



Article

# Identification and Expression Profile of *NCED* Genes in *Arachis hypogaea* L. during Drought Stress

Ao Chen <sup>1</sup> , Jingyan Li <sup>2</sup>, Heping Wang <sup>2</sup> and Puyan Zhao <sup>2,\*</sup>

<sup>1</sup> Guangzhou Dublin International College of Life Sciences and Technology, South China Agricultural University, Guangzhou 510642, China; aochen594666@gmail.com

<sup>2</sup> College of Horticulture, South China Agricultural University, Guangzhou 510642, China; lijingyan0119@163.com (J.L.); 15890700537@163.com (H.W.)

\* Correspondence: zhaopuyan@scau.edu.cn

**Abstract:** Peanut (*Arachis hypogaea* L.) is an important crop that provides essential proteins and oils for human and animal consumption. 9-cis-epoxycarotenoid dioxygenase (NCED) have been found can play a vital role in abscisic acid (ABA) biosynthesis and may be a response to drought stress. Until now, in *Arachis hypogaea*, no information about the *NCED* gene family has been reported and the importance of *NCED*-related drought tolerance is unclear. In this study, eight *NCED* genes in *Arachis hypogaea*, referred to as *AhNCEDs*, are distributed across eight chromosomes, with duplication events in *AhNCED1* and *AhNCED2*, *AhNCED3* and *AhNCED4*, and *AhNCED6* and *AhNCED7*. Comparative analysis revealed that *NCED* genes are highly conserved among plant species, including *Pisum sativum*, *Phaseolus vulgaris*, *Glycine max*, *Arabidopsis thaliana*, *Gossypium hirsutum*, and *Oryza sativa*. Further promoter analysis showed *AhNCEDs* have ABA-related and drought-inducible elements. The phenotyping of *Arachis hypogaea* cultivars NH5 and FH18 demonstrated that NH5 is drought-tolerant and FH18 is drought-sensitive. Transcriptome expression analysis revealed the differential regulation of *AhNCEDs* expression in both NH5 and FH18 cultivars under drought stress. Furthermore, compared to the *Arachis hypogaea* cultivar FH18, the NH5 exhibited a significant upregulation of *AhNCED1/2* expression under drought. To sum up, this study provides an insight into the drought-related *AhNCED* genes, screened out the potential candidates to regulate drought tolerance and ABA biosynthesis in *Arachis hypogaea*.



**Citation:** Chen, A.; Li, J.; Wang, H.; Zhao, P. Identification and Expression Profile of *NCED* Genes in *Arachis hypogaea* L. during Drought Stress. *Int. J. Mol. Sci.* **2024**, *25*, 5564. <https://doi.org/10.3390/ijms25105564>

Academic Editor: De-Guo Han

Received: 17 March 2024

Revised: 30 April 2024

Accepted: 1 May 2024

Published: 20 May 2024



**Copyright:** © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

**Keywords:** peanut; 9-cis-epoxycarotenoid dioxygenase; abscisic acid; drought-tolerant; drought-sensitive

## 1. Introduction

Drought is one of the most serious abiotic stresses, can cause a series of injury symptoms in plants and decline the global crop yields [1,2]. In order to adapt to drought stress, plants have improved their physiological and biochemical metabolism, forming a complex and effective signal network, including ABA biosynthesis pathway [3].

In plants, 9-cis-epoxycarotenoid dioxygenase (NCED) function as a pivotal enzyme in abscisic acid (ABA) biosynthesis and can drive the specific cleavage of 9-cis-epoxycarotenoids to produce the essential ABA precursor, xanthoxin [4–6]. Long-term studies have shown that the endogenous hormone abscisic acid (ABA) is intricately linked to regulating drought resistance in some plants [7–12]. Furthermore, in some plants, *NCED* genes related to ABA synthesis were critical involvement in drought resistance mechanisms [13–16]. For instance, the overexpression of *OsNCED5* in *Oryza sativa* has been shown to increase ABA levels, enhance tolerance to drought stress, and accelerate leaf senescence [14].

The peanut (*Arachis hypogaea* L.), a widely cultivated crop, is an important source of dietary protein and oils [17,18]. The growth and development of *Arachis hypogaeas* are easily affected by drought stress, especially during the seedling stage [19]. In addition, drought is particularly detrimental to *Arachis hypogaea* kernel production [20–24]. Under drought conditions, *Arachis hypogaea* plants exhibit various physiological and morphological alterations

such as leaf curling, wilting, and senescence [25]. These changes reflect adaptive strategies to minimise water loss and cope with the hydric stress [25].

Until now, although the diversity, distribution, and regulation of the *NCED* gene family have been investigated in some plant species like *Populus davidiana*, *Gossypium hirsutum*, and *Oryza sativa* and its participation in drought resistance has been explicated [13–15], there is limited information on *Arachis hypogaea*. In order to explore the drought-related *NCED* in *Arachis hypogaea*, this study used bioinformatic methods to identify *Arachis hypogaea NCED* genes and explored their basic characteristics. Additionally, the expression patterns of *AhNCED* genes related to drought stresses between drought-tolerant and drought-sensitive *Arachis hypogaea* varieties were also investigated. Finally, this study emphasised the immediate response of drought-related *AhNCED* genes, screened out the potential candidate to regulate drought tolerance and ABA biosynthesis.

## 2. Results

### 2.1. Identification and Characterisation of *AhNCEDs*

To identify the *NCED* genes in the *Arachis hypogaea* genome, *NCED* genes from *Pisum sativum*, *Phaseolus vulgaris*, *Glycine max*, *Arabidopsis thaliana*, *Gossypium hirsutum*, and *Oryza sativa* were used as queries to BLAST against the Peanut Genome Resource (PGR) database. A total of eight *AhNCED* genes were identified. The protein sequences of *AhNCEDs* range from 546 to 642 amino acids, with molecular weights in the range of 6.16–7.14 kD (Table 1). In addition, the theoretical pI of the *AhNCEDs* varies from 5.66 to 8.49, indicating different charges under different pH conditions (Table 1). Specifically, the average GRAVY value of these proteins was negative, suggesting that they were hydrophilic proteins (Table 1). The instability coefficients of the *AhNCEDs* range from 31.72 to 43.00, with a maximum of 43.00 for the most unstable *NCED* protein, indicating that most of them are stable proteins (Table 1).

**Table 1.** Characteristics of the eight *AhNCEDs* in *Arachis hypogaea* L. The protein sequences of *NCEDs* from *Arachis hypogaea* were used as reference sequences to target and blast the candidate *NCEDs* in the Peanut Genome Resource (PGR) database (<http://peanutgr.fafu.edu.cn/Download.php>, accessed on 20 December 2023). Ah: *Arachis hypogaea*.

| Protein | Accession Numbers | Encoding Amino Acid No. | Molecular Weight (kD) | Theoretical (pI) | GRAVY | Instability Index | Aliphatic Index |
|---------|-------------------|-------------------------|-----------------------|------------------|-------|-------------------|-----------------|
| AhNCED1 | AH18G19580.1      | 605                     | 6.71                  | 8.49             | −0.38 | 42.04             | 75.44           |
| AhNCED2 | AH07G23190.1      | 601                     | 6.68                  | 8.49             | −0.39 | 42.98             | 75.12           |
| AhNCED3 | AH03G48330.1      | 642                     | 7.12                  | 6.39             | −0.40 | 39.93             | 76.68           |
| AhNCED4 | AH13G50990.1      | 642                     | 7.14                  | 6.39             | −0.41 | 41.83             | 77.13           |
| AhNCED5 | AH08G22610.1      | 547                     | 6.16                  | 5.66             | −0.32 | 31.72             | 82.10           |
| AhNCED6 | AH10G21080.1      | 562                     | 6.27                  | 6.99             | −0.34 | 42.71             | 79.27           |
| AhNCED7 | AH20G27640.1      | 559                     | 6.22                  | 7.59             | −0.36 | 43.00             | 77.26           |
| AhNCED8 | AH10G21240.1      | 546                     | 6.17                  | 5.54             | −0.26 | 35.49             | 82.25           |

In addition, the gene subfamilies and relationships were further investigated. A phylogenetic tree was constructed using *NCED* protein sequences from *Pisum sativum* (PsNCED), *Phaseolus vulgaris* (PvNCED), *Gossypium max* (GmNCED), *Arabidopsis thaliana* (AtNCED), *Gossypium hirsutum* (GhNCED), and *Oryza sativa* (OsNCED) (Figure 1). The analysis revealed that *NCED* proteins in these species could be classified into five subgroups: I, II, III, IV, and V. Based on the evolutionary analysis of protein sequences (Figure 1), these eight *AhNCEDs* were also classified into four subgroups, labelled as I, III, IV, and V, with four, two, one, and one members, respectively (Table 1). Genes in the same subgroup exhibited high sequence similarity and close genetic relationships, indicating the presence of homologous structures. Notably, the selected proteins displayed a relatively high bootstrap value, particularly in the Fabaceae family, indicating their high conservation among these species.

(Figure 1). Consequently, the bootstrap values close to 100 in the phylogenetic analysis indicate a high level of confidence in the evolutionary relationship between *AhNCED1* and *AhNCED2* and their close relatives *PsNCED1*, *PvNCED1*, and *GmNCED1–2* within the Fabaceae family (Figure 1). Furthermore, *A. thaliana* in the Brassicaceae family, *G. hirsutum* in the Malvaceae family, and *O. sativa* in the Poaceae family also exhibited a relatively high bootstrap value in these five subgroups, suggesting that the *NCED* gene belongs to an ancient gene family that is conserved across different plant species during their evolutionary differentiation (Figure 1).

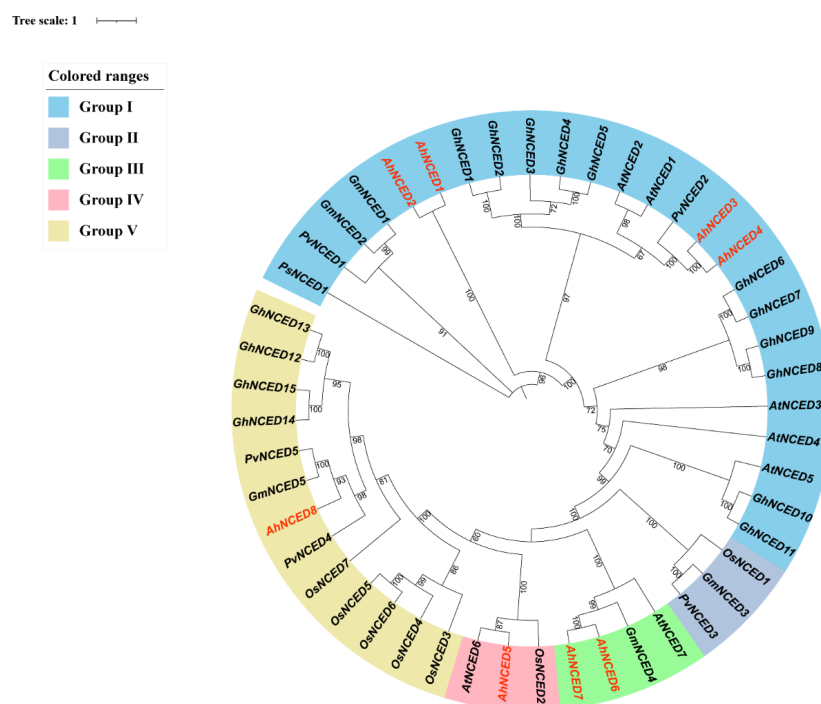


**Figure 1.** Phylogenetic relationship of the *NCEDs*. Five subgroups (I–V, highlighted with different colours) based on the distribution of genes in the rootless evolutionary tree were divided. *NCED* gene sequences from *Arachis hypogaea* were obtained from the Peanut Genome Resources database (<http://peanutgr.fafu.edu.cn/Download.php>, accessed on 20 December 2023), while sequences for *Pisum sativum*, *Phaseolus vulgaris*, *Gossypium max*, *Arabidopsis thaliana*, *Gossypium hirsutum*, and *Oryza sativa* were sourced from the NCBI GeneBank. Accession numbers of genes used for alignment and phylogenetic trees are *AhNCED1* (AH18G19580.1), *AhNCED2* (AH07G23190.1), *AhNCED3* (AH03G48330.1), *AhNCED4* (AH13G50990.1), *AhNCED5* (AH08G22610.1), *AhNCED6* (AH10G21080.1), *AhNCED7* (AH20G27640.1), *AhNCED8* (AH10G21240.1), *AtNCED1* (NP\_193569.1), *AtNCED2* (NP\_174302.1), *AtNCED3* (NP\_188062.1), *AtNCED4* (NP\_177960.1), *AtNCED5* (NP\_189064.1), *AtNCED6* (NP\_191911.1), *AtNCED7* (NP\_195007.2), *GmNCED1* (XP\_040874167.1), *GmNCED2* (XP\_014623805.1), *GmNCED3* (XP\_003516508.1), *GmNCED4* (XP\_003522713.2), *GmNCED5* (XP\_025984636.1), *PvNCED1* (XP\_007149219.1), *PvNCED2* (XP\_007144972.1), *PvNCED3* (XP\_007158058.1), *PvNCED4* (XP\_007137213.1), *PvNCED5* (XP\_007137215.1), *OsNCED1* (XP\_015619611.1), *OsNCED2* (XP\_015619349.1), *OsNCED3* (XP\_015611401.1), *OsNCED4* (XP\_025875749.1), *OsNCED5* (XP\_025875712.1), *OsNCED6* (XP\_015648368.1), *OsNCED7* (XP\_025881983.1), *GhNCED1* (XP\_016731660.2), *GhNCED2* (XP\_016699306.1), *GhNCED3* (XP\_016688155.1), *GhNCED4* (XP\_016724314.1), *GhNCED5* (XP\_016683538.1), *GhNCED6* (XP\_016703654.1), *GhNCED7* (XP\_016696809.1), *GhNCED8* (XP\_016714944.2), *GhNCED9* (XP\_016692107.2), *GhNCED10* (XP\_016731456.1), *GhNCED11* (XP\_016670575.2), *GhNCED12* (XP\_040968868.1), *GhNCED13* (XP\_016689604.2), *GhNCED14* (XP\_040971654.1), *GhNCED15* (XP\_016683086.1), *PsNCED1* (XP\_050905956.1). *Ah*: *Arachis hypogaea*; *At*: *Arabidopsis thaliana*; *Gm*: *Glycine max*; *Ps*: *Pisum sativum*; *Pv*: *Phaseolus vulgaris*; *Os*: *Oryza sativa*.

## 2.2. Analysis of Conserved Motifs and Domains of AhNCEDs

Consistent patterns in the quantity and assortment of conserved motifs were observed within each AhNCED protein subgroup. AhNCED1 and AhNCED2 from subgroup I shared eight motifs, AhNCED3 and AhNCED4 from subgroup I shared ten motifs, while AhNCED6 and AhNCED7 from subgroup III each contained five identical motifs (Figure 2). Additionally, the other four AhNCED proteins each displayed a set of seven distinct motifs, specifically motifs 1–5 and motif 8, with AhNCED8 being the exception as it lacks motif 6 (Figure 2). The subgroups' shared and distinct motif patterns suggest significant structural conservation and specific functions within the AhNCEDs.

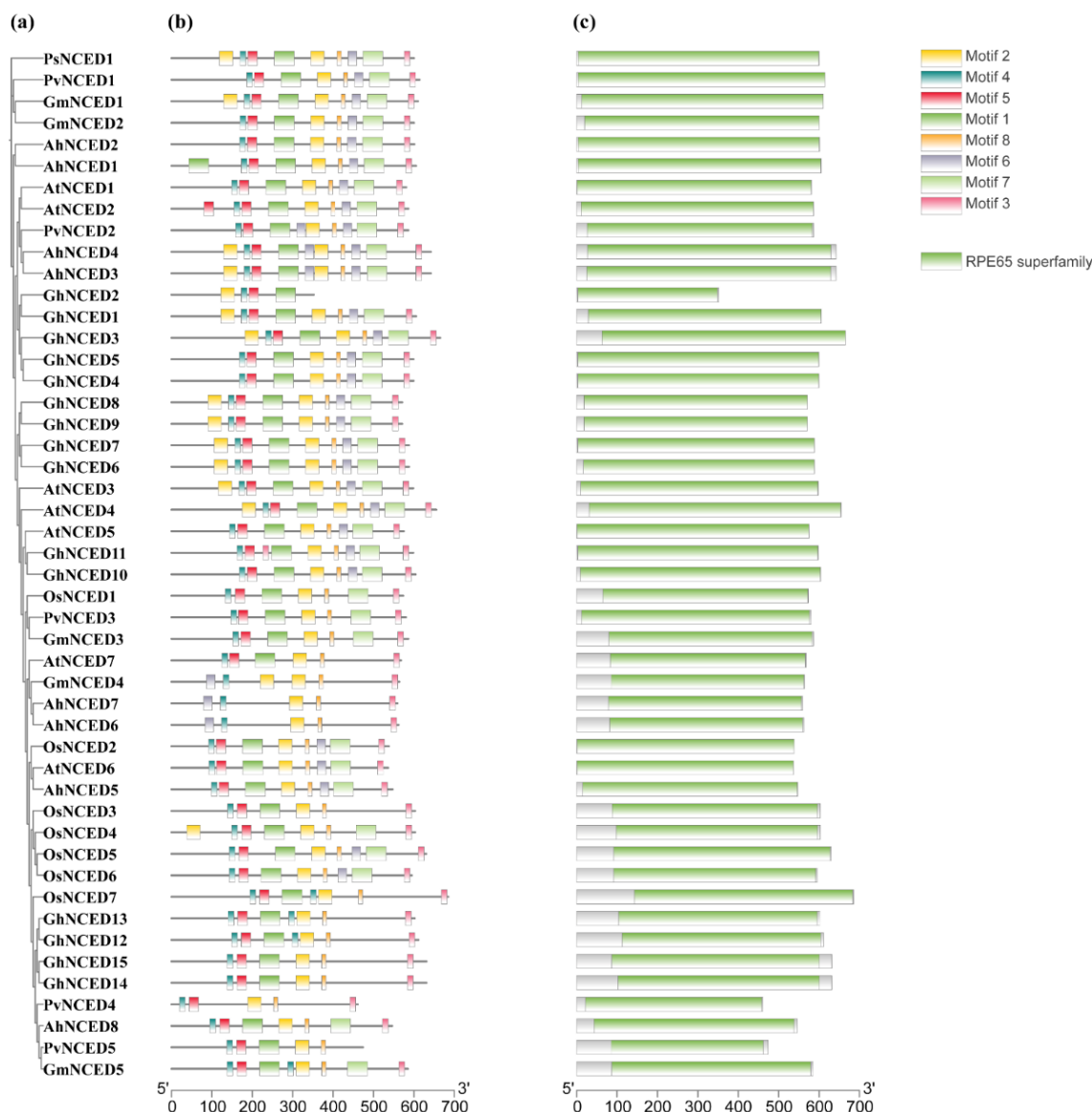


**Figure 2.** Phylogenetic relationship, conserved motifs, and domains of the NCED proteins. The motifs and domains are arranged schematically based on the pattern of the phylogenetic tree. (a) A maximum-likelihood phylogenetic tree was constructed using IQ-tree software v.1.6.8 with the JTT+R5 model and 1000 bootstrap replicates. (b) Conserved motifs of NCED proteins were identified using the MEME web server. Different coloured boxes highlight the eight different motifs. (c) Domain structure of NCED proteins. Ah: *Arachis hypogaea*; At: *Arabidopsis thaliana*; Gm: *Glycine max*; Ps: *Pisum sativum*; Pv: *Phaseolus vulgaris*; Os: *Oryza sativa*.

All AhNCED proteins possess the RPE65 domain, indicating that AhNCEDs are highly conserved across species from different families (Figure 2). The exclusive presence of the RPE65 domain in the NCED family underscores its critical role in the functionality and structural integrity of NCED proteins. The RPE65 domain predominantly occupies positions within the NCED protein sequence (Figure 2), highlighting its importance for the activity and stability of the NCED enzyme. The multiple sequence alignment of AhNCED domains revealed numerous conserved amino acids, particularly histidine (H), glycine (G), and threonine (T), signifying a high degree of conservation in the domain sequences of AhNCEDs (Figure 3).

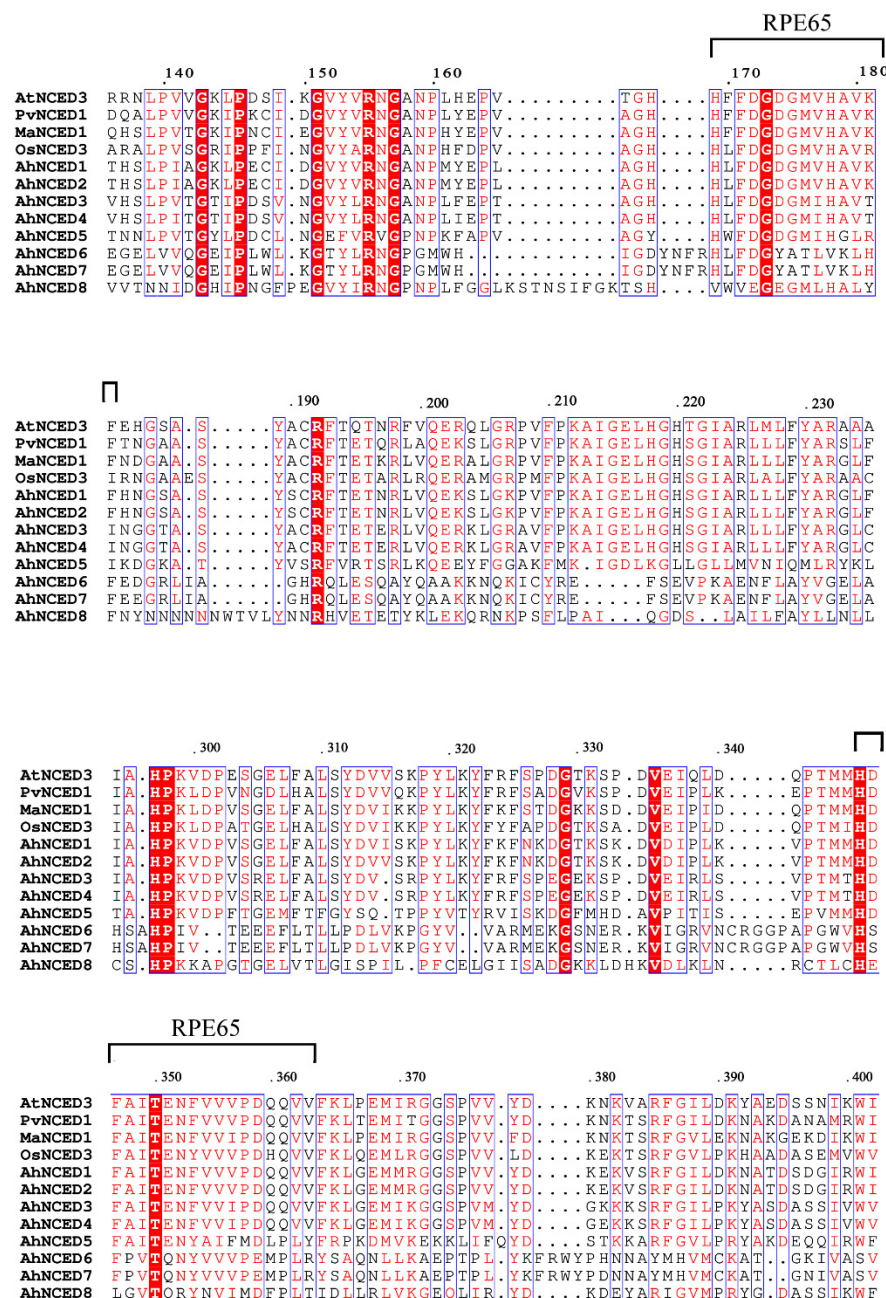


**Figure 3.** The RPE65 domain in AhNCED proteins. Sequence alignment of eight AhNCED proteins with four species (*Arabidopsis thaliana*, *Morus alba*, *Oryza sativa*, *Phaseolus vulgaris*) known for their NCED genes [26–30]. The identified RPE65 domains are in black parentheses. Dots represent the positions of the numbers in the protein sequence. Ah: *Arachis hypogaea*; At: *Arabidopsis thaliana*; Ma: *Morus alba*; Os: *Oryza sativa*.

### 2.3. Gene Structure Prediction of AhNCEDs

By comparing with gene structure maps, it is evident that *NCED* genes in different subgroups within the same species exhibit significant differences in the number and length of their exons and introns (Figure 4). Conversely, genes within the same subgroup share similar lengths and have comparable numbers and lengths of introns and exons (Figure 4), resulting in identical gene types. For instance, the subgroups I and II consist of only one or two exons (Figure 4). Interestingly, genes in the same subfamily, such as *AhNCED1*, *AhNCED2*, *PsNCED1*, *PvNCED1*, and *GmNCED1*, possess only a single exon, highlighting a unique structural characteristic within this group (Figure 4). All *NCED* genes in subgroup III contain 14 exons (Figure 4). The *NCED* genes in subgroup III contain five or six exons, while the *NCED* gene in subgroup IV comprises 14 exons (Figure 4). Subgroup V includes thirteen *NCED* genes, with eight genes containing 13 exons, four genes containing 12 exons (*OsNCED7*, *AhNCED8*, *GhNCED12*, *GhNCED13*), and one gene containing 10 exons (*PvNCED4*) (Figure 4). Therefore, the gene structures of the same subgroup exhibit similarities, including the number and length of introns and exons.

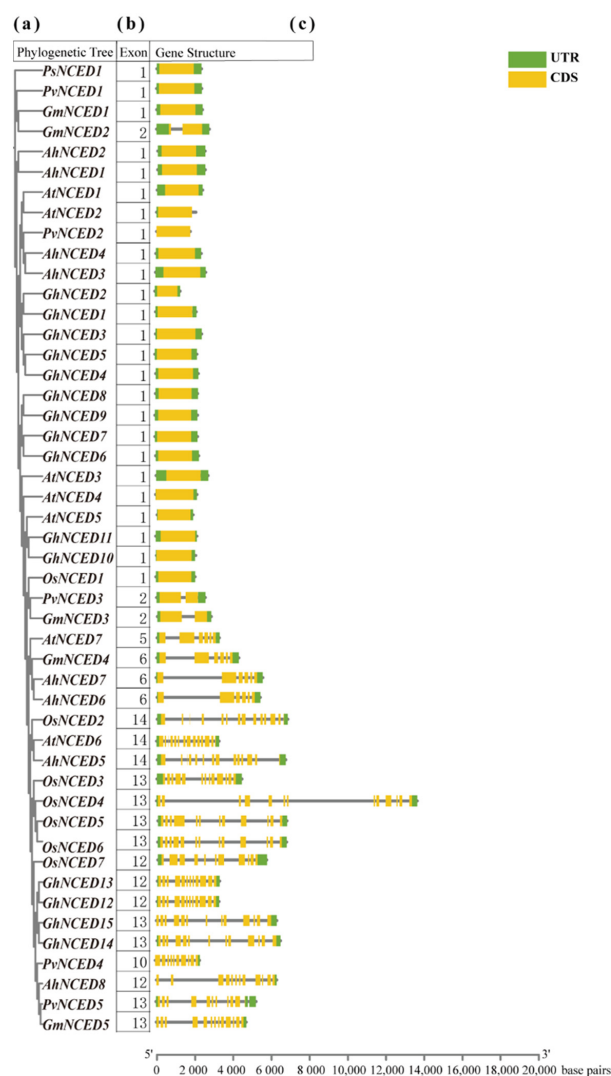


**Figure 4.** Phylogenetic relationship and gene structure of the *NCED* genes. (a) Five subgroups based on the tree shape of rootless evolutionary trees were divided. (b) The number of exons in the *NCED* genes. (c) The coding sequence and intronic regions are depicted using yellow and green boxes. Ah: *Arachis hypogaea*; At: *Arabidopsis thaliana*; Gm: *Glycine max*; Ps: *Pisum sativum*; Pv: *Phaseolus vulgaris*; Os: *Oryza sativa*.

#### 2.4. Stress-Related Cis-Elements in the Promoters of AhNCEDs

Cis-acting elements located in the promoter regions of genes initiate and regulate gene expression. The analysis revealed that the 2000 bp sequence upstream of the translational start site of each *AhNCEDs* contains 163 cis-acting elements related to hormones and stress responses (Figure 5; Table S2). The potential cis-elements of *AhNCEDs* are implicated in multiple responses to hormones salicylic acid (SA), methyl jasmonate (MeJA), gibberellin (GA), abscisic acid (ABA), auxin (IAA), and stress (light, anaerobic, defence and stress, drought, low-temperature) (Figure 5). Interestingly, nearly all the promoters of *Arachis hypogaeaNCED* genes contained cis-acting elements related to plant hormones and stress responsiveness (Figure 5). Additionally, a large number of SA-related (4.91%), MeJA-related (6.13%), GA-related (3.07%), ABA-related (19.63%), and IAA-related (1.84%) cis-acting elements are found in the *AhNCEDs* promoters (Figure S2). Furthermore, *AhNCED3–4* and *AhNCED8* contained drought-inducible elements, each harbouring four, four and one such element(s), respectively (Figure 5; Table S2). Moreover, a total of 32 ABA-related elements were identified, with *AhNCED1*, *AhNCED2*, *AhNCED3*, *AhNCED4*, *AhNCED6*, and *AhNCED7* containing the highest numbers of these elements, which were eight, six, five, four, four, and three elements, respectively (Figure 5; Table S2). Further details on the elements and functions of individual genes are listed in Supplementary Table S2.

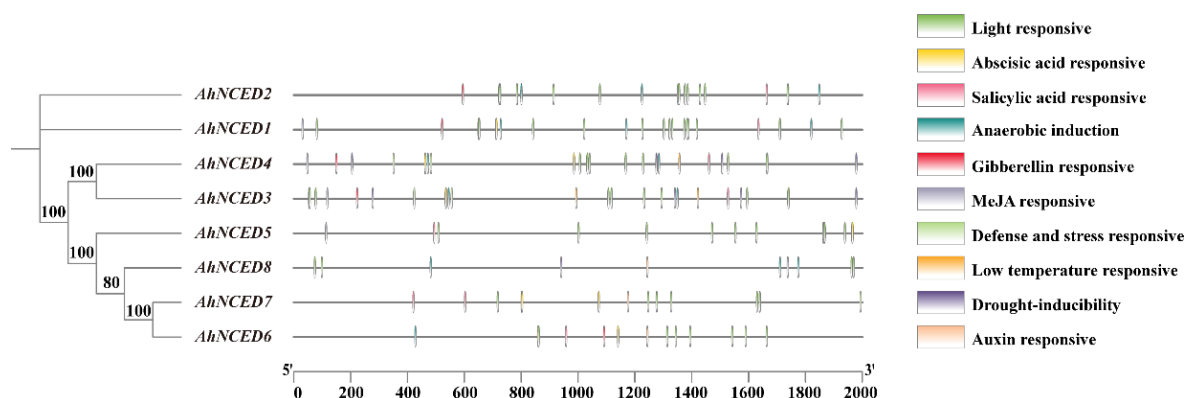


**Figure 5.** Distribution of hormone responsiveness or stress-related cis-acting regulatory elements in the promoter of *AhNCEDs* (2000 bp upstream region). Ah: *Arachis hypogaea*.

#### 2.5. Chromosomal Localisation of AhNCEDs

For chromosomal location analysis, 38 NCED genes are located on 23 chromosomes, with 12 (*Gossypium hirsutum*), 3 (*Arabidopsis thaliana*), and 8 (*Arachis hypogaea*) chromosomes, respectively. The chromosome analysis of the NCED gene family across different species revealed an uneven distribution of genes across various chromosomes (Figure 6). For instance, eight *AhNCED* genes were found on seven chromosomes, with Chr10 containing the highest number of *AhNCED* genes (Figure 6). Interestingly, *AhNCED6* and *AhNCED8* were closely clustered on Chr10, indicating possible tandem repeat gene duplication (Figure 6). The *AtNCED* genes exhibited a relatively uniform distribution on chromosomes one, three, and four, whereas in *Gossypium hirsutum*, NCED genes are distributed inconsistently across different chromosomes (Figure 6). A total of fifteen *GhNCED* genes were identified on twelve chromosomes (Figure 6). Furthermore, the NCED genes in these three species are distributed on three (*Arabidopsis thaliana*), seven (*Arachis hypogaea*), and twelve (*Gossypium hirsutum*) chromosomes, with a number that approaches half of the chromosome complement, indicating the widespread distribution of NCED genes in the species.

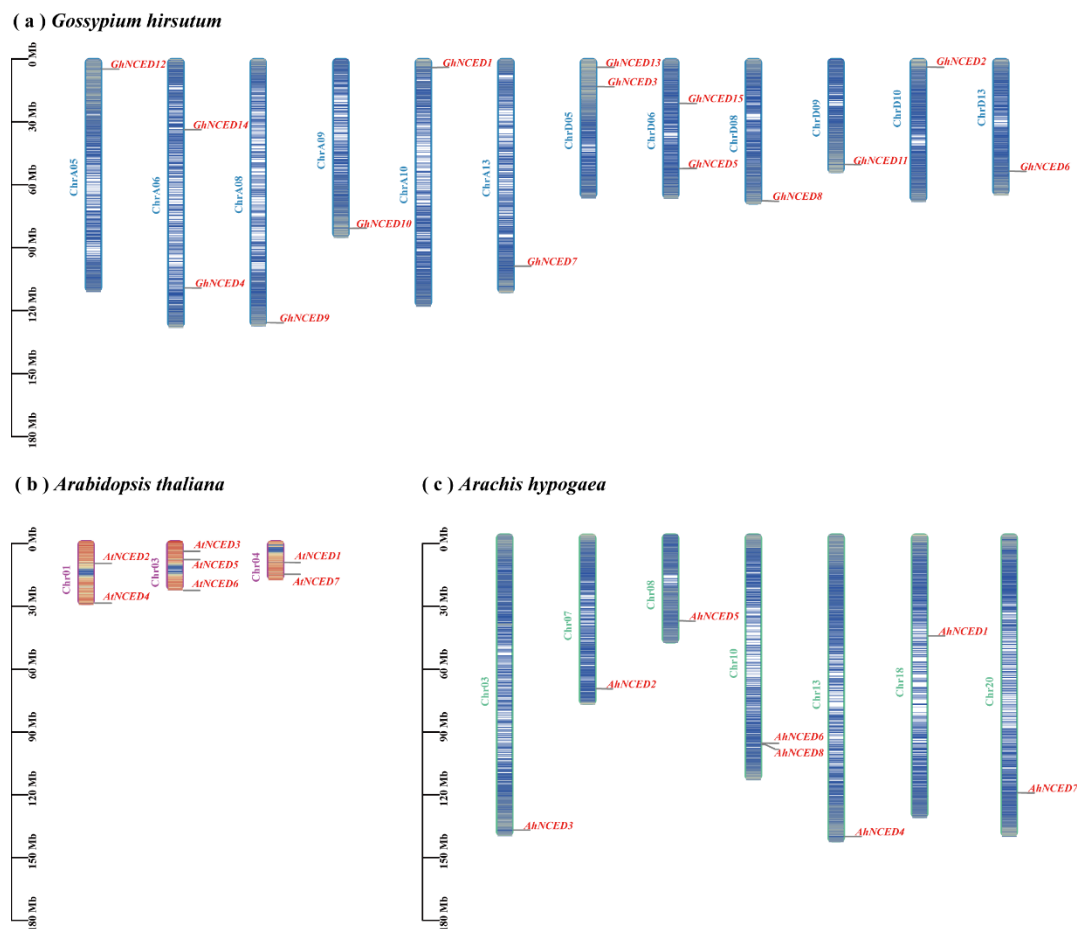


**Figure 6.** Chromosome localisation of NCED genes in *Arachis hypogaea* (green), *Arabidopsis thaliana* (pink), and *Gossypium hirsutum* (blue). Panels (a–c) depict the gene distribution in each respective species. *Arachis hypogaea* has a total of 40 chromosomes, with NCED genes located on 8 distinct chromosomes. *Arabidopsis thaliana* has 10 chromosomes, with NCED genes present on 5 of them. *Gossypium hirsutum* has 52 chromosomes, with NCED genes on 13 chromosomes. Ah: *Arachis hypogaea*; At: *Arabidopsis thaliana*; Gh: *Gossypium hirsutum*.

## 2.6. Collinearity Analysis of AhNCEDs

Collinearity analysis provides valuable information for identifying homologous gene pairs between species. Using Tbttools-II [31], a multicollinearity analysis of the NCED gene family was performed in the model plant *Arabidopsis thaliana*, the Fabaceae family *A. hypogaea*, and the drought-tolerant cotton *Gossypium hirsutum* (Figure 7). Five collinear genes were found between *Arachis hypogaea* and *Arabidopsis thaliana* and eighteen pairs between *Arachis hypogaea* and *Gossypium hirsutum* (Figure 7). The number of NCED genes was higher in polyploid species (*Arachis hypogaea* and *Gossypium hirsutum*) than in diploid species (*Arabidopsis thaliana*), indicating that polyploidisation may have contributed to gene family expansion. Most NCED genes had one-to-one orthologs between species, but some had multiple orthologs, indicating that they originated from a common ancestor. Collinearity analysis revealed the occurrence of tandem duplications in *Arachis hypogaea* in the collinearity plots (Figure 7), suggesting a potential tandem repeat gene duplication event in AhNCED1–2 and AhNCED3–4 as well as AhNCED6–7. For instance, AhNCED2 (on Chr07) and AhNCED1 (on Chr18) correspond to AtNCED4, GhNCED6, and GhNCED7; AhNCED3 (on Chr03) corresponds with AtNCED2, GhNCED1, GhNCED3, and GhNCED5. In a similar manner, AhNCED4 (on Chr13) corresponds with AtNCED2, GhNCED1, GhNCED3, and GhNCED5; AhNCED6 (on Chr10) and AhNCED7 (on Chr20) both correspond with GhNCED15 (Figure 7 and Figure S3). The sequence alignment confirmed the three pairs of

genes initially identified as potential tandem repeats to be three distinct sets of duplicated genes (Figure S3). The collinearity analysis suggests that the *NCED* gene family underwent conservation and diversification during the evolution of *Arachis hypogaea*, *Arabidopsis thaliana*, and *Gossypium hirsutum*.

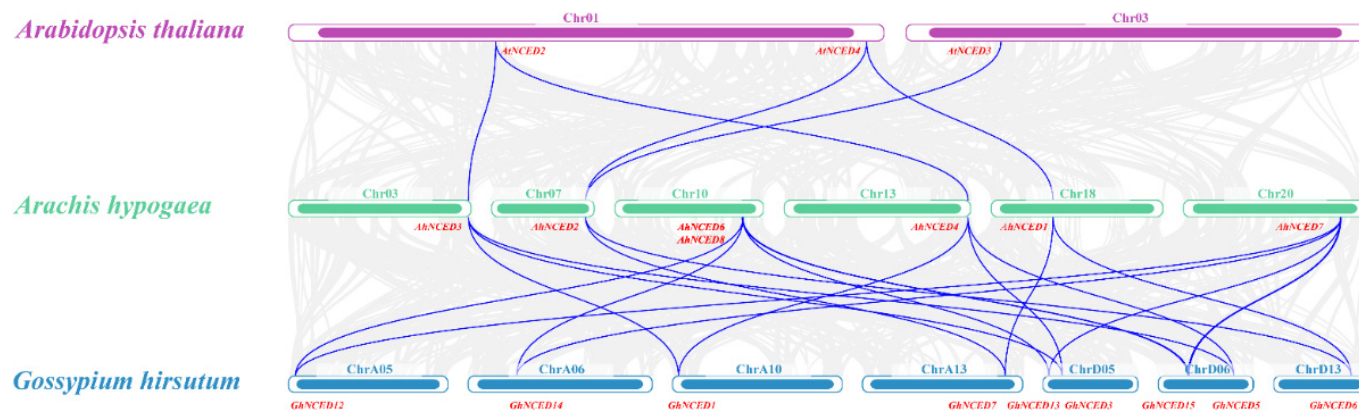


**Figure 7.** The collinearity relationship among *NCED* genes in *Arachis hypogaea*, *Arabidopsis thaliana*, and *Gossypium hirsutum*. The chromosomes of each species are represented by pink (*Arachis hypogaea*), green (*Arabidopsis thaliana*), and blue (*Gossypium hirsutum*) colours. Purple lines indicate the syntenic *NCED* gene pairs between the species, and the highlighted gene names are labelled on the chromosomes. Ah: *Arachis hypogaea*; At: *Arabidopsis thaliana*; Gh: *Gossypium hirsutum*.

### 2.7. Prediction of AhNCED Proteins Structures

Utilizing VP14 for protein structure simulation, it was observed that the majority of *NCED* proteins fold into a seven-bladed  $\beta$ -propeller structure, accompanied by four  $\alpha$ -helices forming an  $\alpha$ -helical domain at the top of the  $\beta$ -propeller (Figure 8). This structure is characterised by conserved features composed of three phenylalanine (Phe) amino acids, with some regions formed by two amino acids (Figures 8 and S4).

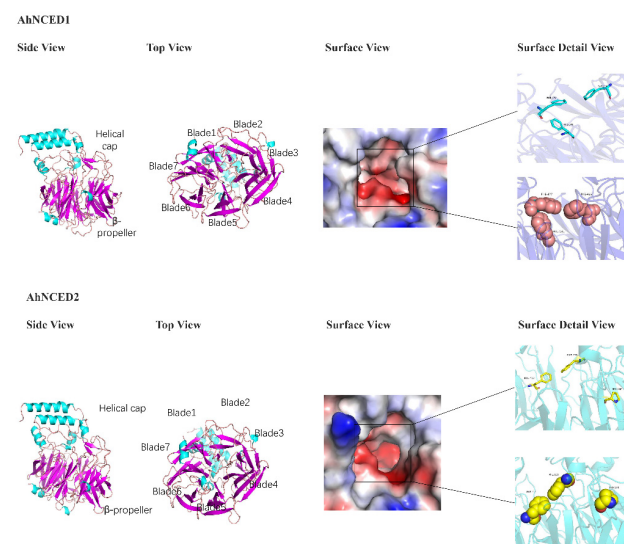


**Figure 8.** Protein structure simulation diagrams of the 9-cis-epoxycarotenoid dioxygenase 1 and 2 in *Arachis hypogaea* (AhNCED1 and AhNCED2). Blue denotes alpha helices, while purple indicates beta sheets. The red areas represent the active sites of the proteins. Amino acids in the active site pockets are depicted in both stick and sphere formats. These diagrams highlight the structural features of the *NCED* enzymes in two phenotypically distinct *Arachis hypogaea* cultivars, the drought-tolerant NH5 and the drought-sensitive FH18, which share the same genotype. Ah: *Arachis hypogaea*.

### 2.8. Expression Profile of *AhNCEDs* under Drought Stress

The transcriptome data (NCBI database SRA accession number: PRJNA657965) were studied by treating with 20% PEG6000 at time points of 0 h, 4 h, 8 h, and 24 h [32]. It is clear that the expression levels of *AhNCED1* and *AhNCED2* were significantly increased, exhibiting a fold change under 20% PEG6000-induced drought stress (Tables S2 and S3). Specifically, the expression levels of *AhNCED1* and *AhNCED2* gradually decreased as the treatment time extended (Figure 9). The expression levels of *AhNCED6* and *AhNCED8* reached their maximum at 4 h, *AhNCED3* and *AhNCED4* peaked at 8 h, and *AhNCED5* peaked at 0 h. These results suggest that *AhNCED1* and *AhNCED2* may significantly respond to plant drought stress, exhibiting temporal variations in their regulation.

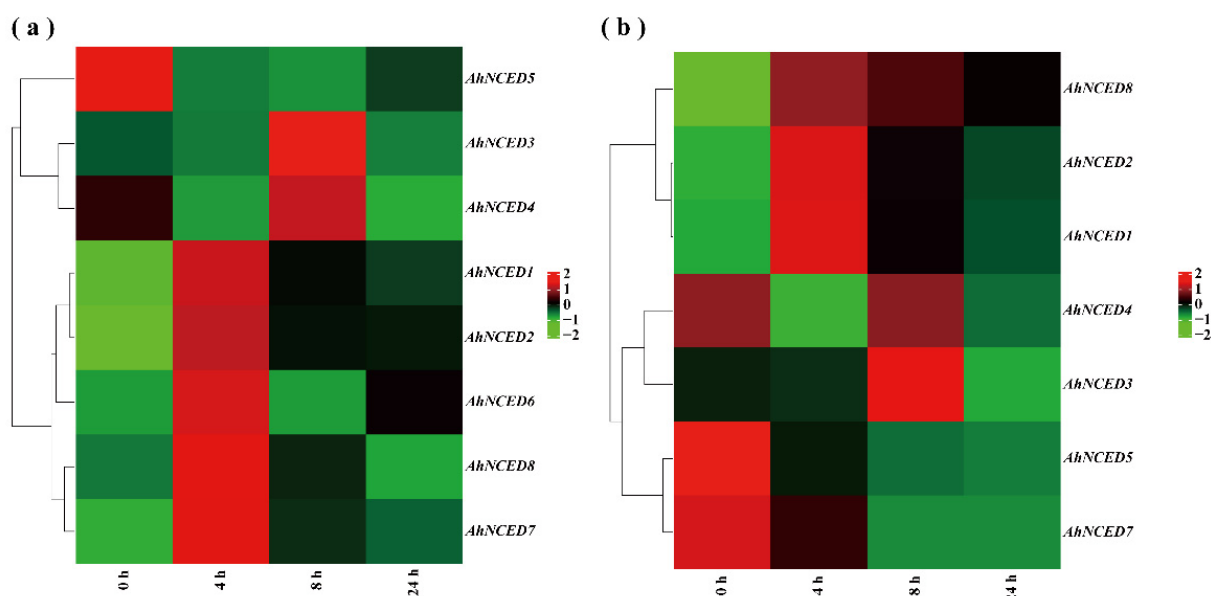


**Figure 9.** Transcriptome expression analysis of *AhNCED* genes in *Arachis hypogaea* cultivars under drought stress: (a) in the drought-tolerant cultivar NH5, and (b) in the drought-sensitive cultivar FH18. This heatmap displays the differential expression of *AhNCED* genes over time following drought exposure. The x-axis represents the time after drought exposure in hours (h), while the y-axis shows the gene names. Expression levels are indicated by a colour scale ranging from −2 (low expression, shown in blue) to +2 (high expression, shown in red). Ah: *Arachis hypogaea*.

From the results of Heatmaps A and B, *AhNCED1* and *AhNCED2* displayed similar expression patterns under various levels of drought stress, consistently showing higher expression levels. The overall expression levels of *AhNCED3* and *AhNCED4*, and *AhNCED6* and *AhNCED8* were relatively high. In contrast, *AhNCED5* genes exhibited overall low expression levels, with no significant differences between the two *Arachis hypogaea* cultivars (Figure 9). The expression patterns and levels of different genes varied between the two *Arachis hypogaea* cultivars (NH5 and FH18).

### 2.9. Phenotyping of Two *Arachis Hypogaea* Varieties under Drought Stress

After 10 days of drought treatment, the drought-tolerant *Arachis hypogaea* variety NH5 exhibited minor stress symptoms compared to the control group, which was treated with distilled water. These symptoms included slight leaf curling, indicating its relatively strong drought resistance (Figure 10A,B).

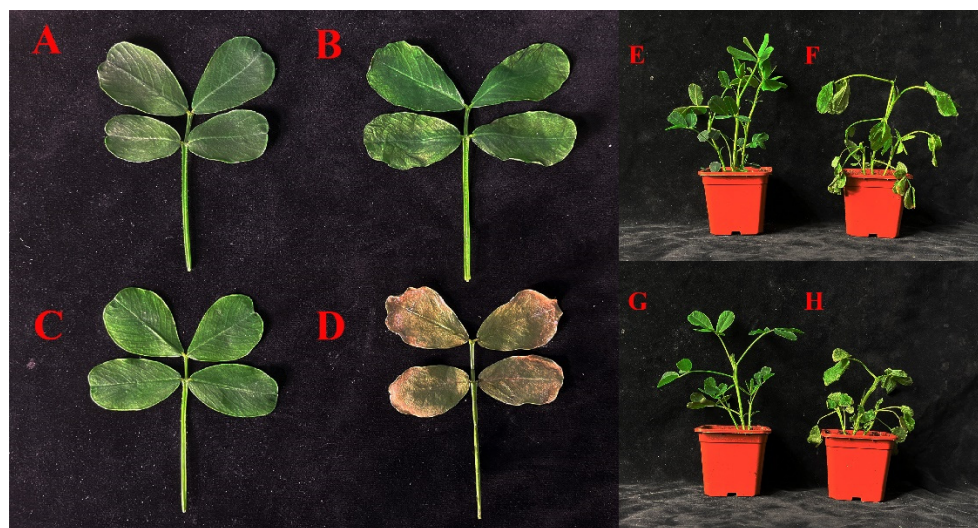


**Figure 10.** Phenotyping of *Arachis hypogaea* leaves and entire plants under normal and 10-day drought conditions. Drought-tolerant variety (NH5) grown under normal conditions (A,E), and drought treatment (B,F). Drought-sensitive variety (FH18) grown under normal conditions (C,G), and drought treatment (D,H).

In contrast, the drought-sensitive *Arachis hypogaea* variety FH18 showed significant stress symptoms after the drought treatment compared to the control group. These symptoms were characterised by notable leaf curling and wilting, with leaves becoming dry and brittle and changing colour from green to brown (Figure 10C,D). These changes suggest a poor adaptation of FH18 to drought conditions.

About the overall plants, the 10-day drought treatment led both *Arachis hypogaea* varieties to exhibit phenotypic changes, including a reduction in overall size and thinner stems (Figure 10E–H). However, the stress symptoms of FH18 were more severe than those observed in NH5, further emphasising the significant differences in drought resistance between them.

#### 2.10. Expression Analysis of *AhNCEDs* in *Arachis Hypogaea* Varieties under Drought Stress

Owing to the fact that the two genes are in tandem repeats and exhibit a high degree of sequence similarity, it is not feasible to design fluorescent quantitative PCR primers that can distinguish between them. Consequently, the symbol ‘/’ is employed to denote the two individual genes. The quantitative PCR results and heatmap expression profiles were broadly consistent, indicating that *AhNCED1/2* exhibited the most significant expression at all eight-time points, markedly higher than the other six genes (Figure 11b,c). *AhNCED3/4* and *AhNCED5* displayed moderate expression levels, while *AhNCED6/7* and *AhNCED8* showed lower expression levels (Figure 11d,e).

Under drought stress, both NH5 (drought-tolerant) and FH18 (drought-sensitive) varieties showed synchronous expression patterns. The *AhNCED1/2* genes demonstrated notable upregulated expression over the 10 days, whereas *AhNCED3/4* only showed increased expression within the first 8 h, peaked and then declined (Figure 11b). The expression of *AhNCED5* generally decreased over time, while *AhNCED6/7* and *AhNCED8* peaked at 4 h and then fell to an almost non-detectable level (Figure 11c–e).

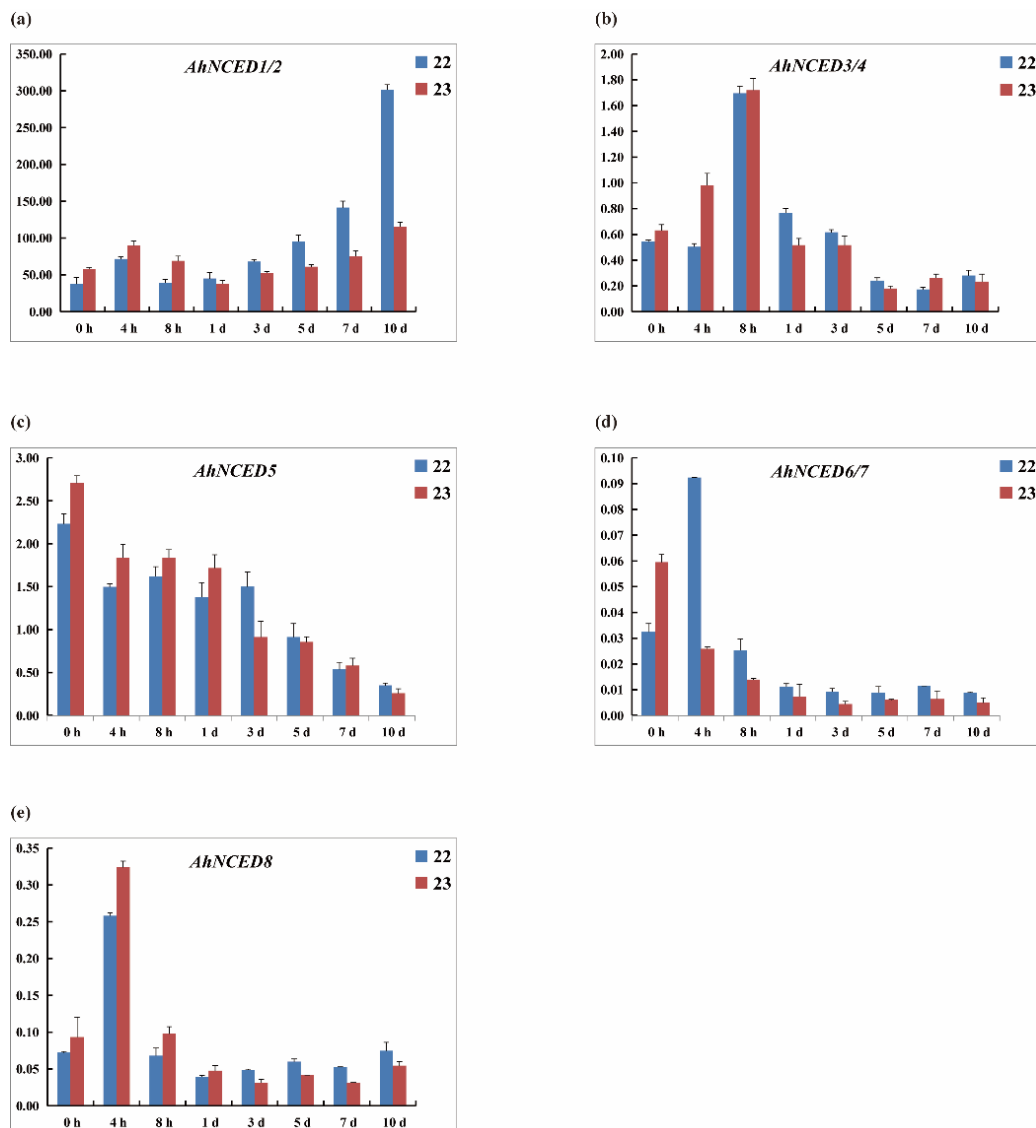


**Figure 11.** Dynamic expression of *AhNCEDs* in drought-tolerant (NH5) and drought-sensitive (FH18) *Arachis hypogaea* leaves under drought stress. The expression profiles were monitored over 10 days at intervals of 0, 4, 8, 24 h, and 3, 5, 7, and 10 days, highlighting the temporal changes in *AhNCEDs* expression in response to drought conditions. 22: NH5; 23: FH18. Ah: *Arachis hypogaea*.

In both the NH5 (drought-tolerant) and FH18 (drought-sensitive) varieties, the relative expression levels of different genes simultaneously showed consistency across the two varieties. The expression levels in NH5 were initially lower than in FH18 but became higher over time (Figure 11a). For *AhNCED1–4* at 0, 4, and 8 h, the expression levels in NH5 were lower than in FH18, but at the other five time points (1, 3, 5, 7, and 10 days), NH5 exhibited higher expression than FH18 (Figure 11a,b). For *AhNCED5* and *AhNCED8*, in the first four time points (0 h, 4 h, 8 h, 1 d), NH5 had relatively lower expression than FH18 but higher expression in the latter four time points (Figure 11c,e). For *AhNCED6/7*, in the first two time points (0 h, 4 h), NH5 showed relatively higher expression than FH18 but lower expression in the subsequent six time points (Figure 11d).

After the fifth day, different expression levels of the *AhNCED1/2* gene were observed in two *Arachis hypogaea* cultivars. In NH5 (drought-tolerant), the expression levels at 5 days, 7 days, and 10 days were approximately 2.55, 3.77, and 8.04 times higher, respectively, compared to the 0 h timepoint (standard water) (Figure 11a). In contrast, FH18

(drought-sensitive) showed about 1.05, 1.30, and 1.98 times the expression levels at 5 days, 7 days, and 10 days, respectively, compared to 0 h (standard water) (Figure 11a).

### 3. Discussion

A wealth of research has demonstrated the ubiquity of the *NCED* gene family in plants, which are highly conserved across diverse lineages [21]. An unrooted phylogenetic tree was constructed to study the distribution of these genes, which was then classified into five subgroups (I–V) (Figure 1 and Figure S1). The number of motifs, the number of exons in a gene, and the gene lengths were similar within the same subgroup (Figures 2 and 4). Additionally, all *NCED* genes only contain the RPE65 domain. The correspondence between *AhNCED1* and *AhNCED2*, *AhNCED3* and *AhNCED4*, and *AhNCED6* and *AhNCED7* suggests that these genes share a common ancestor and have been retained throughout evolution (Figure 7). These gene pairs belonged to the same subgroup in the phylogenetic tree (Figure 1), and exhibited high similarity regarding their gene structures, conserved motifs, and domains (Figures 2 and 4), indicating that their evolution has been conserved.

Tandem repeat gene duplication occurs in *Arachis hypogaea*. Gene replication refers to a gene duplicating in the genome, which can occur through various mechanisms such as haploid genome polyploidisation, chromosome recombination, and gene transposition [33,34]. This process enhances genome diversity and complexity, providing resources for gene evolution [35,36]. Based on the phylogenetic analysis, it was observed that *AhNCED1* and *AhNCED2*, *AhNCED3* and *AhNCED4* as well as *AhNCED6* and *AhNCED7*, clustered together (Figure 1), and were found to be homologous to *Arabidopsis thaliana* and *Gossypium hirsutum* in the collinearity analysis (Figure 7 and Figure S3). These results strongly support the occurrence of tandem repeat gene duplication events in *AhNCED1–2* and *AhNCED3–4* as well as *AhNCED6–7*. *NCED* gene replication is not exclusive to *Arachis hypogaea*, as it has also been observed in other plants, such as upland *Gossypium hirsutum* and *Arabidopsis thaliana* [12]. Moreover, duplication events can effectively improve plants' ability to adapt to various environments [37], and *NCED* gene duplication is likely to enhance drought tolerance in *Arachis hypogaea*. Interestingly, four *NCED* genes (*GhNCED3*, *GhNCED13*, *GhNCED5*, *GhNCED15*) were present on ChrD05 and ChrD06 of *Gossypium hirsutum*, respectively, with duplicated homologs in *Arachis hypogaea* (Figure 7). Therefore, the presence of these genes on the two chromosomes in *Gossypium hirsutum* is likely due to genome duplication events, as *Gossypium hirsutum* and *Arachis hypogaea* may share a common ancestor, and these genes have been retained during evolution. Based on the analysis of conserved structural domains, sequence alignment results, and collinearity analysis, the analysis revealed that *AhNCED* contained only one RPE65 domain and three pairs of tandemly duplicated genes (Figures 2c, 3 and 7; Figure S3). This suggests that a gene family contraction may have occurred in *Arachis hypogaea* during its evolutionary process. Gene duplication events in *AhNCED* genes enhance plant adaptability and offer valuable traits for agricultural biotechnology to improve resilience to climate stress.

The presence of ABA-related and drought-inducibility elements in the promoters of *NCED* genes provides further evidence for their crucial roles in drought response processes. Hormone signalling, particularly ABA, MeJA, and SA, plays a vital role in plant stress resistance [38]. The induction of ABA biosynthesis by *NCED* in response to drought is observed in many other plants, including *Populus davidiana*, *Gossypium hirsutum*, and *Oryza sativa* [13–15]. In *A. hypogaea*, *AhNCED1* and *AhNCED2* possess the highest number of ABA-related elements, with six and eight elements, respectively (Figure 5; Table S2). In addition, *AhNCED1* contains two MeJA elements and one SA element, while *AhNCED2* contains one SA element (Table S2). ABA can interact with hormones such as MeJA and SA [16]. Therefore, the expression levels of *AhNCED1* and *AhNCED2* were significantly upregulated under 20% PEG6000-induced drought stress in the NH5 (drought-tolerant) and FH18 (drought-sensitive) cultivars (Figure 9). These genes exhibit high expression levels in the heatmaps, confirming their essential roles in defence responses to various stresses and plant hormones (Figure 9). Understanding the interaction between *AhNCED*

genes and ABA synthesis can aid in developing crops with superior drought tolerance, addressing the increasing global frequency of drought conditions.

The *AhNCEDs* responds to drought stress, exhibiting differential expression patterns among its members and across cultivars. Specifically, we observed temporal differences in the expression levels of the *NCED* genes. Notably, *AhNCED1–2* showed a gradual decrease in expression (Figure 9), yet its expression exhibited a high fold change in drought-tolerant NH5 and drought-sensitive cultivar FH18 (Tables S3 and S4). Interestingly, the fold changes in NH5 were less pronounced than those in FH18, suggesting that NH5 may not require as high expression levels of these genes to cope with drought stress. This pattern mirrors observations in other plant species, where drought-tolerant cultivars often exhibit lower expression levels than drought-sensitive ones. For example, the *StDRO1* gene in potato and the asparagine synthase and *AMPD* genes in rice display similar trends [39,40]. Due to the identical amino acid sequences in the promoter regions, approximately 300 bp upstream, of *AhNCED1/2*, *AhNCED3/4*, and *AhNCED6/7*, three pairs of similar primers were designed (Table S4). Based on qRT-PCR results, a significant finding of our study is the temporal dynamics of gene expression in NH5 compared to FH18. Initially, *AhNCED* genes in NH5 showed lower expression levels than in FH18. However, as the stress period progressed, NH5 demonstrated a relative increase in expression levels, particularly for *AhNCED1/2* (Figure 11). This pattern, where NH5 starts with lower expression but eventually surpasses FH18, indicates a delayed but robust activation of drought response mechanisms in the drought-tolerant variety (Figure 11).

This study underscores the crucial role of *AhNCED1* and *AhNCED2* in Huayu2's drought tolerance, with significant upregulation observed under drought conditions (Figures 9b and 11). These two genes exhibited the highest expression increase among the *AhNCEDs*, particularly in NH5, highlighting their crucial role in enhancing drought resistance (Figures 9b and 11). In contrast, the drought-sensitive FH18 showed more moderate gene expression changes (Figures 9b and 11). Post 10-day drought treatment, phenotypic analysis revealed minor stress symptoms in NH5, like slight leaf curling, aligning with its strong genetic drought resistance (Figure 10A,B). In contrast, FH18, a drought-sensitive variety, exhibited severe stress symptoms, including leaf curling, wilting, and discolouration (Figure 10C,D), correlating with lower *AhNCED1/2* expression. Both varieties showed phenotypic changes like reduced size and thinner stems (Figure 10E–H), but FH18 symptoms were more severe, highlighting the distinct drought resistance between the varieties. These results emphasise that *AhNCED1* and *AhNCED2* are important in drought tolerance and shed light on the genetic basis of drought resistance in *Arachis hypogaea*, supporting efforts to genetically improve crops for greater resilience against climate change.

## 4. Materials and Methods

### 4.1. Plant Material and Treatments

The seeds of *Arachis hypogaea* cultivars (NH5 and FH18, also named Huayu22 and Huayu23, respectively) was gifted by Ms. Lina Yang from the College of Geography and Oceanography at Minjiang University, Fuzhou, Fujian Province, China. *Arachis hypogaea* seeds were sowed and cultivated in the greenhouse at a  $23 \pm 2$  °C, under long-day conditions featuring a 16 h light/8 h dark cycle, with a light intensity of  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ . After 21 days, *Arachis hypogaea* seedlings were chosen for water interruption treatment and foliar application of 20% PEG6000. Samples were collected at different time points (0 h, 4 h, 8 h, 24 h, 3 d, 5 d, 7 d, 10 d) [32]. Then, the samples were either used immediately or shock-frozen in liquid nitrogen and stored at  $-80$  °C.

### 4.2. Sequence Retrieval

The protein sequence of NCEDs from *Pisum sativum*, *Phaseolus vulgaris*, *Glycine max*, *Arabidopsis thaliana*, *Gossypium hirsutum*, and *Oryza sativa* were used as reference sequence targeted to blast the candidate NCEDs in the Peanut Genome Resource (PGR) database (<http://peanutgr.fafu.edu.cn/Download.php>, accessed on 18 December 2023) and NCBI

database (<https://blast.ncbi.nlm.nih.gov/>, accessed on 18 December 2023). Additionally, the ExPASy web tool was employed to predict parameters such as length, molecular weight (MW), isoelectric point (pI), and grand average of hydropathicity (GRAVY) for the protein sequences (<https://www.expasy.org/>, accessed on 20 December 2023).

#### 4.3. Phylogenetic Analysis of NCED Protein Sequences

The domain architecture of NCED protein sequences was analysed using the National Center for Biotechnology Information Conserved Domain Database (<https://www.ncbi.nlm.nih.gov/cdd>, accessed on 20 December 2023). All amino acid sequences lacking the required conserved structure were removed from the original sequence, resulting in a comprehensive list of NCED gene family members. Based on protein domain prediction results, a total of 48 protein sequences, including 8 AhNCED, 1 PsNCED, 5 PvNCED, 5 GmNCED, 7 AtNCED, 15 GhNCED and 7 OsNCED protein sequences, were retrieved from the NCBI database. Multiple sequence alignment of these 48 NCED protein sequences was performed using IQ-tree (<http://www.iqtree.org/>, accessed on 21 December 2023), and a maximum likelihood (ML) phylogenetic tree was constructed using the IQ-tree with JTT+R5 model (best-fit model) and 1000 bootstrap test. A *Pisum sativum* NCED protein sequence was selected as an outgroup to determine the evolutionary relationship between the NCED genes. The NCED genes were named based on their position on the phylogenetic tree. The resulting phylogenetic tree was visualised using the Interactive Tree of Life (iTOL) web tool [41].

#### 4.4. Protein Motif and Domain Analysis and Multiple Sequence Alignments of AhNCEDs

The conserved domains of NCED proteins were detected using CDD while identifying NCED genes. Full-length protein sequences were analysed using the MEME web to identify conserved motifs. All the gene structures of NCED genes were identified and visualised using TBtools-II [31] based on the genome annotation gff3 files. To identify conserved motifs in NCED genes, the MEME v5.5.1 web server was employed using Zoop for site distribution and setting the maximum number of motifs to 8. Finally, the phylogenetic tree, conserved motifs, and conserved domains were combined and visualised using the Gene Location Visualise Advanced of TBtools-II (South China Agricultural University, Guangzhou, China) [31]. The software also envisioned and merged genetic results and phylogenetic trees. Multiple sequence alignments were constructed using MEGA software using the ClustalW algorithm [42] and manually verified using the ESPript 3.0 web tool (<http://esprict.ibcp.fr/ESPript/ESPript/>, accessed on 20 December 2023). Identifying the RPE65 domain and annotating the multiple sequences were conducted as previously described [29].

#### 4.5. Evolutionary Analysis of NCED Genes

The genome annotation GFF3 files of *Pisum sativum*, *Phaseolus vulgaris*, *Glycine max*, *Arabidopsis thaliana*, *Gossypium hirsutum*, and *Oryza sativa* were downloaded from the NCBI database. And the genome annotation GFF3 files of *A. hypogaea* were downloaded from the Peanut Genome Resource (PGR) database (<http://peanutgr.fafu.edu.cn/Download.php>, accessed on 20 December 2023). TBtools-II [31] was used to analyse the structure of NCED genes in each species in combination with the evolutionary tree. The NCBI GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>, accessed on 20 December 2023) was also used to retrieve the exon quantity of the NCED genes. Adobe Illustrator software v26.0.3 (64-bit) was used for visualisation.

#### 4.6. Cis-Elements Analysis of AhNCEDs Promoters

To identify stress-related cis-acting regulatory elements in the promoter sequences, the 2000 bp upstream regions (from the translation starting sites) of the NCED genes were extracted and examined using the PlantCARE web tool (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 21 December 2023). Predictions were

made for ten stress-related cis-elements associated with the hormones salicylic acid (SA), methyl jasmonate (MeJA), gibberellin (GA), abscisic acid (ABA), auxin (IAA), and stress (light, anaerobic, defence and stress, drought, low-temperature). The *PsNCED1* gene was also included as an outgroup to expand the analysis. Multiple sequence alignment of the eight *Arachis hypogaea* NCED protein sequences was performed using IQ-tree (<http://www.iqtree.org/>, accessed on 21 December 2023), and a maximum likelihood (ML) phylogenetic tree with statistical support was constructed using the VT+I model (best-fit model) and 1000 bootstrap test. The cis-acting elements and the phylogenetic tree were then used to generate a figure using TBtools-II [31], which visualises the relationship between the NCED gene family and the stress-related cis-acting regulatory elements in the promoter sequences.

#### 4.7. Chromosomal Localisation of AhNCEDs

The Gene Density Profile function of TBtools-II was utilised with a bin size of 100,000 and the genome annotation GFF3 files to obtain the gene density information of *Arachis hypogaea*, *Arabidopsis thaliana*, and *Gossypium hirsutum*. The gene location visualisation function of TBtools-II [31] was used to determine the chromosomal locations of NCED genes in these three species, with the genome annotation GFF3 files. The results were then plotted on the chromosome location of NCED genes, and the chromosome blanks were filled with gene density information.

#### 4.8. Synteny Analysis of AhNCEDs

The genome annotation files of *Arachis hypogaea*, *Arabidopsis thaliana*, and *Gossypium hirsutum* were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/assembly/>, accessed on 20 December 2023). To explore the synteny relationship of NCED genes among different species, synteny analysis was performed using the One Step MCScanX module of TBtools-II [31], with an E-value threshold of  $10^{-10}$  and five retained blast hits. Collinear gene pairs were identified using the BLASTP algorithm with an E-value threshold of  $10^{-10}$ , and the results were visualised using the Multiple Synteny Plot module of TBtools-II [31]. The Clustal Omega website (<https://www.ebi.ac.uk/Tools/msa/clustalo/>, accessed on 22 December 2023) was used to perform multiple sequence alignment of nucleotide sequences of the tandem repeat sequences.

#### 4.9. Modelling of AhNCEDs Proteins

Using PDB: VP14 (GeneBank database number: AAB62181.2) as a template and employing SWISS-MODEL (<https://swissmodel.expasy.org/interactive>, accessed on 23 December 2023), a homology model of NCED was constructed, and tertiary structure predictions were conducted for the NCED protein sequences identified in the transcriptome [43]. Based on the target–template alignment, the highest-quality templates were determined and selected for model construction [43]. The protein structures, along with the pocket amino acids, were visualised using PyMOL (<https://pymol.org/2/>, accessed on 23 December 2023).

#### 4.10. Heatmap of AhNCEDs Gene Expression Patterns

Furthermore, two *A. hypogaea* varieties, NH5 and FH18 were sourced from 23 main commercial *Arachis hypogaea* cultivars for physiological characterisation and transcriptomic analysis [32]. The SRA data for NH5 and FH18 under drought stress were obtained from the NCBI database (SRA accession number: PRJNA657965), and the FPKM (fragments per kilobase million) values were extracted for *AhNCED* gene expression analysis [32]. The heat map values of *AhNCEDs* were visualised using the online website Omicshare (<http://www.omicshare.com>, accessed on 23 December 2023).

#### 4.11. RNA Extraction and Gene Expression Analysis

The second leaves of NH5 and FH18 were subjected to drought stress using 20% PEG6000. The experiment spanned eight time points: 0, 4, 8, 24 h, and 3, 5, 7, and 10 days. Each time point was replicated three times to ensure statistical reliability. The protocol extracted RNA from the *Arachis hypogaea* tissues using the Papure Plant RNA kit (Magen Biotech, Guangzhou, China). RNA integrity was confirmed by agarose gel electrophoresis, and quantification was performed using a Thermo NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, RNA was reverse-transcribed into cDNA using HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). The qPCR primers were listed in Table S4 and were designed using the Primer3Plus website (<https://www.primer3plus.com/index.html>, accessed on 23 December 2023). qPCR was conducted on a Bio-Rad CFX96 Real-Time PCR System (BioRad, Hercules, CA, USA), following this program: an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Actin was the internal reference gene; each sample was analysed in triplicate. Data were processed using the  $2^{-\Delta\Delta C_t}$  method [44].

Furthermore, parts of the NH5 and FH18 plants were subjected to a 10-day whole-plant drought stress treatment using 20% PEG6000 (Sangon Biotech, Shanghai, China). After this period, leaves were collected to observe phenotypic changes. Additionally, morphological changes in NH5 and FH18 were assessed and compared between the control group (standard water) and the drought stress group (subjected to a 10-day whole-plant drought treatment using 20% PEG6000).

#### 4.12. Statistical Analysis

Statistical analyses were conducted utilizing SPSS software (version 26.0, SPSS Institute, USA). Comparative evaluations of mean values derived from the various treatments were executed via Student's t-test. Significant disparities are denoted by asterisks, with \*, \*\*, and \*\*\* signifying *p* values less than 0.05, 0.01, and 0.001, respectively, indicating increasing levels of statistical significance.

### 5. Conclusions

This research identified eight *NCED* genes in *Arachis hypogaea*, analysed their phylogenetic relationships, conserved protein motifs and domains, gene structures, cis-acting elements, and chromosomal localisation and carried out synteny analysis. The presence of ABA-related and drought-inducible elements in the promoters of *AhNCEDs* underscores their crucial roles in drought response. Notably, tandem repeat gene duplication in *Arachis hypogaea*, particularly in *AhNCED1* and *AhNCED2*, *AhNCED3* and *AhNCED4*, and *AhNCED6* and *AhNCED7*, suggests an evolutionary adaptation. Moreover, the research revealed that *AhNCED1* and *AhNCED2*, markedly upregulated in both NH5 (drought-tolerant) and FH18 (drought-sensitive) cultivars, play a vital role in the rapid drought response of *Arachis hypogaea*. Additionally, compared to the FH18, the NH5 variety exhibits a delayed yet robust activation of drought response mechanisms, which was particularly evident in the *AhNCED1* and *AhNCED2*. In summary, this research provides valuable insights into the *NCED* genes of *Arachis hypogaea*, contributing to future efforts to enhance the agronomic traits of *Arachis hypogaea* plants.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms25105564/s1>, Figure S1. Phylogenetic relationship of *NCEDs*. Figure S2. Different bar lengths in the chart represent the number of various functional cis-acting elements. Figure S3. Three pairs of tandem duplicated *AhNCEDs*. Figure S4. Protein structure modelling of the *AhNCED3–8* proteins. Table S1. Cis-acting elements of *AhNCEDs*. Table S2. Fold change of *AhNCEDs* expression in drought-tolerant variety (NH5) under drought stress treatment. Table S3. Fold change of *AhNCEDs* expression in drought-sensitive variety (FH18) under different time points of drought stress treatment. Table S4. Primers for quantitative PCR.

**Author Contributions:** A.C. and P.Z. designed the manuscript; A.C. wrote the manuscript; A.C., J.L. and H.W. performed the experiments; A.C., J.L. and H.W. carried out the data analysis; P.Z. contributed the funding support and tutorial. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All data are presented in this article.

**Acknowledgments:** We are grateful to Hongbo Zhao (South China Agricultural University) and Lina Yang (Minjiang University) for their critical reading and valuable suggestions.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Golfazani, M.M.; Taghvaei, M.M.; Lahiji, H.S.; Ashery, S.; Raza, A. Investigation of protein–protein interaction network and the expression pattern of genes involved in the ABA biogenesis and antioxidant system under methanol spray in drought-stressed rapeseed. *3 Biotech* **2022**, *12*, 217.
2. Hou, P.; Wang, F.; Luo, B.; Li, A.; Wang, C.; Shabala, L.; Ahmed, H.A.I.; Deng, S.; Zhang, H.; Song, P.; et al. Antioxidant enzymatic activity and osmotic adjustment as components of the drought tolerance mechanism in *Carex duriuscula*. *Plants* **2021**, *10*, 436. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Trovato, M.; Brini, F.; Mseddi, K.; Rhizopoulou, S.; Jones, M.A. A holistic and sustainable approach linked to drought tolerance of Mediterranean crops. *Front. Plant Sci.* **2023**, *14*, 1167376. [\[CrossRef\]](#) [\[PubMed\]](#)
4. García-Gómez, B.E.; Salazar, J.A.; Nicolás-Almansa, M.; Razi, M.; Rubio, M.; Ruiz, D.; Martínez-Gómez, P. Molecular bases of fruit quality in *Prunus* species: An Integrated Genomic, Transcriptomic, and Metabolic Review with a Breeding Perspective. *Int. J. Mol. Sci.* **2020**, *22*, 333. [\[CrossRef\]](#) [\[PubMed\]](#)
5. García-Pastor, M.E.; Giménez, M.J.; Serna-Escolano, V.; Guillén, F.; Valero, D.; Serrano, M.; García-Martínez, S.; Terry, L.A.; Alamar, M.C.; Zapata, P.J. Oxalic acid preharvest treatment improves colour and quality of seedless table grape ‘magenta’ upregulating on-vine abscisic acid metabolism, relative *VvNCED1* gene expression, and the antioxidant system in berries. *Front. Plant Sci.* **2021**, *12*, 740240. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Ke, D.; Guo, J.; Li, K.; Wang, Y.; Han, X.; Fu, W.; Miao, Y.; Jia, K.P. Carotenoid-derived bioactive metabolites shape plant root architecture to adapt to the rhizospheric environments. *Front. Plant Sci.* **2022**, *13*, 986414. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Hu, B.; Cao, J.; Ge, K.; Li, L. The site of water stress governs the pattern of ABA synthesis and transport in peanut. *Sci. Rep.* **2016**, *6*, 32143. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Chen, K.; Li, G.J.; Bressan, R.A.; Song, C.P.; Zhu, J.K.; Zhao, Y. Abscisic acid dynamics, signalling, and functions in plants. *J. Integr. Plant Biol.* **2020**, *62*, 25–54. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Bharath, P.; Gahir, S.; Raghavendra, A.S. Abscisic acid-induced stomatal closure: An important component of plant defense against abiotic and biotic stress. *Front. Plant Sci.* **2021**, *12*, 615114. [\[CrossRef\]](#)
10. Tang, X.; Fei, X.; Sun, Y.; Shao, H.; Zhu, J.; He, X.; Wang, X.; Yong, B.; Tao, X. Abscisic acid-polyacrylamide (ABA-PAM) treatment enhances forage grass growth and soil microbial diversity under drought stress. *Front. Plant Sci.* **2022**, *13*, 973665. [\[CrossRef\]](#)
11. Wu, C.; Lin, M.; Chen, F.; Chen, J.; Liu, S.; Yan, H.; Xiang, Y. Homologous Drought-Induced 19 Proteins, PtDi19-2 and PtDi19-7, enhance drought tolerance in transgenic plants. *Int. J. Mol. Sci.* **2022**, *23*, 3371. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Xu, H.; Wang, X.; Wei, J.; Zuo, Y.; Wang, L. The regulatory networks of the circadian clock involved in plant adaptation and crop yield. *Plants* **2023**, *12*, 1897. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Li, Q.; Yu, X.; Chen, L.; Zhao, G.; Li, S.; Zhou, H.; Dai, Y.; Sun, N.; Xie, Y.; Gao, J.; et al. Genome-wide identification and expression analysis of the *NCED* family in cotton (*Gossypium hirsutum* L.). *PLoS ONE* **2021**, *16*, e0246021. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Huang, Y.; Jiao, Y.; Xie, N.; Guo, Y.; Zhang, F.; Xiang, Z.; Wang, R.; Wang, F.; Gao, Q.; Tian, L.; et al. *OsNCED5*, a 9-cis-epoxycarotenoid dioxygenase gene, regulates salt and water stress tolerance and leaf senescence in rice. *Plant Sci.* **2019**, *287*, 110188. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Lee, S.U.; Mun, B.G.; Bae, E.K.; Kim, J.Y.; Kim, H.H.; Shahid, M.; Choi, Y.I.; Hussain, A.; Yun, B.W. Drought stress-mediated transcriptome profile reveals *nced* as a key player modulating drought tolerance in *Populus davidiana*. *Front. Plant Sci.* **2021**, *12*, 755539. [\[CrossRef\]](#)
16. Avico, E.H.; Acevedo, R.M.; Duarte, M.J.; Rodrigues Salvador, A.; Nunes-Nesi, A.; Ruiz, O.A.; Sansberro, P.A. Integrating Transcriptional, Metabolic, and Physiological Responses to Drought Stress in *Ilex paraguariensis* Roots. *Plants* **2023**, *12*, 2404. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Li, W.; Yoo, E.; Lee, S.; Sung, J.; Noh, H.J.; Hwang, S.J.; Desta, K.T.; Lee, G.A. Seed weight and genotype influence the total oil content and fatty acid composition of peanut seeds. *Foods* **2022**, *11*, 3463. [\[CrossRef\]](#) [\[PubMed\]](#)

18. Wang, Z.; Huai, D.; Zhang, Z.; Cheng, K.; Kang, Y.; Wan, L.; Yan, L.; Jiang, H.; Lei, Y.; Liao, B. Development of a high-density genetic map based on specific length amplified fragment sequencing and its application in quantitative trait loci analysis for yield-related traits in cultivated peanut. *Front. Plant Sci.* **2018**, *9*, 827. [\[CrossRef\]](#)
19. Qin, M.; Li, X.; Tang, S.; Huang, Y.; Li, L.; Hu, B. Expression of *AhATL1*, an ABA Transport Factor Gene from Peanut, Is Affected by Altered Memory Gene Expression Patterns and Increased Tolerance to Drought Stress in *Arabidopsis*. *Int. J. Mol. Sci.* **2021**, *22*, 3398. [\[CrossRef\]](#)
20. Hu, B.; Hong, L.; Liu, X.; Xiao, S.N.; Lv, Y.; Li, L. Identification of different ABA biosynthesis sites at seedling and fruiting stages in *Arachis hypogaea* L. following water stress. *Plant Growth Regul.* **2013**, *70*, 131–140. [\[CrossRef\]](#)
21. Pruthvi, V.; Narasimhan, R.; Nataraja, K.N. Simultaneous expression of abiotic stress responsive transcription factors, *AtDREB2A*, *AtHB7* and *AtABF3* improves salinity and drought tolerance in peanut (*Arachis hypogaea* L.). *PLoS ONE* **2014**, *9*, e111152. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Razi, K.; Muneer, S. Drought stress-induced physiological mechanisms, signaling pathways and molecular response of chloroplasts in common vegetable crops. *Crit. Rev. Biotechnol.* **2021**, *41*, 669–691. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Dorner, J.W.; Cole, R.J.; Sanders, T.H.; Blankenship, P.D. Interrelationship of kernel water activity, soil temperature, maturity, and phytoalexin production in preharvest aflatoxin contamination of drought-stressed peanuts. *Mycopathologia* **1989**, *105*, 117–128. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Ratnakumar, P.; Vadez, V. Groundnut (*Arachis hypogaea*) genotypes tolerant to intermittent drought maintain a high harvest index and have small leaf canopy under stress. *Funct. Plant Biol.* **2011**, *38*, 1016–1023. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Ding, H.; Zhang, Z.; Kang, T.; Dai, L.; Ci, D.; Qin, F.; Song, W. Rooting traits of peanut genotypes differing in drought tolerance under drought stress. *Int. J. Plant Prod.* **2017**, *11*, 349–360.
26. Qin, X.; Zeevaart, J.A. The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 15354–15361. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Iuchi, S.; Kobayashi, M.; Taji, T.; Naramoto, M.; Seki, M.; Kato, T.; Tabata, S.; Kakubari, Y.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. *Plant J.* **2001**, *27*, 325–333. [\[CrossRef\]](#)
28. Hwang, S.; Chen, H.; Huang, W.; Chu, Y.; Shii, C.; Cheng, W. Ectopic expression of rice *OsNCED3* in *Arabidopsis* increases ABA level and alters leaf morphology. *Plant Sci.* **2010**, *178*, 12–22. [\[CrossRef\]](#)
29. Liu, J.; Yuan, X.; Quan, S.; Zhang, M.; Kang, C.; Guo, C.; Zhang, Z.; Niu, J. Genome-wide identification and expression analysis of *nced* gene family in pear and its response to exogenous gibberellin and paclobutrazol. *Int. J. Mol. Sci.* **2023**, *24*, 7566. [\[CrossRef\]](#)
30. Zhu, P.; Li, R.; Fan, W.; Xia, Z.; Li, J.; Wang, C.; Zhao, A. A mulberry 9-cis-epoxycarotenoid dioxygenase gene *MaNCED1* is involved in plant growth regulation and confers salt and drought tolerance in transgenic tobacco. *Front. Plant Sci.* **2023**, *14*, 1228902. [\[CrossRef\]](#)
31. Chen, C.; Wu, Y.; Li, J.; Wang, X.; Zeng, Z.; Xu, J.; Liu, Y.; Feng, J.; Chen, H.; He, Y.; et al. TBtools-II: A “one for all, all for one” bioinformatics platform for biological big-data mining. *Mol. Plant* **2023**, *16*, 1733–1742. [\[CrossRef\]](#)
32. Jiang, C.; Li, X.; Zou, J.; Ren, J.; Jin, C.; Zhang, H.; Yu, H.; Jin, H. Comparative transcriptome analysis of genes involved in the drought stress response of two peanut (*Arachis hypogaea* L.) varieties. *BMC Plant Biol.* **2021**, *21*, 64.
33. Flavell, R.B. Perspective: 50 years of plant chromosome biology. *Plant Physiol.* **2021**, *185*, 731–753. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Baker, E.A.; Woollard, A. The road less travelled? Exploring the nuanced evolutionary consequences of duplicated genes. *Essays Biochem.* **2022**, *66*, 737–744. [\[PubMed\]](#)
35. Bavishi, A.; Lin, L.; Schroeder, K.; Peters, A.; Cho, H.; Choudhary, M. The prevalence of gene duplications and their ancient origin in *Rhodobacter sphaeroides* 2.4.1. *BMC Microbiol.* **2010**, *10*, 1–13. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Yegorov, S.; Good, S. Using paleogenomics to study the evolution of gene families: Origin and duplication history of the relaxin family hormones and their receptors. *PLoS ONE* **2012**, *7*, e32923. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Flagel, L.E.; Wendel, J.F. Gene duplication and evolutionary novelty in plants. *New Phytol.* **2009**, *183*, 557–564. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Ku, Y.S.; Sintaha, M.; Cheung, M.Y.; Lam, H.M. Plant hormone signaling crosstalks between biotic and abiotic stress responses. *Int. J. Mol. Sci.* **2018**, *19*, 3206. [\[CrossRef\]](#) [\[PubMed\]](#)
39. Degenkolbe, T.; Do, P.T.; Kopka, J.; Zuther, E.; Hinch, D.K.; Köhl, K.I. Identification of drought tolerance markers in a diverse population of rice cultivars by expression and metabolite profiling. *PLoS ONE* **2013**, *8*, e63637. [\[CrossRef\]](#) [\[PubMed\]](#)
40. Sun, C.; Liang, W.; Yan, K.; Xu, D.; Qin, T.; Fiaz, S.; Kear, P.; Bi, Z.; Liu, Y.; Liu, Z.; et al. Expression of potato *StDRO1* in *Arabidopsis* alters root architecture and drought tolerance. *Front. Plant Sci.* **2022**, *13*, 836063. [\[CrossRef\]](#)
41. Letunic, I.; Bork, P. Interactive Tree of Life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* **2021**, *49*, 293–296. [\[CrossRef\]](#)
42. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [\[CrossRef\]](#) [\[PubMed\]](#)

43. Messing, S.A.; Gabelli, S.B.; Echeverria, I.; Vogel, J.T.; Guan, J.C.; Tan, B.C.; Klee, H.J.; McCarty, D.R.; Amzel, L.M. Structural insights into maize viviparous14, a key enzyme in the biosynthesis of the phytohormone abscisic acid. *Plant Cell* **2010**, *22*, 2970–2980. [[CrossRef](#)] [[PubMed](#)]
44. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)] [[PubMed](#)]

**Disclaimer/Publisher’s Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.