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Research Article

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Genome-wide investigation of the *NCED* gene family in alfalfa (*Medicago sativa* L.): identification, analysis and expression

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Abstract

Background Alfalfa (*Medicago sativa* L.) is an essential leguminous forage with high nutrition and strong adaptability. The 9-cis-epoxycarotenoid dioxygenase (NCED) family genes encode 9-cis-epoxycarotenoid dioxygenase, which is the key rate-limiting enzyme in ABA biosynthesis. However, few reports have been reported on the phylogenetic analysis and gene expression profiling of *NCED* family genes in alfalfa.

Result A total of 35 *NCED* genes were identified in the alfalfa genome, distributed across 21 chromosomes. The number of amino acids encoded by members of the alfalfa *NCED* family ranged from 242 to 610, and all were hydrophilic proteins. Subcellular localization prediction showed that most MsNCED proteins are localized to peroxisomes and chloroplasts. Collinearity analysis indicated that segmental duplication was the key driving force for the expansion of this gene family during evolution. A range of cis-acting elements were detected in *MsNCED* gene promoter regions, involved in a variety of hormone and abiotic stress responses. qRT-PCR

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analysis revealed that *MsNCED1*, *MsNCED8*, and *MsNCED31* were significantly induced by low-temperature, salt, and drought stresses, suggesting that these genes may be involved in the abiotic stress responses of alfalfa. These findings provide a theoretical foundation for further functional characterization of the *NCED* gene family in alfalfa.

Conclusion In this study, members of the *NCED* gene family were identified in alfalfa, and their structural characteristics and possible functions were systematically analyzed. These findings lay a foundation for further investigation into the molecular functions of *NCEDs* in alfalfa.

Keywords Alfalfa, *NCED* gene family, Abiotic stress, Expression patterns

Introduction

Plant hormones are endogenous signaling molecules that regulate synthesis and metabolism of plant compounds. Abiotic stresses such as low temperature, salinity, and drought severely restrict plant growth and productivity. Abscisic acid (ABA) plays a central role in plant responses to diverse environmental stresses. ABA is one of the major plant hormones and has a sesquiterpene structure [1]. It occurs broadly throughout organs and tissues of higher plants and regulates growth, development, stress responses, and multiple physiological processes [2]. ABA is especially important for seed germination, maturation and dormancy, and fruit ripening, making it one of the most intensively studied plant hormones [3-5].

9-cis-epoxycarotenoid dioxygenase (NCED) is a key rate-limiting enzyme in the ABA biosynthesis pathway in plants [6]. It is widely involved in plant growth and

development processes and responses to abiotic stresses, and can catalyze the cleavage of 9-cis-epoxycarotenoids to produce xanthoxin. Xanthoxin is then oxidized to ABA in two steps catalyzed by a flavin-dependent dehydrogenase and abscisic aldehyde oxidase [7]. Studies have shown that the expression level and enzyme activities of the *NCED* gene directly affect the ABA content in plants [8]. These genes typically exist as multi-gene families and are widely distributed across various plant species, with their members exhibiting markedly differential expression patterns under stress conditions [9, 10]. The expression level of *SlNCED9* in tomato increases under cold stress, while the expression levels of *SlNCED1*, *SlNCED2*, and *SlNCED4* increase after drought treatment [11]. The expression level of *AtNCED*3 in *Arabidopsis thaliana* increases significantly under drought stress, enhancing drought tolerance by promoting endogenous ABA accumulation [12]. Overexpression of *OsNCED3* in rice enhances ABA accumulation and drought tolerance, and the loss-of-function mutant of *OsNCED4* shows increased sensitivity to low temperature and salt stress [13].

The first *NCED* gene was cloned from maize [14]. Tan et al. identified nine homologs of *vp14* in *Arabidopsis thaliana* [15]. Among these nine homologs, four encode *CCDs* (*CCD1*, *CCD4*, *CCD7*, and *CCD8*), and five encode *NCEDs* (*NCED2*, N*CED3*, *NCED5*, *NCED6*, and *NCED9*) [16]. With the rapid development of whole-genome technology, *NCED* genes have been gradually identified in many plants, such as rice [17], wheat [18], tomato [11], and soybean [9].

As the leguminous forage with the largest global planting area, alfalfa (*Medicago sativa* L.) is known as the ‘king of forage’ due to its high yield and rich contents of

protein, vitamins, and minerals [19]. Northern China is the main alfalfa-producing region, but high-quality arable land available for forage cultivation is very limited. Abiotic stresses such as cold, salt, and drought are the major factors limiting forage growth and development, thereby restricting the development of the forage industry and herbivore livestock husbandry. Elucidating the molecular mechanisms underlying forage crop responses to cold, salt, and drought stresses is of great theoretical and practical significance for improving stress tolerance and promoting the sustainable development of the herbivorous livestock industry.

Although *NCED* gene functions have been extensively characterized in many species, their roles in alfalfa remain unreported. Here, we used bioinformatic approaches to identify and characterize *NCED* family members in the alfalfa genome. Analyses included protein physicochemical properties, chromosomal distribution, phylogeny, gene structure, conserved motifs, cis-acting elements, collinearity, and predicted subcellular localization. We also examined *NCED* expression under cold, salt, and drought stress. These results provide a theoretical basis for understanding alfalfa growth and development, identifying key stress-resistance genes, and elucidating the evolutionary patterns of this gene family.

Results

Identification and basic information analysis of *NCED* genes in alfalfa

Through the screening and identification of alfalfa gene family members by Blast and HMM, 35 *NCED* gene family members were finally identified. According to their positions on chromosomes, they are named *MsNCED1* to *MsNCED35.*

The physicochemical property analysis of the protein encoded by the *MsNCED* gene of alfalfa showed that the prediction results (Table 1) indicated that the sequence length of the MsNCED protein was 242 to 610 amino acids, and the molecular weight was 27,996.90 Da to 68,232.41 Da. Six proteins have isoelectric points greater than 7, which are basic proteins, while the isoelectric points of the remaining proteins are all less than 7, which are acidic proteins. Six proteins have an instability coefficient greater than 40, which indicates they are unstable proteins, while the majority of proteins are stable proteins. The hydrophobicity index of all proteins is negative, and they belong to hydrophilic proteins. The results of subcellular localization prediction analysis show that most members of the *MsNCED* gene family are located in peroxisomes and chloroplasts, *MsNCED1*, *MsNCED14*, and *MsNCED28* are in the cytoplasm, *MsNCED25* is in the endoplasmic reticulum, *MsNCED29* is outside the cell, and *MsNCED32* is in the mitochondria. This indicates that different NCED proteins may function in different microenvironments.

Table 1 Analysis of basic information about the *MsNCED* gene family

Gene ID	Gene	Chromosome number	Number of amino acids(aa)	Molecular weight (MW,Da)	PI	GRAVY	Instability index (II)	Subcellular localization
MS.gene063722.t1	*MsNCED1*	chr1.1	425	46939.50	5.69	-0.207	32.72	Cytoplasm
MS.gene061617.t1	*MsNCED2*	chr1.2	581	63927.11	6.48	-0.266	34.97	Peroxisome
MS.gene073883.t1	*MsNCED3*	chr1.3	581	63911.05	6.34	-0.27	35.31	Peroxisome
MS.gene053436.t1	*MsNCED4*	chr1.4	581	63957.10	6.34	-0.276	35.31	Peroxisome
MS.gene039190.t1	*MsNCED5*	chr2.1	610	68188.39	6.49	-0.449	45.83	Chloroplast
MS.gene068670.t1	*MsNCED6*	chr2.2	610	68209.41	6.59	-0.458	45.79	Chloroplast
MS.gene063446.t1	*MsNCED7*	chr2.3	605	67665.75	6.37	-0.447	46.06	Chloroplast
MS.gene062834.t1	*MsNCED8*	chr2.4	610	68232.41	6.49	-0.457	45.86	Chloroplast

MS.gene014640.t1	*MsNCED9*	chr3.1	587	67062.69	6.54	-0.342	36.1	Peroxisome
MS.gene40296.t1	*MsNCED10*	chr3.2	583	66547.00	6.34	-0.336	36.79	Chloroplast Peroxisome
MS.gene063184.t1	*MsNCED11*	chr3.3	583	66559.03	6.43	-0.34	36.57	Peroxisome
MS.gene023313.t1	*MsNCED12*	chr4.1	608	67383.90	5.89	-0.219	42.78	Chloroplast
MS.gene34924.t1	*MsNCED13*	chr4.1	608	67366.78	5.82	-0.239	42.65	Chloroplast
MS.gene023312.t1	*MsNCED14*	chr4.2	504	55938.73	5.28	-0.151	37.41	Cytoplasm
MS.gene86482.t1	*MsNCED15*	chr5.1	581	64724.45	6.55	-0.171	34.19	Chloroplast
MS.gene86479.t1	*MsNCED16*	chr5.1	501	55217.16	5.93	-0.221	30.61	Peroxisome
MS.gene82475.t1	*MsNCED17*	chr5.2	581	64740.48	6.93	-0.177	33.03	Chloroplast
MS.gene82473.t1	*MsNCED18*	chr5.2	581	63998.19	6.48	-0.278	35.54	Peroxisome
MS.gene88323.t1	*MsNCED19*	chr5.3	581	64779.55	6.56	-0.16	32.18	Chloroplast
MS.gene88321.t1	*MsNCED20*	chr5.3	343	38267.27	9.47	-0.165	26.77	Chloroplast
MS.gene88317.t1	*MsNCED21*	chr5.3	501	55288.28	5.83	-0.197	31.37	Peroxisome
MS.gene72718.t1	*MsNCED22*	chr5.4	569	63354.66	6.69	-0.241	35.91	Chloroplast
MS.gene72720.t1	*MsNCED23*	chr5.4	581	63873.02	6.34	-0.273	35.48	Peroxisome
MS.gene056166.t1	*MsNCED24*	chr8.1	540	60934.79	6.19	-0.323	31.92	Peroxisome
MS.gene056165.t1	*MsNCED25*	chr8.1	440	50427.95	6.47	-0.244	34.59	Endoplasmic reticulum
MS.gene056164.t1	*MsNCED26*	chr8.1	537	61191.43	7.64	-0.242	33	Chloroplast
MS.gene016668.t1	*MsNCED27*	chr8.2	540	61057.95	6.3	-0.336	31.8	Peroxisome
MS.gene016669.t1	*MsNCED28*	chr8.2	242	27996.90	6	-0.443	33.93	Cytoplasm
MS.gene45745.t1	*MsNCED29*	chr8.2	267	30777.64	8.29	-0.272	31.9	Extracellular
MS.gene23406.t1	*MsNCED30*	chr8.2	538	61427.58	7.06	-0.321	31.75	Peroxisome
MS.gene45747.t1	*MsNCED31*	chr8.3	540	60912.79	6.3	-0.322	32.52	Peroxisome
MS.gene45746.t1	*MsNCED32*	chr8.3	567	64624.25	8.25	-0.32	35.03	Mitochondrion
MS.gene016670.t1	*MsNCED33*	chr8.3	575	65657.46	8.5	-0.33	32.36	Chloroplast
MS.gene95249.t1	*MsNCED34*	chr8.4	536	60637.48	6.36	-0.335	31.74	Peroxisome
MS.gene95248.t1	*MsNCED35*	chr8.4	430	49579.87	6.44	-0.346	33.12	Chloroplast Endoplasmic reticulum

Chromosomal localization of the *NCED* gene family in alfalfa

Based on the gene annotation information of alfalfa, chromosomal location analysis of the identified 35 *MsNCED* genes revealed (Fig. 1) that the 35 genes were distributed on 21 chromosomes, among which two genes were distributed on chr4.1, chr5.2, chr5.3, chr8.1 and chr8.4 respectively. Three genes are distributed on chr5.4 and chr8.3, four genes on chr8.2, and only one gene is distributed on the remaining chromosomes. It indicates the diversity and complexity of the *MsNCED* gene.

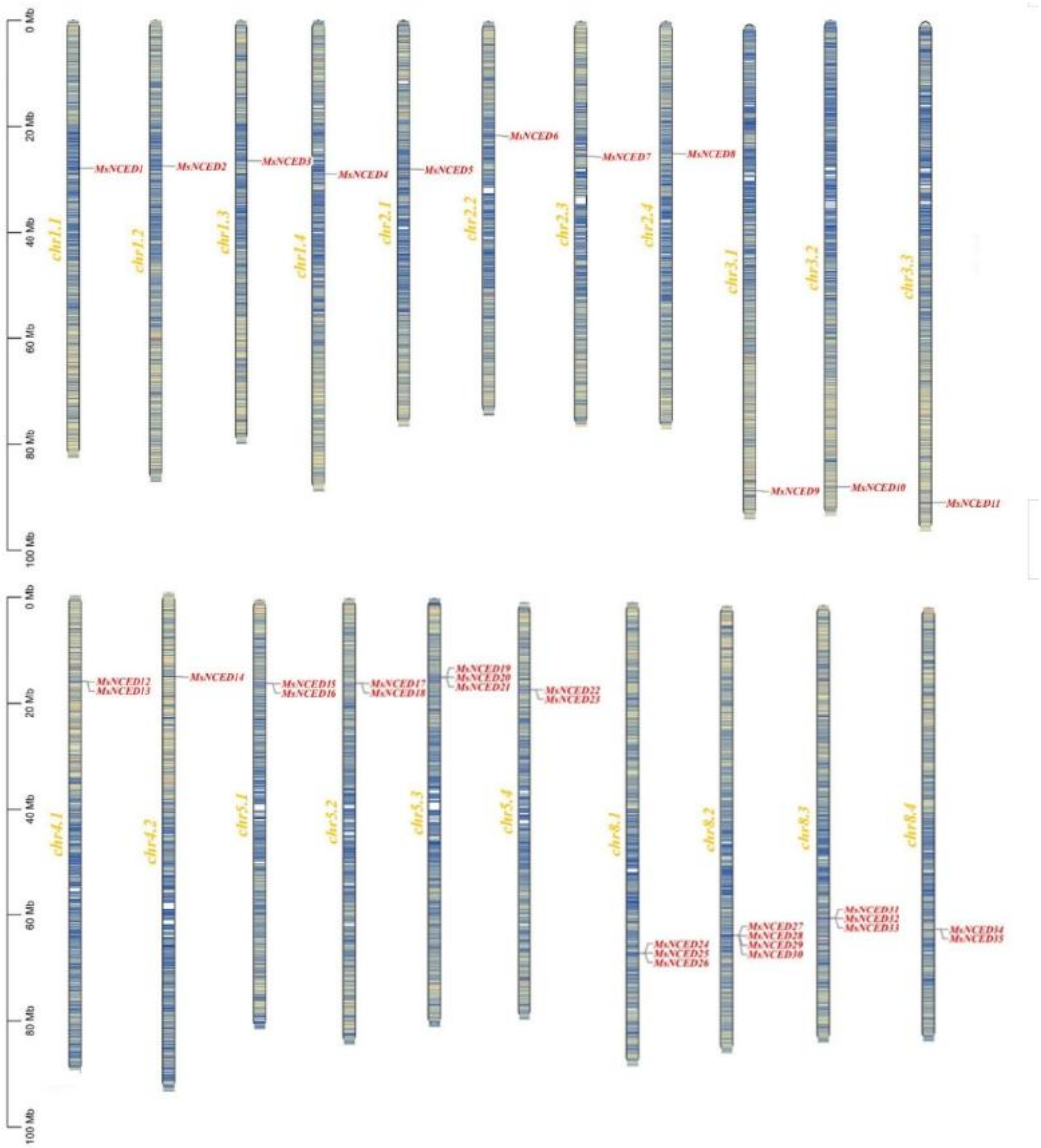


Fig. 1 Chromosomal location of *MsNCED* gene family members

Secondary and tertiary structure prediction analysis of *NCED* family proteins in alfalfa

The secondary structure of the protein was predicted through the SOPMA online website. The results (Table 2) showed that the proportion of irregular curls was the highest, reaching up to 61.80%, followed by α -helices, with the highest proportion reaching 17.23%, and β -turns were the lowest, with the highest proportion reaching

115 6.98%.

116 **Table 2** The secondary structure of *MsNCED* protein in alfalfa

Gene ID	Gene name	Protein	Alpha helix (%)	Beta turn(%)	Extended strand(%)	Random coil(%)
MS.gene063722.t1	*MsNCED1*	MsNCED1	14.82	5.41	24.24	55.53
MS.gene061617.t1	*MsNCED2*	MsNCED2	14.46	5.68	22.55	57.31
MS.gene073883.t1	*MsNCED3*	MsNCED3	14.11	6.02	20.48	59.38
MS.gene053436.t1	*MsNCED4*	MsNCED4	14.63	5.16	21.51	58.69
MS.gene039190.t1	*MsNCED5*	MsNCED5	12.13	5.9	20.82	61.15
MS.gene068670.t1	*MsNCED6*	MsNCED6	12.3	5.74	20.16	61.8
MS.gene063446.t1	*MsNCED7*	MsNCED7	12.56	6.12	20.66	60.66
MS.gene062834.t1	*MsNCED8*	MsNCED8	13.11	6.23	22.95	57.7
MS.gene014640.t1	*MsNCED9*	MsNCED9	13.46	4.26	20.95	61.33
MS.gene40296.t1	*MsNCED10*	MsNCED10	14.41	4.8	20.24	60.55
MS.gene063184.t1	*MsNCED11*	MsNCED11	13.88	4.12	21.78	60.72
MS.gene023313.t1	*MsNCED12*	MsNCED12	14.8	5.1	20.56	59.54
MS.gene34924.t1	*MsNCED13*	MsNCED13	12.5	5.59	21.38	60.53
MS.gene023312.t1	*MsNCED14*	MsNCED14	14.88	5.56	24.21	55.36
MS.gene86482.t1	*MsNCED15*	MsNCED15	14.8	4.99	22.38	57.83
MS.gene86479.t1	*MsNCED16*	MsNCED16	11.98	6.39	23.35	58.28
MS.gene82475.t1	*MsNCED17*	MsNCED17	13.77	5.16	21.86	59.21
MS.gene82473.t1	*MsNCED18*	MsNCED18	14.11	5.51	22.38	58
MS.gene88323.t1	*MsNCED19*	MsNCED19	13.77	5.85	23.06	57.31
MS.gene88321.t1	*MsNCED20*	MsNCED20	14.87	6.41	17.78	60.93
MS.gene88317.t1	*MsNCED21*	MsNCED21	12.57	6.79	25.15	55.49
MS.gene72718.t1	*MsNCED22*	MsNCED22	14.24	5.1	22.32	58.35
MS.gene72720.t1	*MsNCED23*	MsNCED23	13.6	5.16	22.2	59.04
MS.gene056166.t1	*MsNCED24*	MsNCED24	13.89	5.93	24.07	56.11
MS.gene056165.t1	*MsNCED25*	MsNCED25	14.32	6.82	26.36	52.5
MS.gene056164.t1	*MsNCED26*	MsNCED26	12.48	5.4	21.79	60.34
MS.gene016668.t1	*MsNCED27*	MsNCED27	13.7	5.74	23.7	56.85
MS.gene016669.t1	*MsNCED28*	MsNCED28	9.09	4.55	28.51	57.85
MS.gene45745.t1	*MsNCED29*	MsNCED29	17.23	7.49	21.35	53.93
MS.gene23406.t1	*MsNCED30*	MsNCED30	14.5	5.95	24.54	55.02
MS.gene45747.t1	*MsNCED31*	MsNCED31	15.37	5.37	23.52	55.74
MS.gene45746.t1	*MsNCED32*	MsNCED32	13.93	5.82	22.05	58.2
MS.gene016670.t1	*MsNCED33*	MsNCED33	14.09	5.04	22.09	58.78
MS.gene95249.t1	*MsNCED34*	MsNCED34	14.55	5.41	24.81	55.22
MS.gene95248.t1	*MsNCED35*	MsNCED35	14.42	6.98	26.28	52.33

117 The tertiary structure of the protein was predicted by the SWISS-MODEL. The

118 results (Fig. 2) showed that the structures of *MsNCED1*, *MsNCED16*, and *MsNCED21*

were similar, and the structures of *MsNCED2*, *MsNCED3*, *MsNCED4*, *MsNCED18*, and *MsNCED23* were similar. The structures of *MsNCED5*, *MsNCED6*, *MsNCED7* and *MsNCED8* are similar. The structures of *MsNCED9*, *MsNCED10* and *MsNCED11* are similar. The structures of *MsNCED12* and *MsNCED13* are similar. The structures of *MsNCED15*, *MsNCED17* and *MsNCED19* are similar. The structures of *MsNCED24*, *MsNCED26*, *MsNCED27*, *MsNCED31*, *MsNCED32* and *MsNCED33* are similar, while those of *MsNCED30*, *MsNCED34* and *MsNCED35* are also similar. The structures of the other family members are not similar.

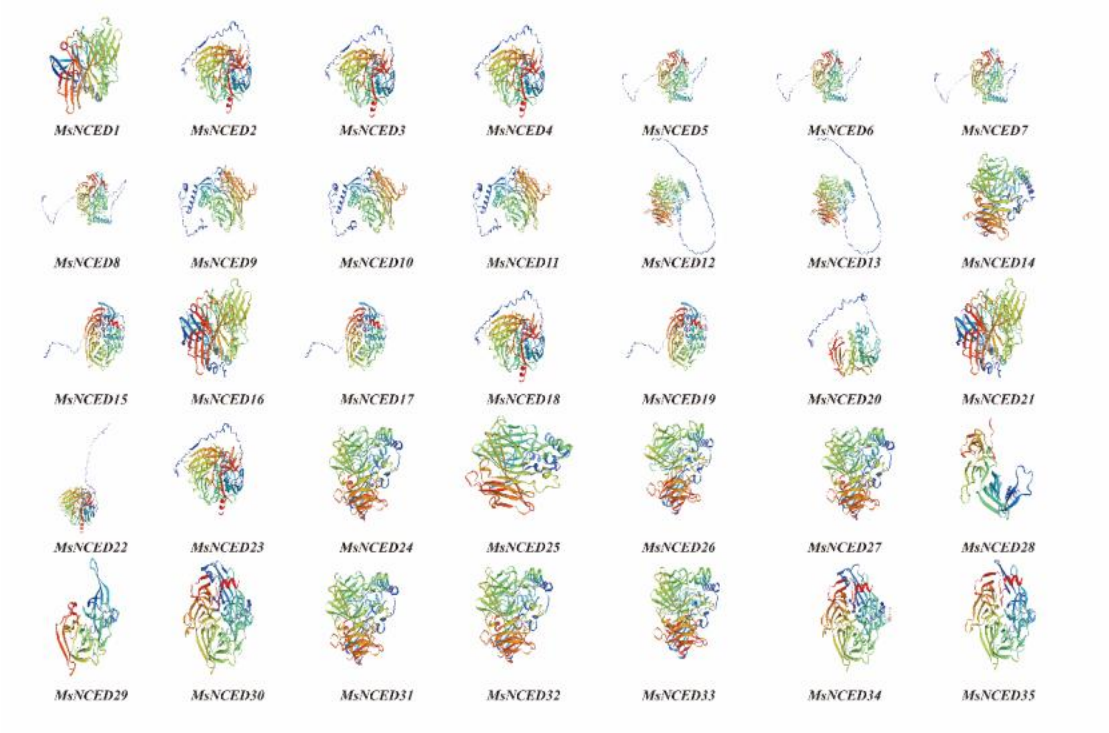


Fig. 2 The tertiary structure of *MsNCED* protein in alfalfa

Conserved motifs and gene structure analysis of the *NCED* gene family in alfalfa

To investigate the diversity of *MsNCED* protein motifs, conserved motifs analysis of *MsNCED* protein sequences was performed using the online tool MEME (Fig. 3). A total of 10 conserved motifs were discovered among the members of the *NCED* family (Fig. 3A). The conserved motifs ranged from 21 to 50 amino acids in length. *MsNCED9*,

MsNCED10, and *MsNCED11* lacked motif 4, motif 9, and motif 10, respectively. *MsNCED1* lacked motif1 and motif10. The absence of motif8 in *MsNCED26* indicates that these motifs have unique functions. Although the number and types of motifs varied among MsNCED proteins, most members shared similar motif compositions. Conserved domain analysis confirmed that all MsNCED proteins contained typical NCED conserved domains (Fig. 3B). Gene structure analysis indicated that MsNCED family members consisted of 1 to 15 exons (Fig. 3C), with *MsNCED32* and *MsNCED33* containing the largest number of exons. Genes with closer phylogenetic relationships exhibited more similar exon structures and conserved domain compositions, further indicating the evolutionary conservation of the NCED gene family.

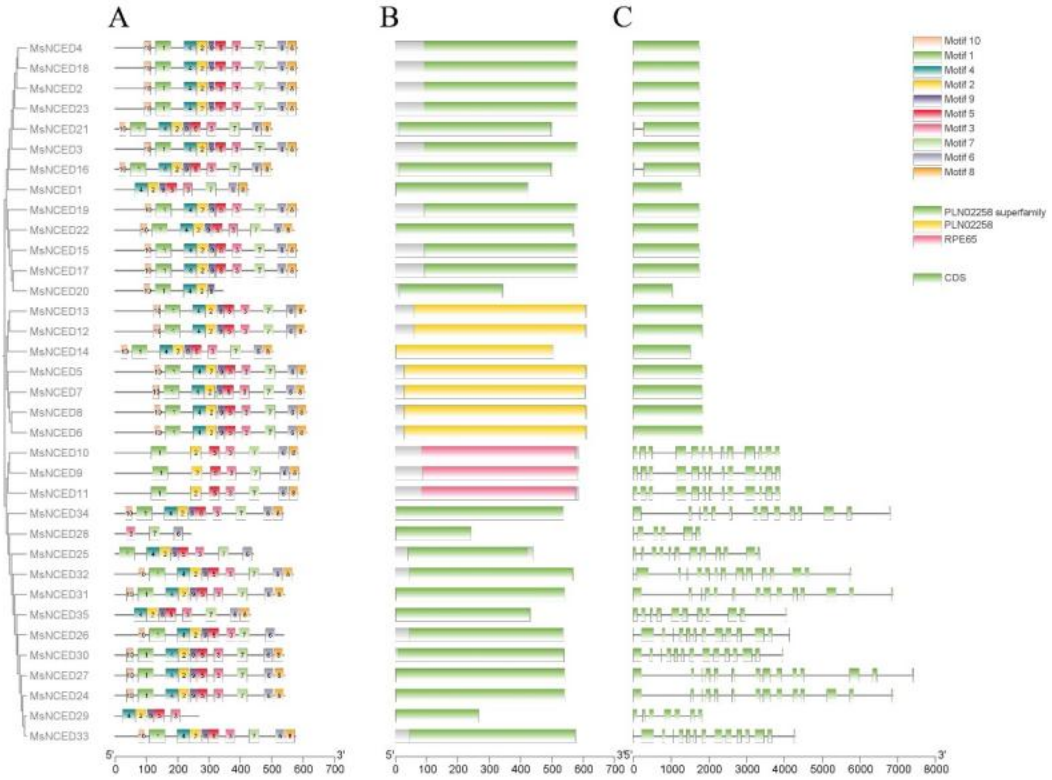


Fig. 3 *MsNCED* gene family conserved motif and *MsNCED* gene structure analysis

Phylogenetic analysis of the *NCED* gene family in alfalfa

To further elucidate the evolutionary relationships of the *MsNCED* gene family, a

comparative analysis of NCED proteins was conducted among alfalfa, soybean and *Arabidopsis thaliana* (Fig. 4; Table S1). The phylogenetic tree was constructed using the NJ method in MEGA11. Based on phylogenetic relationships, NCED proteins are classified into 7 subfamilies. Among them, the 35 *MsNCED* members of alfalfa and the 16 *GmNCED* members of soybean displayed differentiated distributions across each subfamily, indicating that the *NCED* family has undergone significant functional differentiation during the evolution of leguminous plants. The number of genes varied significantly among the subgroups. Subgroup I contained the largest number of genes (18 members), whereas subgroup VII contained the fewest, with only one member. Members of the same subfamily were closely related and likely shared similar biological functions.

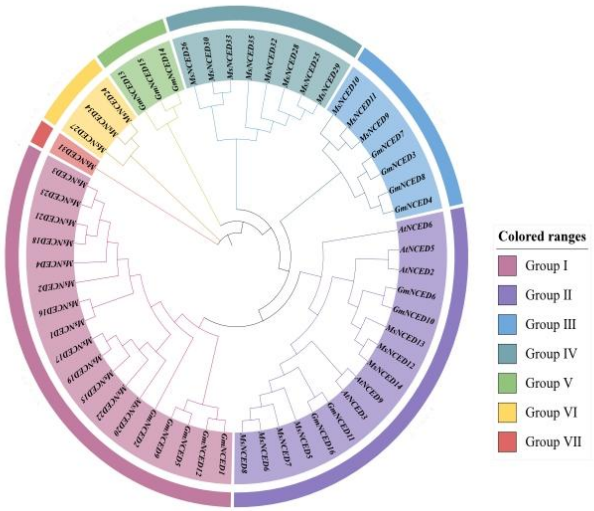


Fig. 4 Phylogentic analysis of the *NCED* gene family in *Arabidopsis thaliana, Medicago sativa*, and *Glycine max*

Collinearity analysis of the *NCED* gene family in alfalfa

The complexity and adaptability of plant genomes largely depend on the expansion of gene families, and gene duplication is considered a major driving force behind this

expansion. Gene duplication profoundly influences genome evolution and contributes to gene diversity. Therefore, interspecific and intraspecific collinearity analyses were conducted on the *NCED* gene family. Intraspecific collinearity analysis revealed widespread segmental duplication events within the *MsNCED* genome. Segmental duplication events were identified on chromosomes 1, 2, 3, 4, 5, and 8, whereas no segmental or tandem duplications were detected on the remaining chromosomes (Fig. 5).

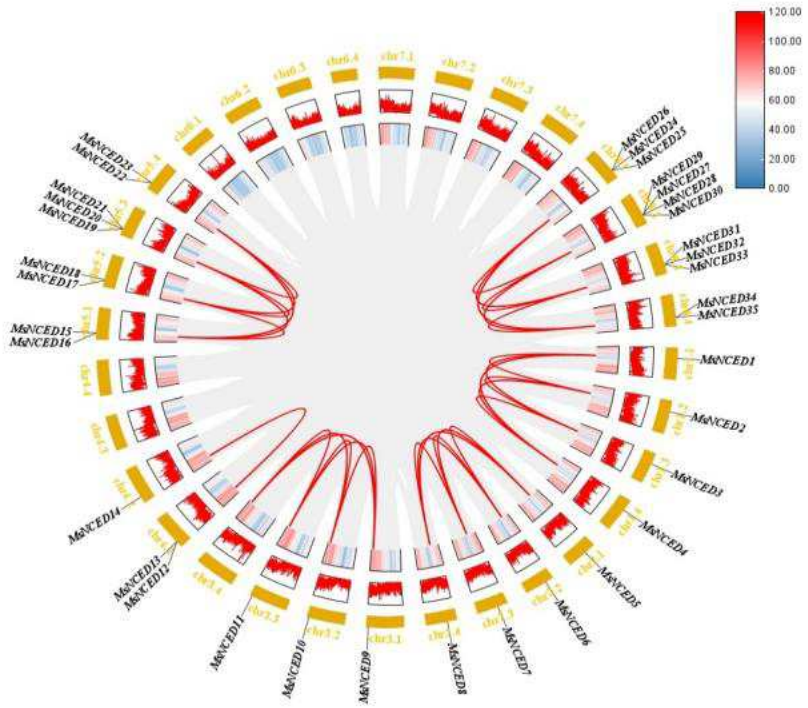


Fig. 5 The collinearity relationship among alfalfa *MsNCED* genes

To further investigate the evolutionary relationships and duplication events of the *MsNCED* gene family, syntenic relationships between *NCED* genes in *Medicago sativa* and those in *Arabidopsis thaliana*, *Medicago truncatula*, and *Glycine max* were analyzed (Fig. 6). The results showed that there were 8 syntenic gene pairs between *Medicago sativa* and *Arabidopsis thaliana*, 15 pairs between *Medicago sativa* and

Medicago truncatula, and 28 pairs between *Medicago sativa* and *Glycine max.* This indicates that *Medicago sativa* has a high degree of synteny and sequence and similarity with *Arabidopsis thaliana*, *Medicago truncatula*, and *Glycine max* during the evolutionary process.

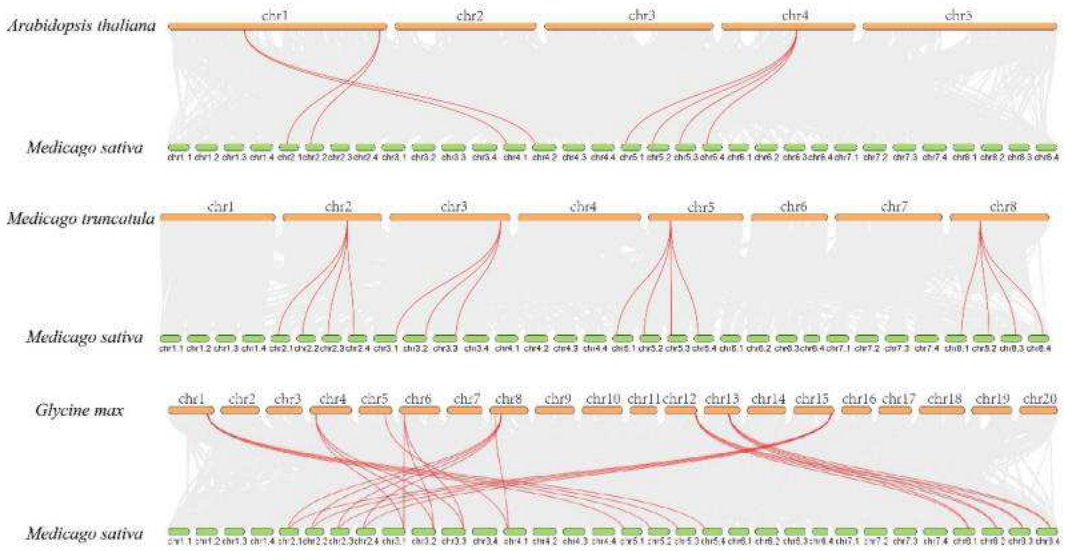


Fig. 6 Collinearity analysis of *MsNCED* genes between *M. sativa*, *A. thaliana*, *M. truncatula*, and *G. max*

Cis-acting element analysis of the *NCED* gene family in alfalfa

Cis-acting elements in gene promoters can bind to various transcription factors to regulate the expression of downstream genes, and analysis of these elements can provide insights into the potential functional differences among genes (Fig. 7). To investigate the function of the *MsNCED* genes, the 2000 bp promoter region upstream of the *MsNCED* gene were selected for cis-acting element analysis (Table S2). A total of 29 types of cis-acting elements were identified, mainly including light-responsive, phytohormone-responsive, stress-responsive, and development-related elements (Table S3). Among them, light-responsive elements appeared most frequently, and some genes contained multiple light-responsive elements. Among these, 26 genes contained MeJA-

responsive elements, 14 genes contained low-temperature-responsive elements, and 25 genes contained abscisic acid-responsive elements (ABRE). This indicates that these genes play a crucial role in responding to abiotic stresses and regulating their own growth and development.

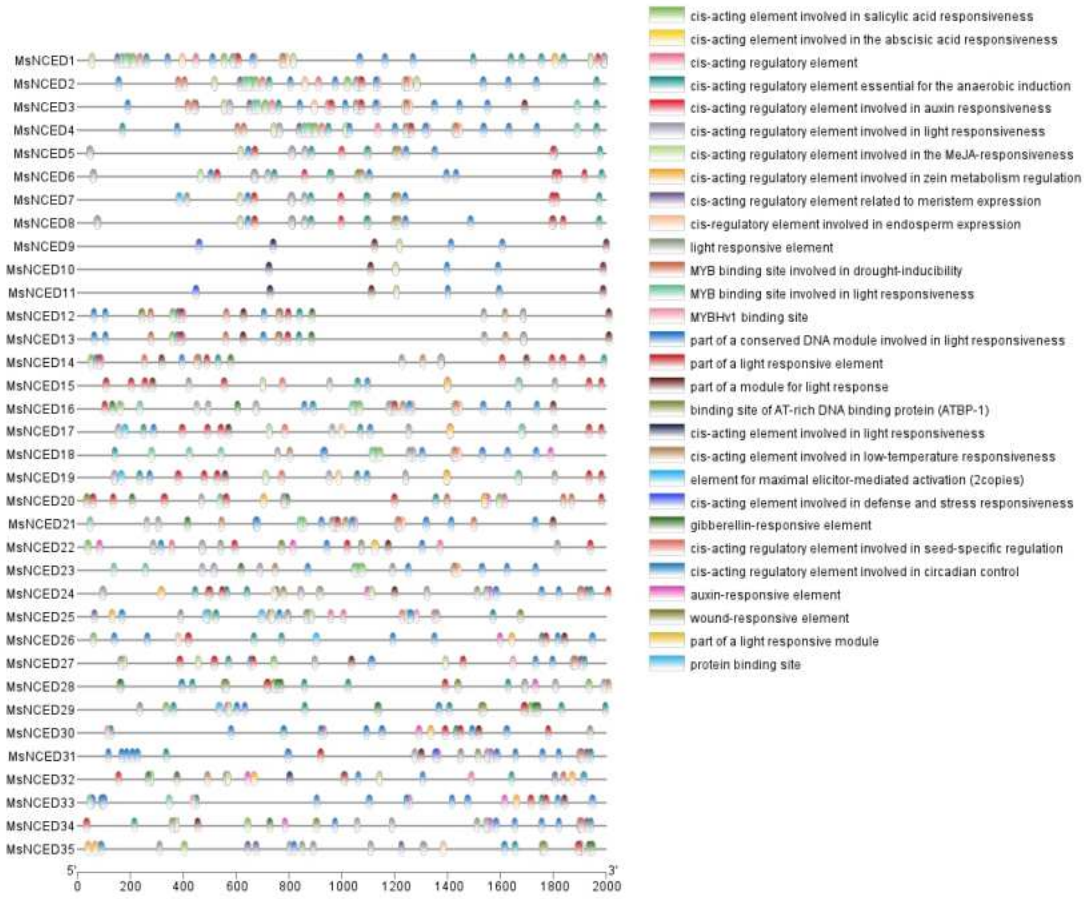


Fig. 7 Cis-elements of the alfalfa *MsNCED*

Expression analysis of the *NCED* gene family under abiotic stresses in alfalfa

To further explore the expression patterns of *MsNCED* genes in aboveground and underground parts under low-temperature stress, salt stress, and drought stress, 8 *MsNCED* genes were selected for qRT-PCR validation (Table S4).

Under low-temperature stress in the aboveground parts of alfalfa (Fig. 8A), the expression levels of *MsNCED1*, *MsNCED8*, and *MsNCED31* showed an upward trend,

while *MsNCED10*, *MsNCED26*, and *MsNCED34* showed a downward trend. The expression levels of *MsNCED1* and *MsNCED8* reached their peaks at 12 h and then decreased over time. The expression level of *MsNCED18* decreased significantly at 4 h, and that of *MsNCED25* decreased significantly at 12 h, showing an upward trend at other time points. Under low-temperature stress in the underground parts of alfalfa (Fig. 8B), the expression levels of *MsNCED1*, *MsNCED8*, *MsNCED18*, *MsNCED25*, and *MsNCED26* showed an upward trend. The expression level of *MsNCED10* was the lowest at 12 h, and those of *MsNCED31* and *MsNCED34* were the lowest at 4 h.

Under salt stress in aboveground alfalfa tissues (Fig. 8C), *MsNCED1*, *MsNCED8*, *MsNCED26*, and *MsNCED31* expression increased significantly at 24 h. *MsNCED34* expression declined over the time course. *MsNCED10* peaked at 4 h and fell below its 0 h level at 12 h and 24 h. *MsNCED18* peaked at 8 h and decreased markedly at 12 h and 24 h. *MsNCED25* rose from 4 h to 12 h and then declined significantly at 24 h. Under salt stress in underground alfalfa tissues (Fig. 8D), *MsNCED1* and *MsNCED8* expression trended upward, whereas *MsNCED18* and *MsNCED34* trended downward. *MsNCED10*, *MsNCED25*, and *MsNCED31* showed their greatest expression at 8 h, while *MsNCED26* reached its maximum at 24 h.

Under drought stress in aboveground alfalfa tissues (Fig. 8E), *MsNCED10*, *MsNCED26*, and *MsNCED31* expression increased, whereas *MsNCED1* and *MsNCED34* expression decreased. *MsNCED8* peaked at 4 h and then declined, while *MsNCED18* peaked at 2 h before declining. *MsNCED25* was lowest at 2 h, rose thereafter, and reached its maximum at 8 h. Under salt stress in underground alfalfa

231 tissues (Fig. 8F), *MsNCED1*, *MsNCED8*, *MsNCED18*, *MsNCED25*, and *MsNCED31*
232 expression increased. *MsNCED10* peaked at 4 h and then fell markedly. *MsNCED26*
233 and *MsNCED34* peaked at 6 h and declined substantially by 8 h.

234 Through the analysis of the *NCED* gene family in alfalfa under three abiotic stress
235 treatments, the expression of *MsNCED1*, *MsNCED8*, and *MsNCED31* genes in alfalfa
236 can be induced under low-temperature, salt, and drought stress treatments.

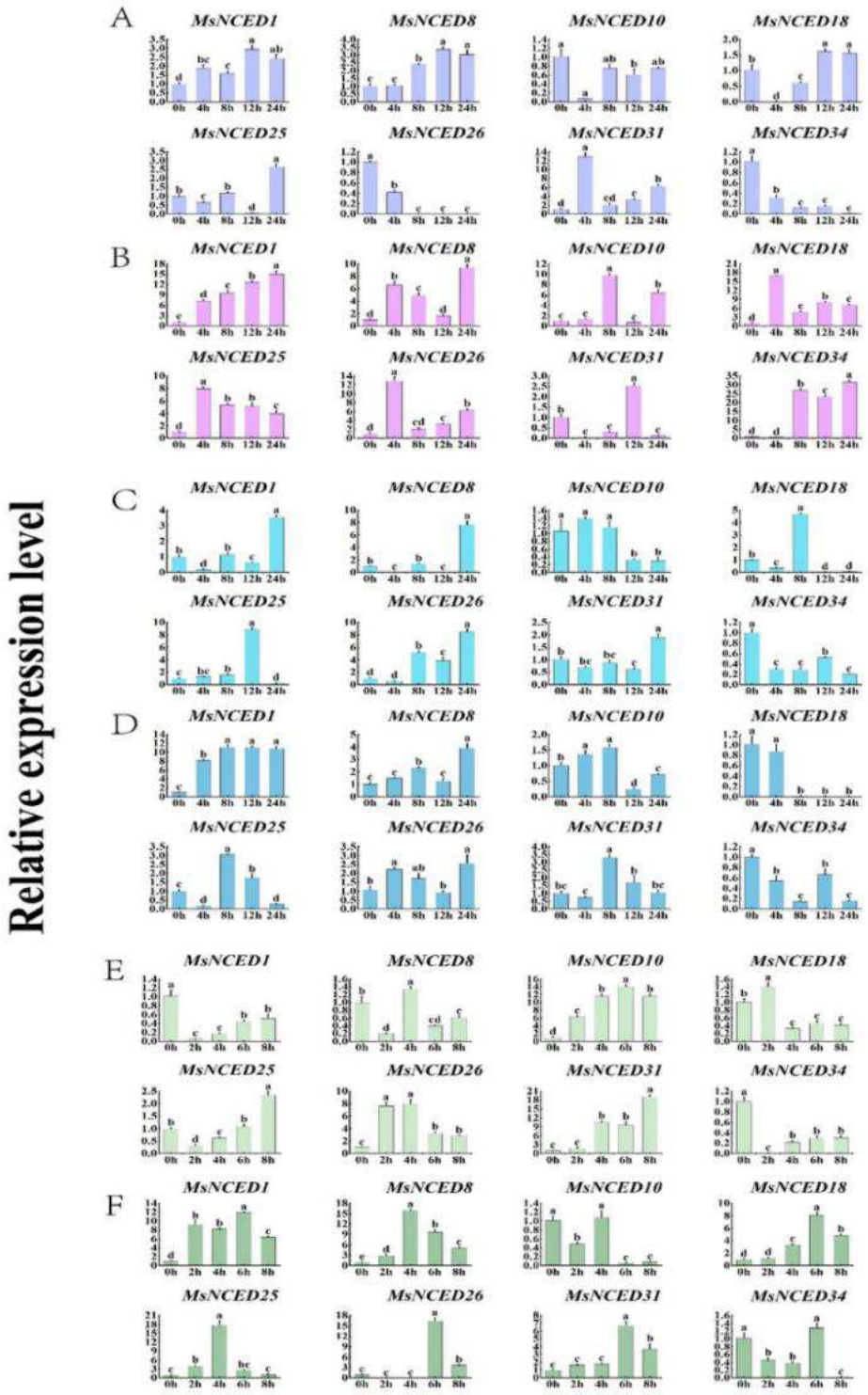


Fig.8 Changes in the expression levels of some members of the *MsNCED* gene family under different abiotic stresses

A: Expression levels of *MsNCED* genes in aboveground tissues under cold stress. B: Expression levels of *MsNCED* genes in underground tissues under cold stress. C: Expression levels of *MsNCED* genes in aboveground tissues under salt stress. D: Expression levels of *MsNCED* genes in underground tissues under salt stress. E: Expression levels of *MsNCED* genes in aboveground tissues under drought stress. F:

Expression levels of *MsNCED* genes in underground tissues under drought stress. Different lowercase letters in the figure indicate significant differences among the values ($p < 0.05$).

Discussion

Alfalfa is frequently subjected to abiotic stresses such as cold, salt, and drought, resulting in yield reduction and impaired quality during growth. Therefore, identifying genes associated with stress tolerance in alfalfa is of great significance for its genetic improvement. ABA is a signaling molecule that plays an important role in plants stress responses [20]. The *NCED* gene family encodes 9-cis-epoxycarotenoid dioxygenase, which is the key rate-limiting enzyme in ABA synthesis. The *NCED* gene family plays a crucial role in plant growth, development and responses to abiotic stress.

In this study, a total of 35 members of the *NCED* gene family were identified in the entire genome of alfalfa. The number differed significantly from those reported in species such as soybean and *Arabidopsis thaliana* [9, 15]. Such differences in gene numbers might be closely associated with the disparities in gene duplication mechanisms among different species. Tandem duplication and segmental duplication of genes are the main driving forces for the expansion of gene families [21]. As an autotetraploid species, alfalfa has undergone multiple whole genome duplication events during evolution, which might be an important reason for the significantly larger number of *NCED* family members compared to diploid species [21, 22]. Further sequence analysis revealed that the *NCED* gene family in alfalfa displayed high structural variability, which was reflected in the significant differences in the physicochemical properties of MsNCED proteins, including amino acid length, theoretical isoelectric point, and molecular weight. Moreover, all MsNCED proteins

were hydrophilic, and their secondary structures mainly consisted of random coils and α - helices. This relatively conserved secondary structure might contribute to maintaining the conformational stability of the catalytic center of the NCED enzyme, thereby ensuring its efficient catalysis of the rate limiting step in ABA biosynthesis when plants respond to abiotic stresses such as drought and salinity, and playing a key physiological function. This structural characteristic might reflect its functional adaptation.

The subcellular localization of all five *AtNCED* family members in *Arabidopsis thaliana* is the plastid. Among them, *AtNCED5* specifically binds to the thylakoid membrane, *AtNCED9* exists in a soluble form in the stroma, while the remaining members are dynamically distributed between the stroma and the thylakoid membrane-binding compartments [15]. *Paeonia lactiflora PlNCED1* and *PlNCED2* are localized to the nucleus and cytoplasm, respectively, where they function as active enzymes in ABA biosynthesis and regulate transcription factors [23]. The *GmNCED* members in soybean are mainly located in the cytoplasm and chloroplasts [9]. In *Medicago sativa*, predicting the subcellular localization of the 35 identified *MsNCED* proteins revealed that the *MsNCED* gene family is subcellularly localized in different locations. Most members are localized in peroxisomes and chloroplasts, followed by the cytoplasm, endoplasmic reticulum, extracellular space, and mitochondria. This localization distribution pattern differs from the research results of the above-mentioned other species, presumably related to the structural variations that occurred during the evolution of the *MsNCED* gene family proteins.

Phylogenetic analysis of NCED protein sequences from *Arabidopsis*, soybean, and alfalfa indicated that alfalfa *NCED* family members cluster predominantly into four subgroups, and genes within the same subgroup tend to share similar functions [21]. All five AtNCED proteins were assigned to Group II, suggesting that MsNCED proteins in this clade may perform functions analogous to their Arabidopsis counterparts. *AtNCED3* is known to enhance drought tolerance by promoting endogenous ABA accumulation [12], while *AtNCED6* and *AtNCED9* play key roles in ABA biosynthesis during seed development [24]. In Group I, soybean *NCED* genes *GmNCED2* and *GmNCED12* are involved in responses to dehydration and salt stress, whereas *GmNCED14* and *GmNCED15* are upregulated under drought. In addition, *GmNCED13* and *GmNCED14* can promote seed germination at elevated temperatures [9].

Gene duplication is an important driving force for species evolution and gene family expansion [25]. Chromosomal localization analysis revealed that *MsNCED* genes are unevenly distributed across 21 chromosomes, possibly reflecting chromosomal rearrangements during evolution [26]. Only one pair of collinear genes has been identified in barley [27], and six pairs of collinear genes were found in soybean through interspecies analysis [9]. Multigene families are generated through gene segment duplication [28]. In this study, segmental duplication was widely present in the *MsNCED* gene family, which may facilitate the emergence of new gene functions. This indicates that segment duplication may play an important role in the expansion of *MsNCED* gene family and potentially enhance genetic diversity and environmental

adaptability.

Motifs and conserved domains reflect the conservation of protein sequences [29, 30]. The differences in conserved motifs and gene structures among *MsNCED* genes partially reflect the evolutionary relationships within this gene family. The *MsNCED* gene family was divided into five subfamilies based on phylogenetic analysis. Each subfamily possessed distinct motif compositions, although the conserved sequences were largely similar, suggesting that these genes may play diverse roles in response to abiotic stresses. The *MsNCED* gene family exhibited structural variation, with members containing 1 to 15 exons. Exons can influence the interaction between regulatory elements and coding sequences, thereby enabling plants to rapidly respond to growth and developmental signals or abiotic stresses [31].

Low-temperature stress severely disrupts plant growth and development. *NCED* genes contribute to plant cold responses by regulating ABA synthesis, are induced by low temperatures in various crops, and show potential for improving cold tolerance through breeding. Studies showed that cold stress strongly upregulates *CsNCED2* expression in tea [32]. Rice *OsNCED5* localizes to chloroplasts, is highly induced by low temperatures, and enhances seedling cold tolerance by modulating ROS homeostasis; *OsNCED5* knockout mutants exhibit reduced ABA levels and increased cold sensitivity [8]. In *Prunus mume*, *PmNCED3*, *PmNCED8*, and *PmNCED9* are markedly upregulated under low temperatures, consistent with roles in cold adaptation [33]. In wheat, *TaNCED5* expression rises significantly in leaves and tiller buds during vernalization, suggesting a tissue-specific role in ABA-mediated cold responses [34].

Moreover, members of the *NCED* family display distinct expression patterns under low temperatures, underscoring the functional complexity and diversity of *NCED* genes in the regulatory network of plant cold adaptation.

Salt stress limits crop growth and yield. The *NCED* gene family plays a central role in plant responses to salt stress by regulating endogenous ABA biosynthesis, thereby activating downstream stress-responsive signaling pathways that enhance salt and alkali tolerance. In rice, OsNCED3 elevates endogenous ABA levels and improves seedling alkali tolerance through osmotic adjustment, ion homeostasis, and reactive oxygen species (ROS) scavenging, collectively increasing survival under alkali stress [35]. Five *HvNCED* members have been identified in the barley genome; under salt stress, *HvNCED1* is strongly upregulated while *HvNCED3* and *HvNCED4* are downregulated, indicating functional divergence and a complex regulatory network [20].

Drought stress induces *NCED* expression, activates ABA biosynthesis, and thereby promotes stomatal closure and the expression of stress-responsive genes, reducing transpiration loss [36]. The *NCED* gene family is ubiquitous in terrestrial plants, and individual members show functionally divergent expression patterns under drought. In *Arabidopsis*, drought induces *AtNCED3*; its overexpression elevates ABA levels, up-regulates stress genes, lowers transpiration rate, and enhances drought tolerance [37]. In rice, drought induces *OsNCED3*, which modulates endogenous ABA to improve drought resistance; RNAi-mediated silencing of *OsNCED3* causes drought sensitivity and reduced biomass, whereas its overexpression markedly enhances

drought resistance [38]. Huang et al. reported that high-salt and drought strongly induced *OsNCED5* expression in both shoots and roots of rice [13]. In foxtail millet, *SiNCED3* is likewise drought induced, suggesting a role in adaptive drought regulation [39]. Together, these studies reveal conserved *NCED* functions alongside species-specific regulatory mechanisms in drought responses.

Materials and methods

Identification of the *NCED* gene family in alfalfa

The whole-genome sequence, gene annotation, and protein sequence files of *Medicago sativa* (cultivar 'Xin Jiang Da Ye') were downloaded from the online website Figshare (https://figshare.com/projects/whole_genome_sequencing_and_assembly_of_Medicago_sativa). The NCED protein sequences of *Arabidopsis thaliana* were obtained from the NCBI database. The NCED protein sequences of *Medicago truncatula* were downloaded from the MODMS database (https://modms.lzu.edu.cn/), and the NCED protein sequences of *Glycine max* were retrieved from Phytozome (https://phytozome.jgi.doe.gov/).

Using TBtools and *Arabidopsis* protein sequences as queries, BLASTP was employed to identify candidate NCED protein sequences in the Medicago sativa genome. The hidden Markov model (HMM) file for the NCED protein domain (PF03055) was retrieved from the Pfam database (https://pfam.xfam.org/), and HMM searches in TBtools were used to detect sequences in the alfalfa genome that contain the NCED domain. Conserved domain analyses were performed using SMART

(http://smart.embl.de) and NCBI's online Batch CD-Search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) to confirm the presence of domains characteristic of the *NCED* gene family. Ultimately, 35 *NCED* family genes were identified in alfalfa (Table S5).

Physicochemical properties analysis, chromosomal localization, and structural prediction of MsNCED proteins in alfalfa

The chromosomal distribution of the *MsNCED* gene family was visualized using TBtools. Physicochemical properties of the alfalfa NCED family proteins-including isoelectric point, molecular weight, and instability index-were analyzed using the ExPASy online platform (https://www.expasy.org/). Subcellular localization predictions were performed with WOLF PSORT (https://wolfpsort.hgc.jp/). Protein secondary structure was predicted using the SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl) online tool, and tertiary structures were modeled using SWISS-MODEL (https://swissmodel.expasy.org/).

Phylogenetic tree analysis of the *NCED* gene family in alfalfa

Multiple sequence alignments of NCED protein sequences from *Arabidopsis thaliana*, alfalfa, and soybean were performed using MUSCLE implemented in MEGA11. The phylogenetic tree was constructed using the neighbor-joining method. The phylogenetic tree was visualized and annotated using the iTOL online platform (https://itol.embl.de/).

Gene structure and conserved motifs of the *NCED* gene family in alfalfa

Conserved motifs of MsNCED proteins were identified using the MEME online

tool (https://meme-suite.org/meme/), with the number of motifs set to 10. The gene structures and conserved domains of the alfalfa *NCED* gene family were visualized using the TBtools base on the obtained information.

Cis-acting element analysis of the *NCED* gene family in alfalfa

A 2000 bp nucleotide sequence upstream of the start codon of each *MsNCED* gene was extracted from the alfalfa genome sequence. These sequences were submitted to the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for cis element prediction. The predicted cis-acting elements were visualized using TBtools.

Collinearity analysis of the *NCED* gene family in alfalfa

Collinearity and gene duplication events of *NCED* gene family members between alfalfa and other species were analyzed using One Step MCScanX module in TBtools, and the results were visualized.

Plant materials and stress treatments

In this study, alfalfa seeds ('Caoyuan No. 4') were bred at Inner Mongolia Agricultural University, China. Alfalfa seeds with uniform morphology and fullness were selected, placed in Petri dishes lined with filter paper moistened with distilled water, and germinated in a light incubator at 25 °C. The germinated seedlings were transplanted into pots filled with a mixture of nutrient soil and vermiculite (2:1). The plants were grown under long-day conditions (16 hours of light /8 hours of darkness) and watered regularly.

After four weeks of growth, alfalfa plants ('Caoyuan No. 4') were subjected to salt,

low-temperature, and drought stress treatments, respectively. For salt stress treatment, alfalfa plants were treated with 200mmol/L NaCl, and samples were collected at 0, 4, 8, 12 and 24h after treatment. For low-temperature stress treatment, plants were placed in a growth chamber at 4 °C, and samples were collected at 0, 4, 8, 12, and 24 h. For drought stress treatment, plants were subjected to air drying, and samples were collected at 0, 2, 4, 6, and 8 h. All sample collections were performed with three biological replicates. After sampling, the samples were immediately snap-frozen in liquid nitrogen and transferred to -80 ℃, and stored for subsequent analysis.

RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from alfalfa samples using TRIzol Reagent and stored at -80 ℃. The obtained RNA was reverse transcribed into cDNA using the EasyQuick RT MasterMix kit, and the cDNA was stored at -20 °C for subsequent analysis. Quantitative primers were designed using NCBI-Blast (Tab. 3), with amplification lengths ranging from 80 to 200 bp, and *Msβ-actin* was used as the internal reference primer. qRT-PCR was performed according to the instructions of the SuperStar Blue Universal SYBR Master Mix kit. With *Msβ-actin* as the internal reference gene and 3 replicates for each sample, the relative expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method [40].

Table 3 qRT-PCR primers

Gene	Forward primer	Reverse primer
MsNCED1	GTGGTGAGCCGTTTTTCGTG	CTTCACCTCCGCCACAATCT
MsNCED8	GTTGATGGGACCCACGGAAT	CACCGTTAGGTGTGACACGA
MsNCED10	AGGATTGGGGTAATGCCACG	AGATCTGCAGCCCCTCACTA
MsNCED18	CACGTGGACCTTACCACCTC	ACAGAGTGTGGCTTTTCCGT
MsNCED25	AGGGTGGGACCTAATCCGAA	GGAAGAGAATGACGCACCCA
MsNCED26	GGACACCTTCCGGATTGCTT	CGACGGGAGAAAACTTCGGA
MsNCED31	ACACTGGCAGGAAACAACGA	AGCCTCAGAGCCAAACCTTC
MsNCED34	GGCTCTCTTCCGGATTGCTT	CCACCGGAGAAAACTTCGGA

Msβ-actin	TTTGAGACTTTCAATGTGCCCGCC	TAGCATGTGGGAGTGCATAACCCT

Statistical analysis

Experimental data were processed using Excel 2019. Statistical analysis was performed using SPSS. One-way ANOVA followed by the least significant difference (LSD) test was used to compare differences among groups. Bar charts were generated using Origin.

Conclusion

In this study, 35 NCED family members were identified in the alfalfa genome, distributed across 21 chromosomes. Exon counts per *NCED* gene ranged from 1 to 15. All MsNCED proteins were predicted to be hydrophilic, and subcellular localization analyses indicate that most were localized to peroxisomes and chloroplasts. Collinearity analysis among alfalfa, *Arabidopsis thaliana*, and soybean revealed syntenic gene pairs. *MsNCED1*, *MsNCED8*, and *MsNCED31* were positively regulated by all three stresses, suggesting they may play key roles in abiotic stress responses. These findings provide useful leads for further functional study of *NCED* genes in alfalfa and enhance our understanding of their roles in stress adaptation.

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Author's contributions

ZZQ designed and performed the experiments; TY, HXW and CQ performed the main experiments; XJ, BL and FQW analyzed all the data; TY wrote manuscript. All authors reviewed and approved the manuscript.

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Data availability

We used previously published genomic data, and no datasets were generated or analysed in the current study.

Declarations

Ethics approval and consent to participate

This study is not a clinical trial. The ‘Caoyuan No.4’ alfalfa was grown and collected from Inner Mongolia Agricultural University, China, and all samples from the cultivar were adopted for all experiment. No specific permits are required for sample collection in this study. For experimental research and field studies on plants, including the collection of plant material, we comply with relevant institutional, national, and international guidelines and legislation. We declared that wild plants have not been collected in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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