scientific reports

Check for updates

OPEN

Overexpression *MsJAR1* gene increased lateral branches and plant height in alfalfa (*Medicago sativa* L.)

Rui Dai[1,2], Qi Chen[1,2], Xiao-yu Wang[1,2], Xiao-wei Huo[1,2], Jian-wei Li[1,2], Jiang-bo An[1,2], Fu-gui Mi[1,2,3], Feng-ling Shi[1,2] & Zhi-qiang Zhang[1,2,3]✉

Alfalfa (*Medicago sativa* L.) is an important legume forage known for its high yield, quality, and adaptability. However, due to its cross-pollination and tetraploid inheritance characteristics, biotechnologies such as genetic modification and gene editing are challenging to be utilized in the practice of alfalfa breeding. In this study, a *MsJAR1* gene involved in jasmonic acid (JA)pathway was overexpressed into 'Zhongmu No.1' alfalfa, and the effects of *MsJAR1* gene on alfalfa development were analyzed. Subcellular localization results indicated that the MsJAR1 protein was located to chloroplast. Compared to wildtype (WT), *MsJAR1* overexpressed alfalfa plants displayed smaller leaves, reduced internode distance, increased lateral branches and plant height. Moreover, anatomical analysis revealed that xylem and phloem area decreased in leaves but increased in stems of *MsJAR1* overexpressed alfalfa. Except intercellular CO_2, photosynthetic rates, stomatal conductance, and transpiration were significantly higher than in *MsJAR1* overexpressed alfalfa. Comparative transcriptomics results showed that 12,046 DEGs were identified between transgenic alfalfa (OE-1 and OE-2) and WT. The DEGs were significantly enriched in ribosome, glycolysis / gluconeogenesis and tricarboxylic acid cycle pathways. Therefore, it is speculated that the *MsJAR1* gene affects the growth and development of alfalfa by regulating photosynthesis-related pathways.

Keywords Alfalfa, *MsJAR1* gene, Functional analysis, Transcriptome

Alfalfa, a leguminous plant, features a well-developed root system capable of nitrogen fixation, thereby improving soil quality. Its stems are erect, reaching heights of approximately 30 to 100 cm, with leaves arranged in trifoliate compound form[1]. As a leguminous plant, alfalfa can form a symbiotic relationship with rhizobia, facilitating nitrogen fixation and thereby effectively enhancing soil fertility. Furthermore, alfalfa can be propagated through cuttings, a method that preserves the desirable traits of the maternal plant, thereby facilitating variety improvement and large-scale cultivation of alfalfa[2]. Recognized globally as a significant forage crop due to its outstanding nutritional quality and adaptability, alfalfa thrives best in temperatures ranging from 22 °C to 30 °C. It can withstand temperatures as low as -15 °C to -30 °C, exhibiting high cold tolerance. When provided with sufficient humidity, it can survive in temperatures between 37 °C and 42 °C[3]. Alfalfa is considered as a promising commercial bioenergy crop due to its ability of improving soil quality through biological nitrogen fixation, making it more conducive for the growth of subsequent crops[4]. Challenges such as tetraploid nature, self-incompatibility, and allogamy mechanisms pose significant obstacles to understanding its essential agronomic traits in alfalfa, which hinders the identification of key genes and slows down progress in applying biotechnological breeding techniques. Emerging breeding methods, such as transgenic approaches, show significant potential for enhancing crop quality, stress resistance, and yield, leading to the development of diversified, superior varieties[5]. With the rapid advancement of modern biotechnology, the application of transgenic technology is becoming more widespread and easier[6]. Stable genetic transformation systems have been established in forage crops such as alfalfa, *Medicago truncatula* L., and *Lolium perenne* L. Functional genes have been extensively explored through

[1]College of Grassland Science, Inner Mongolia Agricultural University, Inner Mongolia Autonomous Region, Hohhot 010010, China. [2]Key Laboratory of Grassland Resources, Ministry of Education, Inner Mongolia Agricultural University, Hohhot 010010, China. [3]Technology Engineering Center of Drought and Cold-resistant Grass Breeding in North of the National Forestry and Grassland Administration, College of Grassland Science, Inner Mongolia Agricultural University, Hohhot 010010, China. ✉email: zhangzq1989@imau.edu.cn

genetic transformation and widely applied in alfalfa breeding to improve growth, development, yield, stress resistance, and adaptability[7].

The study involved the cloning of the BAN homologous gene (*OvBAN*) from saponin and the introduction of its cDNA into alfalfa through Agrobacterium-mediated transformation. The overexpression of the *OvBAN* gene significantly increased the proanthocyanidin (PA) content in alfalfa[8]. The study introduced the *ZxABCG11* gene from the xerophytic plant Zygophyllum xanthoxylum into the alfalfa genome through Agrobacterium-mediated transformation. The overexpression of the *ZxABCG11* gene resulted in the upregulation of wax-related genes, which significantly increased the deposition of epidermal wax in alfalfa. Consequently, this led to reduced cuticular permeability, improved water-holding capacity, and enhanced photosynthetic ability in transgenic alfalfa. Therefore, the overexpression of the *ZxABCG11* gene can simultaneously enhance biomass yield, drought resistance, and heat tolerance in alfalfa by increasing epidermal wax deposition[9]. Other study introduced the dehydrin *CsLEA* from the desert grass Cleistogenes songorica into alfalfa using Agrobacterium-mediated transformation. The overexpression of the *CsLEA* gene resulted in enhanced drought and salt tolerance in transgenic alfalfa. Furthermore, the incorporation of the bar gene into the genome may contribute to increased herbicide resistance[10]. Transcription factors including *NAC*, *AP2*, and *ERF* were found playing an important regulatory role in aphid control[11,12]. Overexpression of the *MIM396* gene increases alfalfa resistance to *Spodoptera frugiperda* larvae.

JAR1 (jasmonic acid-amido synthetase) is an enzyme that catalyzes the conversion of jasmonoyl isoleucine (JA-ile) into its bioactive form. The bioactive JA-Ile induces the JA signaling pathway and plays an important role in regulating plant defense and the synthesis of secondary metabolites[13]. The *JAR1* gene is an important component in regulating the JA signaling pathway. JA-Ile, as a bioactive conjugate, binds to specific receptors to activate JA-mediated defense responses, enhancing plant tolerance to abiotic and biotic stresses[14]. The *JAR1* gene was initially identified in soybean and has since been characterized in a wide range of plant species, including Arabidopsis, rice, grape, peach, sorghum, mulberry and poplar[15–17]. The *JAR1* gene is involved in multiple signaling pathways, including plant hormone signaling, light signaling, and defense responses. Studies have shown that *TaJAR1B*, a functional *JAR1* homolog, is highly expressed in the internodes of mature wheat plants. It plays a role in plant growth and development and regulates wheat's response to salt stress[18]. In Arabidopsis, overexpression of the *JAR1* gene increases JA-Ile content, thereby improving plant tolerance to drought stress[19]. In addition, this study reveals that in homozygous Arabidopsis *JAR1* mutants, the expression level of *JAR1* gene is significantly reduced, to only 41.2% of that in wild-type plants, leading to increased sensitivity of the mutants to methyl jasmonate[20]. Previously, we identified a *JAR1* gene in alfalfa through preliminary comparative transcriptome analysis. Gene cloning and expression pattern analysis revealed that the *MsJAR1* gene exhibits the highest expression levels in mature leaves[21]. To further investigate the function of this gene, we compared morphology traits, anatomical structure, photosynthetic parameters, and transcriptome profiles between *MsJAR1* overexpressed alfalfa and WT plants. Our findings hold significant implications for understanding the role of the *JAR1* gene and its potential applications in breeding programs, providing valuable insights for future research.

Result

Subcellular localization of MsJAR1

In *Nicotiana benthamiana*, JAR1 were predicted to localize in chloroplast (http://psort.hgc.jp/). To examine the subcellular localization of MsJAR1, laser confocal microscopy was employed following injection method, with empty vector pAN580-GFP as control. Results revealed that pAN580-*MsJAR1* predominantly localized in chloroplasts (green fluorescence), co-localizing with chloroplast autofluorescence (red fluorescence); whereas GFP fluorescence of the empty vector appeared diffusely distributed throughout the cell (Fig. 1).

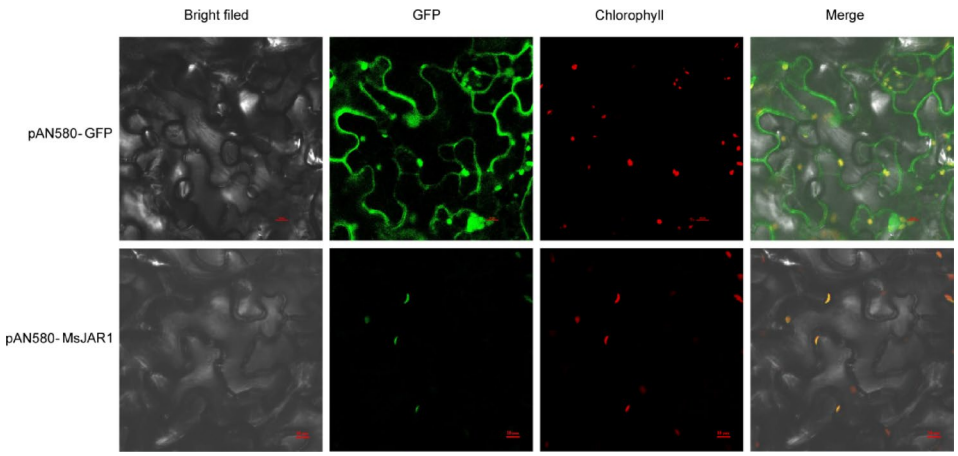

Fig. 1. Subcellular localization of MsJAR1 protein.

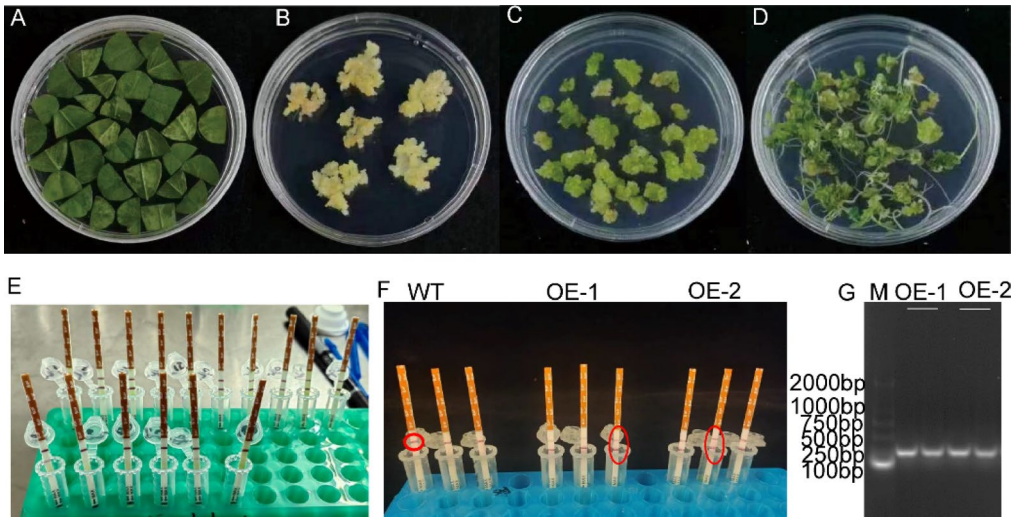

Fig. 2. The regeneration process and positive verification of alfalfa. (**A**) co-cultivation; (**B**) callus tissue; (**C**) differentiate to form resistant shoots; (**D**) take root; (**E**) Bar Rapid Test; (**F**) selected two transgenic alfalfa lines; (**G**) PCR validation of Bar gene sequence, M: maker 2000.

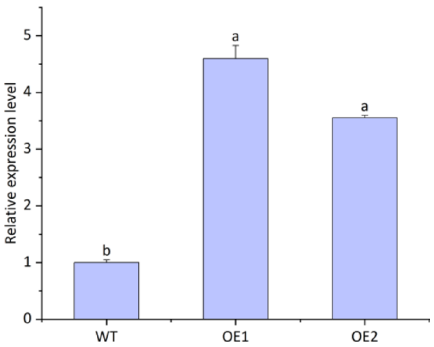

Fig. 3. qRT-PCR analysis of *MsJAR1* gene.

Generation and validation analysis of transgenic alfalfa plants

The process of introducing the *MsJAR1* gene into the alfalfa variety 'Zhongmu No.1' via Agrobacterium-mediated transformation involves the following steps: First, explants are co-cultivation with Agrobacterium to enable T-DNA transfer and promote callus formation. (Fig. 2A, B). In the next step, phosphinothricin (PPT) resistance screening is performed to select callus tissues that exhibit resistance. Resistant shoots are then observed emerge from the edges of the selected callus tissues. (Fig. 2C). These resistant shoot callus tissues are then transferred to rooting media (Fig. 2D), and after acclimatization, transferred to pots for further cultivation. Following the generation of transgenic alfalfa plants, the Bar Rapid Test (Artron Bio Research Inc) was utilized to assess the presence of positive reactions in the adapted plants (Fig. 2E). Subsequently, two transgenic alfalfa lines were selected from these positive plants (Fig. 2F), and genomic DNA was extracted from each for use as templates in PCR validation. PCR validation was conducted using herbicide-specific primers, resulting in the detection of fragments matching the target Bar gene sequence in both regenerated alfalfa plants (Fig. 2G). These results confirm the successful integration of the *MsJAR1* gene into the genomes of two alfalfa lines, designated as transgenic alfalfa (OE-1 and OE-2). These transgenic alfalfa plants will be further utilized for experimental research. qRT-PCR analysis demonstrated that the expression levels of the *MsJAR1* gene in the OE-1 and OE-2 lines were significantly higher than those observed in the WT ($P < 0.05$), reaching approximately three to four times the expression level of WT (Fig. 3).

Effect of the *MsJAR1* gene on the phenotype of alfalfa

After propagating the transgenic alfalfa through cuttings, phenotypic identification was performed on various traits, including leaf morphology, internode distances, plant heights during the vegetative growth stage, and the number of lateral branches observed in the late vegetative growth stage. The results indicated that the transgenic alfalfa primarily exhibits reduced leaf size (Fig. 4A), shortened internode distance (Fig. 4B), increased branching (Fig. 4C), and enhanced plant height (Fig. 4D). According to measurements based on plant phenotypes, in the transgenic alfalfa (OE-1 and OE-2), both leaf area and internode distance were significantly lower than in WT

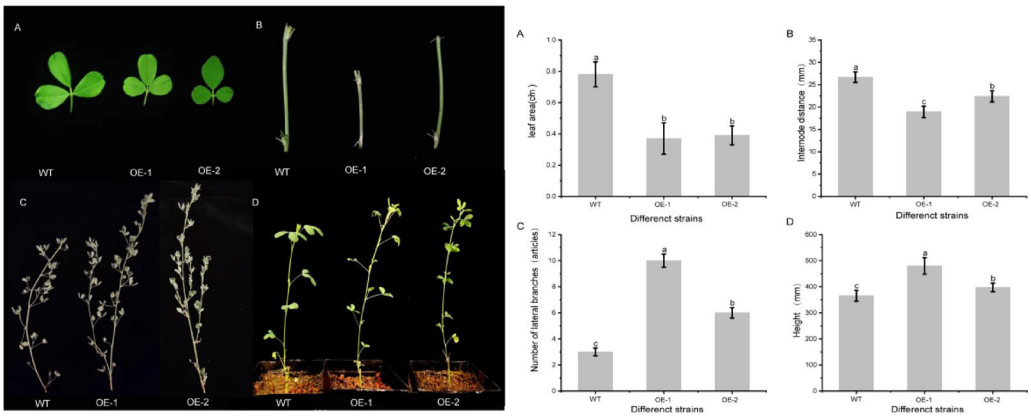

Fig. 4. Phenotypic Characterization and Analysis of *MsJAR1* Transgenic Alfalfa. Left-hand side (**A**) The leaf phenotypes of alfalfa; (**B**) Phenotype of internode distance in alfalfa; (**C**) Phenotype of lateral branch number in alfalfa; (**D**) Phenotype of plant height in alfalfa. Right-hand side (a) Blade size; (b) Comparison of intersection distances; (c) Number of lateral branches; (d) plant height; $P < 0.05$.

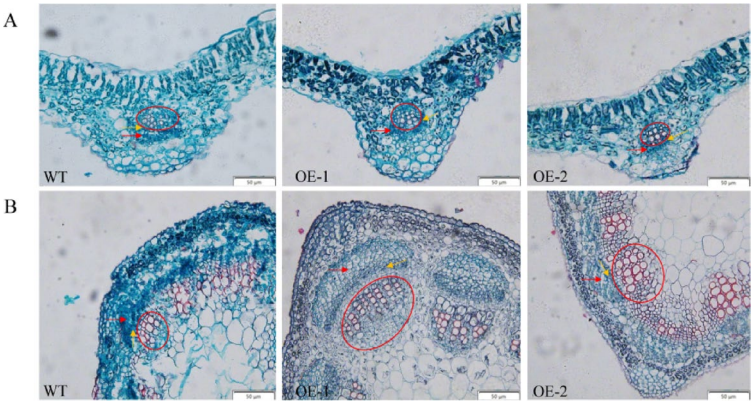

Fig. 5. Structure of alfalfa stems and leaves in cross section. (**A**) Leaf anatomical structure; (**B**) Stem anatomical structure. Red circles represent xylem; red arrows represent phloem; yellow arrows represent cambium; the cambium is located between the xylem and the phloem.

(Fig. 4 ab). However, the number of lateral branches and plant heights were significantly higher than in the control group (Fig. 4cd).

Effect of the *MsJAR1* gene on leaf and stem anatomical structure

The results indicated that the area of the phloem and xylem in the leaves of *MsJAR1* transgenic alfalfa (OE-1 and OE-2) was significantly smaller than that of the WT (Fig. 5A), while the area of the phloem and xylem in the stems of OE-1 and OE-2 was significantly larger than that of WT (Fig. 5B). Specifically, the areas of the phloem, cambium, xylem, and vascular bundle in leaves of OE-1 and OE-2 were significantly lower than those of WT ($P < 0.05$). The phloem and cambium areas of OE-2 leaves were 0.47 times and 0.42 times of WT leaves, respectively. Conversely, the areas of the phloem, cambium, xylem, and vascular bundle in the stems of OE-1 and OE-2 were significantly higher than those of WT ($P < 0.05$), with the phloem area in the stem of OE-1 being the most significant, at 2.3 times of WT (Table 1). In summary, the *MsJAR1* gene can regulate plant growth and development by affecting the area of the xylem and phloem.

Effect of the *MsJAR1* gene on photosynthetic parameters in alfalfa

The research findings indicated that under identical growth conditions, *MsJAR1* overexpressed alfalfa, designated as OE-1 and OE-2, exhibited higher stomatal conductance (GS), net photosynthetic rate (Pn) and transpiration rate (TR) compared to WT plants. Specifically, the average GS, Pn, and TR of the transgenic alfalfa were higher than those of WT (2.9-fold, 1.7-fold, and 1.5-fold, respectively) (Fig. 6A, C, D). It was noteworthy that the stomatal internal CO_2 concentration (Ci) in *MsJAR1* overexpressed alfalfa was significantly lower than that in WT, approximately half of the transgenic lines (Fig. 6B). These observations suggested that overexpression of the *MsJAR1* gene might enhance carbon dioxide consumption during photosynthesis, thereby providing more energy sources for plants growth. This finding held significant implications for physiological regulation during plant growth ($P < 0.05$).

Project	Sample	Phloem (μm²)	Cambium (μm²)	Xylem (μm²)	Vascular bundle (μm²)
Leaft	WT	3635.69 ± 209.80[a]	3482.80 ± 349.69[a]	4089.62 ± 290.31[a]	11021.09 ± 664.54[a]
	OE-1	2082.39 ± 110.52[b]	1732.11 ± 98.62[b]	2194.69 ± 109.46[b]	6009.19 ± 258.55[b]
	OE-2	1700.89 ± 156.02[b]	1468.01 ± 203.30[b]	2452.78 ± 331.41[b]	5621.69 ± 501.55[b]
Stem	WT	5407.79 ± 274.54[c]	4810.27 ± 507.51[c]	10,573.39 ± 1224.70[c]	21,056.18 ± 1,691.75[c]
	OE-1	12,309.13 ± 1153.43[a]	12,963.45 ± 1243.01[a]	19,592.15 ± 2589.74[a]	44,864.72 ± 3702.66[a]
	OE-2	5621.67 ± 793.92[b]	5770.53 ± 735.07[b]	12,834.96 ± 903.82[b]	23,879.11 ± 1818.14[b]

Table 1. Comparison of the structure of paraffin sections of alfalfa. The total area of the cambium, xylem, and phloem is approximately equal to the area of the vascular bundle.

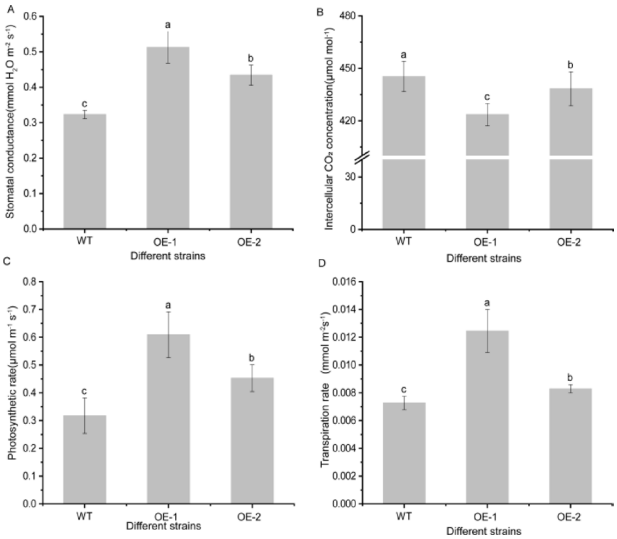

Fig. 6. Photosynthetic parameters of alfalfa. (**A**) Stomatal conductance (GS); (**B**) Intercellular CO_2 concentration (Ci); (**C**) Photosynthetic rate (Pn); (**D**)Transpiration rate (TR).

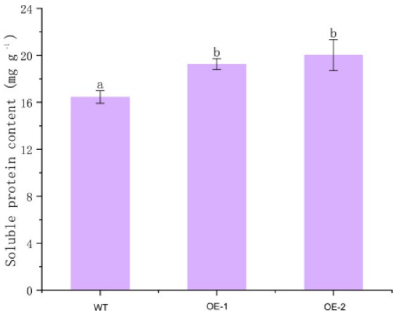

Fig. 7. Soluble protein of alfalfa.

Effect of the *MsJAR1* gene on soluble protein in alfalfa

The results indicated that the soluble protein content of the transgenic alfalfa plants was significantly higher than that of the WT (Fig. 7). This finding reflects that the transgenic alfalfa plants have certain advantages in photosynthesis as well as growth and development ($P < 0.05$).

Transcriptome sequencing and analysis

A total of 68.02 Gb clean data was obtained after elimination of low-quality reads, and the clean data of each sample reached 6.25 Gb with Q30 above 91.51%. The sequencing results of this study demonstrate high accuracy and good quality, rendering them suitable for subsequent data analysis (Table 2).

Sample	Clean data	Q30 (%)	GC%
WT-1	46,265,108	92.26%	43.41%
WT-2	43,121,102	91.51%	43.18%
WT-3	44,440,540	91.87%	43.02%
OE-1-1	56,842,142	93.02%	42.89%
OE-1-2	47,635,746	92.4%	42.56%
OE-1-3	53,282,968	92.94%	42.73%
OE-2-1	61,729,652	92.65%	42.64%
OE-2-2	55,245,532	93.16%	42.59%
OE-2-3	54,743,062	92.91%	42.69%

Table 2. Transcriptome data quality control and alignment statistics.

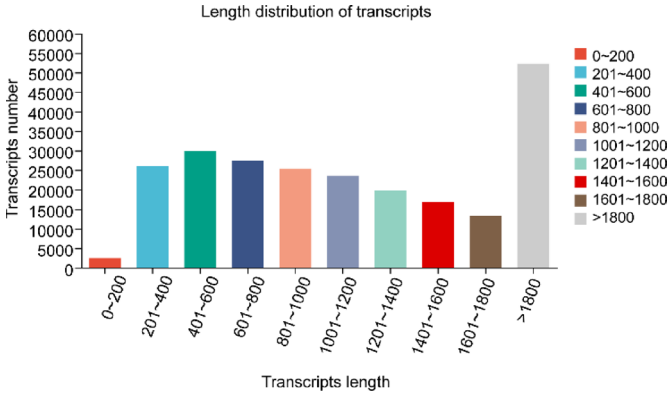

Fig. 8. The known length distribution of transcripts.

Name	Number of expressed genes	Expressed transcript number	Total genes	Total transcripts
GO	95,730	85,908	140,782	140,782
KEGG	44,371	38,877	58,852	58,852
EggNOG	97,724	87,505	132,284	132,284
NR	108,884	97,921	163,657	163,657
Swiss-Prot	88,577	79,360	118,652	118,652
Pfam	90,196	81,405	124,717	124,717
Total_anno	108,897	97,929	163,701	163,701
Total	109,180	98,134	164,632	164,632

Table 3. Database gene annotation results.

Transcriptome assembly and gene annotation

According to the transcript length distribution depicted in the sequencing results, a total of 236,472 transcripts were identified, with 52,230 transcripts exceeding 1,800 bp, accounting for 22.1% of the total transcripts (Fig. 8). After gene alignment, annotation, and functional classification, a total of 97,929 transcripts were annotated. The alignment results with six major databases (Table 3), with annotations found in the GO, KEGG, EggNOG, NR, Swiss-Prot, and Pfam databases for 140,782 (85.51%), 58,852 (35.75%), 132,284 (80.35%), 163,657 (99.41%), 118,652 (72.07%), and 124,717 (75.76%) genes, respectively.

Integrated analysis of differentially expressed genes (DEGs) in WT and MsJAR1 transgenic plants

The leaf samples used for transcriptome analysis underwent correlation analysis. The correlation coefficients among samples within the WT group ranged from 0.977 to 1, within the OE-1 group from 0.918 to 1, and within the OE-2 group from 0.782 to 1. The close correlation among samples within each group indicates good reproducibility and high similarity among samples, demonstrating the reliability of the experiment and the rationality of sample selection (Fig. 9A).

To investigate the DEGs between transgenic and wild-type alfalfa plants, a threshold of log2 (fold change) ≥ 2 and FDR ≤ 0.05 was used. DEGs analysis was performed between two comparison groups: OE-1 vs. WT and

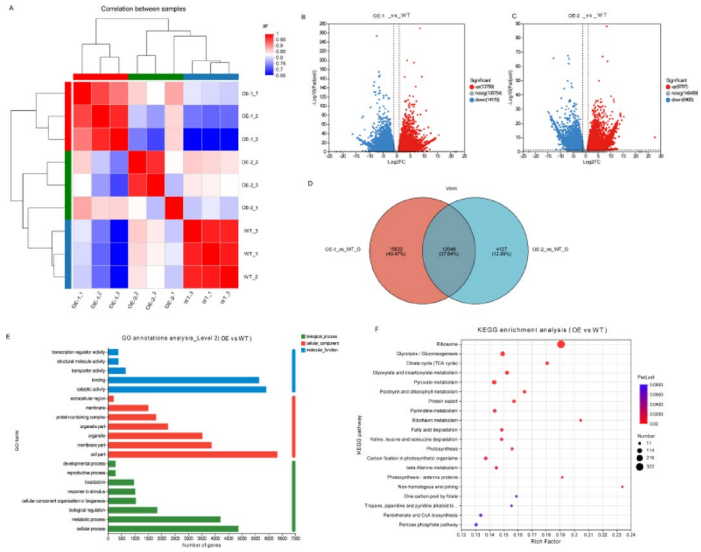

Fig. 9. Transcriptome analysis of WT and *MsJAR1* transgenic alfalfa. (**A**) Correlation analysis between WT and *MsJAR1* transgenic plants; (**B**–**D**) Volcano plots and Venn diagrams of DEGs overexpressing *MsJAR1*; (**E**,**F**) GO annotation and KEGG enrichment analysis of DEGs overexpressing *MsJAR1*.

OE-2 vs. WT. 27,878 genes were found to differentiate in OE-1 vs. WT, of which, there were 13,759 up-regulated DEGs and 14,119 down-regulated DEGs (Fig. 9B). 16,173 genes were found to differentiate in OE-2 vs. WT, of which, there were 9,767 up-regulated DEGs and 6,406 down-regulated DEGs (Fig. 9C). Further analysis revealed that there were 12,046 differential genes shared between the two transgenic lines and the WT, designated as OE vs. WT (Fig. 9D).

The GO annotation revealed that DEGs were annotated to 20 subclasses, of which Biological process contained 8 classes such as: "Developmental process", "Multi-organism process", "Response to stimulus", "Cellular component organization or biogenesis", "Biological regulation", "Metabolic process" and "Cellular process" ; Cellular component contains 7 categories, which are "Extracellular region", "Organelle part", "Membrane part" and "Cell part" ; and the Molecular function contains 5 categories, which are: "Transcription regulator activity", "Structural molecule activity", "Transporter activity", "Binding" and "Catalytic activity" (Fig. 9E). The results of the KEGG enrichment showed that there were 129 pathways enriched in the group of OE vs. WT, including "Ribosome" (322 DEGs), "Glycolysis / Gluconeogenesis" (103 DEGs), "Citrate cycle" (47 DEGs), "Glyoxylate and dicarboxylate metabolism" (66 DEGs), "Pyruvate metabolism" (84 DEGs), "Porphyrin and chlorophyll metabolism" (42 DEGs), "Protein export" (45 DEGs), "Pyrimidine metabolism" (49 DEGs), "Riboflavin metabolism" (18 DEGs), "Fatty acid degradation" (44 DEGs), "Valine, leucine and isoleucine degradation" (41 DEGs), "Photosynthesis" (34 DEGs) and "Carbon fixation in photosynthetic organisms" (53 DEGs) (Fig. 9F).

DEGs analysis in photosynthesis pathway

In the "Photosynthesis" metabolic pathways, we identified 6 DEGs encoding Photosystem II. Among these, 2 DEGs were up-regulated and 4 DEGs were down-regulated. Additionally, 3 DEGs encoding the cytochrome b6 / f complex were identified, with 1 DEG being up-regulated and 2 DEGs being down-regulated. Furthermore, 2 DEGs encoding Photosystem I, of which, there were 1 DEGs up-regulated and 1 DEGs down-regulated. Moreover, 4 DEGs encoding ATP synthase were detected, with 1 DEG up-regulated and 3 DEGs down-regulated (Fig. 10).

DEGs analysis in glycolysis/gluconeogenesis pathway and citrate cycle pathway

In the "Glycolysis / Gluconeogenesis" metabolic pathways, there were 6 DEGs encoding Phosphofructokinase (PFK). Among these, 2 DEGs were up-regulated while 4 DEGs were down-regulated. Additionally, 9 DEGs encoding the Pyruvate kinase (PK) were identified, with 4 DEGs being up-regulated and 5 DEGs being down-regulated. Furthermore, 8 DEGs encoding Pyruvate dehydrogenase complex, of which, there were 2 DEGs up-regulated and 6 DEGs down-regulated. In the "Citrate cycle" metabolic pathways, 3 DEGs encoding Isocitrate dehydrogenase (ICDH) were detected, with 4 DEGs down-regulated. Furthermore, 8 DEGs encoding Malate Dehydrogenase (MDH1) were identified, with 4 DEGs up-regulated and 4 DEGs down-regulated (Fig. 11).

Enzyme assays involved in glycolysis/gluconeogenesis and the citric acid cycle

Activity assays were conducted on three key enzymes involved in glycolysis / gluconeogenesis and the citric acid cycle pathways. It was found that in transgenic alfalfa plants (OE-1 and OE-2), the activities of all three enzymes were significantly higher compared with the WT ($P < 0.05$). The PFK activity in OE-1 and OE-2 was higher than that in WT (92.2 $U \cdot g^{-1}$ and 64.5 $U \cdot g^{-1}$, respectively) (Fig. 12A). The PK activity in OE-1 and OE-2 was higher

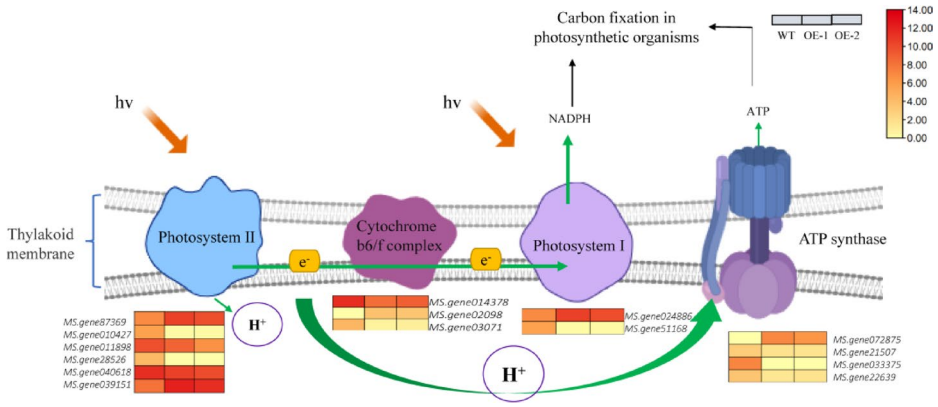

Fig. 10. Pathways of photosynthesis.

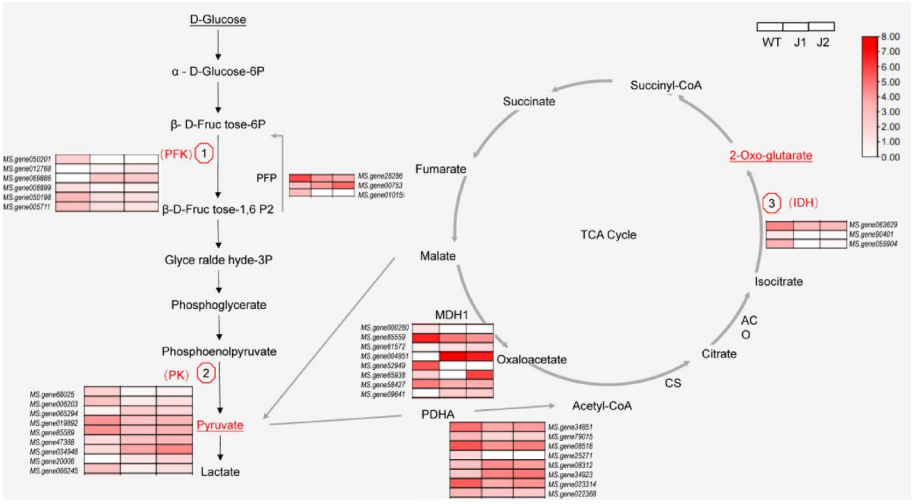

Fig. 11. Pathways of glycolysis and tricarboxylic acid cycle.

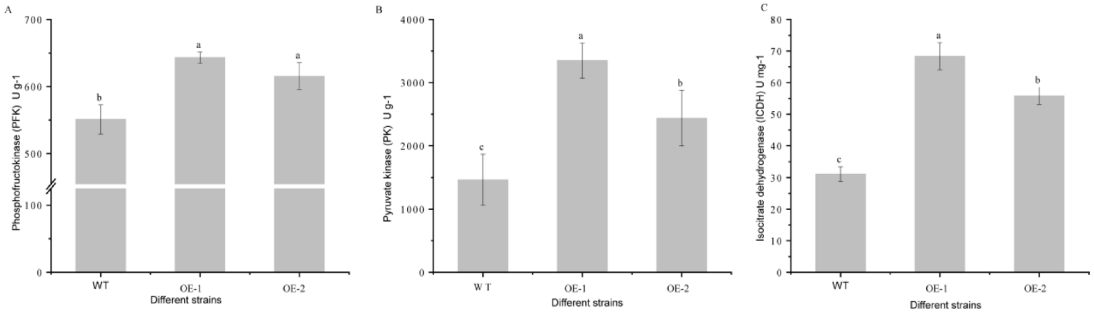

Fig. 12. Enzyme assays involved in glycolysis pathways and tricarboxylic acid cycle. (**A**) Measurement of phosphofructokinase enzyme activity; (**B**) measurement of pyruvate kinase enzyme activity; (**C**) measurement of isocitrate dehydrogenase enzyme activity.

than that in WT (1,884.6 $U{\cdot}g^{-1}$ and 972.9 $U{\cdot}g^{-1}$, respectively) (Fig. 12B). Furthermore, the ICDH activity in OE-1 and OE-2 was higher than that in WT (37.2 $U{\cdot}mg^{-1}$ and 24.6 $U{\cdot}mg^{-1}$, respectively) (Fig. 12C).

Correlation analysis between plant phenotype, photosynthetic parameters, enzyme activities and key differential genes

The results indicated that there were significant correlations between plant phenotypes, photosynthetic parameters, enzyme activities and key differentiation genes ($P<0.05$). Different genes in the photosynthetic

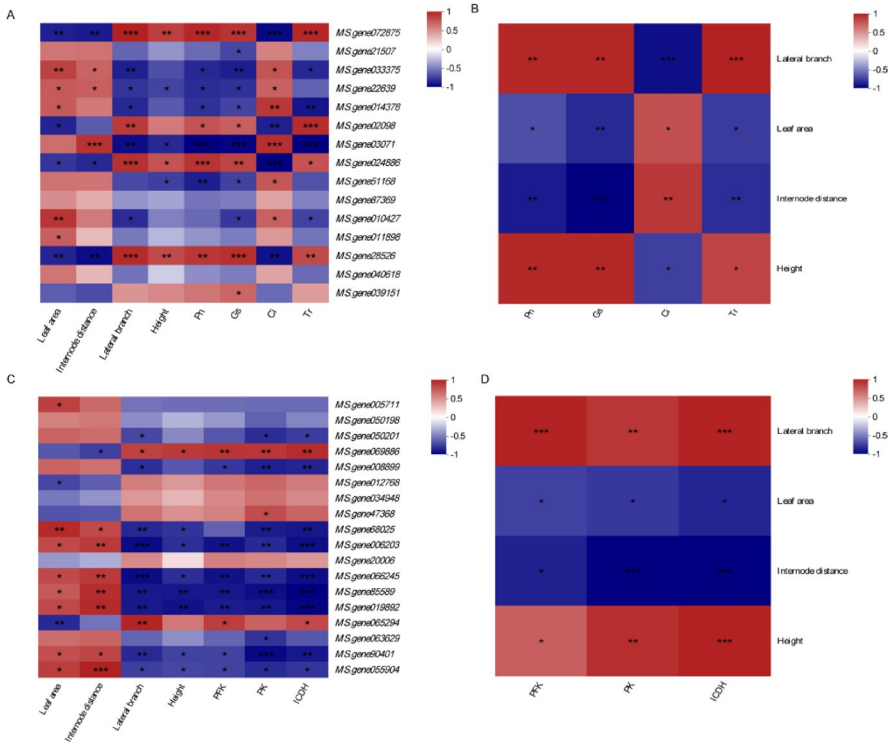

Fig. 13. Correlation Analysis of Plant Phenotypes. (**A**) Differential genes correlate with phenotypic and photosynthetic parameters; (**B**) Correlation of plant phenotypes with photosynthesis parameters; (**C**) Differential genes correlate with phenotype and enzyme activity; (**D**) Plant phenotype and enzyme activity correlation.

pathway were screened based on significance levels. Among the 15 key differential genes identified, 10 genes were positively correlated with leaf area, internode distance and Ci, and negatively correlated with the number of lateral branches, plant height, Pn, Gs and Tr. Conversely, the remaining five genes exhibited opposite correlations. Specifically, *MS.gene072875*, *MS.gene024886*, and *MS.gene028526* showed significant correlations with plant phenotype and photosynthetic indicators (Fig. 13A). Additionally, Pn, Gs, and Tr were significantly positively correlated with lateral branch number and plant height but negatively correlated with leaf area and internode distance. Ci exhibited significant negative correlations with alfalfa lateral branches and plant height but significant positive correlations with leaf area and internode distance (Fig. 13B).

Within the glycolysis and citric acid cycle pathways, 18 key genes regulated the activity of PFK, PK, and ICDH. Among these 18 key differential genes, 12 genes were positively correlated with leaf area and internode distance but negatively correlated with lateral branch number, plant height, the activities of PFK, PK, and ICDH. Specifically, *MS.gene006203*, *MS.gene066245*, *MS.gene85589*, *MS.gene019892*, *MS.gene90401*, and *MS.gene055904* exhibited significant correlations with plant phenotype and enzyme activity (Fig. 13C). Furthermore, the enzyme activities of PFK, PK, and ICDH were significantly positively correlated with the number of alfalfa lateral branches and plant height ($P < 0.05$) but negatively correlated with leaf area and internode distance (Fig. 13D).

Discussion

Alfalfa, as a significant leguminous forage crop, is renowned for its high yield and nutritional value[22]. However, the characteristics of cross-pollination and autotetraploid inheritance have seriously limited the application of biotechnology breeding in alfalfa. Here, we successfully constructed the transient expression vector pAN580-*MsJAR1* and the overexpression vector pCPB-*MsJAR1*, to explor the function of the *MsJAR1* gene through genetic transformation techniques. As the executors of gene functions, proteins must be precisely positioned in the correct cellular compartments to maintain the efficient operation of various biochemical processes[23]. The *MsJAR1* gene was fused to the green fluorescent protein (GFP) gene. The fusion structure was introduced into tobacco cells by microinjection, and *MsJAR1* protein was found to be localized in chloroplasts, consistent with the localization results of similar genes in our previous studies and in other plants. These findings indicated that *MsJAR1* is capable of exerting regulatory effects upon entering the chloroplast. Observations on transgenic alfalfa plants showed that compared with the WT, the leaves and internode distances of transgenic alfalfa were reduced, and the number of lateral branches and plant height were increased. During plant growth and development, hormonal regulation promotes longitudinal cell growth, resulting in shortened internode length. This change causes branches to be closer to each other, thus increasing the number of nodes between them[24]. This

mechanism increases the number of side branches and increases the height of the plant. These traits significantly impact plant growth, development and yield[25].

The phloem and xylem are vital plant tissue structures that play crucial roles in plant growth and function[26]. Notable observations were made through paraffin section examination of both WT and transgenic alfalfa specimens. It was observed that there was a reduction in the phloem, cambium, xylem, and vascular bundles, while the corresponding areas in the stem region showed a significant increase[27]. This suggests that the leaf structure may be weakened, while the stem is reinforced due to the increase in phloem and xylem. This phenomenon contributes to maintaining the plant's structure and enhancing its ability to adapt to adverse environmental conditions[28]. The expansion of stem tissues, including phloem and xylem areas, suggests that resources, including water and nutrients, may be redistributed to the stem, facilitating its growth and development. This enhances the efficiency of water and nutrient utilization, thereby promoting lateral branch growth. Based on anatomical analysis observations of transgenic alfalfa, it can be inferred that this gene may be involved in regulating plant growth and development.

The photosynthetic process of plants plays an important role in maintaining atmospheric oxygen levels and carbon cycling, which is why it is of fundamental importance in agricultural production[29]. Studies have shown that zinc deficiency will result in a decrease in tomato photosynthesis parameters, affect the growth and development of tomato plants, and lead to a decrease in tomato yield[30]. In rice, studies of *JAR1* mutants have shown that embryo length varies under different light conditions[31]. In particular, *JAR1* mutants exhibit a significant increase in embryonic length under red and blue light conditions. Other studies have shown that photoreceptors A and B can induce *JAR1* expression, confirming that the rice *JAR1* gene is required for photomorphogenesis and JA response. Increased lateral branching in plants contributes to the shading effect of internal leaves, affecting photosynthetic efficiency. Analysis of the photosynthetic parameters of transgenic alfalfa plants showed that its net photosynthetic rate, transpiration rate, and stomatal conductance were significantly higher than those of WT plants, while Ci was significantly lower than that of WT plants. Proteins play a crucial role in the photosynthesis of plants. In this study, the content of soluble proteins in transgenic plants is significantly higher than that in the WT, suggesting that the *MsJAR1* gene regulates plant photosynthesis and controls plant growth and development by influencing the content of soluble proteins.

This study conducted transcriptome sequencing and analysis on transgenic alfalfa and wild-type alfalfa. A total of 68.02 Gb of clean data were obtained, with the percentage of Q30 bases exceeding 91.51%. Transcriptome sequencing revealed 12,046 DEGs between OE vs. WT. The KEGG enrichment analysis indicated significant enrichment of differentially expressed genes mainly in metabolic pathways and photosynthesis-related pathways. Photosynthesis, as the foundation of plant growth and development, enables plants to convert light energy into chemical energy, producing ATP and NADPH, and fixing carbon dioxide into organic compounds. The study identified interactions among photosynthesis, glycolysis, and the tricarboxylic acid cycle, where photosynthesis provides energy and reducing power for glycolysis and the tricarboxylic acid cycle, thereby promoting metabolic pathways[32]. In addition, glycolysis and the tricarboxylic acid cycle also provide substrates and intermediates required for photosynthesis, thereby mutually regulating plant growth through carbon metabolism. Glycolysis, as a key pathway for maintaining cellular energy balance and providing carbon sources in plants, ensures effective glucose utilization, enhancing their growth, development, and ability to adapt to environmental changes[33]. Phosphofructokinase is a crucial regulatory enzyme in glycolysis, and studies have shown a positive correlation between glycolytic rate and its activity. The study found that the PFK activity in transgenic alfalfa plants was significantly higher than in wild-type plants ($P<0.05$), indicating their involvement in plant sugar metabolism and playing a crucial role in carbohydrate metabolism. Pyruvate kinase (PK) is a key enzyme in the final step of glycolysis, and research has shown that 90% of glucose in plants is converted into pyruvate through glycolysis, highlighting the importance of this pathway. Furthermore, other studies found that tobacco plants deficient in PK exhibited delayed flowering and serrated leaf margins under low - light conditions. It was observed that transgenic alfalfa plants showed higher PK activity, suggesting their important regulatory role during the flowering process. The tricarboxylic acid cycle (TCA cycle) is a crucial pathway in plant metabolism, and its efficiency directly correlates with nitrogen assimilation and the ability to cope with various stresses[34]. Isocitrate dehydrogenase (ICDH) catalyzes the conversion of isocitrate to α-ketoglutarate, serving as a key enzyme in the TCA cycle[35]. Other studies found that the lack of functional isocitrate dehydrogenase in Arabidopsis inhibits plant growth[36]. This study discovered that transgenic alfalfa plants exhibit higher ICDH enzyme activity compared to wild-type plants.

This study analyzed the phenotypic traits of alfalfa plants along with parameters related to photosynthesis, enzyme activity, and key differentially expressed genes. The results revealed that differentially expressed genes involved in photosynthesis were positively correlated with leaf area, internode distance, and intercellular CO_2 concentration (Ci), while negatively correlated with lateral branch number, plant height, net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (Tr). Conversely, net photosynthetic rate, stomatal conductance, and transpiration rate were significantly positively correlated with lateral branch number and plant height, while significantly negatively correlated with leaf area and internode distance. Other studies found that overexpression of the *CAO* gene in Arabidopsis affects the size of the photosynthetic antenna complex, regulates the efficiency of light harvesting, and consequently influences plant growth and development[37]. Among the differentially expressed genes encoding PFK, PK, and ICDH, it was observed that they were positively correlated with leaf area and internode distance, while negatively correlated with lateral branch number, plant height, and the activities of PFK, PK, and ICDH. Furthermore, net photosynthetic rate, stomatal conductance, transpiration rate, as well as the activities of PFK, PK, and ICDH, were significantly positively correlated with lateral branch number and plant height, while significantly negatively correlated with leaf area and internode distance. Previous studies have shown that Bacillus subtilis G2 can enhance photosynthetic efficiency and carbohydrate conversion, increasing carbohydrate yield by releasing more chemical energy through the glycolytic pathway

and the tricarboxylic acid cycle, thereby sustaining normal plant growth and development[38]. It was found that the upregulation of PFK, PK, and ICDH enzyme activities led to corresponding changes in photosynthetic parameters, ultimately affecting plant growth and development.

These studies on differentially expressed genes contribute to a deeper understanding of the molecular mechanisms underlying plant growth, providing an important theoretical basis for breeding and improvement. Through combined analysis of enzyme activity determination, photosynthetic parameters, and anatomical structure, it is demonstrated that the *MsJAR1* gene may regulate photosynthesis, glycolysis, and the tricarboxylic acid cycle within the plant, thereby influencing plant development. Additionally, transcriptome sequencing and analysis were conducted on both WT and transgenic alfalfa, revealing numerous differentially expressed genes. These studies offer a crucial theoretical foundation for a comprehensive understanding of the molecular mechanisms governing plant growth.

Conclusion

In summary, the MsJAR1 protein primarily was located to chloroplast. Compared to the wild-type (WT), *MsJAR1* overexpressed alfalfa plants (OE-1 and OE-2) exhibited a series of characteristic changes: reduced leaf area, increased lateral branching, decreased internode spacing, and increased plant height. The area of the leaf's xylem and phloem decreases, while the area of the stem's xylem and phloem increases. These changes arise from alterations in gene and enzyme activity induced by the *MsJAR1* gene, which regulates metabolic pathways such as ribosome, sugar metabolism, tricarboxylic acid cycle, and photosynthesis, thereby influencing the phenotype of the plants.

Materials and methods

Plant materials and treatments

This study employed the cultivar 'Zhongmu NO.1' of alfalfa developed by our laboratory (College of Grassland and Resources, Inner Mongolia Agricultural University). Seeds were germinated either in regular soil (in pots with a diameter of 10 cm) or in Hoagland solution in a growth chamber, maintained at a temperature of 25°C under a photoperiod of 14 hours light and 10 hours dark. Transgenic lines overexpressing *MsJAR1* in 'Zhongmu NO.1' alfalfa (OE-1 and OE-2) along with wild-type (WT) control were obtained through genetic transformation. Vigorous plants were selected for propagation through cuttings, and they were subsequently cultured in a growth chamber at 25 °C and 50% RH. Watering was done every 2–3 days as needed. After two months of cultivation, alfalfa plants were obtained for subsequent agronomic trait assessment, relevant indices measurement, and transcriptome sequencing analysis. For transcriptome sequencing, leaf samples were collected from transgenic and wild-type alfalfa during the vegetative growth stage, with three biological replicates per treatment group to ensure experimental reliability.

Construction of the *MsJAR1* plant expression vector and alfalfa transformation

To amplify the coding sequence of *MsJAR1*, primers named JAR1-TF and JAR1-TR (Schedule 4) were used. The PCR product was introduced into the vector pCPB-GFP using BamH I restriction sites by homologous recombination. pCPB-GFP harbors a CaMV 35 S promoter and an GFP reporter gene. Next, the pCPB- *MsJAR1* recombinant vector was transformed into Agrobacterium strain GV3101 via the freeze–thaw method, and Agrobacterium-mediated trans-formation of alfalfa.

Freshly grown trifoliate clover leaves were placed into culture bottles. They were agitated with Solution Tween 20 per thousand for 15 min, followed by rinsing with sterile water until no foam was visible. Subsequently, they were treated with 75% ethanol for 30 s and surface sterilized with 40% sodium hypochlorite for 10 min. After five rinses with sterile water, the leaves were air-dried on sterile filter paper. The surface of the explants was gently scratched to create several wounds before being immersed into the Agrobacterium infiltration solution (MS medium plus 4.43 g·L^{-1}, 2, 4-dichlorophenoxyacetic acid (2,4–D) 4 mL, 6-Benzylaminopurine (6–BA) 0.2 mL, pH 5.8). After shaking to ensure thorough infiltration, the mixture was poured into polyethylene plastic bags and left to stand for 10 min. Vacuum packaging was then conducted using a vacuum sealer at a medium temperature setting for 75 s, followed by 5 min of 40 kHz ultrasonic treatment. Subsequently, the samples were air-dried on sterile filter paper before being placed on co-cultivation solid medium and incubated at 25 °C in darkness for 3–5 days (MS medium plus 4.43 g·L – 1, sucrose 30 g·L^{-1}, Agar 7.5 g·L^{-1}, 2,4-dichlorophenoxyacetic acid (2,4-D) 4mL, 6-Benzylaminopurine (6-BA) 0.2mL, pH 5.8). Next, transfer to callus induction medium supplemented with 1 ml cefotaxime and 400 µL PPT and culture for 30 days. The formed resistant callus was cultured on differentiation medium (MS medium plus 4.43 g·L^{-1}, sucrose 30 g·L^{-1}, Agar 7.5 g·L^{-1} kinetin 1mL, 6-Benzylaminopurine (6-BA) 0.5mL, pH 5.8) under a 25 °C, 16-hour photoperiod for 7–10 days. Subsequently, the differentiated resistant shoots were cultured on rooting medium (MS medium plus 2.22 g·L^{-1}, sucrose 15 g·L^{-1}, Agar 3.5 g·L^{-1}, NAA 1mL, pH 5.8) for 10 days. Finally, the regenerated clover plants were transplanted into soil and grown in the growth chamber.

Construction of *MsJAR1* plant transient expression vector and subcellular localization

To amplify the coding sequence of *MsJAR1*, primers named JAR1-TF and JAR1-TR (Schedule 4) were used. The PCR product was ligated into the pAN580-GFP vector, which harbors the CaMV 35 S-promoter and a GFP, by Xba I and BamH I restriction sites. The pAN580-*MsJAR1* fusion plasmid was transformed into agrobacterium tumefaciens GV3101 and injected into tobacco cells by the injection method, followed by incubation for 24 h in darkness and 48 h in light. The tobacco cells were observed and photographed using a confocal laser scanning microscope (Nikon). Chloroplast autofluorescence (red) was observed at 543 nm, while GFP fluorescence was observed at 488 nm.

Light and action parameters
Fully expanded leaves at the same plant position were selected in each treatment, and the net photosynthetic rate (Pn), transpiration rate (E), stomatal conductance (Gs) and intercellular CO2 concentration (Ci) were measured using a LI-6400XT photosynthetic gas exchange system from 9:00 a.m. to 11:30 a.m. The leaf chamber temperature was set at the normal temperature (25℃). Each treatment was replicated independently four times.

Phenotypic trait indicators
The transgenic alfalfa plants obtained through genetic transformation with *MsJAR1* were subjected to cutting treatment. Three replicates of alfalfa grown for approximately 60 days were selected for each group. The growth status, including plant height, branch number, leaf length, leaf width, and internode distance, was observed and measured.

Histology-based phenotypic characterization
The middle stems and leaves of the plants were immersed in FAA fixative and kept for 7 d at 4 ℃. The stems and leaves were subjected to dehydration with a series of xylene and ethanol at various concentrations. The tissue was embedded in paraffin for approximately 16 h at 60 ℃. Subsequently, the obtained paraffin blocks were sectioned into 8 μm thick slices by using a microtome. The slices were then mounted on slides, deparaffinized, stained overnight with 1% safranin followed by 1% fast green for 10 s, sealed, and incubated in a 40 ℃ thermostat for 2 days. Then subjected to microscopic observation.

Soluble protein (SR), phosphofructokinase (PFK), pyruvate kinase (PK) and isocitrate dehydrogenase (ICDH) enzyme assays
We used the Total protein quantitative assay kit (Nanjing Jiancheng of Bioengineering Institute), and according to the instruction manual, we adopted the Coomassie Brilliant Blue method. The measurement was carried out at a wavelength of 595 nm using a UV spectrophotometer. Using reagent kits (Beijing Solabio Technology Co., Ltd.) to measure phosphofructokinase (PFK, EC2.7.1.11), pyruvate kinase (PK, EC2.7.1.40), and isocitrate dehydrogenase (ICDH, EC1.1.1.41) in the transgenic lines (OE-1 and OE-2) and WT under conditions of 340 nm, 340 nm, and 505 nm wavelengths using a UV spectrophotometer. Calculations of the respective contents were performed according to the instructions provided, with each genotype having three biological replicates.

RNA extraction, RNA sequencing and gene expression analysis
Total RNA was extracted from nine samples using Trizol reagent (Invitrogen, USA). The quality and concentration of the RNA were assessed using NanoDrop, and integrity was verified through 1% agarose gel electrophoresis. Three biological replicates were included for each experimental condition to ensure statistical robustness. Nine cDNA libraries were constructed from 1 μg of RNA from each sample, following the Easy Quick RT Master Mix (CWBIO) protocol. The prepared cDNA libraries were sequenced on the Illumina HiSeq2000 platform, and the acquired data were uploaded to the NCBI database. The qRT-PCR program consisted of a 30 s reaction at 95 °C, followed by 40 cycles of PCR, with each cycle consisting of a 10 s reaction at 95 °C and a 30 s reaction at 60 °C. The *MsActin* gene was used as the internal reference gene in qRT-PCR[39]. Using the Bio-Rad CFX Maestro software 1.0 in conjunction with the LinReg PCR program, the amplification efficiency can be determined. The experiment was conducted using biological triplicate samples, and the data were analyzed using the 2^−ΔΔCT method.

Expression levels of each gene were quantified by normalizing total gene counts with the effective library size. RNA-seq reads were aligned to the reference genome using HISAT2, and gene expression levels were quantified using RSEM. Differential expression analysis was performed using DESeq2 with a significance threshold of $P < 0.05$. The TPM expression levels and counts for all unigenes were estimated in each replicate using RSEM. Low-quality reads were filtered out using Trimmomatic with a quality score threshold of Q30. Gene expression levels were normalized using the TPM method. Gene function was annotated based on the following databases: NR (NCBI non-redundant protein sequences), Pfam (Protein family), KOG/COG/eggNOG (Clusters of Orthologous Groups of proteins), Swiss-Prot (a manually annotated and reviewed protein sequence database), KEGG (Kyoto Encyclopedia of Genes and Genomes), and GO (Gene Ontology).

Analysis of differentially expressed genes (DEGs)
Differentially expressed genes (DEGs) were identified using DESeq2, focusing on unigenes with a significant p-value ($P < 0.05$). The annotated DEGs were subsequently utilized in functional enrichment analysis. Functional enrichment analysis, including Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment and Gene Ontology (GO) category annotation of significant DEGs, was conducted using the clusterProfiler package with an adjusted p-value threshold of < 0.05.

Statistical analysis
The data were processed and graphed using Excel 2019, Origin 2021, IBM SPSS Statistics 25.0, and Adobe Illustrator CS6. Transcriptome data analysis was performed using HISAT2, RSEM, DESeq2 and Trimmomatic.

Data availability
The datasets generated during and/or analyzed during the current study are available in the NCBI repository. The MsJAR1 gene (OK602801) and the transcriptome data (PRJNA1095380) are both accessible in NCBI.

Received: 18 November 2024; Accepted: 5 May 2025

Published online: 15 May 2025

References

1. Lei, R., Jonathan, B., Bruce, C., Jushan, L. & Bill, B. Forage yield trend of alfalfa cultivars in the Canadian prairies and its relation to environmental factors and harvest management. *Grass Forage Sci.* **76**(3), 390–399 (2021).
2. Núñez, A. L. M. E., García, A., Meseguer, R. & Pons, X. Alfalfa winter cutting: effectiveness against the alfalfa weevil, Hypera postica (Gyllenhal) (Coleoptera: Curculionidae) and effect on its rate of parasitism due to bathyplectes spp. (Hymenoptera: Ichneumonidae). *Crop Prot.* **152**(0), 105858–105858 (2022).
3. Edmar Teixeira, J. et al. Moot. Assessing land suitability and spatial variability in Lucerne yields across new Zealand. *Eur. J. Agron.* **148**(0), 126853–126853 (2023).
4. Jiawen, C., Xiaoshan, W., Zhenwu, W. & Biao, J. Medicago truncatula (model legume), Medicago sativa (alfalfa), Medicago polymorpha (bur clover), and Medicago ruthenica. *Trends Genet.* **38**(7), 782–783 (2022).
5. Yingxiao, Z., Brian, J. I. & Yiping, Q. CRISPR ribonucleoprotein-mediated genetic engineering in plants. *Plant. Commun.* **2**(2), 100168–100168 (2021).
6. Zulqurnain, K. *Conventional Breeding, Genetically Modified Crops, and Genome Editing*. 3–36 (Apple Academic Press eBooks, 2023).
7. Liu, Z. P. et al. Progress and existing problems of forage breeding in China. *Bull. Natl. Nat. Sci. Foundation China*. **7**(04), 528–536 (2023).
8. Dong, W., Ma, H., Chen, C. & Li, Y. Overexpression of the OvBAN gene enhances the Proanthocyanidin content in Transgenic alfalfa (Medicago sativa L). *Vitro Cell. Dev. Biology – Plant.* **56**(4), 548–557 (2020).
9. Liu, L. et al. Overexpression of ZxABCG11 from Zygophyllum Xanthoxylum enhances tolerance to drought and heat in alfalfa by increasing cuticular wax deposition. *Crop J.* **11**(4), 1140–1151 (2023).
10. Zhang, J. et al. Co-transforming bar and CsLEA enhanced tolerance to drought and salt stress in Transgenic alfalfa (Medicago sativa L). *Biochem. Biophys. Res. Commun.* **472**(1), 75–82 (2016).
11. Silke, J. et al. A functional genomics approach to dissect spotted alfalfa aphid resistance in Medicago truncatula. *Sci. Rep.*, **10**(1) (2020).
12. Jianping, Y., Rumeng, Q., Kexin, W., Yanrong, L. & Wanjun, Z. Enhancing alfalfa resistance to Spodoptera herbivory by sequestering microRNA396 expression. *Plant Cell Rep.* **42**(4), 805–819 (2023).
13. Christian, M. et al. A chemical inhibitor of jasmonate signaling targets JAR1 in Arabidopsis thaliana. *Nat. Chem. Biol.* **10**(10), 830–836 (2014).
14. Svyatyna, K. et al. Light induces jasmonate-isoleucine conjugation via OsJAR1-dependent and -independent pathways in rice. *Plant. Cell. Environ.* **37**(4), 827–839 (2013).
15. Patrycja, W., Anna, C., Ewelina, S. & Maciej, O. The GH3 amidosynthetases family and their role in metabolic crosstalk modulation of plant signaling compounds. *Phytochemistry* **194**, 113039–113039 (2022).
16. Zhang, X. F., Jiao, F., Nie, H., Liu, M. L. & Cheng, J. L. Cloning induced expression and temporal specificity of MaGH3. 6 gene in mulberry. *Sci. Seric.* **39**(02), 196–203 (2013).
17. Luo, H. L., Luo, L. P., Xiong, D. J. & Yang, B. Y. In silico cloning and bioinformatics analysis of GH3 in Capsicum annuum. *Acta Agriculturae Boreali-Sinica*. **28**(06), 24–29 (2013).
18. Pham Anh, T., Talia, S., Gurkamal, K., Ginelle, G. & Belay, T. A. Molecular and functional characterization of a jasmonate resistant gene of wheat (Triticum aestivum L). *J. Plant Physiol.* **270**, 153637–153637 (2022).
19. Mahmud, S. et al. Constitutive expression of JASMONATE RESISTANT 1 induces molecular changes that prime the plants to better withstand drought. *Plant. Cell. Environ.* **45**(10), 2906–2922 (2022).
20. Edward, E. F. & Clarence, A. R. Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc. Natl. Acad. Sci.* **87**(19), 7713–7716 (1990).
21. Dai, R. et al. Cloning and expression analysis of MsJAR1 in Medicago sativa. *Acta Agrestia Sinica*. **32**(5), 1370–1377 (2024).
22. Dilnur, T. & Shuga, A. M. Alfalfa (Medicago sativa L.): genotypic diversity and transgenic alfalfa for phytoremediation. *Front. Environ. Sci.* **10**(2022).
23. Guo, A. et al. SlGH3.15, a member of the GH3 gene family, regulates lateral root development and gravitropism response by modulating auxin homeostasis in tomato. *Plant Sci.* **330**(0), 111638–111638 (2023).
24. Fengxia, W. et al. ZmTE1 promotes plant height by regulating intercalary meristem formation and internode cell elongation in maize. *Plant Biotechnol. J.* **20**(3), 526–537 (2021).
25. Christopher, J. S. et al. Moving toward short stature maize: the effect of plant height on maize stalk lodging resistance. *Field Crops Res.* **300**, 109008 (2023).
26. Zijian, L. Modeling plant uptake of organic contaminants by root vegetables: the role of diffusion, xylem, and phloem uptake routes. *J. Hazard. Mater.* **434**, 128911–128911 (2022).
27. Chattha, W. S. et al. Xylem-phloem cell area aid in salinity tolerance of tomato plant. *Sci. Hort.* **328**, 112919 (2024).
28. Da Silva, M. C. & Shelp, B. J. Xylem-to-phloem transfer of organic nitrogen in young soybean plants 1. *Plant Physiol.* **92**(3), 797–801 (1990).
29. Noreen, Z. et al. Plant photosynthesis under heat stress: effects and management. *Environ. Exp. Bot.* **206**, 105178–105178 (2023).
30. Mst Salma, A. et al. Regulation of Zn uptake and redox status confers Zn deficiency tolerance in tomato. *Sci. Hort.* **273**, 109624–109624 (2020).
31. Maren, R., Michael, R. & Makoto, T. Rice JASMONATE RESISTANT 1 is involved in phytochrome and jasmonate signalling. *Plant. Cell. Environ.* **31**(6), 783–792 (2008).
32. Yi Zhang. et al. One-carbon metabolism in plants: characterization of a plastid Serine hydroxymethyltransferase. *Biochem. J.*, **430**(1):97–105 (2010).
33. Curtis, V. G. Evolving concepts in plant glycolysis: two centuries of progress. *Biol. Rev.* **74**(3), 277–309 (2007).
34. Bernard, G., Jiao, J., Vicki, L. K. & William, C. P. Photosynthesis and carbon partitioning in transgenic tobacco plants deficient in leaf cytosolic pyruvate Kinase1. *Plant Physiol.* **120**(3), 887–896 (1999).
35. Youjun, Z. & Alisdair, R. F. On the role of the tricarboxylic acid cycle in plant productivity. *J. Integr. Plant Biol.* **60**(12), 1199–1216 (2018).
36. Sijia, Z., Qian, Z., Yuhan, Y., Cui, Y. & Tianzi, H. Cytosolic isocitrate dehydrogenase regulates plant stem cell maintenance in response to nutrient deficiency. *Plant Physiol.* **192**(4), 3069–3087 (2023).
37. Ryouichi, T. et al. Overexpression of chlorophyllide a oxygenase (CAO) enlarges the antenna size of photosystem II in Arabidopsis thaliana. *Plant J.* **26**(4), 365–373 (2001).
38. Thomas, H. C. et al. The role of fructose 2,6-bisphosphate in the regulation of carbohydrate metabolism. *Curr. Top. Cell. Regul.* **23**, 57–86 (1984).
39. Zhang, Z. et al. MsZEP, a novel zeaxanthin epoxidase gene from alfalfa (Medicago sativa), confers drought and salt tolerance in transgenic tobacco. *Plant Cell Rep.* **35**(2), 439–453 (2015).

Author contributions

All authors prepared, read, and approved the final manuscript. DR conceived the study and wrote the paper with the input of all authors. HXW and LJW was involved in the cultivation of the plant material, DR and CQ were involved in the genetic transformation of the plants, DR performed the subsequent validation experiments of the transgenic alfalfa, AJB and LJW was involved in the data analysis and editing of the article, WXY was involved in the editing of the article, SFL, MFG and ZZQ were involved in the review and editing of the article.

Funding

The author(s) declare financial support was received for the research, publication of this article. This research was supported by the Inner Mongolia Autonomous Region Higher Education The National Natural Science Foundation of China (32460341),"Young Science and Technology Talents Support Project" (NJYT23009), First-Class Discipline Special Fund of Inner Mongolia, China (YLXKZX-NND-03) and Alfalfa Molecular Breeding System Construction and Germplasm Creation (BR22-11-12).

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-01227-4.

Correspondence and requests for materials should be addressed to Z.-q.Z.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.