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Identification and Analysis of Expression Patterns of the Caleosin Genes in Hickory (*Carya cathayensis* Sarg.)

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Abstract: The deciduous tree hickory (*Carya cathayensis*) holds economic significance in China due to its high oil content, particularly in unsaturated fatty acids. Oil bodies are crucial for storing triacylglycerol (TAG), with caleosin serving as a predominant oil body protein that aids in oil body formation and stability maintenance. Our study utilized bioinformatics techniques to identify caleosin genes within *Carya cathayensis*, *Carya illinoinensis*, and *Juglans regia*. Three caleosin genes were discovered in the genomes of *Carya cathayensis*, *Carya illi-noinensis*, and *Juglans regia*. These genes encode hydrophilic proteins. Additionally, all caleosin proteins feature a single Ca^{2+}-binding EF-hand, a conserved "proline knot" motif, and a C-terminal hydrophilic region with four potential phosphorylation sites. The caleosin proteins in *Carya cathayensis* consist of α-helix, β-corner, extended chain, and random curl structures. Cis-acting elements related to stress response and hormone signaling were identified in *Carya cathayensis*, *Carya illinoinensis*, and *Juglans regia*, with distinct cis-acting elements implicated in seed-specific regulation in *Carya cathayensis*. Additionally, subcellular localization analysis confirmed that CcaCLO1 and CcaCLO2 were localized within oil bodies. Transcriptome analysis and quantitative real-time polymerase chain reaction (qRT-PCR) data demonstrated a significant up-regulation of CcaCLO1 expression during the developmental stages of the *Carya cathayensis* embryo. Furthermore, qPCR findings indicated that caleosins from *Carya cathayensis* were responsive to salt stress, with a significant up-regulation of CcaCLO1 following exposure to salt stress treatment. Consequently, caleosin genes in *Carya cathayensis*, *Carya illinoinensis*, and *Juglans regia* share similar physicochemical characteristics and conserved motifs. Specifically, CcaCLO1 in *Carya cathayensis* primarily responds to embryo development and salt stress. These findings offer foundational insights for future investigations into the regulatory mechanisms of oil accumulation and response to salt stress in hickory.

Keywords: *Carya cathayensis*; caleosin; expression pattern; subcellular localization; salt stress

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1. Introduction

Lipids are essential for various cellular functions, including energy homeostasis, membrane remodeling, and lipid signaling [1]. In plant cells, lipids are primarily found in the form of triacylglycerol (TAG), which is synthesized in the endoplasmic reticulum. These TAGs form individual oil bodies on the endoplasmic reticulum membrane, which, via the acquisition of specific proteins, support the growth and expansion of oil bodies [2]. These oil bodies play a role in providing energy for seed germination and early seedling growth and development [3]. The oil body is one of the smallest organelles in plant cells, typically measuring between 0.5–2.5 μm in diameter; however, in some species, such as soybean (*Glycine max*), the diameter of the oil body can reach 5–7.5 μm [4]. In contrast to organelles such as mitochondria and chloroplasts, which are surrounded by a phospholipid

bilayer, oil bodies are characterized by a phospholipid monolayer and a high concentration of triacylglycerol, along with embedded proteins.

Proteomics analyses have identified 100–150 proteins in oil bodies isolated from mammalian cells, primarily associated with metabolic pathways concerning lipid synthesis, degradation, membrane transport, and protein degradation [5]. In seed plants, the predominant surface mosaic proteins of oil bodies are oleosin (OLEO), caleosin (CLO), and steroleosin (SLO) [6]. Proteomics have unveiled low-abundance oil body proteins such as LD-ASSOCIATED PROTEIN (LDAP) and LDAP-INTERACTING PROTEIN (LDIP) [7,8]. Notably, oleosin is the most prevalent oil body surface protein, constituting 79% of the total proteins [9]. In seeds, oleosin proteins play a crucial role in maintaining the integrity and stability of oil bodies by preventing their aggregation. The efficient mobilization of TAGs in plant oil bodies necessitates the degradation of oleosin proteins. Therefore, ensuring the degradation of oleosin proteins is crucial for the effective mobilization of TAGs [10,11]. Four important oleosin genes have been identified in *Brassica napus*, and it was found that the overexpression of *BnaOLE1*, *BnaOLE2*, and *BnaOLE4* led to an increase in the oil content of the transgenic lines in *Arabidopsis thaliana*. Moreover, the overexpression of *BnaOLE2* and *BnaOLE4* significantly increased the size of the oil bodies and the linoleic acid content, as well as the thousand-grain weight of *Arabidopsis thaliana* seeds [12].

Caleosin (CLO/PXGs) is another highly abundant oil-body protein that possesses a Ca^{2+} binding domain and is initially identified within the oil bodies of sesame seeds [13]. Caleosin plays important roles in various developmental stages, including plant seed germination, seedling tissue differentiation, leaf senescence, pollen maturation, and seed maturation [14]. Both oleosin and caleosin contribute to the structural stability of oil bodies [15], with caleosin capable of assuming this role in the absence of oleosin [16]. During seed germination, caleosin is involved in the degradation of storage lipids in oil bodies. *Arabidopsis* mutants (*Atclo1-1*, *Atclo1-2*) exhibited a reduction in the number of oil bodies present in vacuole at 48 h and 60 h post-germination compared to wild-type, resulting in a relative delay in the breakdown process of lipids [17]. Introduction of the *caleosin* gene from the oilseed plant *Ricinus communis* into tobacco leaves resulted in an 8.1% increase in oil content compared to wild-type tobacco leaves [18]. In addition, caleosins play a crucial role in plant response to adversity stress. For example, in rice (*Oryza sativa*), the *OsClo5* gene enhances the cold sensitivity of plants by inhibiting jasmonic acid signaling and synthesis [19]. Additionally, the *Atclo3* gene is strongly expressed under abiotic stresses such as drought, high salt, and ABA treatment, and it was further found that *clo3* mutants were sensitive to drought and salt stresses [20]. Thus, these results support the involvement of caleosin in regulating oil body stability and lipid degradation in plant species, as well as playing a role in abiotic stresses on plants.

Hickory (*Carya cathayensis*) and pecan (*Carya illinoensis*) are part of the hickory genus in the pecan family. *Carya cathayensis*, known as Chinese hickory, is economically important for its high phenolic content and antioxidant capacity, the highest among major tree nuts [21]. Hickory has a high oil content of 70%, with over 90% unsaturated fatty acids, including oleic acid, linoleic acid, and linolenic acid [22,23]. The molecular mechanism underlying the high oil content of hickory kernels remains poorly understood. Previous research revealed an increase in the expression of oleosin, caleosin, and steroleosin-related genes during hickory seed kernels seed development [24]. Specifically, seven oleosin genes in the hickory oleosin gene family showed varying levels of up-regulated expression at different stages of embryo development, with *Cca0520S0025* and *Cca0791S0002* showing significant up-regulation during the rapid growth phase of hickory embryo oil accumulation, suggesting its potential involvement in the process of hickory oil accumulation [25]. However, the caleosin genes involved in the oil accumulation of hickory remain unclear. In this study, the commercially cultivated hickory nut species *Carya cathayensis* (representative of the East Asian hickory genus) was taken as the research object, with *Carya illinoensi* (representative of the eastern North American hickory genus) and *Juglans regia* (Walnut) as the reference species. Based on published genomic information, the caleosin genes were identified and

analyzed using bioinformatics. To further screen candidate caleosin genes involved in embryo development of hickory in order to provide basic data for the molecular mechanism of high oil accumulation and withstand adverse conditions in hickory.

2. Results

2.1. Identification of the Carya cathayensi Caleosin Genes

The *Arabidopsis* caleosin genes were used as bait sequences [26], and local blasts were performed among the hickory, pecan, and walnut protein libraries. The results (Table 1) showed that four caleosin proteins were identified in the hickory, and three caleosin proteins were identified in both the pecan and walnut protein libraries.

Table 1. Prediction of physicochemical properties of caleosin proteins.

Species	Gene Name	Gene ID	Protein Length (aa)	MW (kD)	PI	Instability Index	Aliphatic Index	Gravy	Subcellular Localization
Carya cathayensis	CcaCLO1	Cca0849S0013	239	26.87	5.88	44.95	77.62	−0.190	Chloroplast
	CcaCLO2	Cca0601S0075	203	23.00	8.79	38.91	76.85	−0.379	Nucleus
	CcaCLO3	Cca0899S0091	203	23.16	9.26	38.15	69.11	−0.520	Nucleus
	CcaCLO4	Cca0795S0046	142	16.91	6.98	47.74	80.21	−0.628	Cytoplasmic
Carya illinoinensis	CilCLO1	Cil0951S0139	203	23.03	8.89	39.45	75.42	−0.424	Nuclear
	CilCLO2	Cil0001S0015	203	23.22	9.37	37.88	69.61	−0.515	Nuclear
	CilCLO3	Cil0992S0011	239	27.08	5.89	47.72	78.41	−0.299	Chloroplast
Juglans regia	JreCLO1	JreChr05G11280	201	22.92	9.40	40.35	75.22	−0.465	Cytoplasmic Nuclear
	JreCLO2	JreChr06G12167	201	22.80	7.85	41.39	77.61	−0.399	Nuclear
	JreCLO3	JreChr01G11961	239	26.93	5.70	45.71	80.41	−0.198	Chloroplast

The physicochemical properties analysis of the proteins in the hickory caleosin genes revealed a range of amino acids encoded (142aa to 239aa), molecular weights (16.90 kDa to 26.86 kDa), isoelectric points (5.88 to 9.26), and instability coefficients (47.74 to 38.15). All caleosin proteins were identified as hydrophilic proteins. Subcellular localization predictions indicated that CcaCLO2 and CcCLO3 were located in the nucleus, CcaCLO1 in the chloroplasts, and CcaCLO4 in the cytoplasm. However, CLO4 exhibited notable disparities in amino acid length compared to the other three caleosin proteins and lacked significant structural characteristics, thus justifying its omission from further analysis.

The *Carya illinoinensis* caleosin proteins exhibited a range of amino acids encoded (203aa to 239aa), protein molecular weight (23.03 kDa to 27.08 kDa), isoelectric point (5.89 to 9.37), instability index (37.88 to 47.72), and fatty acidity coefficient (69.91 to 78.41). Additionally, the walnut caleosin genes encoded (239aa to 201aa) amino acids, along with protein molecular weight of (22.80 kDa to 26.93 kDa), isoelectric point of (5.70 to 9.40), instability coefficient of (40.35 to 45.71), and Aliphatic index of (75.22 to 80.41). Furthermore, the caleosins of the *Carya illinoensis* and walnut are also hydrophilic proteins. Subcellular localization predictions for *Carya illinoinensis* and walnut caleosins indicated that CilCLO2, CilCLO3, JreCLO1, and JreCLO2 were found in the nucleus, while CilCLO3 and JreCLO3 were located in the chloroplast.

2.2. Phylogenetic Tree Analysis

The evolutionary relationships of caleosin proteins, including those from *Carya cathayensis*, *Carya illinoinensis*, *Juglans regia*, *Arabidopsis thaliana*, *Triticum aestivum*, *Oryza sativa*, and *Glycine max*, were analyzed using MEGA 7.0 software, revealing a total of 45 caleosin proteins (Figure 1). The caleosin proteins can be mainly grouped into three subfamilies. The results indicate that CcaCLO1 (Cca0849S0013) belongs to the group I, while CcaCLO2 (Cca0601S0075) and CcaCLO3 (Cca0899S0091) belong to the group II. CcaCLO1 (Cca0849S0013), CilCLO3 (Cil0992S0011), JreCLO3 (JreChr01G11961) are part of

the group I, while members CcaCLO2 (Cca0601S0075), CcaCLO3 (Cca0899S0091), CilCLO1 (Cil0951S0139), CilCLO2 (Cil0001S0015), JreCLO1 (JreChr05G11280), JreCLO2 (JreChr06G12167) cluster together within the group II subfamily.

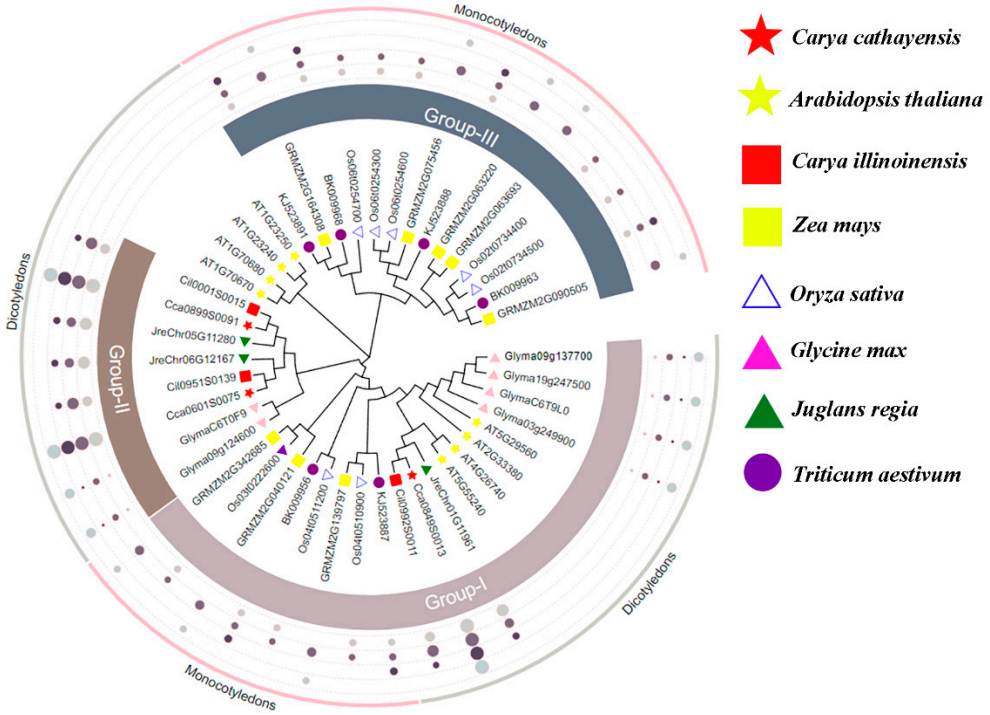

Figure 1. Caleosin phylogenetic tree of *Carya cathayensis*, *Carya illinoinensis*, *Juglans regia*, *Arabidopsis*, *Zea mays*, *Oryza sativa*, *Glycine max*, and *Triticum aestivum*.

Furthermore, the results demonstrate that monocotyledons such as maize, wheat, and rice form a distinct cluster in group III, while dicotyledons, including *Carya cathayensis*, *Carya illinoinensis*, *Juglans regia*, and *Glycine max*, are grouped together in group II. Notably, group I comprises both monocotyledons and dicotyledons.

2.3. Identification of Conserved Structural Domains and Motifs of Caleosin Proteins

A structural domain resembling the oleosin "proline knot" was identified in sesame, situated within the central hydrophobic region of caleosin and contributing to oil body formation. [27]. The "proline knot" of caleosin can be represented as "PX3PSPX2P". Conserved proline junction motifs were examined in *Carya cathayensis*, *Carya illinoinensis*, *Juglans regia*, and *Arabidopsis thaliana*. The N-terminal domain of caleosin contains a single Ca^{2+} bing motif, and the C-terminal domain contains one tyrosine kinase and three casein kinase II phosphorylation sites (Figure 2A).

Analysis of the conserved motifs revealed nine highly conserved motifs among hickory caleosin family members. CcaCLO1, CcaCLO2, and CcaCLO3 all possess motifs 1~3, 5, 7, 8 (Figure 2B). CilCLO3 from *Carya illinoensis* and JreCLO3 from walnut are grouped together with CcaCLO1, and all three contain motifs 1–5, 7–9. Additionally, CilCLO1 and JreCLO2 clustered in a single unit with CcaCLO2, and all three contain motifs 1–3, 5–8. CilCLO2 and JreCLO1 clustered in a single unit with CcaCLO3, and all three contain motifs 1–3, 5–8.

The gene structure analysis revealed that three hickory caleosin proteins exhibited a six-exon configuration. Similarly, all members of the *Carya illinoensis* caleosin family displayed a six-exon structure, while walnut caleosins were characterized by five exons.

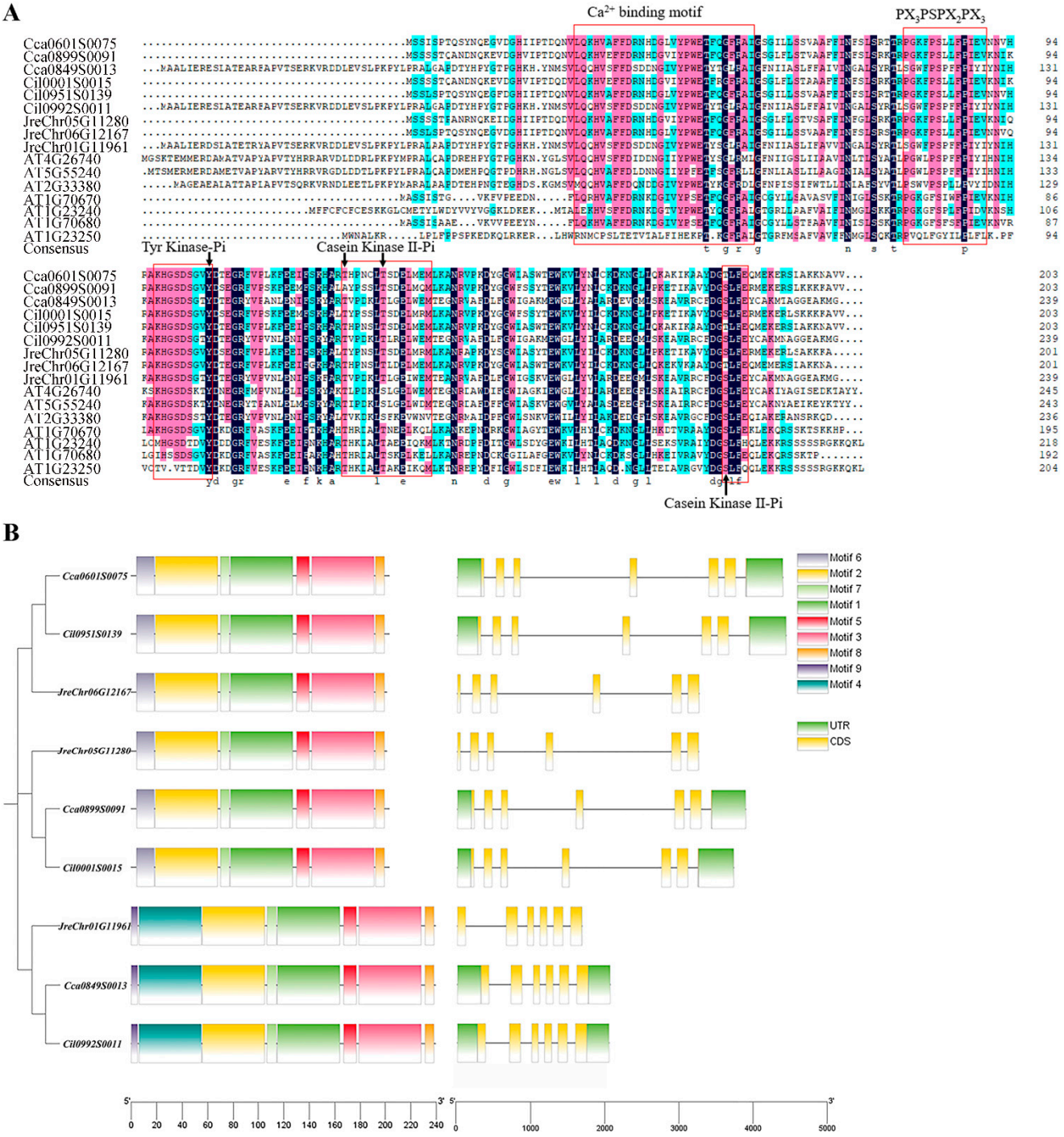

Figure 2. (**A**) Multiple amino sequence alignment of caleosins in *Carya cathayensis*, *Carya illinoinensis*, *Juglans regia*, and *Arabidopsis*. (**B**)Analysis of conserved motif and gene structure of caleosins in *Carya cathayensis*, *Carya illinoinensis*, and *Juglans regia*.

2.4. Analysis of Promoter-Acting Elements of the Caleosin Genes

A total of 13 acting elements were identified in *Carya cathayensis* that were implicated in stress response, hormone signaling, and tissue expression. Specifically, these elements included regulatory elements involved in light responsiveness elements (13), regulatory elements essential for anaerobic induction (3), drought-induced MYB binding sites (2), acting elements associated with defense and stress response (1), and cis-acting elements involved in low-temperature response (1) (Figure 3). Analysis of cis-regulatory elements in promoters of caleosin genes of *Carya cathayensis*, *Carya illinoinensis*, and *Juglans* regiaIn terms

of hormone signaling, there are a total of 10 cis-acting elements involved in response to abscisic acid (ABA) and 10 cis-acting regulatory elements involved in response to jasmonate (MeJA). Moreover, there are three cis-acting elements associated with the gibberellins (GA) response and two cis-acting elements involved in the response to salicylic acid (SA). Additionally, a comparative analysis of cis-acting elements in the promoters of the caleosins in *Carya illinoinensis* and *Juglans regia* revealed the presence of 15 acting elements, encompassing responses to stress, hormone signaling, and tissue expression. Interestingly, a cis-acting regulatory element involved in seed-specific regulation was only found in *Carya cathayensis*.

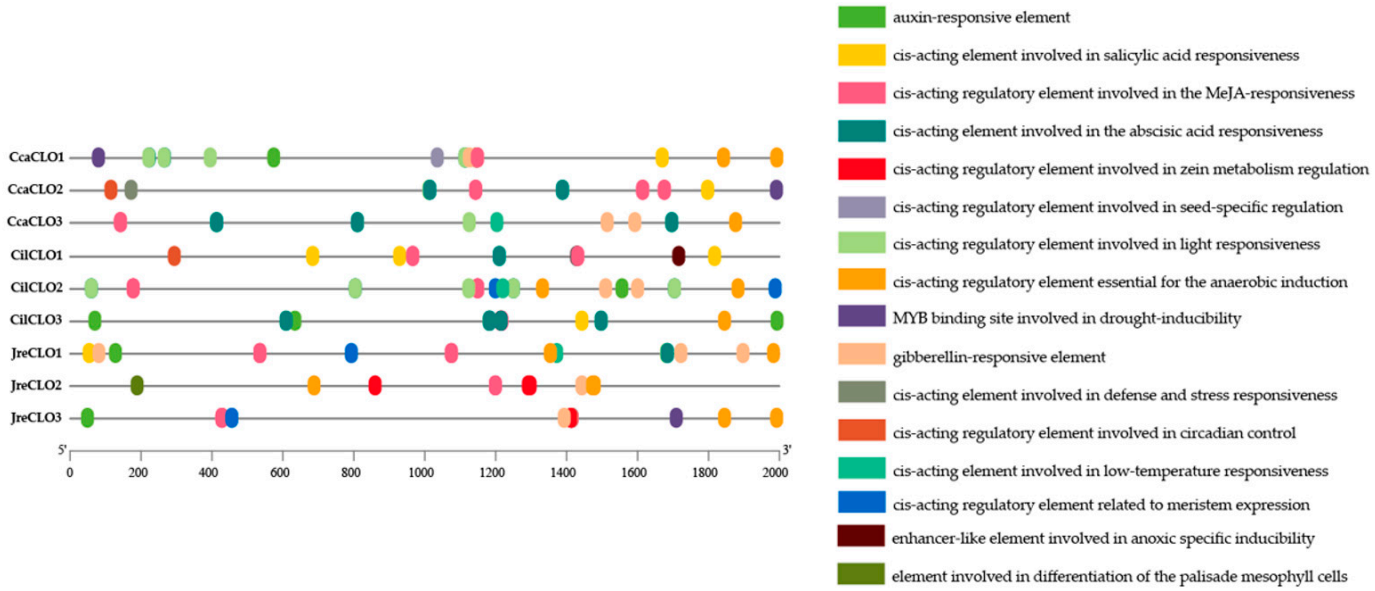

Figure 3. Analysis of cis-regulatory elements in promoters of caleosin genes of *Carya cathayensis*, *Carya illinoinensis*, and *Juglans regia*.

2.5. Structural Analysis of Caleosin Proteins in Carya cathayensis

The caleosin protein structures of *Carya cathayensis* were predicted using the online prediction software SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html, accessed on 26 May 2023). The results indicate that hickory caleosin proteins are composed of four structural elements: α-helix, β-turns, extended chains, and irregular curls. The α-helix and irregular coils represent the highest percentages in CcaCLO1, at 40.59% and 43.10%, respectively. In the beta-turn structure, CcaCLO3 has the highest percentage, while CcaCLO2 exhibits the highest percentage of extended strands (Table 2). Furthermore, the analysis of transmembrane structures indicates that hickory caleosin proteins exhibit characteristics of transmembrane proteins.

Table 2. Secondary structure of caleosins in *Carya cathayensis*.

Protein Name	Alpha Helix/%	Beta Turn/%	Extended Strand/%	Random Coil/%
Cca0849S0013 (CcaCLO1)	40.59	7.11	9.21	43.10
Cca0601S0075 (CcaCLO2)	40.39	6.40	13.30	39.90
Cca0899S0091 (CcaCLO3)	37.93	8.37	12.81	40.89

The tertiary structure of the caleosin proteins in *Carya cathayensis* was investigated using the online prediction software SWISS-MODEL (https://swissmodel.expasy.org/, accessed on 26 May 2023). The modeling results demonstrated a high level of credibility, with GMQE scores exceeding 0.9 for all proteins. The hickory caleosin proteins exist in

their active form as monomers, and analysis revealed the presence of a 'proline knot' in CcaCLO1, CcaCLO2, and CcaCLO3 proteins (Figure 4).

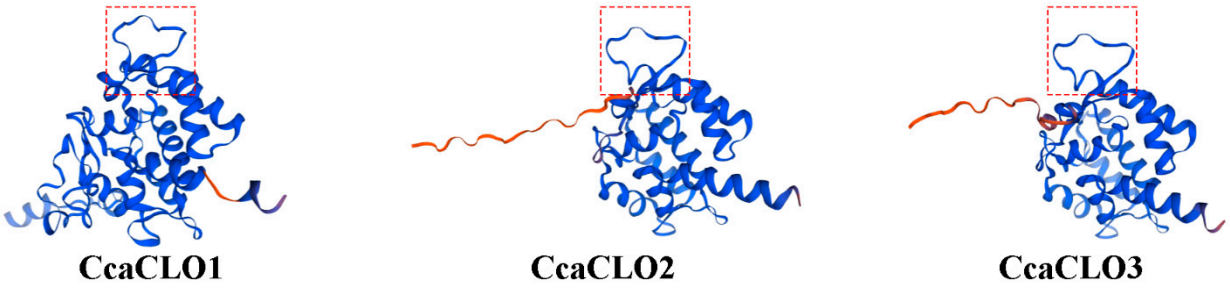

Figure 4. Tertiary structure of caleosins in *Carya cathayensis*. Note: The red boxes represent proline knots.

2.6. Analysis of the Expression Pattern of the Hickory Caleosin Genes during Embryo Development

The expression profile of the caleosin genes identified in the *Carya cathayensis* genome was evaluated for embryos at various stages of development. The findings from the qRT-PCR expression levels were consistent with the trends observed in transcriptome data (Figure 5). The investigation revealed that the gene expression of CcaCLO1 and CcaCLO3 increased as hickory embryos developed and matured, while CcaCLO2 decreased. Additionally, the transcriptome data indicated that the expression of CcaCLO1 was significantly higher than that of CcaCLO2 and CcaCLO3 during hickory embryo development. Furthermore, the qRT-PCR results demonstrated a significant up-regulation of CcaCLO1 starting from the S2 stage, which corresponds to the rapid accumulation stage of oil content [24]. This suggests that the CcaCLO1 gene in hickory is responsive to the embryonic developmental process.

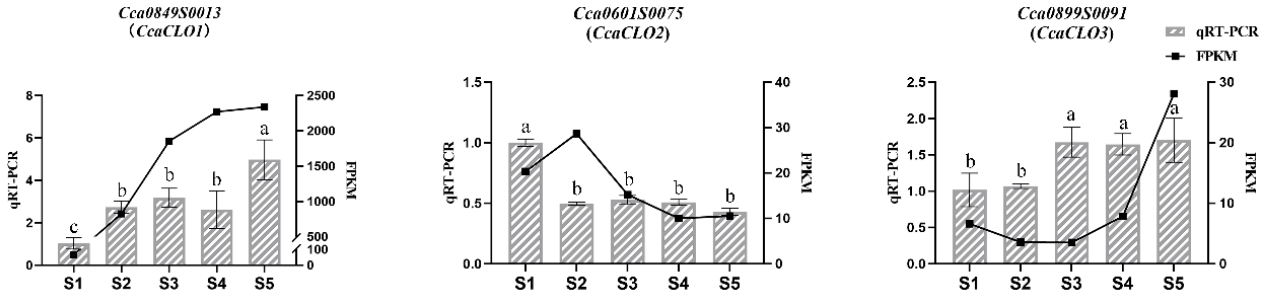

Figure 5. Analysis of the expression patterns of *caleosin* genes at different developmental stages of *Carya cathayensis* embryos. qRT-PCR of *caleosin* genes from hickory during S1, S2, S3, S4 and S5 periods. n = 3. The mean ± SD was showed in the figure. The different letter on the top of bars indicates that the difference was significant according to one-way ANOVA (p value < 0.05).

2.7. Subcellular Localization of CcaCLO1 and CcaCLO2

We previously showed that CcaCLO1 is localized to the oil body using the *Arabidopsis* transient transformation system [28]. To further verify *Carya cathayensis*, we chose to use the callus of *Carya cathayensis* for transient transformation. Given the low efficiency of transient transformation of hickory callus, we only found CcaCLO1 localized to the oil body, which is in line with our previous results. Moreover, using the transient transformation system of *Arabidopsis*, we also found that CcaCLO2 was localized to the oil body. As a positive control for oil body localization, we utilized Nile red, an oil body-specific dye. Our results demonstrated that the green fluorescence emitted by CcaCLO1 and CcaCLO2 co-localized with the red fluorescence of Nile red, indicating that CcaCLO1 and CcaCLO2 are localized within the oil body (Figure 6).

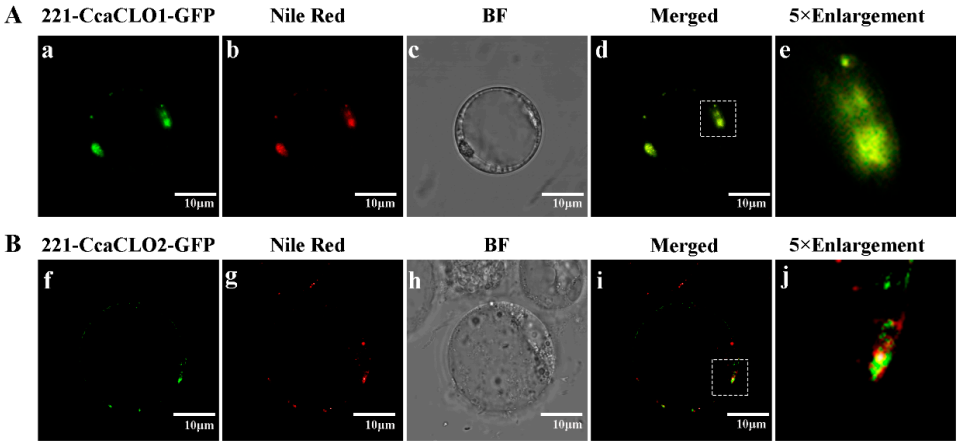

Figure 6. Subcellular localization of CcaCLO1 and CcaCLO2. (**a**,**f**): GFP fluorescence field. (**b**,**g**): RFP fluorescence field of Nile Red. (**c**,**h**): Bright field. (**d**,**i**): Merged with GFP and RFP fluorescence field. (**a**–**e**), colocalization of CcaCLO1-GFP with Nile Red. (**f**–**j**), colocalization of CcaCLO2-GFP with Nile Red. (**A**), CcaCLO11 protein fused to GFP at C termini was transiently expressed in hickory callus cells. (**B**), CcaCLO2 protein fused to GFP at C termini were transiently expressed in *Arabidopsis* suspending cells. Bar. 10 μm.

2.8. Analysis of the Expression Pattern of the Hickory Caleosin Genes under Salt Stress

In the presence of 150 mM NaCl treatment, the expression levels of Cca0849S0013 (CcaCLO1), Cca0601S0075 (CcaCLO2), and Cca0899S0091 (CcaCLO3) exhibited distinct temporal patterns. Specifically, CcaCLO1 displayed a gradual increase in expression over time, reaching its peak at 12 h post-treatment. In contrast, CcaCLO2 demonstrated a fluctuating expression profile, with peak levels observed at 4 h followed by a subsequent decrease and subsequent increase. Similarly, CcaCLO3 exhibited an initial rise in expression levels, followed by a decline, with peak expression occurring at 4 h post-treatment (Figure 7).

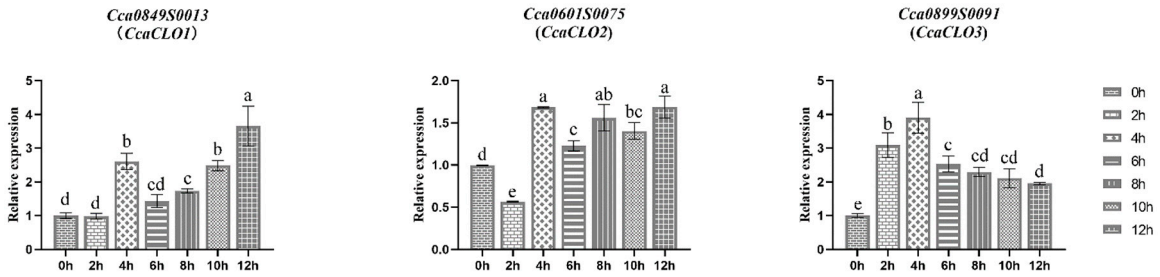

Figure 7. Analysis of the expression pattern of caleosin genes in *Carya cathayensis* under NaCl treatment. n = 3. The mean ± SD was showed in the figure. The different letter on the top of bars indicates that the difference was significant according to one-way ANOVA (p value < 0.05).

3. Discussion

Caleosin genes have been identified in multiple plant species, such as *Arabidopsis thaliana*, *Brassica napus*, *Glycine max*, and *Oryza sativa*. The number of genes identified in each species varies, with 8, 22, 7, and 8 genes, respectively [26,29–31]. These findings indicate the presence of caleosin in diverse plant species, with significant variation in the number of caleosin family members. In the genome of hazelnut of Betulaceae (*Corylus heterophylla*), five caleosin genes were identified [32]. In this study, we used bioinformatics to identify three caleosin proteins in the genomes of hickory, pecan, and walnut. The molecular weights of these caleosin proteins ranged from 20–27 kD, and CcaCLO1, CilCLO3, and JreCLO3 belong to the H-caleosin protein group, in accordance with Shen [26]. Previous research suggested that caleosins evolved differently in Magnoliaceae, Sphagnums, and Bryophytes [33]. Forty-five species of magnoliophyte plants were analyzed in a phylogenetic study, revealing that

caleosins have evolved differently in monocotyledons and dicotyledons. Furthermore, the caleosins in *Carya cathayensis*, *Carya illinoinensis*, and *Juglans regia* are more closely related to each other than to other species.

Higher plant caleosins are characterized by three distinct regions: a hydrophilic N-terminal region containing a single Ca^{2+}-binding EF-hand, a central hydrophobic domain known as a "proline knot", and a C-terminal hydrophilic region with four potential phosphorylation sites [27]. We also found three distinct regions: *Carya cathayensis*, *Carya illinoinensis*, and *Juglans regia*. The conserved structural features of caleosin suggest that its function is likely influenced by calcium binding and phosphorylation. While phosphorylation by casein kinase II has been observed in plants, tyrosine phosphorylation is considered less probable [34].

Caleosins are significant members of the proteins of the oil body, and they play a crucial role in its functions in oil accumulation and embryo development. Liu discovered that doubly protruding seeds (*clo1* × *clo2*) had a significant reduction in oil content and seed weight in *Arabidopsis*. In addition, overexpression of CLO1 in seedlings and BY2 cells increased triacylglycerol content up to 73.6% [35]. The expression of CcaCLO1 (Cca0849S0013) significantly increased during embryo development, indicating a potential response to oil accumulation in hickory. Our previous preoteome results reveal the dynamic proportion of hickory caleosin (CcaCLO1) increased parallelly with embryo development [28]. Based on the transient transformation system of hickory and *Arabidopsis*, it is proved that CLO1 is located in the oil body. Thus, CcaCLO1 (Cca0849S0013) played a function during embryo maturation in hickory. The latest research showed that CALEOSIN 1 (CLO1), CALEOSIN 2 (CLO2), and CALEOSIN 3 (CLO3) interact with ATG8 proteins and possess putative ATG8-interacting motifs (AIMs) and then facilitate lipid droplet microautophagy in *Arabidopsis* seedlings [36]. However, the molecular mechanism of CcaCLO1 in regulating lipid accumulation lipolysis needs further analysis.

Caleosin has been demonstrated to exhibit a response to abiotic stresses, particularly salt stress. Kim observed a decrease in the expression of AtCLO4 in *Arabidopsis* following exposure to salt stress [37]. Furthermore, a study conducted by Charuchinda et al. revealed that subjecting TISTR 8580 cells from Chlorella vulgaris to 0.3 M NaCl resulted in a notable increase in triacylglycerol levels and mRNA expression levels under salt stress conditions [38]. Additionally, Xue's research indicated that enhancing salt tolerance in Dendranthema morifolium was achieved via the overexpression of the caleosin gene BrRD20 [39]. Following the treatment of rice seedlings with NaCl, Jing et al. observed a significant increase in OsClo5 expression with prolonged treatment duration. Furthermore, overexpressing OsClo5 strains exhibited lower seed germination rate, plant stem length, and seedling weight compared to the wild type under 150 mM NaCl treatment. Conversely, OsClo5 silenced strains demonstrated higher seed germination rate, stem length, and seedling weight than the wild type [40]. In the present investigation, it was noted that the genes CcaCLO1 (Cca0849S0013) and CcaCLO2 (Cca0601S0075) exhibited an increased expression pattern in hickory callus tissue subjected to NaCl stress. These findings imply that the up-regulation of these genes in response to salt stress may be indicative of their functional significance.

Carya cathayensis, commonly referred to as Chinese hickory, is a member of the pecan genus within the Juglans family and is recognized as one of the most prolific woody oil-producing plants in China [24,41,42]. The primary focus of this research was to analyze the caleosin proteins present in hickory, with a specific emphasis on identifying the role of CcCLO1 in both oil accumulation and its response to salt stress. Via examination of the phylogenetic tree, it was observed that there are analogous proteins present in both pecan and walnut, namely Cil0992S0011 and JreChr01G11961, respectively. As a result, it can be inferred that the function of CcCLO1 closely resembles that of its homologous proteins in other species. However, due to the absence of a gene transformation system for Chinese hickory or pecan, functional validations were conducted on *Arabidopsis* [42,43]. However, as *Arabidopsis* is not an oil-producing plant, further research is needed to investigate the

network mechanism of CcCLO1 in lipid accumulation, specifically in Chinese hickory or pecan. Additionally, ectopic expression of proteins in different plant species may result in mis-localization [44], necessitating enhancements to the transient transformation system for hickory. Currently, we are employing the procedures outlined in our previous study on bamboo transient transformation for the manipulation of hickory callus. However, the current efficiency levels are suboptimal and are undergoing refinement. Furthermore, future studies could involve screening for regulatory factors interacting with CcCLO1 and upstream regulatory factors using molecular biological methods to elucidate the functions of CcCLO1. Overall, elucidating the high oil content of hickory nuts lays a theoretical foundation.

4. Materials and Methods

4.1. Identification and Physicochemical Properties of the Hickory Caleosin Gene

The hickory genome file was downloaded from the database (http://gigadb.org/dataset/100571, accessed on 26 May 2023), along with the pecan genome file. Additionally, the walnut genome file was downloaded from the walnut database at (http://www.xhhuanglab.cn/data/juglans.html, accessed on 26 May 2023). To identify the hickory caleosin genes, the published protein sequences of the *Arabidopsis* caleosin genes were utilized as bait sequences (https://www.arabidopsis.org/, accessed on 26 May 2023). Local databases for hickory, Carya illinoensis, and walnut were constructed using Bioedit software (version 7.0.9.1), and repeated local BlastP screenings were conducted to identify caleosin candidate genes. After removing duplicate genes, we analyzed the structural domains of the caleosins using the InterPro database (https://www.ebi.ac.uk/interpro/, accessed on 26 May 2023).

The ProParam online prediction tool from the Expasy database (https://web.expasy.org/protparam/, accessed on 26 May 2023) was used to predict the physicochemical properties of the candidate protein. Additionally, the subcellular localization of the candidate proteins was predicted using the WoLF PSORT online prediction software (https://wolfpsort.hgc.jp/, accessed on 26 May 2023).

4.2. Phylogenetic Tree Construction

The caleosin protein sequences of *Oryza sativa*, *Zea mays*, *Glycine max*, *Triticum aestivum*, *Arabidopsis*, *Carya illinoensis*, and *Juglans regia* were downloaded from the UniProt database (https://www.uniprot.org/, accessed on 6 June 2023). The evolutionary tree was constructed using the Neighbor-Joining (NJ) method of MEGA 7.0 software with a proofreading parameter of 1000 bootstrap replicates, following the previous method [45]. The tree was then created using online software (https://www.evolgenius.info/evolview-v2/ accessed on 6 June 2023).

4.3. Caleosin Protein Gene Structure and Conserved Motif Analysis

The genome annotation file GFF was downloaded from the hickory database (http://gi-gadb.org/dataset/100571, accessed on 26 May 2023), the pecan database (http://gigadb.org/dataset/100571, accessed on 26 May 2023), and the walnut database (http://www.xhhuanglab.cn/data/juglans.html, accessed on 26 May 2023), respectively. The conserved amino acid sequence of candidate caleosin proteins was analyzed using MEME Suite 5.5.3 (https://meme-suite.org/meme/doc/meme.html, accessed on 26 May 2023), and the gene structure and conserved motifs were determined using TBtools (v1.120) software for visualization, according to the method of Li [46].

4.4. Analysis of Cis-Acting Elements Upstream of the Calesoin Genes Promoter

The GFF and genome files for hickory, pecan, and walnut were obtained from the hickory, pecan database (http://gigadb.org/dataset/100571, accessed on 26 May 2023) and the walnut database (http://www.xhhuanglab.cn/data/juglans.html, accessed on 26 May 2023). The 2000 bp region upstream of the promoter for hickory, pecan, and walnut

caleosin proteins was extracted using TBtools (v1.120) software and analyzed for cis-acting elements using PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 26 May 2023).

4.5. Structural Analysis of Hickory Caleosin Proteins

The online prediction website SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa%20_sopma.html, accessed on 6 June 2023) was used to analyze the secondary structure of the hickory caleosin proteins. Additionally, the online prediction software TMHMM-2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/, accessed on 6 June 2023) was employed to determine the presence of transmembrane structural domains. The online prediction software SWISS-MODEL (https://swissmodel.expasy.org/, accessed on 6 June 2023.) was used to build and analyze the tertiary structure of the candidate proteins.

4.6. Expression Pattern Analysis of the Hickory Caleosin Genes during Development Stage

In 2022, hickory nuts were collected from Linglong Mountain in the Lin'an District of Hangzhou City, Zhejiang Province. The collection encompassed five distinct growth stages denoted as periods S1 through S5: period S1 (4 August 2022), period S2 (9 August 2022), period S3 (15 August 2022), period S4 (22 August 2022), and period S5 (27 August 2022). Each time point consisted of four trees, with each tree serving as an individual biological replicate, resulting in a total of four biological replicates. The sampling methodology adhered to the protocol outlined by Huang [24]. The procedure involves selecting four trees with small differences in growth at each time point and taking about 20 fruits from each tree. The fruits were subsequently placed on ice and peeled, and the embryos were preserved in 2 mL centrifuge tubes. These tubes were then subjected to freezing with liquid nitrogen and stored at −80 °C.

The expression patterns of the caleosin genes were examined In embryos at various developmental stages based on the previous transcriptome database [24]. qRT-PCR was used for further analysis. Refer to our previous article for specific methods [28]; the hickory *CcARF* gene was utilized as the internal reference gene. The qPCR reaction mixture comprised TB Green (5 μL), cDNA (2 μL), forward and reverse primers (0.2 μL each), and ddH_2O (2.6 μL). The qPCR reaction protocol included an initial denaturation step at 95 °C for 2 min, followed by denaturation at 95 °C for 5 s and annealing at 60 °C for 30 s, with a total of 39 cycles. Each reaction was conducted with three biological replicates. Data processing was carried out to determine the relative expression of genes using the $2^{(-\Delta\Delta CT)}$ method. Statistical analysis was performed using SPSS software (SPSS24, SPSS Inc., Chicago, IL, USA), and the results were presented using GraphPad Prism 9. The primers (forward primer/reverse primer, 5′~3′) were as follows: *Cca0849S0013*, CAGAGGGAAGGTATGTGCCG/CCAATCCATCCAAAGAGGTCG; *Cca0601S0075*, GTGGTATTCTTCTGTCCTCCGTG/CTTCCTTCAGTGTCATAGACACCGG; *Cca0899S0091*, CAACCGTCGCTGCCTTCTT/TCACTACCGTGTTTGGCTCTTTTA; *CcARF*, TGTGGTTGAAGCTAGGGATG/GCGTTGACGGAGTGAGTG.

4.7. Expression Pattern Analysis of Hickory Caleosin Genes under Salt Stress

Experimental materials consisted of hickory callus tissues maintained in our laboratory, which were subjected to treatment with 150 mM NaCl. Three replicates were collected for each treatment at time points of 0 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 12 h. The samples were rapidly frozen with liquid nitrogen and stored at −80 °C RNA extraction from callus tissues was performed using an RNA extraction kit (Hua Yue Yang). Subsequently, the extracted RNA was reverse-transcribed into cDNA using a reverse-transcription kit (Takara, Shiga, Japan) and prepared for analysis. The qRT-PCR primers, detailed in Table 1, were designed using Primer Premier 5.0 software and synthesized by Tsingke Biotechnology (Hangzhou, China).

4.8. Analysis of Subcellular Localization of Hickory Caleosin Proteins

The coding sequence of hickory caleosins was fused with a green fluorescent protein to generate the pBI221-CcaCLO1-GFP and pBI221-CcaCLO2-GFP vectors using seamless Cloning. We introduced the pBI221-CcaCLO1-GFP vector into hickory callus cells using a similar method as in a previous study [29]. The pBI221-CcaCLO2-GFP vector was subsequently introduced into *Arabidopsis thaliana* suspension cells through transient transformation [28]. The subcellular localization of the candidate proteins was observed using laser confocal microscopy (Leica TCS SP8). The excitation wavelength for green fluorescence (GFP) was set to 488 nm, with a receiving wavelength range of 493–525 nm. The excitation wavelength for red fluorescence (Nile Red) was set to 552 nm, with a receiving wavelength range of 558–588 nm. The primers (forward primer/reverse primer, 5′~3′) were as follows:

Cca0849S0013, CAGGGATCCGTCGACACTAGTATGGCTGCTTTGATTGAGAGAGAATCAC/CTCGCCCTTGCTCATGGTACCACCCATCTTAGCCTCTCCGC; Cca0601S0075, TTTTCTGATTAACAGGGATCCATGTCTTCCATATCTCCAAC/CTTGCTCATGGTACCACTAGTCACAACAGCATTCTTTTTAG.

5. Conclusions

This study utilized bioinformatics methods to identify three caleosin genes from hickory, pecan, and walnut, respectively. The phylogenetic analysis revealed that the caleosins in hickory, pecan, and walnut are more closely related to each other than to other species. The caleosin proteins possess a single Ca^{2+}-binding EF-hand, a conserved "proline knot" motif, and four potential phosphorylation sites. Notably, the CcaCLO1 gene in hickory was shown to play a role in the oil accumulation process during embryo development and under salt stress conditions, with localization in the oil body.

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