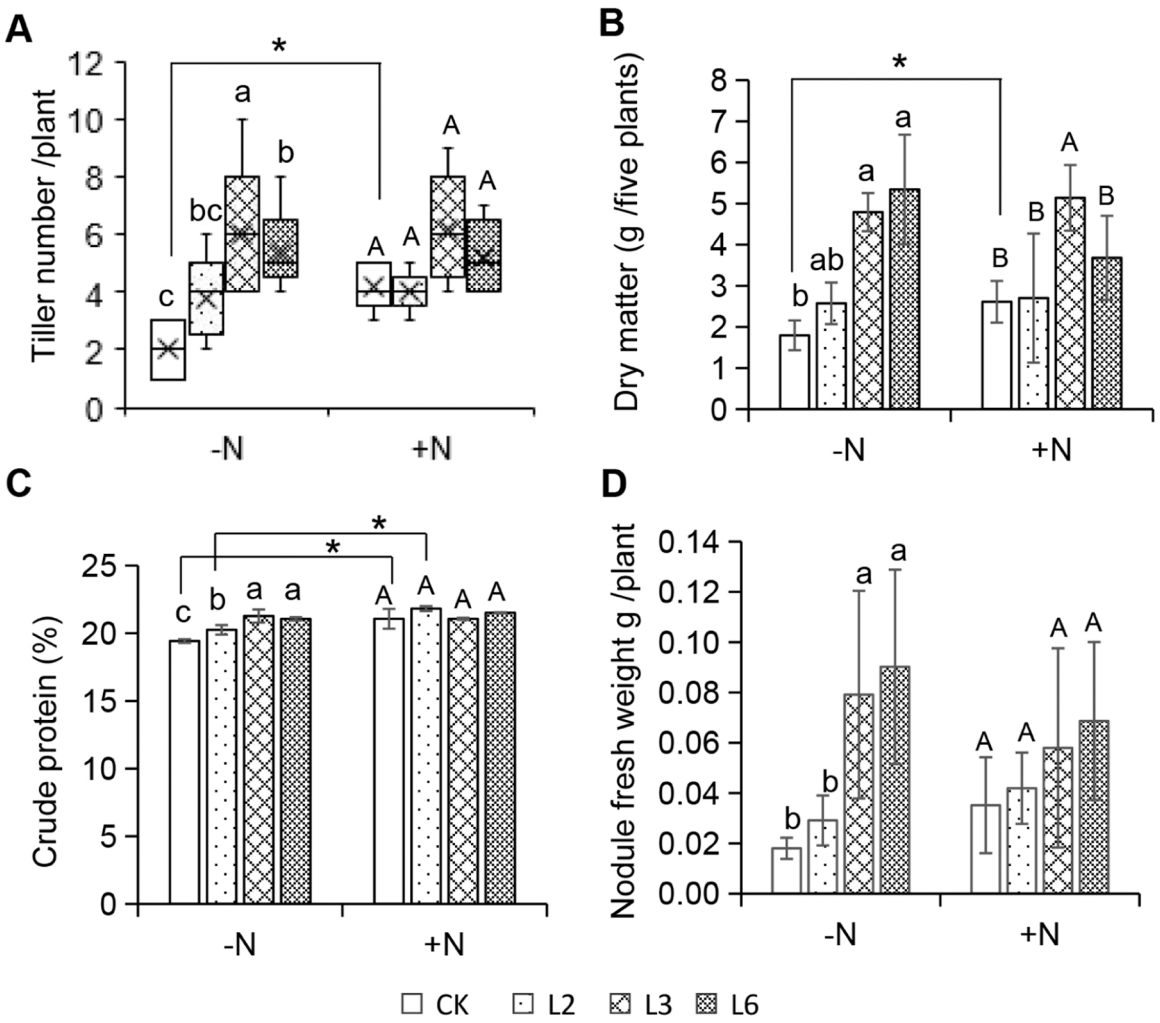

Fig. 5. The phenotypes of the plant and nodulation of CK and Ls under non-nitrogen (-N) and normal (+N) conditions. (A) Analysis of branch number per plant after growing for 28 days under -N and +N conditions. Fifteen plants per line were used for measurement and data were presented as box plots with minimum to maximum plots. Center lines represent the medians, "×" represents the mean value, and down and up whiskers extend to the minimum and maximum values. (B) Comparison of dry matter per five plants. Statistical analysis was conducted with three biological duplicates. (C) Comparison of crude protein content of CK and Ls. (D) Comparison of nodule fresh weight per plant. Values are presented as mean ± SD ($n = 5$). Different letters and an asterisk denote significant differences ($P < 0.05$) by one-way ANOVA analysis.

QQS affects the expression of genes involved in carbon and nitrogen metabolism, lignin biosynthesis as well as amino acid metabolism, contributing to the improved forage quality and nitrogen metabolism and altering amino acid content in transgenic alfalfa.

4. Discussion

Alfalfa is a crucial leguminous forage crop that provides high-quality feed for livestock. In this study, we aimed to improve alfalfa forage quality and crude protein content by generating transgenic plants overexpressing the Arabidopsis *QQS* gene, which is known to regulate carbon/nitrogen relocation and increase total protein content and decrease total starch content in Arabidopsis and food crops [3,18,21,31,32]. Surprisingly, we observed an increase in biomass yield in *QQS* transgenic alfalfa plants compared to the WT, which differs from previous reports in food crops [21,31,32]. Due to the complex genetic background of alfalfa [26], we carefully selected and compared the phenotype, forage quality, and nodulation of *QQS* transgenic plants (Ls) with those of segregated non-transgenic plants used as controls (CK).

Previous studies have shown that ectopic expression of *QQS* in rice and soybean did not affect plant morphology, development, and flowering time [19,21]. However, our results demonstrate that *QQS* overexpression in alfalfa led to an increase in biomass yield, which may be due to the regrowth and perennial characteristics of alfalfa. Interestingly, we also observed improved nodule weight in *QQS* transgenic plants, indicating enhanced nitrogen assimilation. This finding aligns with previous reports that increasing nitrogen assimilation can lead to increased branch number, growth, and protein content in plants [11,39]. We also tested the effect of nitrogen fertilizer on plant growth and protein content, and found that it significantly improved biomass yield and crude protein in CK but not in Ls plants. This suggests that the growth of CK was limited in the absence of nitrogen, while the Ls plants were able to sustain their growth. In contrast, *QQS* transgenic plants did not show any significant phenotypic changes in rice and maize [21], crops that lack a biological nitrogen-fixing system, suggesting that the increased nitrogen fixation in *QQS* transgenic alfalfa plants is responsible for the observed increase in tiller number and plant growth.

Alfalfa's total nitrogen content is mainly attributed to biological nitrogen-fixing by nodules and root nitrogen absorption from the soil [1]. *QQS* overexpression improved total nitrogen and crude protein content per gram of alfalfa above-ground materials, consistent with previous reports [21]. The molecular mechanism of QQS in *Arabidopsis* and crop species (i.e., soybean, rice, corn, tobacco and potato) has been established that QQS could replace NF-YB to interact with NF-YC (i.e.,

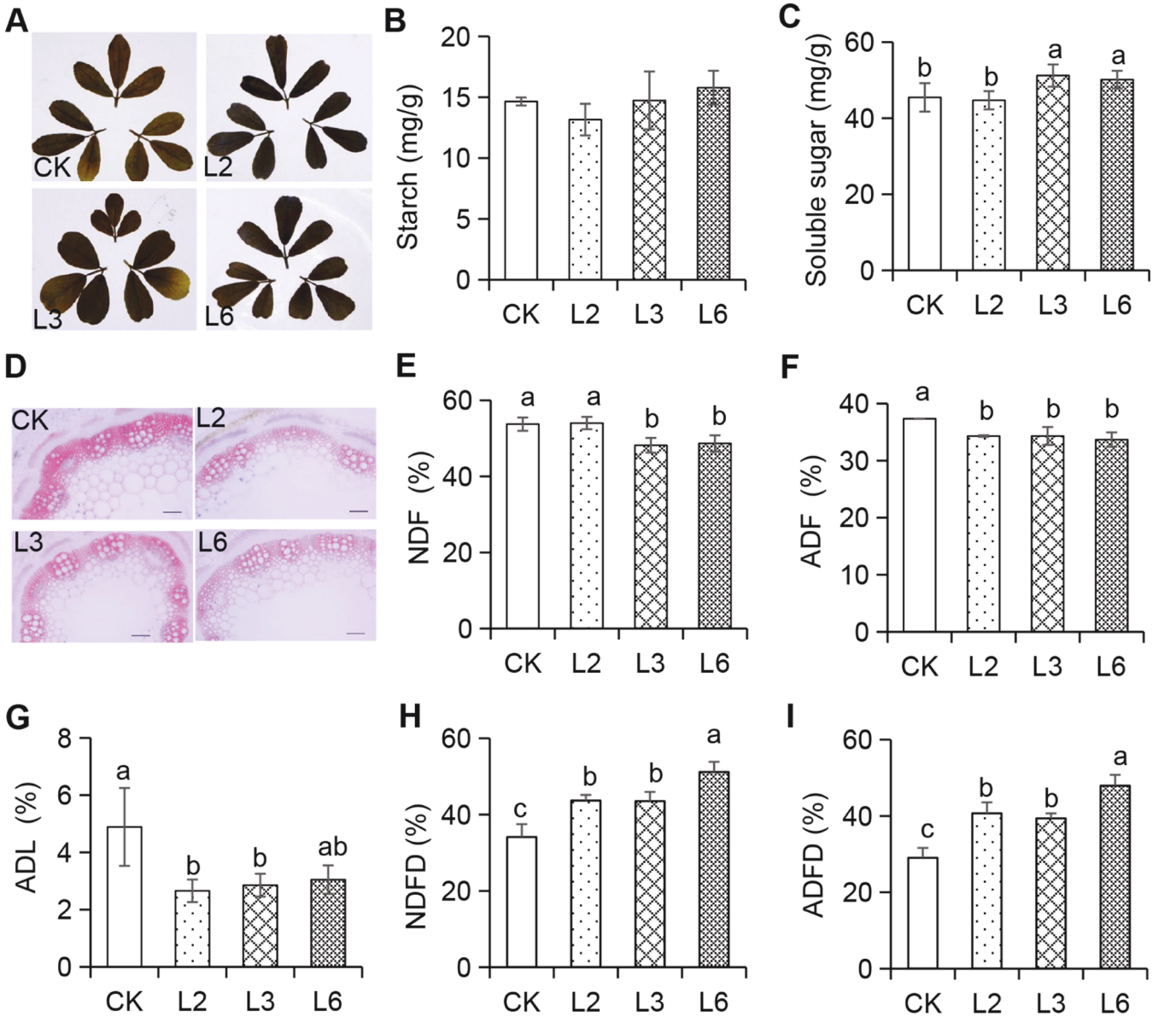

Fig. 6. *QQS* overexpression improves alfalfa forage quality. (A) Starch staining of leaves of CK and Ls using KI/I_2 stain. (B-C) Comparison of starch (B) and soluble sugar content (C). (D) Lignin staining assay of WT and Ls. The bar represents 100 μm. (E-H) Comparison of the NDF (E), ADF (F), and ADL (G) content of CK and Ls plants. (H-I) NDF digestibility (H) and ADF digestibility (I) of CK and Ls after 48 h of digestion in vitro. Values are presented as mean ± SD ($n = 3$). Different letters indicate significant differences ($P < 0.05$) by one-way ANOVA analysis.

NF-YC4) in the NF-Y trimeric complex [3,18,21,31,32]. Recent research showed that the interaction between QQS and the NF-YC subunit, does not need NF-YA subunit could down regulate starch content and increase total protein in *C. reinhardtii*. In *S. cerevisiae*, QQS appears to regulate glycogen level via the interaction of QQS with either only HAP5 (NF-YC homolog) or with HAP2 (NF-YA homolog), HAP3 (NF-YB homolog) or HAP4, and affect protein content via the interaction with the HAP complex that requires HAP2, HAP3, HAP4 and HAP5 [38]. It has reported that QQS interacting protein GmNF-YC4 improved nodule fresh weight per plant and crude protein content [41]. In this study, we also observed that *QQS* transgenic plants had improved alfalfa nodule fresh weight under low-nitrogen conditions. And, according to the results of RNA-seq and RT-qPCR, *MsNF-YC4* is significantly more highly expressed in *QQS* transgenic plants than in WT. Those results indicated that QQS may be interact with MsNF-YC4 to regulated nodule fresh weight and nitrogen content in alfalfa.

We also identified several key genes in nitrogen metabolism, such as glutamine synthetase 1 (*GS1*), proline dehydrogenase (*PDH*), and glycine dehydrogenase (*GDH*), that were significantly up-regulated in *QQS* transgenic plants. Similar to *QQS* transgenic alfalfa, increasing *GS1* expression levels in alfalfa led to higher biomass yield, nodule fresh weight per plant, and crude protein content [15], and ectopic expression of *GS1* in tobacco enhanced the utilization of nitrogen, increased biomass yield and leaf soluble protein in *GS1* transgenic plants [27]. Additionally, *PDH* and *PEPC* play important roles in nitrogen fixation, ammonia absorption, and assimilation of legume root nodules [2,30,33], which may contribute to the amount of nitrogen accumulation in *QQS* transgenic alfalfa plants. Notably, the number of nodule bifurcations was significantly increased in *QQS* transgenic alfalfa plants. In contrast to soybean, where *GmNF-YC4* overexpression did not affect nodule size and morphology [41], these results indicate the existence of other regulatory pathways modulating nodule formation and nitrogen fixation in alfalfa that require further study.

Photosynthesis provides carbohydrates and energy for plant growth

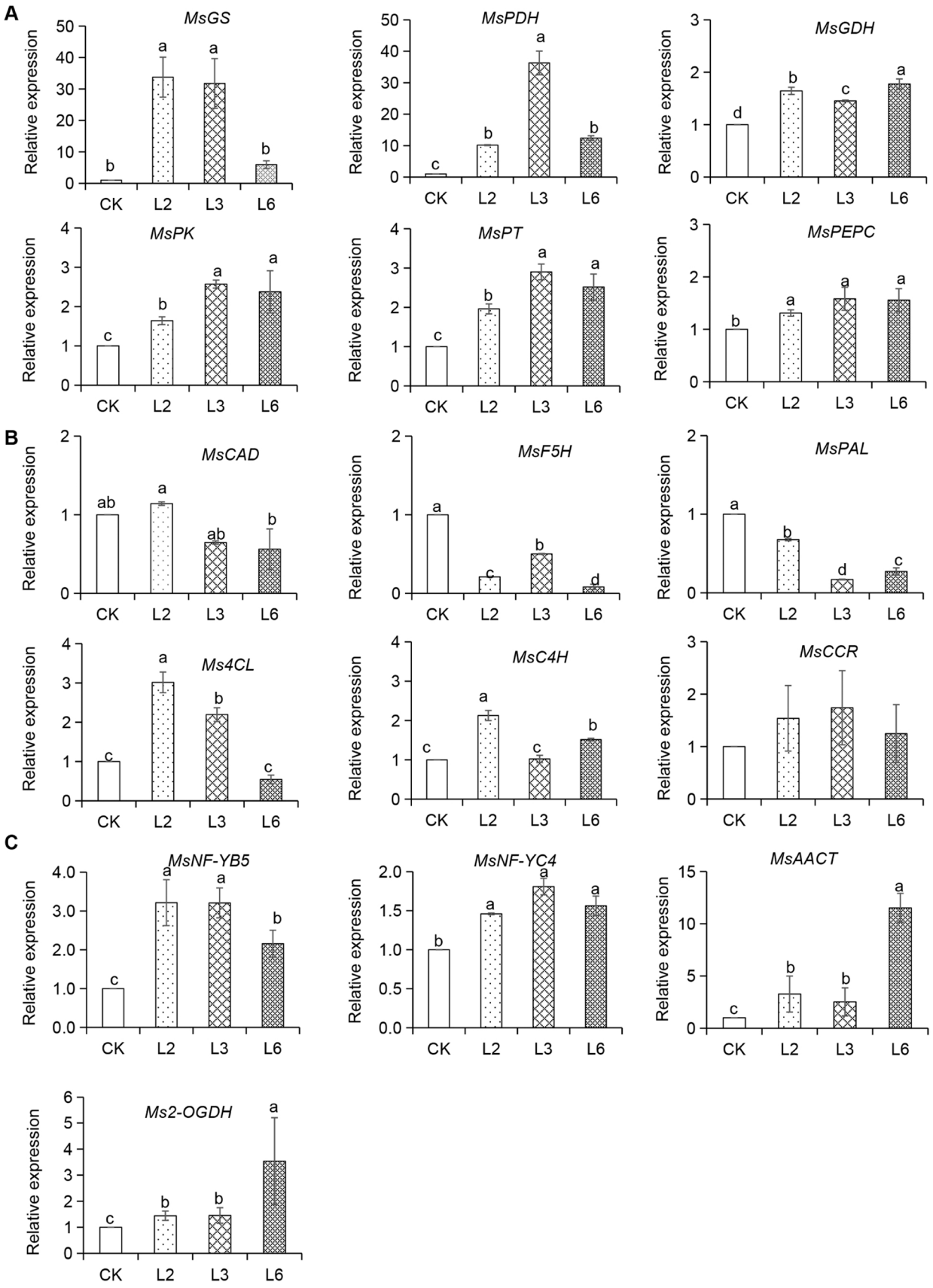

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Fig. 7. Expression of genes associated with carbon and nitrogen metabolism and lignin biosynthesis in CK and Ls alfalfa. (A) Relative expression of genes involved in carbon and nitrogen metabolism, including *GS* (glutamine synthetase), *PDH* (proline dehydrogenase), *GDH* (glycine dehydrogenase), *PK* (pyruvate kinase), *PT* (plastid transketolase), and PEPC (phosphoenolpyruvate carboxykinase). (B) Relative expression of genes associated with lignin biosynthesis, including *CAD* (cinnamyl alcohol dehydrogenase), *F5H* (ferulic acid-5-hydroxylase/coniferaldehyde-5-hydroxylase), *PAL* (phenylalanine ammonia-lyase), *4CL* (4-coumarate-CoA-ligase), *C4H* (cinnamate-4-hydroxylase), and *CCR* (cinnamoyl-CoA reductase). (C) Relative expression of genes including *NF-YC4* (transcription factor nuclear factor Y, subunit C4), *NF-YB5* (transcription factor nuclear factor Y, subunit B5), AACT (acetyl-CoA c-acetyltransferase), and 2-OGDH (2-oxoglutarate dehydrogenase E1 component). Values are shown as mean ± SD ($n = 3$). Statistical significance is represented by different letters ($P < 0.05$) as determined by one-way ANOVA analysis.

and development. Similar to food crops, the leaf photosynthesis rate showed no significant difference between *QQS* transgenic plants and CK in alfalfa, indicating *QQS* did not affect plant carbon fixation [21]. However, *QQS* did alter the carbohydrate composition ratio in alfalfa, leading to an increase in soluble sugar content and a decrease in cell wall cellulose content (NDF and ADF), which may be related to improved nitrogen assimilation in *QQS* transgenic alfalfa.

Sucrose, a photosynthetic product, is transported from leaves to roots to provide energy and carbon skeletons to nitrogen fixation in nodules [12]. The increased demand for root nodules in alfalfa may have affected carbon allocation, resulting in changes in the levels of certain amino acids. According to the results of global gene expression profiles between WT and Ls, DEGs significantly enrich into the biosynthesis and metabolism of amino acids KEGG pathways. Specifically, we found that the Lys level in transgenic alfalfa decreased by more than 30 %, while the levels of other amino acids increased significantly compared to the WT, which may be due to the higher expression of *MsAACT* and *Ms2-OGDH*, two genes enrichment into lysine degradation pathway, in Ls and need further study. Those results suggest that *QQS* altered the nitrogenous metabolites composition and can regulate carbon and nitrogen partitioning in alfalfa.

Interestingly, *QQS* did not decrease starch content in alfalfa, unlike other food crops [21,31,32]. This unexpected finding may be due to the upregulation of genes responsible for promoting starch accumulation, such as pyruvate kinase (*PK*) and plastid transketolase (*PT*) [5,14], which showed significantly higher expression in *QQS* transgenic alfalfa. This may explain the unexpected starch accumulation phenomenon in *QQS* transgenic alfalfa.

While *QQS* did not affect leaf photosynthesis rate, it did alter carbohydrate composition and amino acid levels in alfalfa, possibly through changes in carbon allocation. *QQS* also appears to regulate starch accumulation in a unique way, which warrants further investigation.

The presence of high lignin content in forage is known to have negative effects on forage digestibility and animal performance. In our study, we observed a significant reduction in lignin content in Ls alfalfa, which led to a significant increase in NDFD and ADFD. This effect may be attributed to the downregulation of *PAL* and *F5H*, two key enzymes in the lignin biosynthesis pathway [17], in the tested transgenic lines. Interestingly, we also found that the expression of *4CL* and C4H were up-regulated in Ls individuals, suggesting that *QQS* may have a role in modulating the expression of lignin biosynthesis genes in alfalfa. However, the precise molecular mechanisms by which *QQS* regulates lignin biosynthesis in alfalfa requires further investigation.

In conclusion, our study has shown that *QQS* overexpression improves several important agronomic traits of alfalfa, including biomass yield, soluble sugar content, crude protein content, and forage digestibility, as well as offering increased resistance to low-nitrogen stress. Furthermore, our results suggest that these beneficial traits are heritable and can be passed on to the next generation of plants. These findings highlight the potential of *QQS* as a valuable tool for improving alfalfa forage quality and yield, with potential benefits for animal nutrition and the livestock industry. However, further research is necessary to fully elucidate the underlying mechanisms involved in the QQS-mediated improvements and to assess any potential environmental impacts associated with *QQS* overexpression in alfalfa.

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CRediT authorship contribution statement

KW, YL and WZ conceived and designed research. KW and JY conducted experiments. LL and RT made the *QQS* expression construct. KW, RT, LL, YL and WZ wrote the manuscript. All authors read and approved the paper.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2023.100295.

References

[1] L. Aleman, J. Ortega, M. Martinez-Grimes, M. Seger, F. Holguin, D. Uribe, D. Garcia-Ibilcieta, C. Sengupta-Gopalan, Nodule-enhanced expression of a sucrose phosphate synthase gene member (*MsSPSA*) has a role in carbon and nitrogen metabolism in the nodules of alfalfa (*Medicago sativa* L.), Planta 231 (2) (2010) 233–244, https://doi.org/10.1007/s00425-009-1043-y.

[2] D. Aleksza, G. Horváth, G. Sándor, L. Szabados, Proline accumulation is regulated by transcription factors associated with phosphate starvation, Plant Physiol. 175 (1) (2017) 555–567, https://doi.org/10.1104/pp.17.00791.

[3] Z. Arendsee, L. Li, E. Wurtele, Coming of age: orphan genes in plant, Trends Plant Sci. 19 (11) (2014) 698–708, https://doi.org/10.1016/j.tplants.2014.07.003.

[4] J. Barros, S. Temple, R. Dixon, Development and commercialization of reduced lignin alfalfa, Curr. Opin. Biotechnol. 56 (2019) 48–54, https://doi.org/10.1016/j.copbio.2018.09.003.

[5] Y. Cai, W. Zhang, J. Jin, X. Yang, X. You, H. Yan, et al., *OsPKpa1* encodes a plastidic pyruvate kinase that affects starch biosynthesis in the rice endosperm, J. Integr. Plant Biol. 60 (11) (2018) 1097–1118, https://doi.org/10.1111/jipb.12692.

[6] Z. Dai, Z. Wu, S. Jia, G. Wu, Analysis of amino acid composition in proteins of animal tissues and foods as pre-column o-phthaldialdehyde derivatives by HPLC with fluorescence detection, J. Chromatogr. B 964 (2014) 116–127, https://doi.org/10.1016/j.jchromb.2014.03.025.

[7] Z. Dai, Z. Wu, J. Wang, X. Wang, S. Jia, F. Bazer, G. Wu, Analysis of polyamines in biological samples by HPLC involving pre-column derivatization with o-phthalaldehyde and *N*-acetyl-L-cysteine, Amino Acids 46 (6) (2014) 1557–1564, https://doi.org/10.1007/s00726-014-1717-z.

[8] J. Deng, F. Zhu, J. Liu, Y. Zhao, J. Wen, T. Wang, J. Dong, Transcription factor bHLH2 represses *CYSTEINE PROTEASE77* to negatively regulate nodule

senescence, Plant Physiol. 181 (4) (2019) 1683–1703, https://doi.org/10.1104/pp.19.00574.
[9] C. Diaz, T. Lemaître, A. Christ, M. Azzopardi, Y. Kato, F. Sato, et al., Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in Arabidopsis under low nitrogen nutrition, Plant Physiol. 147 (3) (2008) 1437–1449, https://doi.org/10.1104/pp.108.119040.
[10] M. Dubois, K. Gilles, J. Hamilton, P. Rebers, F. Simith, A colorimetric method for the determination of sugars, 167–167, Nature 168 (4265) (1951), https://doi.org/10.1038/168167a0.
[11] L. Gao, W. Bai, M. Xia, C. Wan, M. Wang, P. Wang, X. Gao, J. Gao, Diverse effects of nitrogen fertilizer on the structural, pasting, and thermal properties of common buckwheat starch, Int. J. Biol. Macromol. 179 (2021) 542–549, https://doi.org/10.1016/j.ijbiomac.2021.03.045.
[12] S. Gebril, M. Seger, F. Villanueva, J. Ortega, S. Bagga, C. Sengupta-Gopalan, Transgenic alfalfa (*Medicago sativa*) with increased sucrose phosphate synthase activity shows enhanced growth when grown under N_2-fixing conditions, Planta 242 (4) (2015) 1009–1024, https://doi.org/10.1007/s00425-015-2342-0.
[13] D. Hannaway, P. Shuler, Nitrogen fertilization in alfalfa production, J. Prod. Agric. 6 (1) (1993) 80–85, https://doi.org/10.2134/jpa1993.0080.
[14] S. Henkes, U. Sonnewald, R. Badur, R. Flachmann, M. Stitt, A small decrease of plastid transketolase activity in antisense tobacco transformants has dramatic effects on photosynthesis and phenylpropanoid metabolism, Plant Cell 13 (3) (2001) 535–551, https://doi.org/10.1105/tpc.13.3.535.
[15] H. Kaur, A. Peel, K. Acosta, S. Gebril, J. Ortega, C. Sengupta-Gopalan, Comparison of alfalfa plants overexpressing glutamine synthetase with those overexpressing sucrose phosphate synthase demonstrates a signaling mechanism integrating carbon and nitrogen metabolism between the leaves and nodules, Plant Direct 3 (1) (2019), e00115, https://doi.org/10.1002/pld3.115.
[16] J. Kjeldahl, Neue methode zur bestimmung des stickstoffs in organischen Körpern, Z. Anal. Chem. 22 (1) (1883) 366–382, https://doi.org/10.1007/BF01338151.
[17] Y. Lei, A. Hannoufa, P. Yu, The use of gene modification and advanced molecular structure analyses towards improving alfalfa forage, Int. J. Mol. Sci. 18 (2) (2017) 298–311.
[18] L. Li, C. Foster, Q. Gan, D. Nettleton, M. James, A. Myers, E. Wurtele, Identification of the novel protein QQS as a component of the starch metabolic network in Arabidopsis leaves, Plant J. 58 (3) (2009) 485–498, https://doi.org/10.1111/j.1365-313X.2009.03793.x.
[19] L. Li, E. Wurtele, The *QQS* orphan gene of Arabidopsis modulates carbon and nitrogen allocation in soybean, Plant Biotechnol. J. 13 (2) (2015) 177–187, https://doi.org/10.1111/pbi.12238.
[20] X. Li, Y. Zhang, A. Hannoufa, P. Yu, Trnsformation with *TT8* and *HB12* RNAi constructs in model forage (*Medicago sativa*, Alfalfa) affects carbohydrate structure and metabolic characteristics in ruminant livestock systems, J. Agric. Food Chem. 63 (43) (2015) 9590–9600, https://doi.org/10.1021/acs.jafc.5b03717.
[21] L. Li, W. Zheng, Y. Zhu, H. Ye, B. Tang, Z. Arendsee, et al., *QQS* orphan gene regulates carbon and nitrogen partitioning across species via NF-YC interactions, Proc. Natl. Acad. Sci. U. S. A. 112 (47) (2015) 14734–14739, https://doi.org/10.1073/pnas.1514670112.
[22] Z. Li, R. Long, T. Zhang, Q. Yang, J. Kang, Molecular cloning and characterization of the *MsHSP17.7* gene from *Medicago sativa* L, Mol. Biol. Rep. 43 (8) (2016) 815–826, https://doi.org/10.1007/s11033-016-4008-9.
[23] X. Li, H. Feng, J. Wen, J. Dong, T. Wang, MtCAS31 aids symbiotic nitrogen fixation by protecting the leghemoglobin MtLb120-1 under drought stress in *Medicago truncatula*, Front. Plant Sci. 9 (2018) 633–647, https://doi.org/10.3389/fpls.2018.00633.
[24] Y. Liu, H. Cen, J. Yan, Y. Zhang, W. Zhang, Inside out: high-efficiency plant regeneration and *Agrobacterium*-mediated transformation of upland and lowland switchgrass cultivars, Plant Cell Rep. 34 (7) (2015) 1099–1108, https://doi.org/10.1007/s00299-015-1769-x.
[25] K. Livak, T. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method, Methods 25 (4) (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[26] C. Lorenzo, P. García-Gagliardi, M. Antonietti, M. Sánchez-Lamas, E. Mancini, C. Dezar, et al., Improvement of alfalfa forage quality and management through the down-regulation of *MsFTa1*, Plant Biotechnol. J. 18 (4) (2020) 944–954, https://doi.org/10.1111/pbi.13258.
[27] I. Oliveira, T. Brears, T. Knight, A. Clark, G. Coruzzi, Overexpression of cytosolic glutamine synthetase. relation to nitrogen, light, and photorespiration, Plant Physiol. 129 (3) (2002) 1170–1180, https://doi.org/10.1104/pp.020013.
[28] A. Pell, R. Pitt, P. Doane, P. Schofield, The development, use and application of the gas production technique at Cornell University, USA, BSAP Occasional Publication 22 (1998) (1998) 45–54, https://doi.org/10.1017/S0263967×00032262.
[29] M. Poret, B. Chandrasekar, R. van der Hoorn, J. Avice, Characterization of senescence-associated protease activities involved in the efficient protein remobilization during leaf senescence of winter oilseed rape, Plant Sci. 246 (2016) 139–153, https://doi.org/10.1016/j.plantsci.2016.02.011.
[30] L. Szabados, A. Savouré, Proline: a multifunctional amino acid, Trends Plant Sci. 15 (2) (2010) 89–97, https://doi.org/10.1016/j.tplants.2009.11.009.
[31] R. Tanvir, L. Wang, A. Zhang, L. Li, Orphan genes in crop improvement: enhancing potato tuber protein without impacting yield, Plants 11 (22) (2022) 3076–3090, https://doi.org/10.3390/plants11223076.
[32] R. Tanvir, W. Ping, J. Sun, M. Cain, X. Li, L. Li, *AtQQS* orphan gene and *NtNF-YC4* boost protein accumulation and pest resistance in tobacco (*Nicotiana abacum*), Plant Sci. 317 (2022) 111198–111207, https://doi.org/10.1016/j.plantsci.2022.111198.
[33] M. Takeya, M. Hirai, T. Osanai, Allosteric inhibition of phosphoenolpyruvate carboxylases is determined by a single amino acid residue in cyanobacteria, 41080–41088, Sci. Rep. 7 (2017), https://doi.org/10.1038/srep41080.
[34] M. Theodorou, R. Lowman, Z. Davies, D. Cuddeford, E. Owen, Principles of techniques that rely on gas measurement in ruminant nutrition, BSAP Occas. Publ. 22 (1998) 55–63, https://doi.org/10.1017/S0263967×00032274.
[35] Z. Tong, C. Xie, Li Ma, L. Liu, Y. Jin, J. Dong, T. Wang, Co-expression of bacterial aspartate kinase and adenylylsulfate reductase genes substantially increases sulfur amino acid levels in transgenic alfalfa (*Medicago sativa* L.), PloS One 9 (2) (2014), e88310, https://doi.org/10.1371/journal.pone.0088310.
[36] P. Van Soest, J. Robertson, B. Lweis, Methods for dietary fiber, neutral detergent fiber and nonstarch polysaccharides in relation to animal nutrition, J. Dairy Sci. 74 (10) (1991) 3583–3597, https://doi.org/10.3168/jds.S0022-0302(91)78551-2.
[37] M. Vlahova, G. Stefanova, P. Petkov, A. Barbulova, D. Petkova, P. Kalushkov, A. Atanassov, Genetic modification of alfalfa (*Medicago sativa* L.) for quality improvement and production of novel compounds 19 (3) (2005) 56–62, https://doi.org/10.1080/13102818.2005.10817286.
[38] L. Wang, A. Tonsager, W. Zheng, Y. Wang, D. Stessman, K. Stenback, et al., Single-cell genetic models to evaluate orphan gene function: the case of QQS regulating carbon and nitrogen allocation, Front. Plant Sci. 14 (2023) 1126139, https://doi.org/10.3389/fpls.2023.1126139.
[39] J. Yu, W. Xuan, Y. Tian, L. Fan, J. Sun, W. Tang, et al., Enhanced OsNLP4–OsNiR cascade confers nitrogen use efficiency by promoting tiller number in rice, Plant Biotechnol. J. 19 (1) (2021) 167–176, https://doi.org/10.1111/pbi.13450.
[40] W. Zhang, T. Wang, Enhanced salt tolerance of alfalfa (*Medicago sativa*) by *rstB* gene transformation, Plant Sci. 234 (2015) 110–118, https://doi.org/10.1016/j.plantsci.2014.11.016.
[41] Q. Zhuang, Y. Xue, Z. Yao, S. Zhu, C. Liang, H. Liao, J. Tian, Phosphate starvation responsive GmSPX5 mediates nodule growth through interaction with GmNF-YC4 in soybean (*Glycine max*), Plant J. 108 (5) (2021) 1422–1438, https://doi.org/10.1111/tpj.15520.
[42] R. Zhai, Y. Feng, H. Wang, X. Zhan, X. Shen, W. Wu, et al., Transcriptome analysis of rice root heterosis by RNA-Seq, BMC Genom. 14 (19) (2013), PMC3556317, https://doi.org/10.1186/1471-2164-14-19.