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Genome-Wide Identification and Expression Analyses of the *PP2C* Gene Family in *Paulownia fortunei*

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Abstract: We explored the composition and roles of the protein phosphatase 2C (*PP2C*) family in *Paulownia fortunei*. The genome *P. fortunei* harbored 91 *PfPP2C* genes, encoding proteins with 120–1107 amino acids (molecular weight range, 13.51–124.81 kDa). The 91 *PfPP2Cs* were distributed in 12 subfamilies, with 1–15 *PfPP2C*s per subfamily. The number and types of conserved structure domains differed among *PP2Cs*, but the distribution of conserved motifs within each subfamily was similar, with the main motif structure being motifs 3, 16, 13, 10, 2, 6, 12, 4, 14, 1, 18, and 8. The *PfPP2C* genes had 2 to 20 exons. There were ABA-response elements in the promoters of 42 *PfPP2C* genes, response elements to phytohormones, and stress in the promoters of other *PfPP2C* genes. A covariance analysis revealed that gene fragment duplication has played an important role in the evolution of the *PfPP2C* family. There were significant differences in the transcript levels of some *PfPP2C* genes in *P. fortunei* affected by witches' broom (PaWB) and after treatment with rifampicin and methyl methanesulfonate. *PfPP2C02*, *PfPP2C12*, *PfPP2C19*, and *PfPP2C80* were strongly related to PaWB. These findings provide a foundation for further studies on the roles of *PP2C*s in PaWB.

Keywords: *Paulownia fortunei*; *PP2C* family; identification; hormone treatment; expression analysis

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1. Introduction

Protein phosphorylation and dephosphorylation are important for the functional expression of proteins and are essential regulatory mechanisms in living organisms [1]. Protein phosphatases catalyze the dephosphorylation of phosphorylated proteins, thereby playing important roles in plants' responses to abiotic stresses and hormones [2]. Protein phosphatases can be classified according to their substrates into protein tyrosine phosphatases (PTPs), serine/threonine phosphatases (STPs), and dual-substrate PTPs (DSPTPs) [3–5]. The STPs can be further divided into phosphoprotein phosphatases (PPP) and protein phosphatase metal-dependent (PPM) proteins according to their crystal structure [6]. The protein phosphatase 2C (*PP2C*) and phosphopyruvate dehydrogenase phosphatase (PDP) families are the two main PPM families [6]. *PP2C*s are Mg^{2+}- or Mn^{2+}-dependent monomeric enzymes that are widely found in Archaea, bacteria, fungi, animals, and plants [7]. Compared with other organisms, plants tend to have more *PP2C* proteins [8]. Plant *PP2C* proteins have a specific structural pattern; most have a conserved catalytic region at the C-terminus, while the N-terminus is less conserved, with a variable-length extension region that contains sequences related to intracellular signaling, such as transmembrane and kinase interaction sequences [9]. *PP2C*s are widely involved in physiological processes, such as abscisic acid (ABA) signaling and trauma signaling, plant growth and development, and plant disease resistance [10]. *Arabidopsis thaliana* has 80 *PP2C* gene family members in 12 subfamilies [11]. The *PP2C* proteins in subfamily A negatively regulate ABA signaling by binding to the ABA receptor proteins PYR/PYL/RCAR, thereby causing physiological responses, such as inhibition of germination and stomatal closure [12,13]. The *AtPP2C* proteins in subfamily B play a regulatory role in the mitogen-activated protein kinase (MAPK) pathway [14].

Members of subfamily C are involved in physiological processes such as plant flower organ development [15]. Members of subfamily D in *A. thaliana* are involved in regulating seed germination in the dark, seed growth, and the ABA signaling pathway by mediating the activity of the plasma membrane H^+-ATPase in cells [16,17]. Less is known about the other subfamilies. However, various studies have shown that *PP2Cs* are also involved in biological responses under abiotic stresses; for example, *AtPP2C31* and *AtPP2CG1* negatively regulate the response to high salt and low temperatures, respectively, in *A. thaliana* [18,19].

Previous studies have shown that members of the *PP2C* family play important regulatory roles in responses to abiotic stresses, such as low temperature, high temperature, and drought, as well as in hormonal regulation. For example, gene microarray expression profiling and real-time fluorescence quantification analyses revealed that members of the subclades C, E, and G of the *PP2C* family in *Vitis vinifera* were up-regulated under stress conditions, while members of subclades A, D, F, H, and K were down-regulated [20]. In *Broussonetia papyrifera*, four members of the 18 *BpPP2Cs* tested were found to be up-regulated under low temperature (4 °C) [21]. Furthermore, two proteins showed increased phosphorylation levels at 6 h of the low-temperature treatment, demonstrating that *PP2Cs* are involved in the cold stress response in plants [21]. In *Brachypodium distachyum*, almost all *BdPP2Cs* were up-regulated under low-temperature stress (4 °C) [22]. In *Oryza sativa*, *OsPP2C09* mediated ABA desensitization, which contributed to root elongation, under drought stress [23]. Under low-temperature, high-temperature, and drought conditions, 10, 8, and 9 *PP2C* genes respectively, were found to be continuously up-regulated in *Poncirus trifoliata* [24]. Six *Phyllostachys heterocycla PP2C* genes, including *PH02Gene33357.t1* and *PH02Gene38274.t1*, were up-regulated under high-salt conditions (200 mmol·L^{-1} NaCl) and by ABA (100 μmol·L^{-1}) [25]. In *Dendrobium catenatum* treated with 20% PEG-6000, 200 mmol·L^{-1} NaCl, 100 μmol·L^{-1} ABA, and 200 μmol·L^{-1} salicylic acid, *DcPP2C5*, *DcPP2C5*, *DcPP2C5*, and *DcPP2C5* were up-regulated under drought and salt stress, and *DcPP2C20*, *DcPP2C38*, and *DcPP2C56* were up-regulated in the roots under ABA and SA treatment [26]. These findings indicated that *D. catenatum PP2Cs* are not only involved in responses to abiotic stresses, but also in responses to hormones [26].

Paulownia fortunei is a deciduous tree in the genus *Paulownia* (family *Scrophulariaceae*) [27]. It is a source of timber and is planted for farmland protection in China [27]. It has fast growth, produces high-quality wood, and shows strong adaptability and resistance [27]. It contributes to alleviating timber shortages, improving the ecological environment, ensuring food security, and improving people's living standards [27]. However, there are some serious problems in its production, such as the occurrence of witches' broom disease (PaWB), which increases tree mortality, slows tree growth, and seriously affects the development of the *Paulownia* industry. Although the genome of *P. fortunei* has been sequenced [28], the members of the *PfPP2C* gene family in this species have not yet been reported. In this study, using the *PP2C* gene sequences from *Arabidopsis thaliana* as search queries, members of the *PfPP2C* gene family in *P. fortunei* were screened and identified using homologous alignment analyses. The genes and their encoded proteins were analyzed using a series of bioinformatic tools. Differences in gene expression between diseased and healthy *P. fortunei* seedlings were determined by analyses of RNA-Seq data. A preliminary investigation of the expression patterns of *PfPP2Cs* in *P. fortunei* under various stress conditions and in PaWB-affected plants reveal potential functions of the *PfPP2C* family, and provide a theoretical basis for exploring their roles in the development of PaWB.

2. Materials and Methods

2.1. Identification, Physicochemical Properties, and Prediction of Subcellular Localization of PP2C Family Members in P. fortunei

The sequences of *Arabidopsis thaliana PP2C* proteins were obtained from the TAIR database (https://www.arabidopsis.org/) (accessed on 24 May 2022). The *P. fortunei* genome database was searched for homologous protein sequences with high structural similarity to the *Arabidopsis thaliana PP2C* family using BlastP. The hidden Markov model

(PF00481) file of the *PP2C* protein structural domain was downloaded from the Pfam database (http://pfam.xfam.org/) (accessed on 25 May 2022), and then used in Biolinux to search the genome of *P. fortunei* using hmmersearch. Candidate protein sequences were those with an e-value of $\leq 10^{-2}$. The candidate protein sequences of the *PP2C* family in *P. fortunei* were those that were detected in both the BlastP and hmmer analyses. The candidate protein sequences were verified by Pfam, and protein structural domains were identified using SMART (http://smart.embl.de/smart/batch.pl) (accessed on 26 May 2022) and CDD (https://www.ncbi.nlm.nih.gov/cdd) (accessed on 26 May 2022). The protein sequences without *PP2C* structural domains were removed to obtain the final set of *PP2C* family members in *P. fortunei*. The number of amino acids, isoelectric point, and molecular weight of putative *PP2C* proteins of *P. fortunei* were predicted using Expasy (https://web.expasy.org/protparam/) (accessed on 22 June 2022). Subcellular localization of the *PP2C* gene family members by using the Cell-PLoc 2.0 online tool (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) (accessed on 14 January 2023).

2.2. Chromosomal Localization and Phylogenetic Analysis of PP2C Genes in P. fortunei

The genome annotation files were downloaded from the *P. fortunei* genome database. Information about chromosome length and the location of *PP2C* genes on chromosomes was extracted from the *P. fortunei* genome annotation files using TBtools software. The Map MG2C online tool (http://mg2c.iask.in/mg2c_v2.0/) (accessed on 10 July 2022) was used to map the distribution of *Paulownia PP2C* genes on chromosomes. The *PP2C* protein sequences were downloaded from the *A. thaliana* genome website and the *Poncirus trifoliata* protein sequence file was downloaded from the citrus genome database (http://citrus.hzau.edu.cn/) (accessed on 19 July 2022). The amino acid multiple sequence alignment analysis of *PP2C* proteins from *A. thaliana*, *P. trifoliata*, and *P. fortunei* was performed using MEGA-X software, and the phylogenetic tree was constructed using NJ in MEGA-X software, with the bootstrap value set to 1000 and other parameters set to default values.

2.3. Conserved Structural Domains, Conserved Motifs, and Gene Structure Analysis of PP2C Family Members in P. fortunei

The online tool Pfam search (http://pfam.xfam.org/search#tabview=tab1) (accessed on 17 August 2022) was used to identify conserved structural domains in *P. fortunei PP2C* proteins, and the results were visualized using TBtools software. The conserved motifs of *PP2C* protein sequences were analyzed using the online tool MEME (https://meme-suite.org/meme/tools/meme) (accessed on 7 August 2022), with the number of motifs set to 20, and the results were visualized using TBtools software. The structure of each member of the *PP2C* gene family, based on its coding sequence, was analyzed online by GSDS (http://gsds.gaolab.org/) (accessed on 12 July 2022).

2.4. Analysis of Promoter Cis-Acting Elements and Covariance in Members of the PP2C Family in P. fortunei

TBtools software was used to extract the 2000-bp upstream sequence of the start codon of each *PP2C* gene as the promoter sequence. The *cis*-acting elements in the promoters of *PP2C* genes were detected using PlantCARE online software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (accessed on 27 August 2022), and the results were visualized using TBtools software [29]. Fasta Satas, File Merge For MCScanX, Text Block Extract, and Filter tools in TBtools were used to obtain files with information about chromosome length and associations between gene family members of *P. fortunei*. Table Row Extract or the Filter tool was used to obtain gene ID display files. The Advanced Circos tool was used to conduct the *P. fortunei PP2C* gene family covariance analysis.

2.5. Expression Analysis of PP2C Genes in P. fortunei

The materials used to generate RNA-Seq data were healthy *P. fortunei* (PF) and infected *P. fortunei* (PFI) grown in the intelligent greenhouse of the *Paulownia* Institute of Henan Agricultural University. At the age of 3 months, seedlings with the same growth status were

selected and treated with rifampicin (Rif) at 30 mg/L or with methyl methanesulfonate (MMS) at 20 mg/L. Leaves of five seedlings were randomly taken after 5, 10, 15, and 30 d of treatment, snap-frozen in liquid nitrogen, and stored at −80 °C. This experiment was conducted with three biological replicates, using healthy seedlings in normal culture and seedlings with PaWB as controls. Affymetrix GeneChip 16K gene IDs with identical sequences were retrieved using the *PP2C* nucleic acid sequence of *P. fortunei* as a probe, and then the RNASeq data for the *PP2C* genes of P. fortunei (PF and PFI) treated or not with Rif or MMS were extracted, log2-transformed using Excel, and used to generate a heat-map using TBtools software.

3. Results

3.1. Identification and Physicochemical Properties of the PP2C Family in P. fortunei

A total of 91 *PP2C* genes were identified in the *P. fortunei* genome. The protein sequences were extracted using TBtools software. Redundant sequences were manually deleted, and the remaining sequences were further validated using tools at the Pfam, SMART, and CDD databases. The 91 *PP2C* genes of *P. fortunei* were finally identified and named *PfPP2C1* through *PfPP2C91* (Table 1). The lengths of the putative proteins encoded by the *PfPP2C* genes ranged from 120 aa (*PfPP2C61*) to 1107 aa, with an average length of 432.39 aa; the predicted molecular weight ranged from 13.51 kDa to 124.81 kDa (average, 47.62 kDa); and the theoretical isoelectric point ranged from 4.60 to 9.51. In total, 20 of the *PfPP2C* proteins were predicted to be basic (including *PfPP2C01* and *PfPP2C90*) and the remaining 71 were predicted to be acidic (including *PfPP2C03* and *PfPP2C47*). Among the 91 *PfPP2C* proteins, 32.97% were predicted to be stable proteins, but the majority (67.03%) were predicted to be unstable. The theoretical instability index ranged from 42.32 to 94.39. The GRAVY values were all less than 0, ranging from −0.586 to −0.101, indicating that all 91 *PP2C* proteins were hydrophilic.

Table 1. Physicochemical properties of *PP2C* family members in *P. Fortunei*.

Gene Name	Gene ID	Number of Amino Acids	Molecular Weight	Theoretical PI	Instability Index	Aliphatic Index	GRAVY	Predicted Location(s)
PfPP2C01	Pfo01g001610.1	294	32,296.66	8.24	40.85	83.54	−0.408	Nucleus
PfPP2C02	Pfo01g002750.1	473	52,519.65	5.22	48.98	91.46	−0.268	Nucleus
PfPP2C03	Pfo01g006380.1	392	43,064.51	4.81	40.50	81.84	−0.283	Nucleus
PfPP2C04	Pfo01g009870.1	655	72,156.19	5.58	31.28	90.37	−0.137	Nucleus
PfPP2C05	Pfo02g010590.1	369	40,963.87	6.89	32.03	86.40	−0.213	Chloroplast/Nucleus
PfPP2C06	Pfo02g010660.1	426	46,080.74	5.87	37.47	93.31	−0.133	Chloroplast
PfPP2C07	Pfo02g014240.1	279	30,485.43	7.12	42.24	83.23	−0.368	Nucleus
PfPP2C08	Pfo02g016010.1	386	43,014.95	8.48	48.79	87.10	−0.317	Nucleus
PfPP2C09	Pfo02g019750.1	397	44,228.38	8.66	45.22	87.63	−0.258	Nucleus
PfPP2C10	Pfo03g000530.1	379	42,820.76	6.44	49.31	89.74	−0.339	Nucleus
PfPP2C11	Pfo03g006870.1	1081	119,853.48	5.03	39.87	89.16	−0.207	Cell membrane/Nucleus
PfPP2C12	Pfo03g008490.1	553	60,968.45	5.33	53.04	93.24	−0.203	Nucleus
PfPP2C13	Pfo03g009450.1	294	32,627.04	7.67	47.43	79.25	−0.473	Nucleus
PfPP2C14	Pfo03g013130.1	377	42,129.12	9.51	46.15	90.69	−0.359	Nucleus
PfPP2C15	Pfo03g013680.1	631	70,004.71	5.50	38.11	79.41	−0.380	Chloroplast/Nucleus
PfPP2C16	Pfo03g015100.1	433	48,321.81	5.24	37.09	82.66	−0.379	Chloroplast/Mitochondrion
PfPP2C17	Pfo04g000480.1	397	44,173.32	8.72	44.84	87.88	−0.247	Nucleus
PfPP2C18	Pfo04g003840.1	280	30,717.54	6.76	35.40	80.79	−0.444	Nucleus
PfPP2C19	Pfo04g006590.1	429	46,293.98	7.49	39.12	87.48	−0.190	Chloroplast
PfPP2C20	Pfo04g006660.1	372	41,415.26	6.42	33.56	85.43	−0.252	Nucleus
PfPP2C21	Pfo05g000400.1	349	38,472.54	4.73	54.69	91.35	−0.101	Nucleus
PfPP2C22	Pfo05g003700.1	397	43,555.96	5.25	44.41	83.73	−0.188	Nucleus
PfPP2C23	Pfo05g003720.1	1107	124,807.73	5.56	46.73	82.18	−0.366	Nucleus
PfPP2C24	Pfo05g010690.1	405	44,340.19	5.25	64.32	78.49	−0.347	Nucleus
PfPP2C25	Pfo05g011250.1	669	74,601.59	5.15	42.67	78.73	−0.456	Chloroplast/Nucleus
PfPP2C26	Pfo06g004460.1	270	30,030.46	6.76	51.43	89.59	−0.260	Nucleus
PfPP2C27	Pfo06g004710.1	196	22,216.76	8.92	53.29	94.39	−0.143	Nucleus
PfPP2C28	Pfo07g001190.1	449	48,913.54	7.16	45.84	76.88	−0.407	Nucleus
PfPP2C29	Pfo07g005140.1	422	45,245.83	8.34	28.48	88.06	−0.159	Nucleus
PfPP2C30	Pfo07g009030.1	801	88,560.94	5.27	46.37	74.59	−0.481	Chloroplast
PfPP2C31	Pfo07g014460.1	293	31,684.04	4.93	36.38	79.52	−0.355	Nucleus
PfPP2C32	Pfo07g014670.1	353	39,022.99	5.20	34.35	75.47	−0.392	Nucleus
PfPP2C33	Pfo07g015080.1	282	30,638.32	5.50	49.48	75.46	−0.321	Nucleus
PfPP2C34	Pfo08g002420.1	348	38,000.29	5.69	49.59	88.76	−0.228	Nucleus
PfPP2C35	Pfo08g010120.1	343	37,581.19	5.17	39.89	70.52	−0.551	Nucleus

Table 1. *Cont.*

Gene Name	Gene ID	Number of Amino Acids	Molecular Weight	Theoretical PI	Instability Index	Aliphatic Index	GRAVY	Predicted Location(s)
PfPP2C36	Pfo08g013880.1	555	60,319.53	4.97	40.94	91.98	−0.166	Nucleus
PfPP2C37	Pfo09g009830.1	526	57,753.16	5.26	41.75	79.94	−0.358	Nucleus
PfPP2C38	Pfo09g014660.1	284	31,332.79	6.84	40.26	90.63	−0.348	Nucleus
PfPP2C39	Pfo09g017020.1	283	31,287.57	6.14	32.44	88.55	−0.332	Chloroplast/Cytoplasm
PfPP2C40	Pfo10g007720.1	358	38,635.14	6.27	54.49	84.41	−0.159	Chloroplast/Nucleus
PfPP2C41	Pfo10g009060.1	425	46,090.32	6.83	59.09	72.68	−0.363	Nucleus
PfPP2C42	Pfo10g009070.1	425	46,090.32	6.83	59.09	72.68	−0.363	Nucleus
PfPP2C43	Pfo10g012720.1	394	44,063.26	6.55	43.71	91.75	−0.293	Nucleus
PfPP2C44	Pfo10g013980.1	491	54,041.38	5.23	41.96	72.06	−0.542	Chloroplast/Nucleus
PfPP2C45	Pfo11g000240.1	379	42,063.30	5.08	63.53	83.43	−0.275	Nucleus
PfPP2C46	Pfo11g001200.1	505	54,945.55	4.75	45.45	88.22	−0.202	Nucleus
PfPP2C47	Pfo11g001760.1	349	38,194.48	4.60	34.90	80.37	−0.296	Nucleus
PfPP2C48	Pfo11g002190.1	601	65,992.20	6.34	51.36	91.70	−0.155	Chloroplast
PfPP2C49	Pfo11g008040.1	388	42,005.64	5.32	56.68	83.69	−0.206	Chloroplast/Cytoplasm
PfPP2C50	Pfo11g011060.1	438	47,647.39	5.22	37.03	83.70	−0.217	Nucleus
PfPP2C51	Pfo11g013940.1	313	34,056.08	4.93	46.41	81.02	−0.337	Nucleus
PfPP2C52	Pfo11g015860.1	403	44,718.89	6.54	44.29	90.77	−0.345	Nucleus
PfPP2C53	Pfo12g008400.1	731	81,194.22	5.62	39.63	77.36	−0.479	Chloroplast/Nucleus
PfPP2C54	Pfo14g000920.1	557	60,286.22	4.86	39.82	89.57	−0.218	Nucleus
PfPP2C55	Pfo14g006170.1	265	28,851.94	8.59	34.98	93.89	−0.114	Nucleus
PfPP2C56	Pfo14g009710.1	348	37,801.87	5.31	51.37	89.60	−0.255	Chloroplast/Nucleus
PfPP2C57	Pfo15g011470.1	461	50,501.81	5.87	41.40	82.47	−0.206	Chloroplast/Nucleus
PfPP2C58	Pfo15g012450.1	390	42,239.89	5.11	53.71	82.54	−0.201	Chloroplast
PfPP2C59	Pfo16g002080.1	471	52,221.33	5.27	52.56	69.92	−0.493	Nucleus
PfPP2C60	Pfo16g008470.1	526	57,751.38	5.05	38.31	80.68	−0.319	Chloroplast/Nucleus
PfPP2C61	Pfo16g013780.1	120	13,508.30	4.68	36.38	89.33	−0.458	Chloroplast/Cytoplasm
PfPP2C62	Pfo17g001200.1	489	53,137.05	8.85	34.27	87.36	−0.150	Chloroplast
PfPP2C63	Pfo17g006640.1	439	47,981.43	6.65	44.24	76.79	−0.424	Chloroplast
PfPP2C64	Pfo18g001250.1	548	59,083.53	4.61	40.50	88.03	−0.126	Chloroplast
PfPP2C65	Pfo18g001790.1	346	37,658.61	6.55	40.99	85.69	−0.249	Nucleus
PfPP2C66	Pfo18g003260.1	387	42,305.59	5.10	61.40	82.17	−0.318	Nucleus
PfPP2C67	Pfo18g003900.1	360	39,673.58	5.00	35.39	73.44	−0.414	Nucleus
PfPP2C68	Pfo18g005750.1	461	49,990.82	8.62	57.48	74.49	−0.279	Chloroplast
PfPP2C69	Pfo18g006540.1	373	40,537.83	6.49	59.32	72.92	−0.463	Nucleus
PfPP2C70	Pfo19g000370.1	397	43,812.26	4.92	61.73	84.08	−0.222	Nucleus
PfPP2C71	Pfo19g001430.1	375	41,296.74	7.55	35.74	80.13	−0.324	Nucleus
PfPP2C72	Pfo19g002000.1	506	55,328.18	4.94	49.31	87.11	−0.198	Nucleus
PfPP2C73	Pfo19g002890.1	378	42,036.04	4.83	35.01	79.89	−0.337	Nucleus
PfPP2C74	Pfo19g003480.1	600	66,319.14	6.20	47.53	88.42	−0.215	Chloroplast
PfPP2C75	Pfo19g005950.1	233	26,049.77	7.05	52.31	72.79	−0.389	Nucleus
PfPP2C76	Pfo20g000210.1	538	59,481.42	6.99	45.87	78.79	−0.353	Chloroplast
PfPP2C77	Pfo20g001120.1	394	43,765.70	6.12	39.81	90.10	−0.275	Nucleus
PfPP2C78	Pfo20g004520.1	427	46,141.06	5.53	63.43	71.66	−0.367	Nucleus
PfPP2C79	Pfo20g005770.1	363	39,132.58	7.02	44.44	80.55	−0.199	Cell membrane/Nucleus
PfPP2C80	Pfo20g008850.1	387	42,878.69	5.11	45.02	67.05	−0.586	Nucleus
PfPP2C81	Pfo20g009100.1	389	42,573.53	7.55	44.69	92.06	−0.119	Nucleus
PfPP2C82	Pfoxxg008780.1	385	42,539.37	9.11	43.32	89.82	−0.331	Nucleus
PfPP2C83	Pfoxxg011050.1	432	48,285.76	5.78	38.35	77.64	−0.458	Nucleus
PfPP2C84	Pfoxxg015210.1	380	42,806.71	5.93	49.34	92.63	−0.236	Nucleus
PfPP2C85	Pfoxxg021750.1	631	69,947.47	5.56	40.22	79.10	−0.394	Nucleus
PfPP2C86	Pfoxxg021830.1	433	48,153.28	5.54	40.15	73.93	−0.310	Nucleus
PfPP2C87	Pfoxxg022160.1	293	31,764.12	4.87	36.59	78.19	−0.369	Nucleus
PfPP2C88	Pfoxxg025250.1	432	48,177.56	5.58	34.23	77.87	−0.419	Nucleus
PfPP2C89	Pfoxxg026240.1	632	70,046.61	5.63	39.96	79.13	−0.392	Nucleus
PfPP2C90	Pfoxxg026500.1	385	42,563.43	9.11	42.32	42.32	−0.318	Nucleus
PfPP2C91	Pfoxxg028980.1	380	42,792.68	5.93	49.56	92.37	−0.237	Nucleus

3.2. Prediction of the Subcellular Localization of PP2C Family Members of P. fortunei

Proteins are distributed throughout the cell to participate in physiological activities, and subcellular localization prediction can clearly show the respective protein prediction sites of *PfPP2C* gene family members (Table 1), thus inferring the functions of related genes. The predicted subcellular cellular localization results showed that the predicted sites of *P. fortunei PP2C* protein were in the nucleus, chloroplast, cytoplasm, mitochondria, and cell membrane (Figure 1). The protein prediction sites had 83.5% (76) of their members in the nucleus, followed by the chloroplasts (24) and the least number of members in the mitochondria (1). Members of the protein prediction site in the nucleus are distributed except for the K subfamily, and the distribution of members in the C, D, I, J, and L subfamilies reaches 100%. Members of protein prediction sites in chloroplasts are distributed in all but the D, I, J, and L subfamilies, with the K subfamily having a 100% distribution. Based on

the results of the study, it can be inferred that most of the action sites of the *PP2C* gene family members of *P. fortunei* are in the nucleus and chloroplasts.

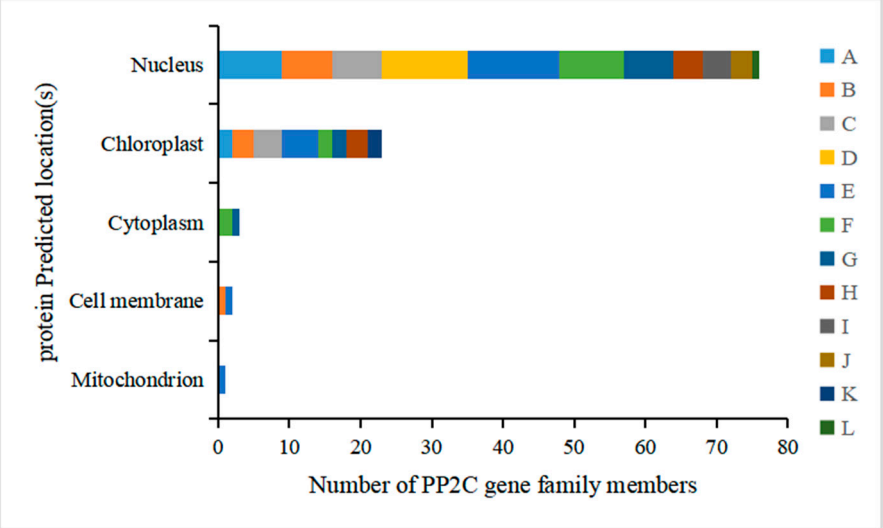

Figure 1. Prediction of subcellular localization.

3.3. Chromosomal Localization of PP2C Genes in P. fortunei

The location of *PP2C* genes was mapped onto the chromosomes of *P. fortunei* (Figure 2). The *PP2C* genes were unevenly distributed on 19 chromosomes. Chromosome 11 had the highest number of *PP2C* genes (8), followed by chromosome Chr03 (7), and then Chr7, Chr18, Ch19, and Chr20 (6 on each). The lowest number of *PP2C* genes (1 gene) was on Chr12. The distribution of *PP2C* genes within the same chromosome was also uneven, with two or more genes forming gene clusters. The results of sequence and chromosomal localization analyses revealed that *PfPP2C41* and *PfPP2C42* encoded the same amino acid sequence but were located at different chromosomal positions. In general, there was no positive correlation between the length of a chromosome and the number of *PP2C* genes it contained. Most genes on the same chromosome did not belong to the same subclade in the evolutionary tree. These results suggested that different genes on the same chromosome may encode proteins with different functions.

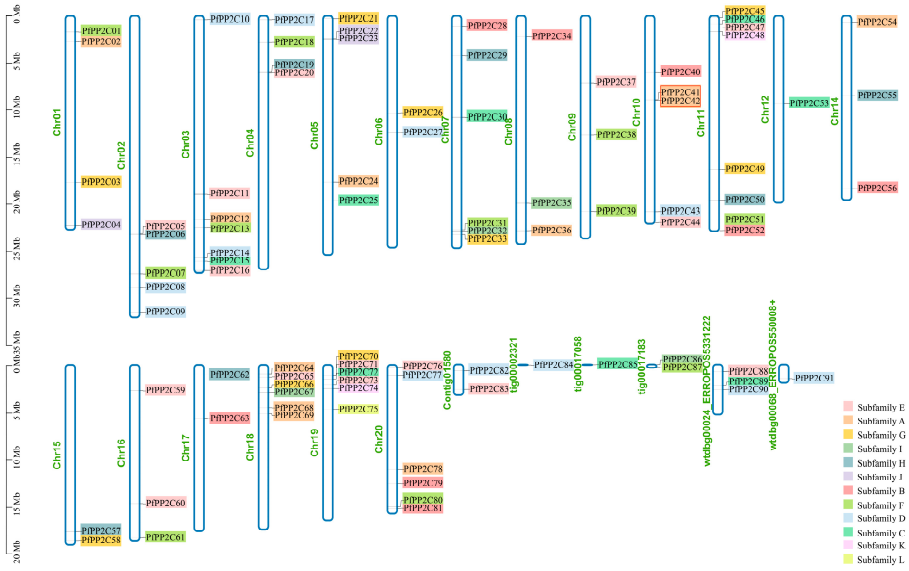

Figure 2. Chromosome mapping of *PP2C* family members in *P. fortunei*.

3.4. Phylogenetic Analysis of Members of the PP2C Family in P. fortunei

Sequence analyses showed that the *PP2C* family genes in *P. fortunei* were poorly conserved at the N-terminal end, but strongly conserved at the C-terminal end, which contained common structural subdomains that are presumed to be functionally similar. To clarify the evolutionary relationships among *PP2C* family members in *P. fortunei*, 80 protein sequences of the *A. thaliana PP2C* family, 53 protein sequences of the *P. trifoliata PP2C* family, and 91 protein sequences of the *P. fortunei PP2C* family were used to construct a phylogenetic evolutionary tree using the neighbor-joining method with MEGA-X software. In the tree (Figure 3), the *PP2C* sequences from the three species were divided into 12 subfamilies, with those of *Paulownia* distributed among the 12 subfamilies. Subfamily E had the highest number of *Paulownia PP2C* genes (15 genes), followed by subfamily D (12 genes), while subfamily L had the lowest number of *Paulownia PP2C* genes (1 gene). The number of *Paulownia PP2C* genes in the other families ranged from 2 to 11. In general, all subfamilies A–L contained *PP2C* genes from *A. thaliana*, *P. trifoliata*, and *P. fortunei*. All three species showed similar distribution ratios of *PP2C* genes in the subfamilies, indicative of relatively consistent evolutionary relationships among *A. thaliana*, *P. trifoliata*, and *P. fortunei*.

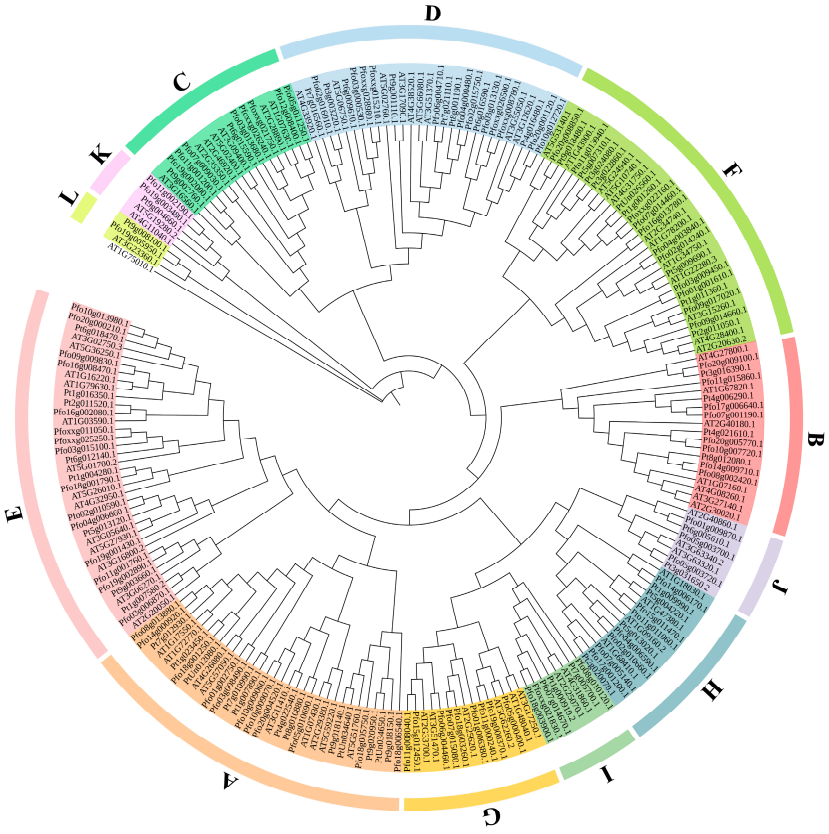

Figure 3. Phylogenetic tree of the *PP2C* gene family in *A. thaliana* (*At*), *P. trifoliata* (*Pt*), and *P. fortunei* (*Pf*).

3.5. Analysis of Conserved Structural Domains and Conserved Motifs of PP2C Family Members of P. fortunei

Analysis of the conserved structural domains revealed that all 91 *PP2C* protein sequences of *P. fortunei* contained conserved *PP2C* structural domains (Figure 4b). Among them, eight Paulownia *PP2C* proteins (*PfPP2C16*, *PfPP2C45*, *PfPP2C52*, *PfPP2C69*, *PfPP2C74*, *PfPP2C81*, *PfPP2C83*, and *PfPP2C88*) contained *PP2C*-2 structural domains; *PfPP2C11* contained the most diverse conserved structural domains (*PP2C*, Pkinase, cNMP_binding, and PK_Tyr_Ser-Thr domains); and *PfPP2C04* contained three conserved structural domains (*PP2C*, PK_Tyr_Ser-Thr, and Pkinase_fungal). The conserved structural domains of Yop-YscD_cpl and FHA were only present in *PfPP2C*48 and *PfPP2C74*, respectively.

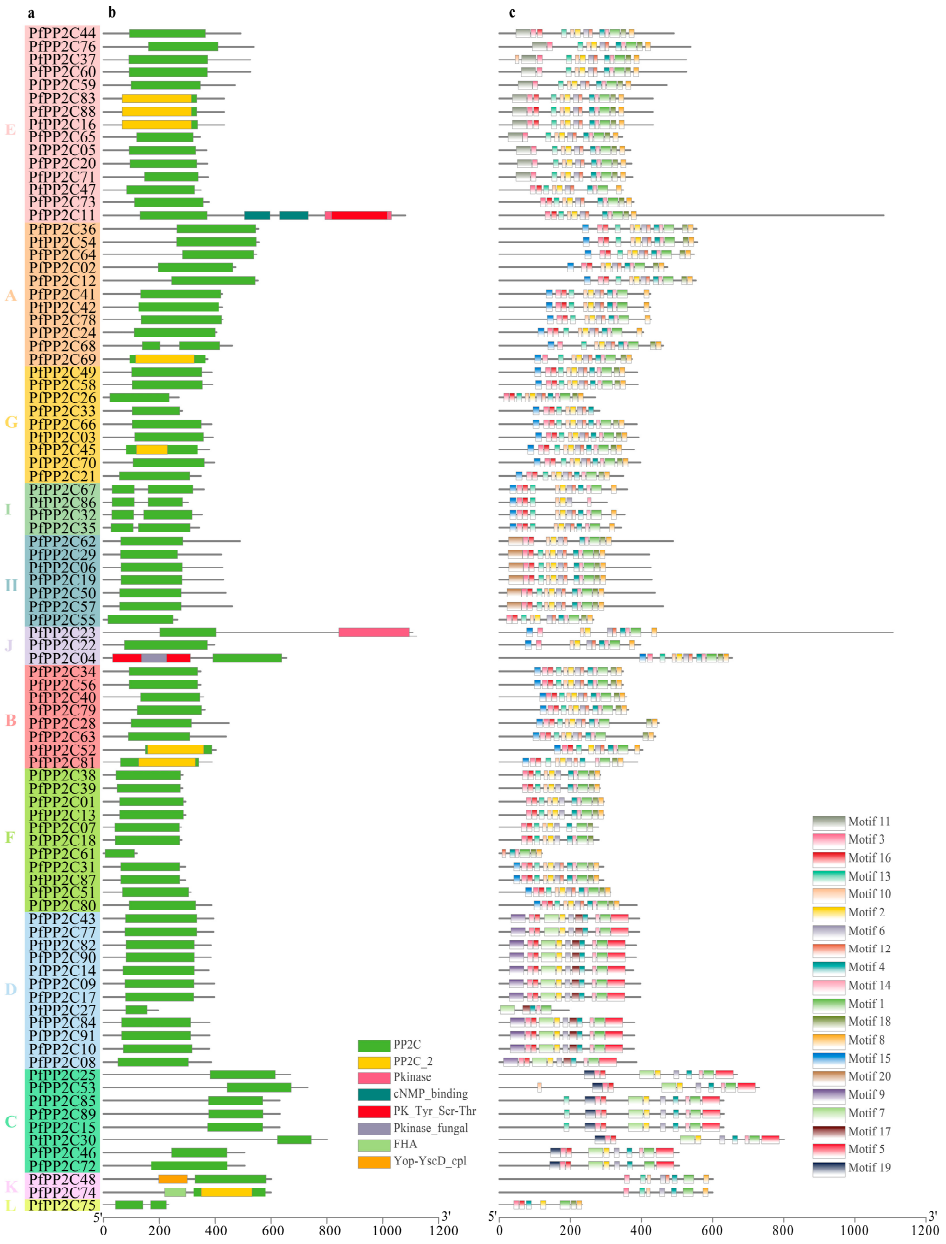

Figure 4. Subfamily grouping (**a**), conserved structural domain analysis (**b**) and conserved motif analysis (**c**) of *PP2C* gene family of *P. fortunei*.

We detected 20 conserved motifs in members of the *PP2C* family in *P. fortune* (Table 2), but the distribution of motifs differed significantly among subfamilies (Figure 4c). Among the 20 conserved motifs, motif 5 was only present in subfamilies C and D; motif 11 was only present in subfamily E; motif 20 was only present in subfamily H; motifs 9 and 17 were only present in subfamily D; and motif 19 was only present in subfamily C. This situation may be indicative of different functions of proteins in the different subfamilies. While each subfamily had unique motifs, subfamilies E, A, G, I, H, J, B, and F all contained the following motif structure: motifs 3, 16, 13, 10, 2, 6, 12, 4, 14, 1, 18, and 8; and the common motif structure in members of subfamilies C and D was motifs 3, 16, 7, 2, 6, 4, 14, 1, and 5. These findings indicated that many *PfPP2C* proteins share a high degree of similarity in the composition of their conserved motifs. *PfPP2C27* and *PfPP2C61* had a number of motifs missing compared with other members of the same subfamily, which may have resulted from sequence losses during tandem duplication of genes.

Table 2. Conserved motif information of the *PP2C* gene family in *P. fortunei*.

Motif	Length	Amino Acid Sequence Information
motif 1	29	LTPEDEFLILASDGLWDVLSNZEAVDJVR
motif 2	16	DLYVANVGDSRAVLCR
motif 3	15	TFFGVFDGHGGPGAA
motif 4	15	GGLAVSRAIGDRYLK
motif 5	50	NPRGGPARRLVKAALFRAAKKREMRYSELKKIDQGVRRHYHDDITVIVIF
motif 6	15	AIQLTVDHKPNREDE
motif 7	41	DVJKKAFSATEEEFLSLVDRQWMIKPZJASVGSCCLVGVIC
motif 8	15	RGSKDBITVIVVDFK
motif 9	41	GRVDGLLWYKDLGHHVNGEFSMAVVQANNLLEDQSQLESGP
motif 10	11	SGTTAVTALVI
motif 11	41	TPGRVFLNGSSKYASLFTQQGKKGVNQDAMIVWENFGGQED
motif 12	11	RERIEAAGGRV
motif 13	15	KKAJKKAFLKTDKEL
motif 14	11	PYLIAEPEVTV
motif 15	18	GRRREMEDAVAAIPDLCG
motif 16	15	FVKDNLFENVLKELK
motif 17	21	RSLHPDDSQIVVLKHKVWRVK
motif 18	15	PDPEAAAKRLVEEAL
motif 19	29	SLGSQNLQWAQGKAGEDRVHVVVSEEHGW
motif 20	41	NEKIEKPTVK YGQAAQSKKGEDYFLIKTDCQRVPGBPSTSF

3.6. Structure of PP2C Genes in P. fortunei

To understand the structure of *PfPP2C* genes, their intron and exon composition was determined (Figure 5b). All the *PfPP2C* genes contained introns and exons in their sequences, with the number of exons ranging from 2 (in *PfPP2C47*, *PfPP2C73*, *PfPP2C26*, *PfPP2C45*, and *PfPP2C70*) to 20 (in *PfPP2C23*) and the number of introns ranging from 1 to 19. There were 15 exons 14 introns in *PfPP2C11*. In total, 36 *PfPP2C* genes (39.5%) contained four exons and 23 contained five exons. In total, 36 *PfPP2C* genes (39.5%) contained three introns and 22 contained four introns. These results indicated that the gene structure of *PfPP2Cs* is relatively well conserved. Apart from genes in subfamilies E and J, those in the other subfamilies contained similar numbers of exons and introns, with a difference of no more than three. For example, all twelve members of subfamily D had four exons and three introns except for *PfPP2C27*, which had five exons and four introns, and all four members of subfamily I had ten exons and nine introns. In addition, the exon distribution and sequence lengths of *PfPP2C* genes belonging to the same subfamily in the phylogenetic tree were not very different and somewhat conserved, suggesting that the genes within these subfamilies have similar functions.

3.7. Analysis of Cis-Acting Elements in Promoters of PP2C Genes in P. fortunei

We identified 20 *cis*-acting elements in the promoter regions of *PP2C* genes in *P. fortunei* (Figure 5c), including hormone-responsive elements, light-responsive elements, and stress-responsive elements. Among all the *PfPP2C* genes, 62.6% (57), 48.4% (44), 47.3% (43), 35.2% (32), and 33.0% (30) had methyl jasmonate (MeJA)-responsive, gibberellin (GA)-responsive, abscisic acid (ABA)-responsive, indole acetic acid (IAA)-responsive, and salicylic acid (SA)-responsive elements, respectively, in their promoter regions; moreover, 96.7% (88), 70.3% (64), 40.7% (37), and 56.0% (51) had response elements related to light regulation, anaerobic induction, meristem expression and abiotic stress/defense, respectively, in their promoter regions. A small number of *PfPP2C* gene promoters also contained specific response elements related to circadian rhythm regulation, cell cycle regulation, endosperm expression, wound response, zein metabolism, tissue growth, and development related to palisade mesophyll cell differentiation. These results suggested that members of the *PP2C* family of *P. fortunei* play important regulatory roles in responses to hormone induction, light regulation, and stress under adverse conditions.

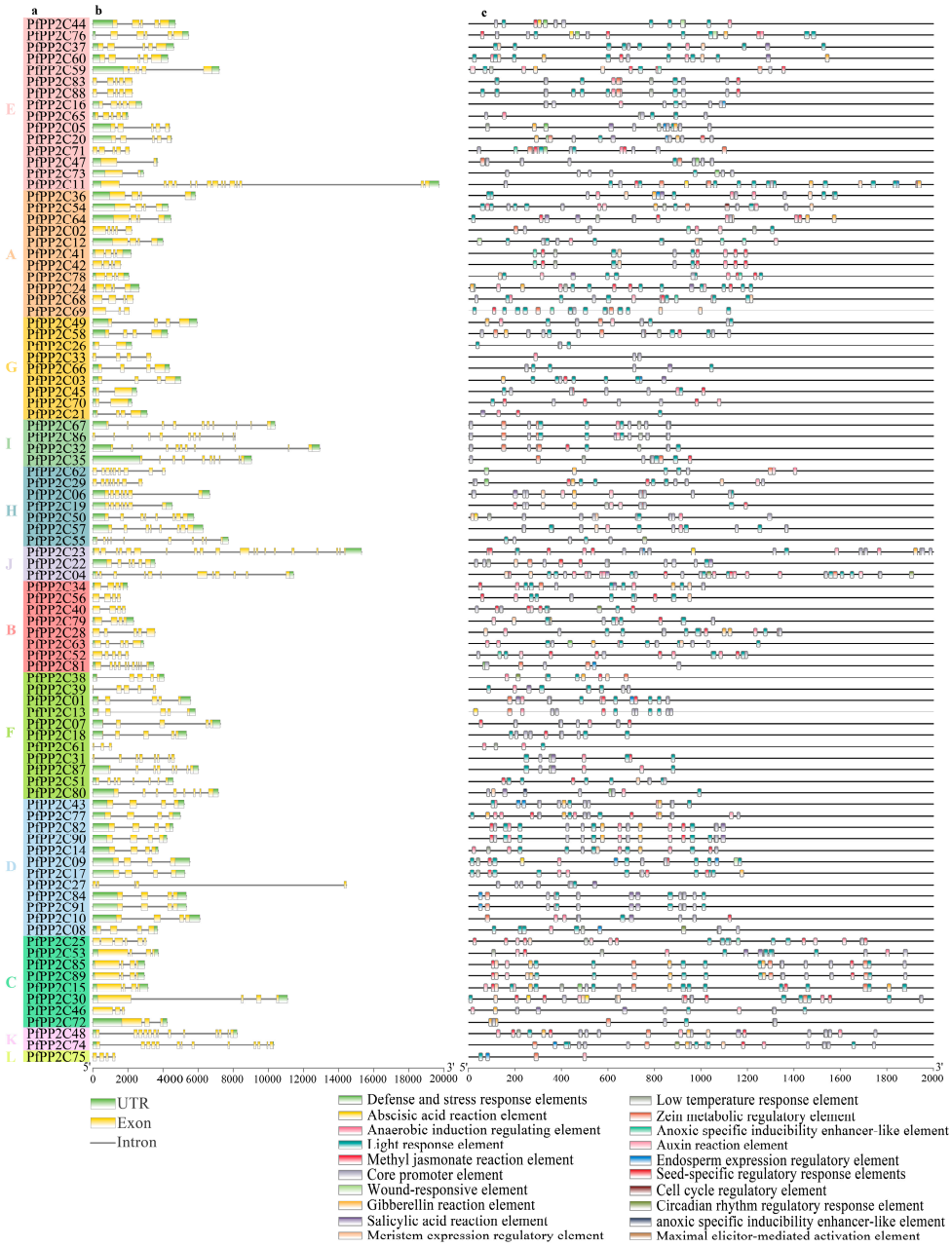

Figure 5. Subfamily grouping (**a**), analysis of the gene structure (**b**) and *cis*-element (**c**) of the *PP2C* gene family in *P. fortunei*.

3.8. Covariance Analysis of Members of the PP2C Family of P. fortunei

To explore the evolution of the *PP2C* family in *P. fortunei*, an intraspecific covariance analysis was conducted (Figure 6a). The results showed that 67 of the 91 *PP2Cs* in *P. fortunei* (74% of all *PP2Cs* in *P. fortunei*) were involved in 56 pairs of gene covariation events, suggesting that gene fragment duplication has played an important role in the evolution of the *PP2C* family in *P. fortunei*. The largest number of *PFPP2C* family members involved in covariance events was on chromosome Chr11 (six genes), followed by Chr3 and Chr19 (five genes each). To further elucidate the evolutionary relationships of *PP2C* genes between different species, a covariance analysis was performed on *P. fortunei* and *A. thaliana* (Figure 6b). We detected 98 pairs of gene covariation events between the two species, of which 54 *AtPP2C* genes (67.5% of all *AtPP2Cs*) and 67 *PfPP2C* genes (73.6% of all *PfPP2Cs*) were involved in gene covariation events. These results indicate a high degree

of homology and similar evolutionary relationships between the *PP2C* family members of *P. fortunei* and *A. thaliana*, which have been highly conserved during evolution.

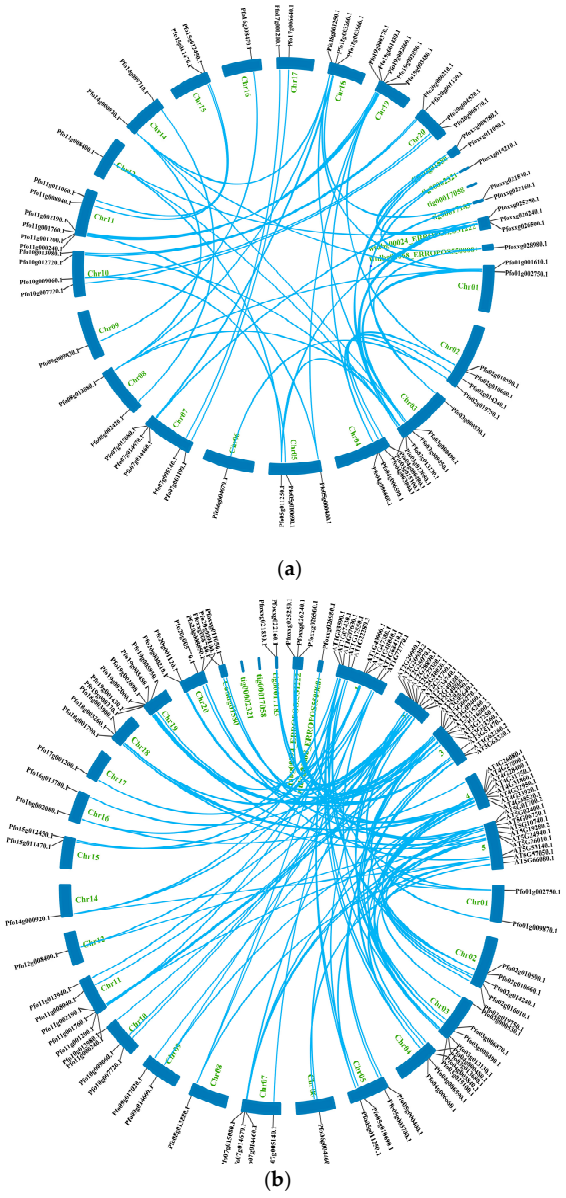

Figure 6. Collinearity analysis of *PP2C* gene families in different species: (**a**) Collinearity analysis of the *PP2C* gene family in *P. fortunei*; (**b**) Collinearity analysis of *PP2C* gene families in *P. fortune* and *A. thaliana*.

3.9. Expression Analysis of PP2C Family Members in P. fortunei

Analyses of RNAseq data from *P. fortune* infected (PFI) or uninfected (PF) with the mycoplasma that causes PaWB revealed that 80 of the 91 *PfPP2C* genes were expressed, and 11 were not (Figure 7a). The transcript levels of *PP2C19*, *PfPP2C57*, *PfPP2C59*, *PfPP2C80*, and *PfPP2C81* significantly increased after the development of PaWB. A total of 10 *PfPP2C* genes showed decreased transcript levels after the development of PaWB in *P. fortunei*, among which *PfPP2C02*, *PfPP2C12*, and *PfPP2C24* showed the most obvious decreases. The transcript levels of the remaining 66 *PfPP2C* genes, including *PfPP2C38* and *PfPP2C55*, did not change significantly. After Rif treatment, 80 *PfPP2C* genes were expressed, while 11 *PfPP2C* genes were not (Figure 7b). As the Rif treatment time extended, the transcript levels of 5 *PfPP2C* genes, including *PfPP2C02*, *PfPP2C12*, and *PfPP2C20*, significantly continued increase, while the transcript levels of 19 *PfPP2C* genes, including *PfPP2C19*, *PfPP2C52*, *PfPP2C69*, and *PfPP2C80*, significantly continued to decrease. The transcript levels of 23 *PfPP2C* genes, including *PfPP2C57* and

PfPP2C68, showed no significant monotonous trend, and the transcript levels of the remaining 19 and 15 genes increased and then decreased and decreased and then increased, respectively. After MMS treatment, 78 *PfPP2C* genes had detectable transcript levels and 13 *PfPP2C* genes had no detectable expression (Figure 7c). Among them, eight *PfPP2C* genes, including *PfPP2C02*, *PfPP2C12*, and *PfPP2C47*, were up-regulated over time under MMS treatment. The transcript levels of *PfPP2C19*, *PfPP2C52*, *PfPP2C69*, *PfPP2C80*, and 14 other genes showed an overall decreasing trend, compared with their respective levels in the control. Another 10 genes showed an increasing and then decreasing transcript levels, and 25 genes showed a decreasing and then increasing transcript levels, while 21 genes did not show significant changes in transcript levels under MMS treatment.

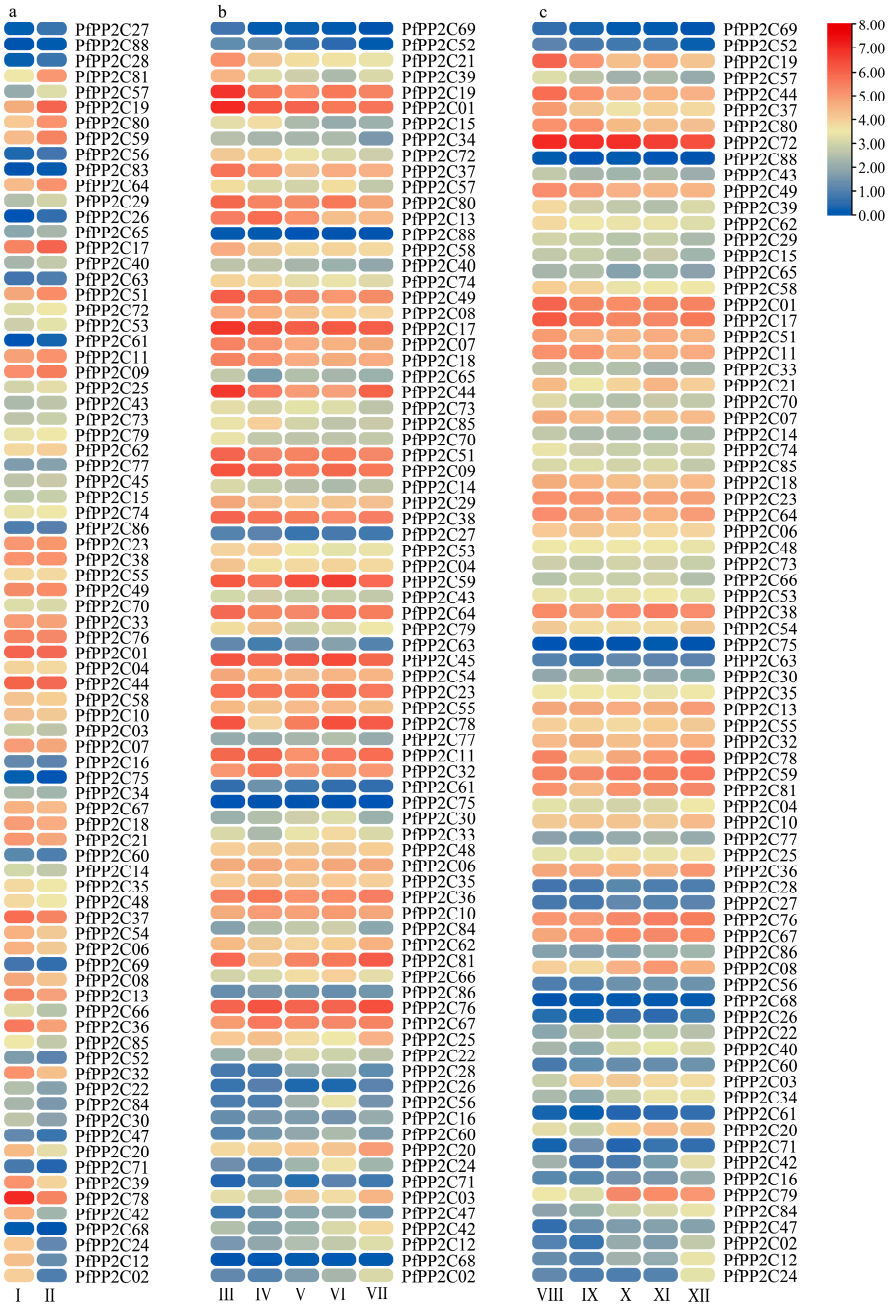

Figure 7. Expression analysis of the *PP2C* gene of *P. fortunei* during the development of witches' broom: (**a**) I is the *P. fortunei* seedling, II is the PaWB seedling; (**b**) III is the PaWB seedling, IV, V, VI, and VII are the PaWB seedlings treated with Rif (30 mg/L) for 5, 10, 15, and 30 days, respectively; (**c**) VIII is the PaWB seedling, IX, X, XI, and XII are the PaWB seedlings treated with MMS (20 mg/L) for 5, 10, 15, and 30 days, respectively.

Summarizing the above results, *PfPP2C19* and *PfPP2C80* were up-regulated in *P. fortunei* affected by PaWB, but down-regulated by Rif and MMS treatments. *PfPP2C02* and *PfPP2C12* were down-regulated in in *P. fortunei* affected by PaWB and up-regulated by Rif and MMS treatments. These findings indicated that these genes play an important regulatory role in the development of PaWB. Further in-depth analyses of their roles will provide further insights into the molecular mechanism of PaWB.

4. Discussion

In plants, *PP2C*s are an important class of protein phosphatases that regulate plant metabolism by catalyzing the dephosphorylation of phosphorylated proteins [30]. The number of *PP2C* family members varies widely among different plant species. For example, there are 80 *PP2C* genes in *A. thaliana* [11], 27 in *V. vinifera* [20], 86 in *B. distachyum* [22], 53 in *P. trifoliata* [24], 125 in *P. heterocycla* [25], 67 in *Dendrobium catenatum* [26], 90 in *O. sativa* [31], 122 in *Vigna radiata* [32], and 81 in *Fagopyrum tataricum* [33]. This suggests that the number of *PP2C* gene family members may be related to the genome size of the species, or may have changed during the course of evolution. In this study, we identified 91 *PP2C* family members in *P. fortunei*. The amino acid length, isoelectric point, and relative molecular weight varied widely among the putative *PfPP2C* proteins, and such variations may be related to their functional diversity. The chromosomal localization analyses revealed that the *PP2C* genes in *P. fortunei* are unevenly distributed on 19 chromosomes, with one to eight *PP2C* genes per chromosome. The distribution of *PP2C* genes was also uneven within the same chromosome, with two or more genes arranged in gene clusters. These patterns of chromosomal localization are similar to those of *PP2C* genes in *P. trifoliata* and *V. radiata* [24,32].

In the phylogenetic evolutionary analysis of *PP2C* genes in *A. thaliana*, *P. trifoliata*, and *P. fortunei*, the *PP2C* genes were divided into 12 subfamilies, each of which harbored *P. fortunei PP2C* genes. Consistent with this, the *PP2C* genes of *A. thaliana* and *P. heterocycla* are also distributed among 12 subfamilies [11,24]. Our results indicate that the *PP2C* genes of *A. thaliana*, *P. trifoliata*, and *P. fortunei* are similarly distributed among the 12 subfamilies, indicative of relatively consistent evolutionary relationships among these three species. Thus, the *PP2C* gene family has been conserved during evolution. We detected clear differences in the number and type of conserved structural domains among *PfPP2C* family members. Our results show that the cNMP_binding and Pkinase_fungal domains are conserved domains unique to *PfPP2C11* and *PfPP2C04*, respectively. We also detected some variability in the distribution of conserved motifs among different subfamilies, and some similarities in the distribution of conserved motifs within each subfamily. Nearly 3/4 of *PfPP2C* proteins have the following motif structure: motifs 3, 16, 13, 10, 2, 6, 12, 4, 14, 1, 18, and 8. The distribution of conserved motifs in members of the A subclade of *PPfPP2C* is similar to that in members of the A subclade in *A. thaliana*. This high degree of affinity suggests that A subclade members in *P. fortunei* participate in the regulation of ABA signaling, similar to their counterparts in *A. thaliana*.

In terms of gene structure, *PfPP2C* genes have between 2 and 20 exons, similar to the members of the *PP2C* families in *P. trifoliata* and *S. italica* [24,34]. The type and number of *cis*-acting elements in the promoter region affect differential gene expression [35]. One of the most important roles of the *PP2C* gene family is in the regulation of ABA signaling [36]. In plants, subfamily A *PP2C*s regulate early events in the ABA signaling pathway [37]. The involvement of *PP2C* proteins in this pathway varies among species, and among different organs and tissues of the same species [37]. For example, in *A. thaliana*, subfamily A *PP2C* proteins negatively regulate the ABA pathway, while *AtPP2C-G1* positively regulates the ABA pathway and the response to high salt stress [38], and *AtPP2C2* can significantly increase the response to ABA when overexpressed [39]. In this study, we detected *cis*-acting elements responsive to various phytohormones, such as MeJA, GA, ABA, IAA, and SA in the promoter regions of *PfPP2C* genes. In total, 7 of the 11 members of subfamily A contained ABA-responsive elements in their promoter regions, suggesting that subfamily

A *PfPP2Cs* are involved in the regulation of the ABA signaling pathway. These results are consistent with those reported in studies on *Zea mays* and *B. papyrifera* [21,40]. Overall, 42 of the *PfPP2C* family members contain ABA-responsive elements in their promoter regions, and half of the *PfPP2C* genes have stress-responsive elements in their promoters. Therefore, we speculate that *PfPP2Cs* may regulate various physiological activities in plants via the ABA signaling pathway.

Previous studies have demonstrated that *PP2Cs* also participate in the disease resistance signaling pathway. When plants are attacked by fungi, bacteria, and viruses, various signaling molecules such as SA and JA are produced, and they trigger the expression of genes encoding components of the disease resistance response [30]. It has been suggested that *PP2Cs* may play a role in plant resistance to biological stresses, such as rust and powdery mildew [41]. In this study, we found that the expression of some *PP2C* genes in *P. fortunei* were significantly affected by PaWB and treatment with Rif and MMS. The expression of *PfPP2C19* and *PfPP2C80* increased during the formation of PaWB in *P. fortunei*, but decreased under Rif and MMS treatments, while *PfPP2C02* and *PfPP2C12* were down-regulated during the formation of PaWB, but up-regulated in response to Rif and MMS treatments. These findings suggest that these genes play an important regulatory role in the development of PaWB, but may have a complex regulatory network. Further research is needed to explore their roles in the development of PaWB.

5. Conclusions

At present, research on *P. fortunei* and its molecular biology lags behind that on other plants, and much less is known about the function of its *PP2C* proteins than about those of model plants, such as *A. thaliana*, *O. sativa*, and *Glycine max*. In this study, we analyzed *P. fortunei PP2C* family members to determine the chromosomal distribution, evolutionary relationships, and gene structure, and the conserved structural domains and conserved motifs in their encoded proteins. We also determined their transcript profiles before and after the development of PaWB, and identified four genes closely related to PaWB development (*PfPP2C02*, *PfPP2C12*, *PfPP2C19*, and *PfPP2C80*) among the 91 *PP2C* genes in *P. fortunei*. The results of this study provide a reference for future studies on the structure, function, and regulatory roles of the *PP2C* gene family in *Paulownia*, and provide clues about the *PP2C* proteins that may participate in the formation of PaWB.

Author Contributions: G.F. conceived and designed the experiments; Z.Z. and P.Z. performed the experiments and wrote the paper; M.D. and Y.C. contributed reagents and analyzed the data. All authors have read and agreed to the published version of the manuscript.

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