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PP2C Mediated ABA Signaling During Early Somatic Embryogenesis in Longan (*Dimocarpus longan* Lour.): Comprehensive Insights into *PP2C* Gene Family and Functional Characterization of *DlPP2C1* in ABA Signaling

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Abstract

The PP2C (protein phosphates 2C) are key regulators of abscisic acid (ABA) signaling that play a crucial role in plant stress responses. In this study, we performed a comprehensive genome-wide analysis and identified 71 *DlPP2C* genes in *Dimocarpus longan* which is an economically important fruit crop. The evolutionary analysis revealed that *DlPP2C* genes were classified into distinct subgroups based on phylogenetic relationships with *Arabidopsis thaliana* and *Oryza sativa*. Structural analysis demonstrated conserved motif composition and gene organization within subgroups, whereas chromosomal distribution and synteny analysis revealed that segmental duplication events contributed to gene family expansion. Promoter analysis findings identified numerous *cis*-acting elements related to hormone and stress responsiveness especially abscisic acid-responsive elements (ABREs), suggesting their potential involvement in ABA signaling pathways. Under exogenous ABA treatments, expression profiling of the *DlPP2C* genes exhibited dynamic, dose and time dependent response with several genes showing peak expression at 10 µM ABA after 16 h, especially the *DlPP2C1* displayed a strong transcriptional response, indicating its potential role as a key regulator. Overexpression and GUS staining assays revealed enhanced activity under ABA treatment, further supporting its involvement in ABA-responsive regulation. Moreover, RNA sequencing analysis revealed a total of 1799 differentially expressed genes, with prevalence of downregulated genes, showing extensive transcriptional reprograming. Functional enrichment analysis demonstrated that these genes were largely associated with plant hormone signaling, stress response and metabolic pathways. Together, these findings propose that *DlPP2C* genes, especially *DlPP2C1*, play a key role in ABA-mediated regulatory networks and provide valuable insights intro stress adaption mechanisms during early somatic embryogenesis in longan.

Keywords: *Dimocarpus longan*; somatic embryogenesis; protein phosphatase 2C; ABA signaling

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1. Introduction

The protein phosphatase 2C (PP2C) commonly known as the metal dependent type protein phosphatases having serine or threonine as their dephosphorylation site. In comparison to other protein phosphatases, the regulatory subunits are absent in PP2C that itself a monomeric enzyme whose activity depends on Mg^{2+} or $Mn^{2+.}$ The PP2C may lost their activity when Mg^{2+} or Mn^{2+} are replaced with other ions such as CA^{2+} and Zn^{2+} [1]. The initial report on *PP2C* gene family was published on *Arabidopsis thaliana* and *Oryza sativa* in early 2008. According to that report a total 80 and 90 *PP2C* genes were reveled in *Arabidopsis thaliana* and *Oryza sativa*, respectively [2]. The previously published studies have shown that the conserved catalytic domains of PP2C in plants are mostly centered at the C terminus whereas the N terminus serve as an extension zone with weak conservation and varying lengths, resulting in different functions to PP2C [3,4]. The clade A PP2C in *Arabidopsis* serve as negative regulator in ABA signal transduction pathways. For instance, the *AtABI1* (a clade A PP2C member), has a conserved sequence at its C terminus which forms a ternary complex with the ABA receptor (PYL) and SnRK2 kinases, thereby modulating downstream stress activities [5].

Longan, also known as "dragon's eye" or *Dimocarpus longan*, is a tropical evergreen tree indigenous to Southeast Asia, particularly southern China, Taiwan, and Vietnam. It is closely related to *Lychee* and *Rambutan* and is a member of the *Sapindaceae* family. Significant progress has been made in the molecular research of *D. longan*, mainly due to genome sequencing initiatives [6]. In longan, somatic embryogenesis has been investigated thoroughly as a model system for regenerating woody plants. To identify the molecular mechanisms underlying embryo development, research has focused on molecular biology and proteomics during somatic embryogenesis, employing methods such as gene cloning, real-time quantitative PCR, and two-dimensional electrophoresis [7,8]. Furthermore, studies on the *PP2C* gene family have demonstrated its functional significance and evolutionary conservation across several plant species [9]. Techniques for tissue culture have been crucial to the conservation and multiplication of *D. longan*. These techniques benefit the mass propagation of superior genotypes and conserve cultivars at risk of extinction [10,11].

Genome sequencing and various bioinformatics software have provided basic and powerful tools for the identification and analysis of gene families. At present, a large number of gene families have been identified and characterized at the whole genome level including TF families in *Dimocarpus longan*, including (bZIP, B3, ARF, ABI, MYB and NAC) [12–17]. *PP2Cs* are well organized as key negative regulators in core ABA signaling pathway, modulating the activities of downstream kinases and transcription factors to fine-tune plant responses to environmental stimuli. Although *PP2C* gene families have been extensively characterized in several model crop species, including Grape (*Vitis vinifera* L.*)* [18], Litchi (*Litchi Chinensis Sonn*.) [19], cucumber [20], strawberries (*Fragaria ananassa*) [21], peanut (*Arachis hypogaea*) [22] and soyabean (Glycine max) [23], but their deification and functional roles in *Dimocarpus longan* remain largely unexplored.

Here a total of 71 *DlPP2C* genes were identified and a comprehensive genome-wide analysis was performed to investigate their structural features, evolutionary relationships and expression patterns. Furthermore, we functionally characterized *DlPP2C1* using transient overexpression and GUS assays and integrated transcriptomic analysis to elucidate its role in ABA responsive regulatory cascade. Our findings provide new insights into molecular mechanism underlaying ABA signaling during early somatic embryogenesis in *Longan* and establish a foundation for future studies on stress adaption and genetic improvement of this economically important fruit crop.

2. Results

2.1. Physicochemical Properties Analysis of DlPP2C Gene Family

Based on the physicochemical properties, the structural features and possible functional properties of the 71 PP2C proteins detected in *Dimocarpus longan* were analyzed. These were the amino acid length, molecular weight (MW), isoelectric point (pI), instability index (II), aliphatic index (AI), grand average of hydropathicity (GRAVY) and predicted subcellular localization. The sizes of the DlPP2C proteins differed significantly ranging between 276 amino acids (*DIPP2C33*) to 1018 amino acids (*DIPP2C67*) with a molecular weight of 30.27 kDa to 114.97 kDa respectfully. Most plant proteins are usually in the 10-100 kDa diameter, however, because of its comparatively large size, *DIPP2C67* might have a more complicated structure organization, possibly enclosing more functional domains or regulatory regions than smaller proteins like *DIPP2C33*.The estimated isoelectric points were between 4.83 (*DIPP2C52*) and 8.91 (*DIPP2C34*), indicating a high variation in the charge features of the proteins. The *pI* values of most of the DlPP2C proteins were below 7 implying that it is mainly acidic proteins. This property could enable them to be soluble and exercise their activity in intracellular organelles like the chloroplasts and the cytoplasm. The instability index was used to estimate protein stability that varied significantly among *DlPP2Cs* with a range of 40.91 (*DIPP2C34*) to 101.12 (*DIPP2C41*). The proteins that have an instability index above 40 are normally regarded as unstable and therefore, a significant percentage of DlPP2C proteins might be less stable in vitro. Conversely, the aliphatic index ranged (AI), a measure of the volume of aliphatic side chains (a measure of stability), went between 68.07 (*DIPP2C1*) and 101.12 (*DIPP2C41*), indicating unpredictable thermostability. It is worth noting that values of AI, is an indicator of increased structural stability, which means that a portion of DlPP2C proteins can maintain stability in different environmental conditions despite increased instability index. GRAVY values are above -0.579 (*DIPP2C5*) and below 0.013 (*DIPP2C56*). The existence of negative GRAVY values implies that majority of the proteins of DlPP2C are hydrophilic, which is a property commonly linked with soluble proteins that have a role in intracellular signaling. This finding is in line with the overall functional role of PP2C proteins in the plant signaling pathways Subcellular localization prediction revealed that DlPP2C proteins are spread through various cellular compartments, such as the nucleus, cytosol, chloroplasts, mitochondria, vacuoles, and the plasma membrane. Such large-scale localization pattern indicates that the DlPP2C proteins are involved in numerous biological processes primarily in signal transduction and stress response, as well as metabolic processes.

Table 1. Physicochemical properties of the *PP2C* gene family in D. longan. First column contains the gene IDs list, number of Amino acids (A.A), Molecular weight (MW), Isoelectric points (pI), Instability index (II), Aliphatic index (AI), Grand Average of Hydropathicity (GRAVY) and predicted subcellular localization (SCL) in subsequent columns.

ID	AA	MW	pI	II	AI	GRAVY	SCL
DlPP2C1	481	53095.49	5.37	46.39	68.07	-0.469	chlo
DlPP2C2	487	53534.05	5.63	41.3	76.28	-0.437	chlo
DlPP2C3	532	58738.96	4.95	38.69	81.43	-0.367	cyto
DlPP2C4	494	54353.02	5.53	40.61	78.16	-0.403	chlo
DlPP2C5	446	50102.44	6.29	31.26	75.61	-0.579	cyto
DlPP2C6	386	42984.75	6.36	40.35	76.53	-0.279	chlo
DlPP2C7	386	42984.75	6.36	40.35	76.53	-0.279	chlo
DlPP2C8	380	41976.53	7.99	48.26	81.08	-0.31	chlo
DlPP2C9	351	38552.76	5.71	44.38	88.29	-0.273	cyto
DlPP2C10	386	42904.35	5.28	41.16	76.04	-0.299	mito
DlPP2C11	386	42904.35	5.28	41.16	76.04	-0.299	mito
DlPP2C12	713	80002.69	5.44	43.35	78.49	-0.517	nucl
DlPP2C13	709	79381.33	5.41	45.21	73.96	-0.601	nucl
DlPP2C14	397	42739.4	7.53	60.46	76.12	-0.28	chlo
DlPP2C15	431	47829.8	5.97	61.46	65.57	-0.581	cyto
DlPP2C16	427	46665.56	5.6	58.58	77.82	-0.417	nucl
DlPP2C17	786	86793.15	5.36	42.48	79.21	-0.474	chlo
DlPP2C18	882	98099.08	5.93	39.87	68.21	-0.56	nucl
DlPP2C19	393	42503.3	6.38	44.89	83.77	-0.188	chlo
DlPP2C20	351	38708.11	5.84	36.22	86.58	-0.329	nucl
DlPP2C21	448	49649.68	8.24	36.49	85.87	-0.376	mito
DlPP2C22	524	57090.5	5.17	47.32	88.95	-0.188	nucl
DlPP2C23	397	43513.15	5.88	46.48	85.31	-0.359	chlo
DlPP2C24	494	54248.02	8.59	44.14	77.91	-0.43	Nucl
DlPP2C25	418	45486.45	5.56	62.73	81.2	-0.278	Nucl

DlPP2C26	455	50300.21	6.12	49.41	83.54	-0.415	Chlo
DlPP2C27	544	59008.92	4.89	44.04	91.69	-0.12	Chlo
DlPP2C28	397	43225.38	4.96	55.72	74.84	-0.434	Nucl
DlPP2C29	546	58749.44	4.9	47.91	93.35	-0.167	Chlo
DlPP2C30	464	51427.31	5.51	65.62	82.28	-0.258	Chlo
DlPP2C31	293	31518.87	5.05	39.49	77.92	-0.327	Cyto
DlPP2C32	356	39302.94	5.24	47.5	75.06	-0.4	Chlo
DlPP2C33	276	30273.96	5.1	30.36	82.28	-0.244	Cyto
DlPP2C34	353	39479.4	8.91	42.23	91.42	-0.219	Chlo
DlPP2C35	658	73053.46	6.11	40.91	93.31	-0.187	Cyto
DlPP2C36	282	31023.03	7.76	40.74	82.98	-0.413	Cyto
DlPP2C37	282	31011.19	5.68	42.61	89.18	-0.296	Cyto
DlPP2C38	283	30887.89	6.75	38.41	82.37	-0.385	Nucl
DlPP2C39	406	44250.34	5.09	53.3	89.11	-0.058	Nucl
DlPP2C40	394	42888.75	5.87	49.59	80.96	-0.307	Nucl
DlPP2C41	187	20712.63	6.15	38.64	101.12	-0.14	Chlo
DlPP2C42	428	45737.3	6.9	32.34	86.33	-0.111	Chlo
DlPP2C43	439	47181.98	8.24	36.67	86.86	-0.174	Chlo
DlPP2C44	438	47673.46	5.17	37.87	82.58	-0.193	Cyto
DlPP2C45	400	44478.72	8.15	57.04	77.5	-0.357	Chlo
DlPP2C46	318	35003.53	8.21	43.44	83.43	-0.393	Cyto
DlPP2C47	303	33325.7	8.22	43.19	84.65	-0.364	Cyto
DlPP2C48	277	31547.17	9.68	55.24	75.63	-0.467	Chlo
DlPP2C49	398	43428.37	5.76	37.62	93.87	-0.114	Chlo
DlPP2C50	398	43331.26	5.76	37.45	91.91	-0.129	Chlo
DlPP2C51	410	44560.11	5.07	35.52	79.98	-0.348	Chlo
DlPP2C52	907	100000.67	4.83	46.17	96.37	-0.09	Plas
DlPP2C53	476	52456.31	5.7	39.43	82.23	-0.214	Chlo
DlPP2C54	155	17163.21	6.81	36.12	62.32	-0.517	Nucl
DlPP2C55	372	41033.06	5.44	41.47	71.34	-0.422	Nucl
DlPP2C56	306	32961.16	5.05	39.31	92.06	0.013	Chlo
DlPP2C57	514	55360.65	6.23	44.45	80.04	-0.227	Mito
DlPP2C58	453	47908.98	8.48	40.16	78.98	-0.09	Chlo
DlPP2C59	394	44304.57	6.9	50.54	96.24	-0.199	Mito
DlPP2C60	391	43315.21	5.85	37.44	94.99	-0.207	Cyto
DlPP2C61	385	43139.22	8.12	42.64	90.34	-0.288	Chlo
DlPP2C62	397	44158.32	8.67	48.55	88.84	-0.307	Chlo
DlPP2C63	397	44176.3	8.94	48.68	90.3	-0.272	Nucl
DlPP2C64	385	42838.69	7	38.99	91.14	-0.289	Chlo
DlPP2C65	374	41591.6	8.86	43.57	87.35	-0.293	Mito
DlPP2C66	383	42310.97	5.74	42.23	89.32	-0.193	Chlo
DlPP2C67	1018	114972.38	5.62	45.74	80.42	-0.382	Vacu
DlPP2C68	243	26329.38	9	42.67	98.35	-0.016	Cyto
DlPP2C69	258	28459.06	4.9	42.67	79.73	-0.252	Extr
DlPP2C70	454	48976.75	4.72	42.62	88.24	-0.137	Chlo
DlPP2C71	555	61220.53	5.55	48.62	87.48	-0.235	Chlo

2.2. Evolutionary Analysis of the PP2C Gene Family

In order to render the patterns of evolution and possible diversification of the *DlPP2C* gene family in terms of functionality, a phylogenetic tree was built using the multiple sequence alignment of the conserved *PP2C* catalytic domain. Whereas PP2C proteins of *Dimocarpus longan*, *Arabidopsis thaliana* and *Oryza sativa*, belonging to dicot and monocot species were analyzed. According to the phylogenetic topology, these PP2C proteins were divided into 13 different groups, each group has a color, which indicates the evolutionary divergence of the proteins and their evolutionary subgroups. *D.longan* contains 71 *PP2C* genes that is similar to 80 reported members of *A. thaliana* but significantly lower than 122 reported members in rice. The phylogenetic tree also revealed that the major clades did not evenly have the *DlPP2C* members. Clade A comprised of the most members 25 *DlPP2C*, and, f clade D had 1 *DlPP2C*. The clades F1 and F2 consisted of four proteins of the *DlPP2C* group and one of them was the smallest, consisting of one *DlPP2C* protein. Interestingly, *DlPP2C* were found in all the clades (A-J), and there is no significant *PP2C* group is lost in longan. The DlPP2C proteins within a clade were further clustered with their homologs of either *A. thaliana* and *O. sativa*, indicating that the large subfamilies of *PP2C* are very conserved across dicot and monocot species. It is a conserved phylogenetic distribution that suggests the split of *PP2C* genes probably preceded the split of those plants' lineages. The fact that all major subgroups have the representatives retained in longan is another argument in favor of the functional conservativity of PP2C proteins. On the whole, phylogenetic connections give important information on the evolutionary conservation and possible functional similarity of DlPP2C proteins with their homologs in model plant species.

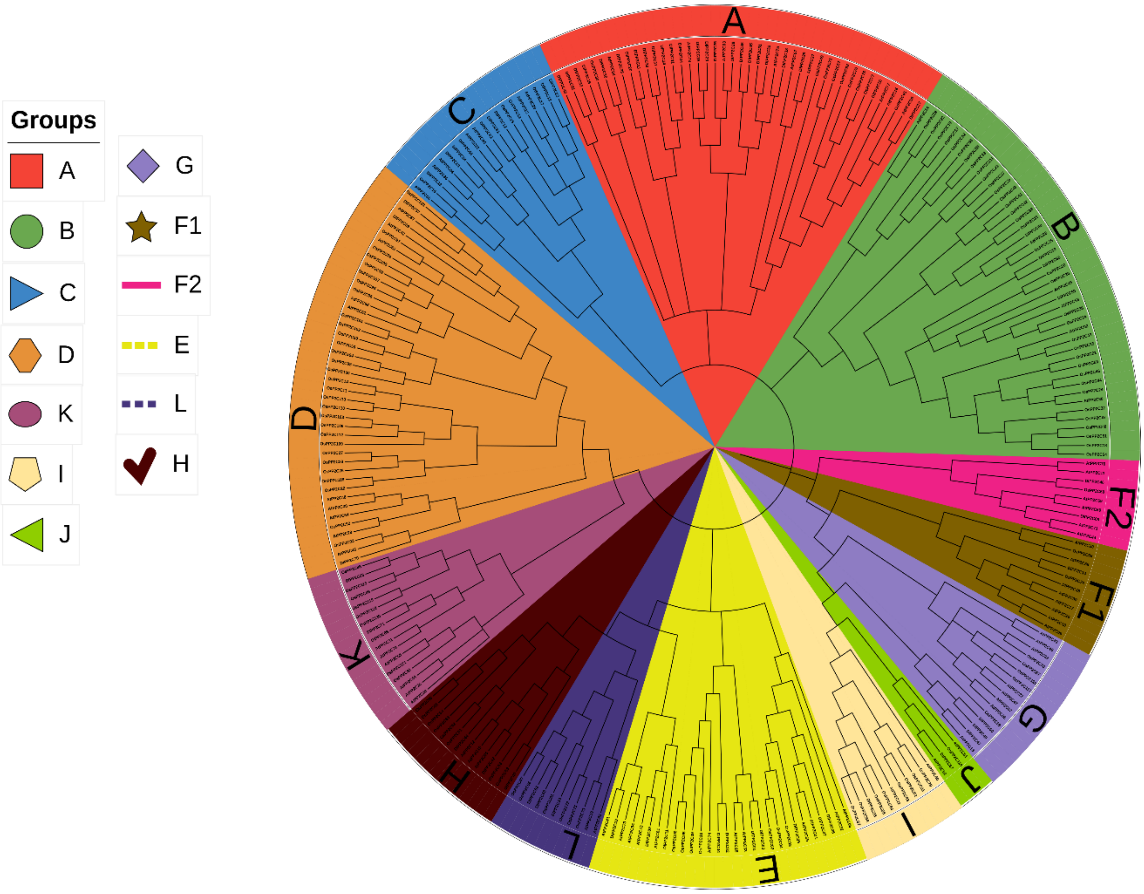

Figure 1. The figure represents a phylogenetic tree of *PP2C* gene family in three species, including *D. longan*, *Arabidopsis thaliana* and *Oryza sativa*. The tree is divided into 13 families, which consist of a unique set of *PP2C* gene family members of the species, displayed in different colors.

2.3. PP2C Gene Clustering and Distribution of Chromosomes in Logan

After the discovery of the *PP2C* gene family, 71 possible *DlPP2C* genes have been mapped onto the *longan* genome and systematically renamed according to the position of the chromosome and physical order, from *DlPP2C1* to *DlPP2C71*. The genes of *DlPP2C* were unevenly distributed in the chromosomes, and there were local instances of gene clustering. It was worth noting that *DlPP2C* genes were evenly spread in various chromosomes even though their abundance was extremely uneven. There was a total of fifteen *DlPP2C* genes were found on chromosomes 1 whereas only two *DlPP2C* genes were observed on chromosome 15 Moreover, there were three *DlPP2C* gene members in chromosomes 3,4, 11 and 14, but two genes on chromosomes 7, 8, 12 and 13. Such a non-random pattern of distribution indicates that specific areas of the chromosomes can be *PP2C*-enriched locus. Further, a total of five 5 *DlPP2C* genes were found in unanchored (UA) scaffolds, which illustrates that the genes are present in the assembled genome, but their actual chromosomal positions had not been determined. Comprehensively, chromosomal localization analysis offers important data that lead to the understanding of the genomic organization of the *DIPP2C* gene family. The difference in the number and distribution of the genes on the chromosomes may reflect lineage-specific expansion patterns that may either be related to evolutionary adaptation and functional specialty of *PP2C* genes in longan.

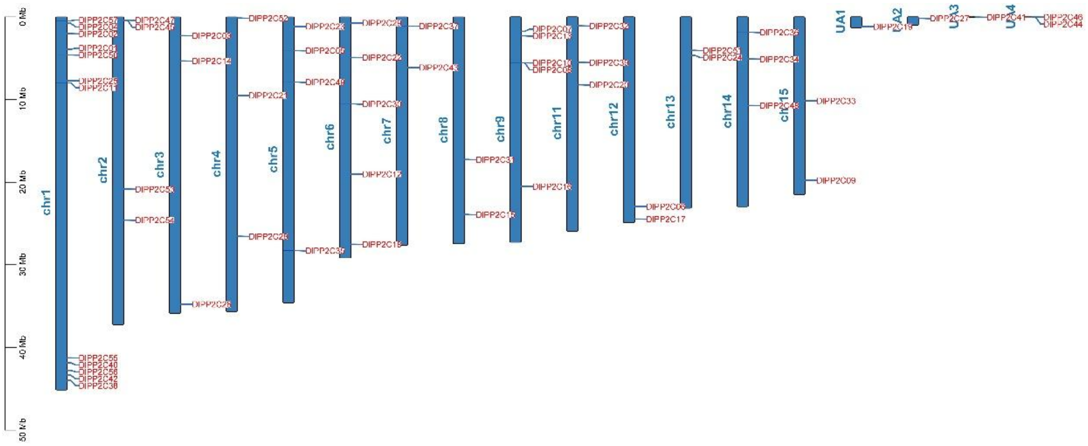

Figure 2. The chromosomal distribution of the DIPP2C gene family, where 13 chromosomes (Chr1-Chr 13) are clearly defined with its genes being positioned in different locations on the chromosome. Unanchored (UA) regions, contain additional DIPP2C genes, which is an indication that they are unassembled or their genomic regions remains unresolved.

2.4. Interaction Network of Longan and Arabidopsis PP2C Genes

In order to further evaluate the pattern of evolutionary conservation and duplication of *DlPP2C* gene family, synteny analysis was conducted between *Dimocarpus longan* and the representative dicot and monocot species, *Arabidopsis thaliana* and *Oryza sativa*. Analysis demonstrated the presence of massive collinearity among the *DlPP2C* genes in the longan genome with many intrachromosomal relationships (Figure 7A). The majority of the duplicated *DlPP2C* gene copies occurred on other chromosomes; thus, it is possible that segmental duplication or whole-genome duplication (WGD) has been the major driving force behind the expansion of the *PP2C* gene family in *longan*. Conversely, the numbers of duplicated gene pairs in close proximity were few, which is another sign that tandem duplication has been a rather minor process. Interestingly, chromosome 9 showed the highest number of collinearity interaction with chromosomes 5, 7 and 8 and so it is possible that the chromosome might be a hot spot of *PP2C* gene expansion. Also, multiple *DlPP2C* genes were found within unanchored (UA) genomic regions. But even though these sequences are not yet attached to particular chromosomes, their existence stating that the sequences can add to the general diversity of the *PP2C* gene family. Incomplete genome assembly or structural complexity may also hide the true picture of *PP2C* gene spread because similar associations with unanchored regions were also found in Arabidopsis thaliana. Overall, the synteny analysis demonstrates the role of the events of segmental and whole-genome duplication on the development and evolution of the *DlPP2C* gene family. The existing results can provide meaningful information about the evolutionary dynamics and structural preservation of *PP2C* genes in the plant species.

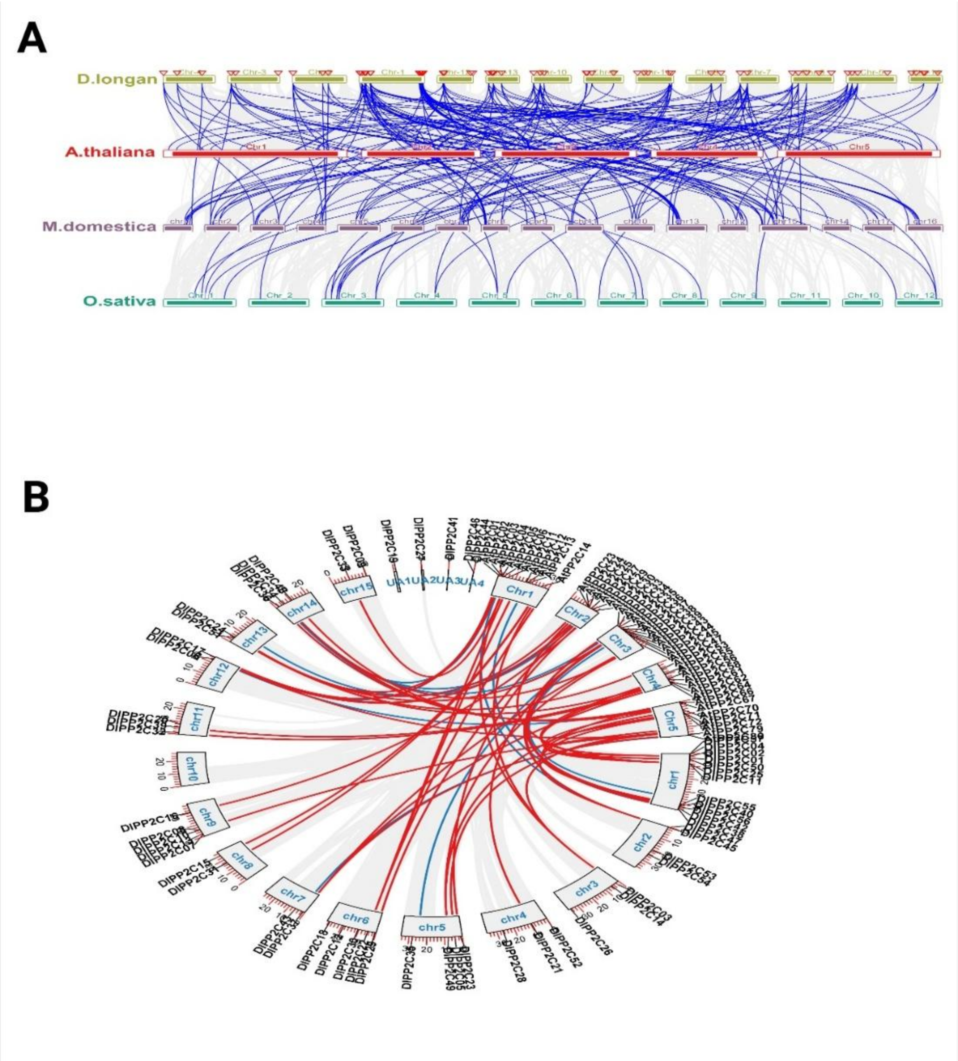

Figure 3. (A). The synteny plot of the chromosomal localization of *Arabidopsis thaliana*, *Dimocarpus longan*, and *Oryza sativa*. The blue lines represent the synteny between *Arabidopsis* and *Longan* and the red lines mark the syntenic associations between *Longan* and *Oryza sativa*. The map highlights the preserved gene arrangement and genomic rearrangements among the species, and provides information on their evolutionary association and the maintenance of the genomic regions throughout the evolution. **(B).** This circos map of the locus of the *PP2C* genes in the chromosomes of *Longan* (denoted as "chr") and *Arabidopsis* (denoted as "Chr"). The red lines represent the *PP2C* genes of Longan and the blue lines are the *PP2C* genes of *Arabidopsis*. The unanchored regions denoted as (UA1, UA2, etc.) refer to loci of the chromosomes, which could have additional *PP2C* genes, but with diminished resolution.

2.5. Gene Structure and Conserved Motifs and Domains Analysis of the DlPP2C Gene Family

Combined phylogenetic, conserved motif, domain analysis, and gene structure analysis allowed further analysis of the structural and evolutionary associations of the *DlPP2C* gene family (Fig. 5). The conserved motif distribution analysis showed that proteins of *DlPP2Cs* have some common motifs similar to the conserved functional regions and necessary to catalytic activity. It is also noteworthy that evolution members of the same subgroup had similar motif composition, which indicated functional preservation, but the differences in the motif arrangement among subgroups could identify functional divergence. Domain analysis also revealed that all DlPP2C proteins have conserved *PP2C* catalytic domains which are critical in their phosphatase activity. These domains in conservation in the family of genes emphasize their central role in conserving core biochemical functions and minor structural differences could help to explain the differences in substrate specificity or regulatory interactions. Analysis of the gene structure represents the arrangement of the coding sequences (CDS) and untranslated regions (UTRs) in *DlPP2C* genes. Regions associated with protein encoding (the CDS regions) had patterns which were relatively conserved across highly related genes. Conversely, it was found that the length and structure of UTRs had varying variants and this can lead to post-transcriptional regulation, such as mRNA stability and translational efficiency. Taken as a whole, the homogeneity in the structure of genes among subgroups, hypothesizes both the functionality and diversification in the *DlPP2C* family.

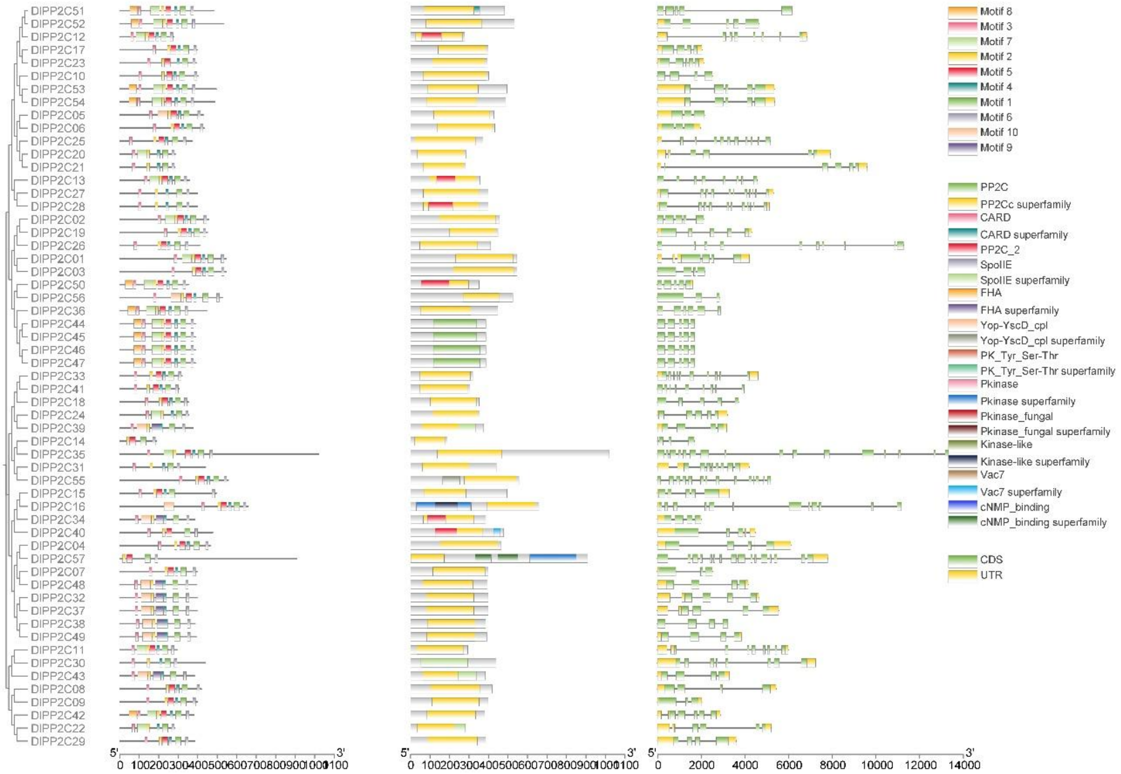

Figure 4. This figure is an illustration of a four-part study (from left to right), of the DlPP2C gene family including evolutionary connections (part 1) Phylogenetic tree, motif distributions (part 2), conserved domains (part 3), and coding sequences (CDS) and untranslated regions (UTRs) (part 4), highlighting the structural and functional diversity of the DlPP2C gene family.

2.6. Protein-Protein Interaction (PPI) Analysis of PP2C Gene Family in Longan

In order to further investigate the functional relationships of the proteins that are involved in DlPP2C, a protein-protein interaction (PPI) network was built to project the possible interactions of *DlPP2C* gene family members with the interacting proteins (Fig. 6). Each node in the network is a protein, and the connecting edges depict predicted or experimentally validated interactions between the proteins. The position of DlPP2C proteins places them in the center of the node, and they have interactions with proteins of various functional categories. The PPI network shows a complex feature and some of the DlPP2C proteins provide a broad connectivity. It is noteworthy that DlPP2C12, DlPP2C18 and DlPP2C29, have more patterns of interaction implying that these proteins may be the key to a number of cell events. Interaction pattern functional annotation showed that DlPP2C proteins could be implicated in a number of biological pathways such as signal transduction, stress response, metabolic control and cellular homeostasis. The high connectivity of the network indicates that the DlPP2C proteins can serve as important parts of the protein complexes in order to organize the responses in the cell. In addition, a group of DlPP2C proteins that included DlPP2C52 and DlPP2C50 were also predicted to interact with transcription factors and regulatory proteins which showed a potential to play a role in the regulation of gene expression.

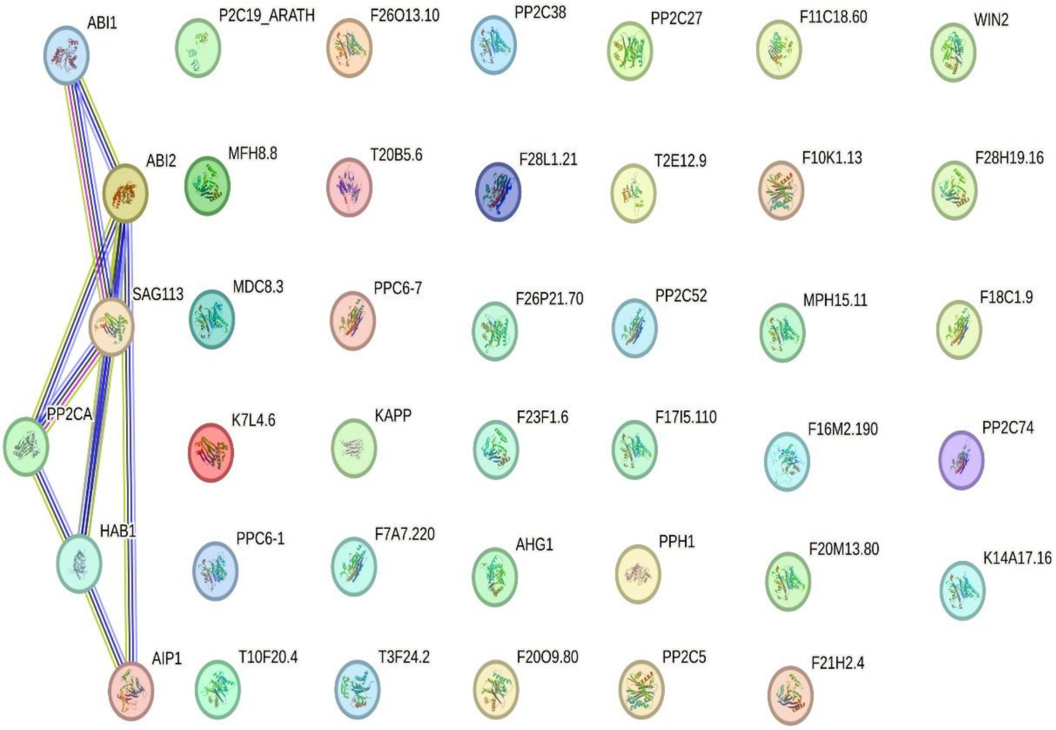

Figure 5. The predicted Protein-protein interaction network of DlPP2C gene family members displaying functional associations based on Arabidopsis thaliana as query. Each node stands for a protein whereas edges represent predicted interactions.

2.7. Cis-Acting Elements Analysis of the PP2C Gene Family in D. longan

In an attempt to understand the possible transcriptional regulation of the *DlPP2C* gene family, 2,000 upstream promoter kilobase regions have been studied in order to identify putative *cis*-acting regulatory factors (Supplementary Table.S1). The promoters of *DlPP2C* genes had a large number of *cis*-elements linked to hormone responsiveness, stress responses and developmental regulation. These included hormone responsive elements including abscisic acid-responsive elements (ABREs) and auxin-responsive elements (AuxREs), which were often found, indicating the possibility of involvement of *DlPP2Cs* in hormone signaling pathways. Specifically, the abundance of ABRE motifs suggests that many genes of DlPP2C participate in the ABA-dependent processes. Moreover, various stress-dependent *cis*-elements were highly distributed such as dehydration-responsive element (DRE), MYB binding sites (MBS-FBR), TC-rich repeats, MeJA-responsive element (MeJARE), and wound responsive element (WRE). The fact that these aspects are common implies that *DlPP2C* genes can be linked to the responses to abiotic stresses, such as drought, salinity and oxidative stress, and biotic stress-related signaling. Furthermore, the promoters contained several *cis*-elements associated with the developmental and cellular regulation, including CMA3, SEF1-BS, PMCD, CRE, and CC-CRE and thus suggested their roles in growth, tissue differentiation, and circadian regulation. It is interesting to note that genes with similar *cis*-elements composition tended to cluster in certain phylogenetic subsets indicating that transcription was regulated. By and large, the analysis of *cis*-regulatory elements indicates that the *DlPP2C* genes have complex regulatory structures that allow them, to combine hormonal and environmental cues. These functional predictions however are found on the analysis of promoters in silico and need further experimental verification.

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2.8. Expression Analysis of the DlPP2C Gene Family in Response Exogenous to ABA Treatment

The heatmap demonstrates the expression profile of the *DlPP2C* genes after ABA (Abscisic Acid) treatment. A number of genes such as *DlPP2C1* and *DlPP2C25* are highly expressed which means that they are tangled in stress or response pathways mediated by ABA (Supplementary Figure.1). On the other hand, other genes like *DlPP2C28* and *DlPP2C38* show lower levels of expression implying that they are not so imperative in ABA signaling. The study of the effect of *DlPP2C* genes during ABA signal response was performed using longan embryogenic callus subjected to various concentrations of abscisic acid (5mM, 10mM and 20mM) at different time intervals (8h, 16h and 24h). The transcriptional response of the genes of interest (*DlPP2C*) to ABA treatment was then evaluated through RT-qPCR expression of the gene (Fig. 6). The results showed that the selected *DlPP2C* genes displayed dynamic and dose-dependent patterns of expression to exogenous ABA treatment. There were many genes that showed significant changes in transcription under various treatment conditions, indicating that they are sensitive to ABA concentration and duration of exposure. Interestingly, ABA treatment of 16h at 10 mM concentration triggered the most significant levels of several *DlPP2C* gene expression, indicating that intermediate levels of concentration and intermediate exposure time is the best in stimulating *PP2C*-mediated signaling response. Particularly, *DlPP2C01* had a strong and consistent response to ABA treatment with its expression being suggestively changed at various time points implying its possible involvement as a key regulator in ABA signaling.

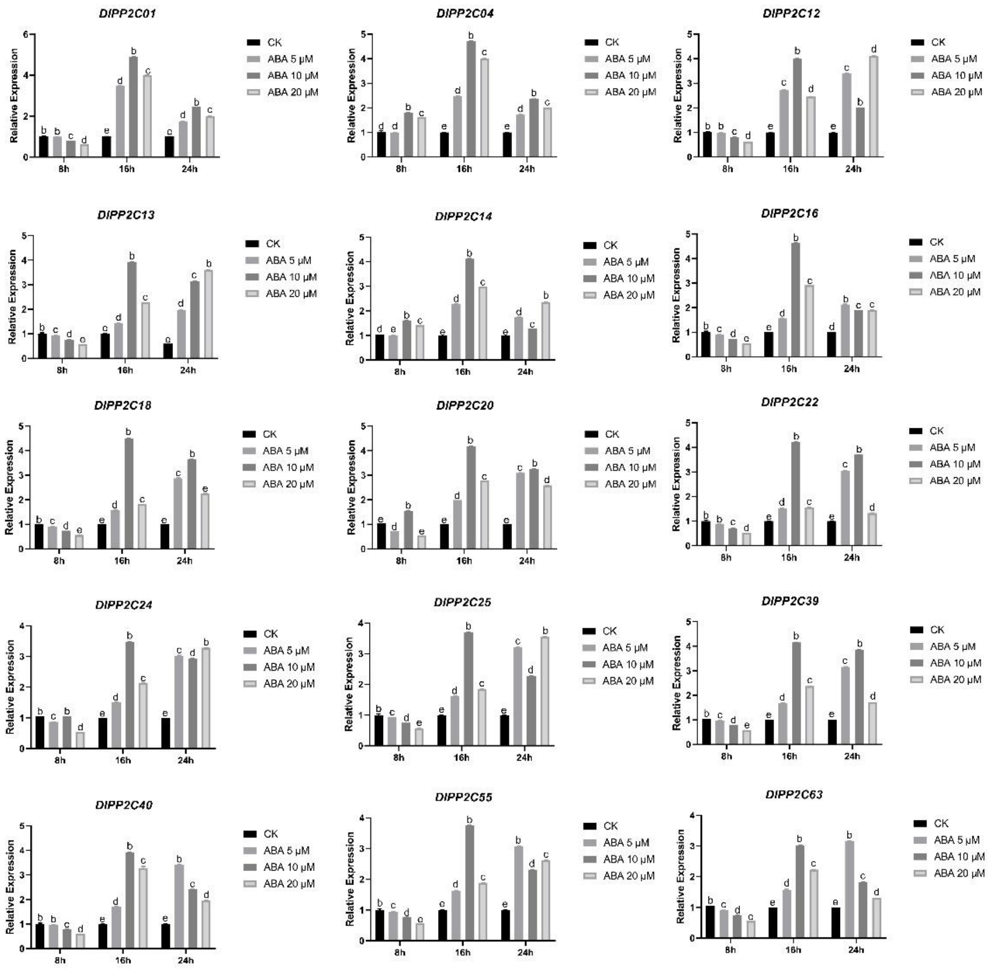

Figure 6. The relative expression of DlPP2C genes in response to exogenous ABA treatments (5,10 and 20 μM) at three-time intervals (8,16 and 24h), during early SE of longan determined by qRT-PCR. Values are the mean (n=3) of three biological replicates. The data is plotted as fold change ($2^{-\Delta\Delta CT}$) relative to control, normalized to UBQ as internal reference. Graphpad prism 8 (v 10.0.0), software was used for plotting the graphs using one

way ANOVA. Different letters above the bars indicate significant differences among treatments based on Tuckey's t-test (p≤0.05).

This was also confirmed by GUS staining of *DlPP2C01* lines of overexpression that exhibited increased activity in the presence of ABA which indicated its efficient role in ABA-responsive regulation (Fig. 7). Moreover, other *DlPP2C* genes demonstrated varied function with respect to expression, some were prematurely induced at 8h and gradually decreased with the time interval (16-24 h) which depicts functional separation among the genes in the gene family. The difference in expression patterns of *DlPP2C* members revealed that they might be having different functions in the regulation during ABA signaling.

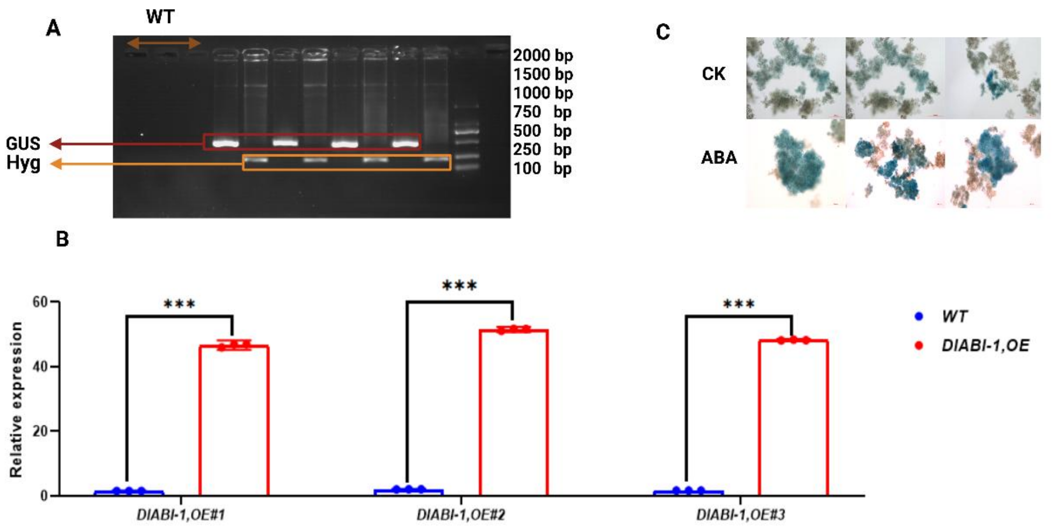

Figure 7. Transient overexpression of D. longan EC and molecular identification. (A). PCR amplification of WT and DlPP2C1 transient overexpression cell lines displaying the GUS (225 bp) and Hyg (475 bp), detection for the verification of amplified sequences. (B). GUS staining of WT and DlPP2C1 transiently overexpressed line. The fluorescence microscopy technique was used for capturing images with field of view set at 20x. (C). Relative expression level of WT and DlPP2C1 transient overexpression cell line determined by qRT-PCR. Asterisks indicate significant differences (WT vs. OE comparison), "*" is $p < 0.05$, "**" is $p < 0.01$, and "***" is $p < 0.001$.

Moreover, transcriptome (RNA-seq) results of the ABA-treated and control longan EC represented vast transcriptional reprogramming. The sample correlation analysis revealed that there were high correlation coefficients between biological replicates that described and presented a good data consistency (Fig. 8A). Principal component analysis (PCA) also revealed that there is a distinct difference in control and ABA-treated groups (Fig. 8B). indicating that ABA treatment caused a considerable amount of transcriptional change. A total of 1799 differentially expressed genes (DEGs) were identified in the course of differential expression (DEA) analysis, comprising of 253 upregulated and 1546 downregulated genes (Fig. 8C). The over-representation of downregulated genes shows that ABA treatment induces widespread transcriptional repression in longan callus. To further investigate the idea of the functional significance of these DEGs, Gene Ontology (GO) enrichment analysis was conducted (Fig. 8D), and the enrichment in biological process was significantly obtained based on stress response, metabolic regulation and cellular process. In addition, KEGG pathway enrichment analysis (Fig. 8E), indicated that the DEGs mainly participated in pathways of phenylpropanoid biosynthesis, MAPK signaling, and plant hormone signal transduction respectively.

The RNA-seq findings were in line with the RT-qPCR results to indicate that *DlPP2C* genes, specifically *DlPP2C1*, are involved in the regulation of ABA-responsive transcriptional networks.

Collectively, these findings postulate the vitality of the ABA-induced *DlPP2C* genes and specify that *DlPP2C1* is of prime importance in the coordination of ABA-induced gene expression and ABA-induced stress adaptation in *longan* callus.

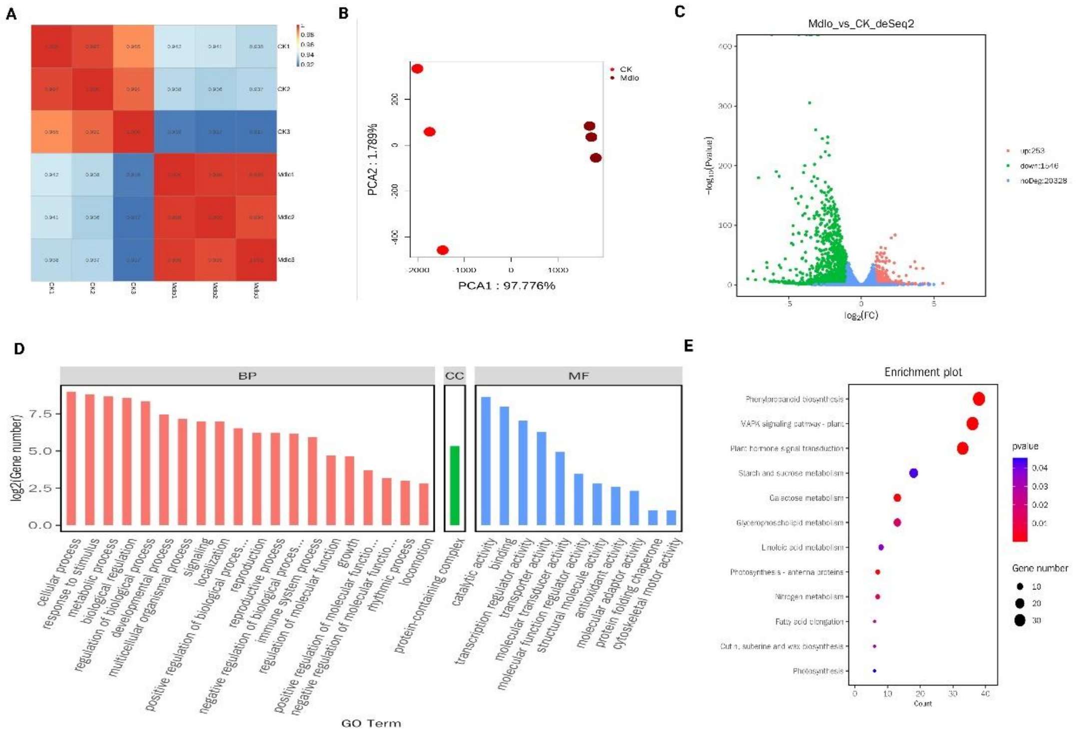

Figure 8. Transcriptomic analysis and data quality assessment. (A) Correlation heatmap between samples. A closer R2 value to 1 indicates better reproducibility between the two samples. (B) PC1 and PC2 are two principal components; different colors represent different groups of biological replicates. (C) Volcano plot on differential expression. Each dot represents a gene. X-axis: log2Fold change of expression; Y-axis: -log10(FDR) or -log10(P-value). (D) GO classification of DEGs. X-axis: Go terms and classifications; Y-axis: Number of DEGs (genes) annotated to the term (right) and percentage of that in all DEGs (genes) (Left). (E) KEGG pathway enrichment on DEGs-Bubble chart. Each dot represents a KEGG pathway. Y-axis: Pathway; X-axis: Rich factor.

3. Discussion

This study gives the first thorough genome-wide analysis of the *PP2C* gene family in Dimocarpus longan, a tropical fruit species of enormous economic and therapeutic relevance. Identifying 71 *PP2C* genes offers vital insights into their probable functions in regulating *Longan's* growth, development, and stress responses [24,25]. Phylogenetic analysis showed that longan *PP2C* genes are organized into six primary clades in a way comparable to *Arabidopsis thaliana* and *Oryza sativa*, which are model dicot and monocot plants, respectively. This clustering demonstrates that the *PP2C* gene family underwent early evolutionary diversity well before the split between monocots and dicots [26]. Chromosomal mapping of the longan *PP2C* genes indicated an unequal distribution among numerous superscaffold, with various clusters of genes detected nearby. The clustering of *PP2C* genes across these scaffolds indicates potential gene duplication events, a primary process by which gene families increase and diversify [27]. Synteny's research further confirmed the findings from the Circos plot by indicating conserved collinear areas between *Longan* and numerous other plant species, including *Arabidopsis* and *Oryza sativa* [28]. This comparative genomic study demonstrated the evolutionary and functional significance of key *PP2C* loci, supporting the concept that these genes have been conserved across numerous plant taxa [29]. Gene structure and conserved motif analysis of longan *PP2C* genes indicated the existence of essential functional domains, including the PP2C catalytic domain and kinase-like regions [30]. These conserved domains demonstrate that longan *PP2Cs* share core structural properties with other PP2C proteins throughout plants [31,32]. Additionally, changes in exon-intron arrangement suggest that some genes may have undergone structural modifications to enable more complex control. The discovery of kinase-like domains in specific *longan PP2C* genes suggests that these genes likely participate in unique signaling pathways that are specific to this species [33]. Protein-protein interaction network analysis identified a closely connected cluster of PP2C proteins likely engaged in the abscisic acid (ABA) signaling pathway, a vital mechanism for *Longan's* adaptation to abiotic challenges such as drought and cold stress [44,45]. The predominance of this cluster fits with the well-established role of *PP2Cs* as negative regulators of ABA-mediated stress responses [34]. The network analysis revealed that these *PP2Cs* are likely interacting to modify ABA signaling in a coordinated manner, which is necessary for Longan's ability to respond to altering environmental conditions [35,36]. *Cis*-acting element analysis showed a variety of hormone-responsive and stress-related regulatory motifs inside the promoters of longan *PP2C* genes. Elements such as ABRE (ABA-responsive), MeJARE (methyl jasmonate-responsive), and others related to gibberellin and auxin signaling were prevalent, supporting the concept that longan PP2Cs are major integrators of numerous hormonal and environmental signals [37,38]. Distribution of these elements illustrated that *PP2C* of *D. longan* have ability to effectively interfere with multiple signaling pathways which allows the plants to respond to both external and internal stimuli. These *cis*-acting areas show how *PP2C* genes are regulated in response to shifting environmental factors, indicating how adaptable these genes are in facilitating adaptive reactions [39].

Abscisic acid (ABA) plays a central role in regulating plant growth, development, and stress adaptation, with clade A protein phosphatase 2C (PP2C) proteins functioning as key negative regulators in the ABA signaling pathway. In this study, several *DlPP2C* genes exhibited dynamic transcriptional responses to exogenous ABA treatment in longan embryogenic callus, with 10 μM ABA at 16 h inducing peak expression levels, particularly for *DlPP2C1*. This time- and dose-dependent response is consistent with previous findings in longan embryogenic tissues, where hormone-induced transcriptional regulation has been shown to occur in a temporally dynamic manner [40,41]. ABA plays a key role in regulating plant growth, development and stress adaption, with clade A PP2C proteins known as key negative regulators in the ABA signaling pathway. Our findings showed that several *DlPP2C* genes exhibited dynamic transcriptional responses to exogeneous ABA treatments in longan EC, especially *DlPP2C* with 10 μM ABA at 16 h induced peak expression levels. This time and dose dependent responses is consistent with previous findings in longan embryogenic callus, where hormonal induced transcriptional regulation has been shown to occur in temporally dynamic manner [42–44]. The *DlPP2C1* which is ABA responsive suggests that it may function similarly to ABI1-like *PP2Cs* in model plants. It was already reported that, clade A PP2Cs such as ABI1 and ABI2 act as negative regulators of ABA signaling by inhibiting SnRK2 kinases in Arabidopsis thaliana, thereby modulating downstream transcriptional responses [45,46]. Remarkably, despite their inhibitory role at the protein level, both *ABI1* and *ABI2* genes are transcriptionally induced by ABA, establishing part of negative feedback regulatory loop [47,48], which permits plants to fine-tune ABA signaling intensity and halt redundant responses under continued stress conditions. The expression patterns revealed in the current study especially *DlPP2C1* aligns with this conserved regulatory model. The higher expression patterns of DlPP2C under exogenous ABA treatment, particularly at intermediate time interval, may reflect its involvement in feedback diminution of ABA signaling [49]. This claim was further supported by the GUS staining findings, where the higher GUS activity in *DlPP2C1* OE line under ABA treatment was recorded which indicates that the gene is transcriptionally activated in response to ABA. In addition to expression and functional validation, RNA sequence analysis revealed an extensive transcriptional reprogramming in response to exogenous ABA treatment, with a predominance of downregulated genes. This universal repression pattern propose that ABA may primarily suppress growth related metabolic process while triggering a subset of stress responsive pathways. Analogous reports have already been published in other plant systems, where ABA induces a large-scale reorganization of gene expression to aid stress adaption [50,51].

4. Materials and Methods

4.1. Identification and Physicochemical Properties of DlPP2C Proteins

The protein sequences of *Arabidopsis thaliana PP2C* family members were obtained from TAIR (https://www.arabidopsis.org/) [52], while the rice PP2C sequences were retrieved EnsemblPlants (https://plants.ensembl.org/) [53]. Firstly, the Arabidopsis thaliana PP2C amino acid sequences were used a query and probe to download the HHZ D. longan third-generation genome from National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA) database (SEE17675476). The TBtools software (v 2.420) [54], was used for searching possible DlPP2C sequences and further screened by two-way Blast at NCBI. The PP2C conserved structural domains of the screened members were reconfirmed by using the HMMER online software (https://www.ebi.ac.uk/Tools/hmmer/search/phmmer) [55]. Finally, the preliminarily identified members were compared with HHZ D. longan third-generation genome to search for any omissions and confirmed the existence of a total of 71 *DlPP2C* gene family members which are renamed in reference to Arabidopsis thaliana nomenclature for the DlPP2C transcription factor family. The online software ExPASy (https://web.expasy.org/protparam/) [56], was used to determine the number of amino acids (AA), molecular weight (MW) and isoelectric point (pI), instability index (II), aliphatic index (AI) and grand average of hydropathicity (GRAVY) of DlPP2C family proteins while the subcellular localization predictions were done by using WoLF PSORT (https://wolfpsort.hgc.jp/) [57].

4.2. Phylogenetic Tree, Conserved Motif and Gene Structure of DlPP2C Family Members

The evolutionary tree between *Arabidopsis thaliana*, *Rice* and *longan* was constructed by using the maximum likelihood (ML) algorithm with TBtools software (V 2.420). The online interactive software iTOL (https://itol.embl.de/) was used edit and visualize the phylogenetic tree [58]. The conserved motifs of DlPP2C proteins were identified using Multiple Em for motif Elicitation (MEME) suite (http://meme-suite.org/). The full-length amino acid sequences of DlPP2C proteins were submitted to MEME with maximum number of motifs was set up to 10, the optimum motif width ranged from 6 to 50 residues and other parameters were kept at default settings. The coding sequences (CDS) were aligned with their corresponding genomic DNA sequences to analyze exon-intron organization of DlPP2C genes while the diagrams were generated using the Gene Structure Display Server (GSDS V 2.0) (http://gsds.gao-lab.org/) [59].

4.3. PP2C Gene clustering, Distribution of Chromosomes, Cis- Elements Analysis and Synteny Visualization of DlPP2C Genes

The genome annotation (GFF/GTF) information files of *Dimocarpus longan* were used to determine chromosomal distribution of *DlPP2C* genes. The TBtools software (version 2.420) was utilized for mapping *DlPP2C* genes onto their respective chromosomes. The PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze the *cis*-acting elements prediction [60]. For synteny analysis between *D. longan*, *Arabidopsis thaliana (L.) Heynh*. and *Oryza sativa subsp. japonica Kato*, the TBtools software v 2.420 (https://github.com/CJ-Chen/TBtools-II) was used.

4.4.. Protein-Protein (PPI) Interaction Network Analysis

The string database (https://string-db.org/) was used to predict the potential interaction relationships of DlPP2C proteins [61]. The DlPP2C protein sequences were first used to identify homologous proteins in *Arabidopsis thaliana* due to limited annotation of *Dimocarpus longan*.

4.5. Plant Materials, ABA Treatments and Expression Analysis

For the current study, we used embryogenic callus (EC) of *D. longan* Lour-Honghezi. Selected 0.2 g EC were treated with ABA in MS medium provided by (Coolaber manufacturer Beijing, China) after 20 days of proliferation, at concentrations of 5 μM, 10 μM and 20 μM. Treated materials were incubated in the dark environment at 25 °C for three-time intervals including 8h, 16h and 24h. EC in MS medium without ABA treatments were used as a control. For each treatment, three biological replicates were performed and samples were collected and frozen in liquid nitrogen and stored in a −80 °C refrigerator.

4.6. The qRT-PCR Analysis

The TransZol Up kit provided by (TransGen, China) was used for the total RNA extraction from samples following their instructions manual. The cDNA synthesis was completed by using Revertaid Master Mix (Thermo Fisher scientific, China) and qRT-PCR was done on the Roche Light Cycler 96 instrument with 10-fold diluted cDNA as amplification template. The UBIQUITIN (UBQ) was used as an internal reference [62]. Data calculations were performed according to $2^{-\Delta\Delta Ct}$ [63], and graphs were generated by using GraphPad Prism 8.0.2 software. The DNAMAN 6.0 software was used to design the qRT-PCR primers (Suppl. Table S2).

4.7. RNA Sequencing and Analysis

Based on the comprehensive assessment, expression outcomes, aimed at capturing the most biologically informative, reproducible and interpretable transcriptomic response, the 10 μM ABA treated samples (labeled as Mdlo) at 16 h with a control group (CK) with three independent biological replicates were subjected to RNA sequencing analysis (Wuhan Baiyuan Biotechnology Co., Ltd.). Total RNA from the six groups of longan embryogenic cultures. The purity and concentration of RNA were assessed using a Nanodrop 2000 spectrophotometer, and RNA integrity was verified using the Agilent 2100/Lab Chip GX [64]. After samples passed quality control, library construction and mRNA transcriptome sequencing were carried out. HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts) software was used to quickly and accurately compare the Clean Reads with the reference genome, and to acquire aligning information for the Reads on the reference genome [65]. The reads were then assembled using String Tie, to reconstruct the transcriptome for subsequent analysis [66]. Annotation of genes for various analysis including DEGs, KEGG [67], GO was performed. and bioinformatics analysis were conducted according to the methods described as followed.

4.8. Transient Transformation of D. longan Embryogenic Callus

The DNAMAN 9.0 software was used to design the specific amplification primers at the 3′and 5 ends of the *Dlo000068* (*DlPP2C1*) CDS sequence which were cloned into the pCAMBIA1301-35-GUS vector (Suppl. Table S3). The bacterial solution comprising the recombinant plasmid was activated and cells were gathered by centrifugation at 7800 r/min for the duration of 10 min. The collected cells were further resuspended by using MS suspension medium containing (30 g/L sucrose, 200 mM AS, and 100 mM, $MgCl_2$) in the infiltration solution. The OD_{600} was adjusted between 0.6-0.8. The 15-d old *D. longan* EC was co-cultured with Rhizobium radiobacter for 30 min, sap was filtered and transferred to MS solid media for 3 d. The wild type (WT) having Empty vector and the pCAMBIA1301-35-GUS were taken as control check whereas transient overexpression DlPP2C cell lines were labeled as DlPP2C01-OE#1,2,3. The transiently transformed pCAMBIA1301 and pCAAMBIA1301:DlPP2C1: GUS(OE1-OE3) cell lines were collected with 0.1g each. After freezing in liquid nitrogen, samples were stored in −80 °C refrigerator for further analysis.

4.9. GUS Staining and PCR Amplification

The transiently transformed pCAMBIA1301 and pCAAMBIA1301:DlPP2C1: GUS(OE1-OE3) cell lines were collected with 0.1g each. After freezing in liquid nitrogen, samples were stored in −80 °C refrigerator for further analysis. Following the instructions mentioned on the GUS staining kit provided by (HUAYUEYANG BIOTECHNOLOGY, China), the GUS staining on the transgenic materials was completed. The fluorescence microscope (LEICA DMI8, Germany) was used to observe and obtaining the images of the GUS-Stained D. longan EC transgenic cells under 20x field of view. For the DNA extraction from both WT and overexpression cell lines after transient transformation, the Plant Genomic DNA Kit (ThermoFisher, USA) was used. The PCR amplification using F/R primers of GUS and Hyg (Suppl. Table S3) were used for the identification of transgenic cell lines.

5. Conclusions

Our findings provide the comprehensive exploration of *PP2C* gene family in Dimocarpus longan, during early somatic embryogenesis. In the context of ABA signaling, during development processes and under stress conditions, the *DlPP2C1* (ABI1) revealed it significant role as a negative regulator within the ABA signaling pathway. The uniformity between RNA-seq data and RT-qPCR findings further chains the participation of DlPP2C genes, particularly *DlPP2C1*, in ABA-mediated regulatory networks. Taken together, the collective evidence form ABA treatment assays, GUS staining findings, and transcriptomic outcomes suggests that DlPP2C functions as an ABA -responsive regulator in *Dimocarpus longan*. Overall, our results provide new insights into the functional role of *DlPP2C* genes in ABA signaling as well as establishes a basis for future investigations into stress adaption and its application to other important plant species having challenging micropropagation systems.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: title; Table S2: Table S3.

Author Contributions: M.A. designed and performed the experiments, carried out statistical analyses, produced the figures and tables, and wrote the manuscript. H.M.U and M.S. assisted in bioinformatics analysis. X.X., C.Z. and S.L. assisted in morphological checks. Y.C. revised the manuscript. Z.L. and Y.L. contributed to the creation of the concept and the funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All relevant data is available within the manuscript and Supplementary materials.

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Conflicts of Interest: The authors declare no competing interests.

Abbreviations

The following abbreviations are used in this manuscript:

ABA	Abscisic acid
PP2C	protein Phosphatase 2C
ABI	Abscisic acid insensitive
GO	Gene Ontology
KEGG	Kyoto encyclopedia of genes and genomes
DEGs	Differentially expressed genes GO Gene ontology

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