Abstract: Coffee is one of the most popular beverages around the world, which is mainly produced from the allopolyploid Coffea arabica. The genomes of C. arabica and its two ancestors C. canephora and C. eugenioides have been released due to the development of next generation sequencing. However, few studies on C. arabica are related to the PIN-FORMED (PIN) auxin efflux transporter despite its importance in auxin-mediated plant growth and development. In the present study, we conducted a genome-wide analysis of the PIN gene family in the three coffee species. Totals of 17, 9 and 10 of the PIN members were characterized in C. Arabica, C. canephora, and C. eugenioides, respectively. Phylogenetic analysis revealed gene loss of PIN1 and PIN2 homologs in C. arabica, as well as gene duplication of PIN5 homologs during the fractionation process after tetraploidy. Furthermore, we conducted expression analysis of PIN genes in C. arabica by in silico and qRT-PCR. The results revealed the existence of gene expression dominance in allopolyploid coffee and illustrated several PIN candidates in regulating auxin transport and homeostasis under leaf rust fungus inoculation and the tissue-specific expression pattern of C. arabica. Together, this study provides the basis and guideline for future functional characterization of the PIN gene family.

Keywords: PIN; auxin efflux carrier; phylogeny; expression; Coffea arabica; Coffea canephora; Coffea eugenioides

1. Introduction

Auxin is one of the most important phytohormones and plays a critical role in regulating plant growth and development [1]. As the main form of auxin, indole-3-acetic acid (IAA) can be passively transported in phloem in a fast non-directional manner [2]. There is also a slow and directional polar auxin
transport (PAT) between cells, which is mediated by auxin influx and efflux carriers [3]. Among these, members of the PIN_FORMED (PIN) family have been well-characterized for their function as auxin efflux carriers [4]. PAT triggers auxin gradient distribution to form maxima, which is closely related with tissue development [2]. In Arabidopsis, eight PINs have been reported with two distinct subcellular localizations. For instance, five PINs (AtPIN1–4 and 7) are located at the plasma-membrane, linking their functions with the cell-to-cell PAT [5]. AtPIN5 and 8 reside within the endoplasmic reticulum (ER), manipulating the cellular auxin homeostasis [6,7]. Interestingly, AtPIN6 is targeted to both plasma membrane and ER, implying that it might mediate cell-to-cell PAT and cellular auxin homeostasis [8].

To date, the eight Arabidopsis PINs have been functionally characterized in regulating plant development. AtPIN1–3 and 7 are directly related to the gravitropic response regarding their PAT function [9–12]. AtPIN1, 3, 4 and 7 are involved in phototropic responses [13,14]. Usually, AtPINs are expressed in auxin-mediated fast developmental tissues. AtPIN1 has multiple functions in regulating floral bud formation, leaf shape and vein patterning [15,16]. AtPIN2 is mainly expressed in cortical and epidermal cells during root tip elongation [17,18]. AtPIN3 regulates apical hook formation/maintenance and lateral root formation [19,20]. AtPIN4 is necessary for apical hook development and root patterning [14,21]. AtPIN5 is involved in the initiation of the lateral root, the growth of root and hypocotyl, and the expansion of cotyledon, based on analysis of PIN5 overexpressing lines [7]. AtPIN6 obtains more functions than other PIN members, such as regulating shoot apical dominance, the development of adventitious root, lateral root primordia, root hair, inflorescence stem, short stamen and nectar [8,22,23]. AtPIN7 controls the development of early embryogenesis and the apical hook [2,14]. AtPIN8 is specifically expressed in pollen, regulating sporophyte and male gametophyte development [5,6,24].

Nowadays, due to the development of next-generation sequencing technology, more and more plant genomes have been assembled and released, which makes genome-wide analysis of the PIN family successful [25]. In total, there are 8, 17, 12, 11, 14, 10, 15, 10, 11, 23 and 20 PIN members characterized in the genomes of Arabidopsis thaliana, cotton (Gossypium hirsutum), maize (Zea mays), Medicago truncatula, pear (Pyrus bretschneideri), pepper (Capsicum annuum), poplar (Populus trichocarpa), potato (Solanum tuberosum), Sorghum bicolor, soybean (Glycine max) and tobacco (Nicotiana tabacum), respectively [4,26–35]. However, few PIN-related studies are reported in coffee species, despite the fact that it is one of the most popular beverages around the world. In the present study, we carried out a genome-wide analysis of the PIN family in the allotetraploid Coffea arabica and its two diploid ancestors, C. canephora and C. eugenioides. Phylogenetic, structural and gene expression analyses were also conducted to reveal the evolution and expression patterns of the coffee PIN family. Together, our results can provide a guideline for future coffee PIN gene-related studies.

2. Results

2.1. Genome-Wide Identification of PIN in Coffee Species

Eight Arabidopsis PIN protein sequences were selected to search against the three coffee genomes (Table S1) [4]. The results showed that 17 PINs (CaPINs) were found in the Coffee arabica genome and 10 in C. canephora (CcPINs) and C. eugenioides (CePINs) (Table 1), in which CaPINs were located on 10 chromosomes and 1 unanchored scaffold with coding sequence (CDS) lengths of about 1074–1995 bp, proteins lengths about 357–664 aa, molecular weight of about 38,862.37–71,668.43 and isoelectric point (pI) of about 6.95–9.11. In Coffea canephora, the CcPINs were mapped on seven chromosomes with CDS length, protein length, molecular weight and theoretical isoelectric points (pI) ranging from 444–1998 bp, 147–665 aa, 16,560.9–71,838.64 and 7–9.3, respectively. CePINs were located on five chromosomes and one unanchored scaffold, and their four characters varied between 1074–1986 bp, 357–661 aa, 38,879.22–71,315.05 and 6.95–9.05, respectively. Moreover, all Arabidopsis and coffee PINs were predicted to be located at the plasma membrane (Table 1, Table S1). CELLO also predicts endomembrane-localized Arabidopsis thaliana PINs to localize to the plasma membrane. Therefore, the exact cellular localization
(plasma membrane or endomembrane) would require experimental validation. These proteins also contained 5–10 transmembrane helices at the N- and C-termini (Figure S1).

2.2. Phylogenetic Analysis of Coffee PIN

The protein sequences of *Arabidopsis* and coffee PIN were selected for phylogenetic analysis. A total 44 of AtPINs/CaPINs/CcPINs/CePINs were separated into six groups (Figure 1A). Three PIN of *C. arabica* and *C. eugenioides* were in group I together with two from *C. canephora* and one from *Arabidopsis*. Group II contained three PIN of *Arabidopsis*, two of *C. arabica* and one of each ancestor. In group III–V, there were two members of *C. arabica*, one of each ancestor and *Arabidopsis*. Besides, seven, three, three and one PIN(s) were from *C. arabica*, *C. canephora*, *C. eugenioides* and *Arabidopsis*, respectively. A chromosomal distribution map was also constructed according to the chromosomal locations of coffee PIN genes (Table 1, Figure 1B). Additionally, coffee PINs were further aligned to examine their conserved domains, and it was observed that they contained two conserved domains at the N- and C-termini (Figure S2). We further calculated the nonsynonymous (Ka) and synonymous nucleotide substitutions (Ks) and their ratio (Ka/Ks) among the coffee PIN gene pairs (Table S2). Apparently, none of the gene pairs was subjected to positive selection (Ka/Ks > 1), suggesting the conserved evolution of coffee PIN genes.

![Figure 1](image-url)  
**Figure 1.** Phylogenetic (A) and chromosomal distribution (B) of the coffee PIN family. The protein sequences of *Arabidopsis thaliana*, *C. arabica*, *C. canephora* and *C. eugenioides* are shown in yellow, pink, blue and green, respectively (A). The chromosome numbers are listed beside chromosomes (yellow) for *C. arabica* (pink), *C. canephora* (blue) and *C. eugenioides* (green). The un represents the unanchored scaffold.
### Table 1. Gene ID, accession, sequence length, molecular weight, theoretical isoelectric points (pI) and CELLO localization of coffee PINs.

| ID   | NCBI Accession   | Chromosome Location              | Coding Sequence (bp) | Predicted Protein (aa) | Molecular Weight | pI     | CELLO Localization |
|------|------------------|----------------------------------|----------------------|------------------------|------------------|--------|-------------------|
| CaPIN1-1 | LOC113742499  | Chr4:5319931-5324277(+)          | 1809                 | 602                    | 66,068.52        | 8.78   | PlasmaMembrane (4.325) |
| CaPIN1-2 | LOC113692256  | Chr6:1975567-19795117(−)         | 1800                 | 599                    | 64,648.69        | 9.11   | PlasmaMembrane (4.541) |
| CaPIN1-3 | LOC113695379  | Chr6:18475803-18479240(−)        | 1800                 | 599                    | 64,660.77        | 9.06   | PlasmaMembrane (4.613) |
| CaPIN2  | LOC113691530  | Chr6:28762761-28766913(+)        | 1857                 | 618                    | 67,154.96        | 9.04   | PlasmaMembrane (4.413) |
| CaPIN3-1 | LOC113716800  | Chr11c:36778779-36782931(+)      | 1995                 | 664                    | 71,668.43        | 7.71   | PlasmaMembrane (3.822) |
| CaPIN3-2 | LOC113708749  | Chr9c:3279598-3277726(+)         | 1074                 | 357                    | 39,130.15        | 7.66   | PlasmaMembrane (4.154) |
| CaPIN4-1 | LOC113708750  | Chr9c:3294659-3297259(+)         | 1077                 | 357                    | 39,279.26        | 7     | PlasmaMembrane (4.359) |
| CaPIN5-1 | LOC113709610  | Chr9c:2787073-2799648(+)         | 1074                 | 357                    | 39,084.12        | 7.66   | PlasmaMembrane (4.072) |
| CaPIN6-1 | LOC113709696  | Chr9c:2809701-2812402(+)         | 1077                 | 357                    | 39,286.23        | 6.95   | PlasmaMembrane (4.384) |
| CaPIN6-2 | LOC113712046  | Chr10c:36822577-38625899(+)      | 1089                 | 362                    | 40,211.78        | 7.99   | PlasmaMembrane (4.578) |
| CaPIN7-1 | LOC113712073  | Chr10c:38697672-38700185(+)      | 1089                 | 362                    | 40,315.95        | 7.99   | PlasmaMembrane (4.577) |
| CaPIN7-2 | LOC113717320  | Chr10c:4265611-4256696(+)        | 1635                 | 544                    | 59,358.58        | 8.67   | PlasmaMembrane (4.589) |
| CaPIN8-1 | LOC113713234  | Chr10c:74114288-37421605(+)      | 1644                 | 547                    | 59,627.88        | 8.52   | PlasmaMembrane (4.621) |
| CaPIN8-2 | LOC113717328  | Chr10c:901613-9040398(−)         | 1080                 | 359                    | 38,908.26        | 9.02   | PlasmaMembrane (4.057) |

| CePIN1-1 | Ce04_g06290  | Chr6:4777301-4782838(+)          | 1809                 | 602                    | 66,017.47        | 8.78   | PlasmaMembrane (4.238) |
| CePIN1-2 | Ce06_g19880  | Chr8:22016777-22044841(−)        | 1800                 | 599                    | 64,662.72        | 9.11   | PlasmaMembrane (4.560) |
| CePIN2   | Ce06_g12940  | Chr8:10631952-10634716(+)        | 1857                 | 618                    | 67,168.99        | 9.04   | PlasmaMembrane (4.450) |
| CePIN3   | Ce11_g08600  | Chr3:26054971-26059237(+)        | 1998                 | 665                    | 71,838.64        | 7.71   | PlasmaMembrane (3.852) |
| CePIN5-1 | Ce10_g14830  | Chr2:25685429-25685861(+)        | 1089                 | 362                    | 40,211.78        | 7.99   | PlasmaMembrane (4.578) |
| CePIN5-2 | Ce08_g0470   | Chr11:2994318-2996247(+)         | 1074                 | 357                    | 39,132.12        | 7.66   | PlasmaMembrane (4.474) |
| CePIN5-3 | Ce09_g03480  | Chr11:3012816-3015442(+)         | 1077                 | 358                    | 39,279.26        | 7     | PlasmaMembrane (4.339) |
| CePIN5-4 | Ce09_g12950  | Chr2:22666072-22671714(−)        | 1635                 | 544                    | 59,372.6         | 8.67   | PlasmaMembrane (4.596) |

| CePIN1-1 | LOC113768376  | Chr4:4999933-5004212(+)          | 1809                 | 602                    | 66,068.52        | 8.78   | PlasmaMembrane (4.325) |
| CePIN1-2 | LOC113775722  | Chr6:26100829-26104135(−)        | 1800                 | 599                    | 64,602.73        | 9.05   | PlasmaMembrane (4.549) |
| CePIN1-3 | LOC113759196  | NW_02086486.1:11455-4437(+)      | 1800                 | 599                    | 64,616.75        | 9.05   | PlasmaMembrane (4.566) |
| CePIN2   | LOC113776283  | Chr12:1410303-1214398(−)         | 1845                 | 614                    | 66,772.54        | 9.03   | PlasmaMembrane (4.414) |
| CePIN3   | LOC113751527  | Chr13:38612463-38666661(+)       | 1986                 | 661                    | 71,315.05        | 7.71   | PlasmaMembrane (3.858) |
| CePIN5-1 | LOC113783693  | Chr9:3448811-3450798(−)          | 1074                 | 357                    | 39,098.15        | 7.66   | PlasmaMembrane (4.085) |
| CePIN5-2 | LOC113783723  | Chr9:3460896-3463464(−)          | 1074                 | 357                    | 39,300.26        | 6.95   | PlasmaMembrane (4.393) |
| CePIN5-3 | LOC113750662  | Chr10:31456928-3154938(−)        | 1089                 | 362                    | 40,293.93        | 7.99   | PlasmaMembrane (4.574) |
| CePIN6   | LOC113750213  | Chr10:36854392-3686063(−)        | 1620                 | 539                    | 59,223.7         | 8.64   | PlasmaMembrane (4.611) |
| CePIN8   | LOC113754254  | Chr11:48522325-4852496(−)        | 1080                 | 359                    | 38,079.22        | 9.02   | PlasmaMembrane (3.985) |
2.3. Cis-Element Prediction and Gene Structure Analysis of Coffee PIN

The upstream sequences (2000 bp) of coffee PINs were analyzed for cis-elements (Table S3). Ten regular cis-regulatory elements, including elements related to anaerobic induction, light response, gibberellin response, methyl jasmonate (MeJA) response, abscisic acid response, defense and stress response, drought-inducibility, low-temperature response, salicylic acid response and auxin response, were annotated (Figure 2A). Moreover, intron analyses indicated that most of PINs contain five or six introns (Figure 2B). Surprisingly, CaPIN5-3 contained an intron of more than 10 kb in length.

![Figure 2. Cis-element (A) and intron (B) analyses of the coffee PIN family. The cis-element analysis was conducted with 2kb upstream from the initiation codon using PlantCARE software. The intron analysis was carried out with genomic and coding sequences of the coffee PIN family.](image)

2.4. In Silico Expression of Coffee PINs under Biotic Stress

To evaluate the expression pattern of PINs under biotic stress, in silico analyses was carried out with inoculation of the coffee leaf rust fungus *Hemileia vastatrix*. The results showed that 5 of the 17 genes were regulated under stress condition (Figure 3). Among these, three were up-regulated and afterwards down-regulated (CaPIN1-1, CaPIN3-1 and CaPIN8-1), while the other two were continuously down-regulated during the entire process (CaPIN1-3 and CaPIN8-2).

![Figure 3. In silico expression of PINs in the leaf of C. arabica after inoculation with the coffee leaf rust fungus Hemileia vastatrix. Hai and dai represent hours and days after inoculation, respectively. Grey squares represent no data.](image)
2.5. Expression Profiles of Coffee PINs in Different Coffee Tissues

We further examined the expression of PINs in different tissues of *C. arabica* by qRT-PCR (Figure 4, Table S4). The results indicate that *CaPIN1-1* was highly expressed in young leaves, roots, stems and buds. *CaPIN1-2* and *CaPIN1-3* shared a similar expression pattern and were highly expressed in buds. *CaPIN2* was mainly expressed in roots. *CaPIN3-1* and *CaPIN3-2* showed similar expression patterns with relatively high expression levels in all five tissues except the fruits. However, *CaPIN3-2* and *CaPIN5-3/6/7* displayed an extremely low expression level in all tested tissues. *CaPIN5-1* was mainly expressed in leaves, but at a low level. *CaPIN5-2/4* showed a high expression in stems. *CaPIN6-1/2* and *CaPIN8-1/2* were mainly expressed in young leaves, buds and stems with similar patterns.

![Figure 4. Expression pattern of PIN genes in different tissues of C. arabica determined by qRT-PCR: L, Y, B, S, R and F represent mature leaf, young leaf, bud, stem, root and fruit, respectively.](image)

3. Discussion

3.1. Evolution of the Coffee PINs

In the present study, 17 PINs were characterized in *C. arabica*, while 9 and 10 members were identified in its two ancestors *C. canephora* and *C. eugenioides*, respectively (Table 1). *C. arabica* is a self-compatible perennial allotetraploid species derived from a spontaneous hybridization between the two closely related diploid ancestors. Theoretically, there should be 20 PIN members in the *C. arabica* genome. According to the phylogenetic results, the numbers in group II, IV, V and VIa proved the theory (Figure 1A). Interestingly, the three coffee species share the same number of PINs in group Ia and III. Moreover, *C. arabica* contains five PIN members in group VIb, twice more than the numbers of each ancestor, which might be due to the complex genome rearrangements during the generation of *C. arabica*, even if its divergence time (0.665 million years) is considerably shorter than that between the two ancestors (4.2 million years) [36]. This phenomena of gene loss in group I and III is closely related with a fractionation bias, which has been reported in *Arabidopsis* and maize [37,38]. The gene loss is most likely due to bulk DNA removal induced by intra-chromosomal recombination [38]. After reaching tetraploidy, the increased chromosome number has also been described to promote the frequency of chromosome recombination [39]. Several chromosomes of the C subgenome in *C. arabica* are significantly longer than those in *C. canephora* (Table 1, Figure 1B). Furthermore, the promoter and coding regions of the coffee PIN genes were affected by this process (Figure 2). Nucleotide sequence changes in promoter regions directly contribute to gene expression [40], and these changes occurring in coding regions will directly alter gene function [41]. Moreover, the neighbor gene pair *CaPIN5-6/7* with the same CDS is a duplication event during the process of fractionation (Figure 1, Table S2).
This expansion might be caused by gene cassette translocation, which is different from early duplication events accompanied with the divergence of a genus or a family [42]. In coffee genomes, the PIN1 and PIN5 groups are expanded when compared with Arabidopsis (Figure 1). The expansion of the PIN1 group is commonly observed in other plants, such as cotton, grasses, pear, pepper, poplar, potato, soybean and tobacco [27–29,31–35]. In contrast, the PIN5 group is expanded in grasses and tobacco [31,33]. *AtPIN1* and *AtPIN5* are defining members of two functionally divergent groups PIN1 directional polar auxin transport and PIN5 regulation of auxin transport between cytosol and ER [5–7]. The expansion of these two genes might contribute to auxin-mediated plant development and environmental adaptation [43].

3.2. Candidate Regulators in the Coffee PIN family

Our in silico analysis revealed that there are five coffee PINs that are regulated after fungal inoculation (Figure 3). For instance, *CaPIN1*-1 and *CaPIN8*-1 reach a peak expression at 12 hai and *CaPIN3*-1 at 24 hai, which might be caused by plant cell wall-mediated immunity [44]. Moreover, the diverse expression of *CaPIN1*-1/2/3 and *CaPIN3*-1 indicate that they might function as main auxin polar transporters in coffee tissues (Figure 4). The peak expression of *CaPIN2* in roots and *CaPIN5*-2/4 in stems suggests that they are the good candidates in regulating root and stem development, respectively. The results also indicate that *CaPIN6*-1/2 and *CaPIN8*-1/2 might control auxin homeostasis in young leaves, stems and buds. In addition, *CaPIN1*-2/3, *CaPIN6*-1/2 and *CaPIN8*-1/2 display slightly different expression patterns within the three gene pairs, indicating the existence of gene expression dominance in allopolyploid coffee that might be controlled by *cis*-elements [45]. The promoter regions showed significant differences within the three gene pairs (Figure 2), implying that PIN expression might be regulated at the transcription level. In addition, three kinds of *cis*-elements were identified in the promoter regions of all coffee PIN genes, including anaerobic induction, light response and drought-inducibility, indicating that all coffee PINs might be involved in these stresses responses. The other seven kinds of *cis*-elements were separately identified in different parts of coffee PINs, which might contribute to their different response to phytohormone and stress signals. Besides, the existence of a duplicated gene pair (*CaPIN5*-6/7) indicates the redundancy and functional loss across the PINs family. However, more evidence is still needed to reveal the mechanisms between duplication and fractionation after reaching tetraploidy of coffee.

4. Materials and Methods

4.1. Sequence Retrieval and Phylogenetic Analysis

The protein sequences of eight Arabidopsis PINs were selected to search against the genomes of *C. arabica*, *C. canephora* and *C. eugenioides* in the NCBI Genome Database by Tblastn method [4,46,47]. Obtained coffee PIN genes were named according to their Arabidopsis homologs and chromosome positions. Their accessions and chromosome locations are listed in Table 1. The protein characteristics, including lengths, molecular weights, and pl, were online predicted by ProtParam tool [48]. Subcellular localization was examined by CELLO software [49]. The TMHMM Server was used to analyze protein transmembrane topology [50]. Phylogenetic analysis was carried out with MEGA7 software [51]. A neighbor-joining (NJ) tree was constructed with bootstrap values tested for 1000 trails. DNAMAN 7 software was used to find the conserved domains of coffee PIN proteins and calculate sequence identities of the coffee PIN gene pairs [52]. Ka, Ks and Ka/Ks values were calculated by using DnaSP5.0 [53]. Ka/Ks > 1 represents positive selection and Ka/Ks ≤ 1 represents purified selection [54]. A chromosomal distribution map was constructed by TBtools [55].

4.2. Cis-Element and Gene Structure Analysis

The promoter (2 Kb upstream of the initiation codon) and genomic sequences of coffee PIN genes were retrieved from the three coffee genomes, respectively. The promoter sequences were upload to the
PlantCARE for identifying putative cis-elements associated with growth, development and stress responses under default settings [56]. The image data were displayed in TBtools [55].

4.3. Plant Materials and RNA Extraction

The plants of *C. arabica* cultivar Bourbon were planted in the germplasm garden of Dehong Tropical Agriculture Research Institute of Yunnan (24.02° N, 97.85° E). Leaf, bud and flower samples were separately collected from 3-year-old plants at the flowering stage in March. Fruit samples were collected 2 months after flowering. Young leaf, stem and root samples were collected from seedlings with a height of 10 cm. Each sample was collected for three times from different plants as biological replicates and immediately placed into liquid nitrogen. RNA samples were extracted by Tiangen RNA prep Pure Plant Kit (Tiangen Biomart, Beijing, China) according to the manufacturer’s protocol and then stored at −80 °C.

4.4. Expression Analysis

For in silico expression analysis, the raw read data by RNA-seq were downloaded from the Sequence Read Archive (SRA) database under the accession of PRJNA353185 [57]. After trimming, the clean reads were mapped to *CaPINs* and the read count for each gene was obtained by RSEM software [58]. All read counts were normalized into fragments per kilobase per million mapped reads (FPKM).

For qRT-PCR expression analysis, RNA samples were reverse transcribed by using the GoScript Reverse Transcription System (Promega, Madison, WI, USA). Each qRT-PCR reaction contained a final volume of 20 µL including 1 µL cDNA template, 0.5 µL gene-specific primers (10 µM), 10 µL TransStart Tip Green qPCR Supermix (Transgen Biotech, Beijing, China), 0.4 µL Passive ReferenceDye (50×) (Transgen Biotech, Beijing, China) and 7.6 µL ddH2O. qRT-PCR reaction was conducted with a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The thermal cycles were 94 °C for 30 s, 40 cycles of 94 °C for 5 s and 60 °C for 30 s, and were followed by a dissociation stage. Each sample was repeated three times as technical repeats. Specific primers for *CaPINs* were designed with Primer 3 (Table 2) [59]. Coffee protein phosphatase and tubulin genes were selected as endogenous control according to a previous study [60]. The ∆∆Ct method was used for calculating relative expression levels [61].

| ID     | Forward Primer | Reverse Primer | Product Length |
|--------|----------------|----------------|----------------|
| CaPIN1-1 | TTTTGTAGGGCTATGAGAC | TCTCTGGACCTCCATCCATCC | 155 |
| CaPIN1-2 | GGGTAGCCTGCTATCCCTT | AATTGTCTGCTCTTATGAC | 173 |
| CaPIN1-3 | AGGGATTCCTTTAGGAC | TTTGAGCTTTTGGGTGTTAG | 211 |
| CaPIN2   | GTCTGTCGTACCCACCTT | TCCACAGCTTCTTTTCTG | 182 |
| CaPIN3-1 | GTTGTAGCTTCCAGCTTGTTT | ACCCTCTTGGCCTAGTTT | 221 |
| CaPIN3-2 | TGCTCTTCAGCCAAGATTT | TGGGCGAACACAACATGGGA | 191 |
| CaPIN3-3 | TATGCGTGATTTACTACACC | GTACTGTTAAGAGGTGTTCGCC | 141 |
| CaPIN5-2 | GCGCTTAAATCCACAAACAG | ATCGAAGTCCTCTTCCCGA | 238 |
| CaPIN5-3 | ATCTGAAATAAAAGATGGGT | TACTTTTGGAGTGAATGCA | 199 |
| CaPIN4   | TCACATCAAAATAAAGGTGCT | GTACCTTATATTAAGTGT | 153 |
| CaPIN5-5 | GTTGTATTTTTCCTTCCTCC | AAATGAGGCTATGGTGGT | 175 |
| CaPIN6   | TAGAGGGGGCGCATTTTAT | TAAACACGCGAGCTAAGT | 180 |
| CaPIN7   | TGGGATCTGTCTTGAATGCT | TTTAAAGCCATCCCGAACAA | 197 |
| CaPIN8-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN8-2 | TGTATGTTGTCCTTGGTTG | GGAAGGCAAGCACCACGAAC | 205 |
| CaPIN5-4 | AATTGTGCAGACGCGCTG | TGACCAAAATTATCTCGTG | 196 |
| CaPIN5-6 | TGGGATCTGTCTTGAATGCT | TTTAAAGCCATCCCGAACAA | 197 |
| CaPIN8-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-2 | TGTATGTTGTCCTTGGTTG | GGAAGGCAAGCACCACGAAC | 205 |
| CaPIN5-4 | AATTGTGCAGACGCGCTG | TGACCAAAATTATCTCGTG | 196 |
| CaPIN5-6 | TGGGATCTGTCTTGAATGCT | TTTAAAGCCATCCCGAACAA | 197 |
| CaPIN8-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-2 | TGTATGTTGTCCTTGGTTG | GGAAGGCAAGCACCACGAAC | 205 |
| CaPIN5-4 | AATTGTGCAGACGCGCTG | TGACCAAAATTATCTCGTG | 196 |
| CaPIN5-6 | TGGGATCTGTCTTGAATGCT | TTTAAAGCCATCCCGAACAA | 197 |
| CaPIN8-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-2 | TGTATGTTGTCCTTGGTTG | GGAAGGCAAGCACCACGAAC | 205 |
| CaPIN5-4 | AATTGTGCAGACGCGCTG | TGACCAAAATTATCTCGTG | 196 |

# Table 2. Primers used for qRT-PCR analysis.

| ID     | Forward Primer | Reverse Primer | Product Length |
|--------|----------------|----------------|----------------|
| CaPIN1-1 | TTTTGTAGGGCTATGAGAC | TCTCTGGACCTCCATCCATCC | 155 |
| CaPIN1-2 | GGGTAGCCTGCTATCCCTT | AATTGTCTGCTCTTATGAC | 173 |
| CaPIN1-3 | AGGGATTCCTTTAGGAC | TTTGAGCTTTTGGGTGTTAG | 211 |
| CaPIN2   | GTCTGTCGTACCCACCTT | TCCACAGCTTCTTTTCTG | 182 |
| CaPIN3-1 | GTTGTAGCTTCCAGCTTGTTT | ACCCTCTTGGCCTAGTTT | 221 |
| CaPIN3-2 | TGCTCTTCAGCCAAGATTT | TGGGCGAACACAACATGGGA | 191 |
| CaPIN3-3 | TATGCGTGATTTACTACACC | GTACTGTTAAGAGGTGTTCGCC | 141 |
| CaPIN5-2 | GCGCTTAAATCCACAAACAG | ATCGAAGTCCTCTTCCCGA | 238 |
| CaPIN5-3 | ATCTGAAATAAAAGATGGGT | TACTTTTGGAGTGAATGCA | 199 |
| CaPIN4   | TCACATCAAAATAAAGGTGCT | GTACCTTATATTAAGTGT | 153 |
| CaPIN5-5 | GTTGTATTTTTCCTTCCTCC | AAATGAGGCTATGGTGGT | 175 |
| CaPIN6   | TAGAGGGGGCGCATTTTAT | TAAACACGCGAGCTAAGT | 180 |
| CaPIN7   | TGGGATCTGTCTTGAATGCT | TTTAAAGCCATCCCGAACAA | 197 |
| CaPIN8-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN8-2 | TGTATGTTGTCCTTGGTTG | GGAAGGCAAGCACCACGAAC | 205 |
| CaPIN5-4 | AATTGTGCAGACGCGCTG | TGACCAAAATTATCTCGTG | 196 |
| CaPIN5-6 | TGGGATCTGTCTTGAATGCT | TTTAAAGCCATCCCGAACAA | 197 |
| CaPIN8-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-2 | TGTATGTTGTCCTTGGTTG | GGAAGGCAAGCACCACGAAC | 205 |
| CaPIN5-4 | AATTGTGCAGACGCGCTG | TGACCAAAATTATCTCGTG | 196 |
| CaPIN5-6 | TGGGATCTGTCTTGAATGCT | TTTAAAGCCATCCCGAACAA | 197 |
| CaPIN8-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-1 | TGTAGTAGAAGCCATTTGCT | ACTCTATTATTCACACCAC | 146 |
| CaPIN6-2 | TGTATGTTGTCCTTGGTTG | GGAAGGCAAGCACCACGAAC | 205 |
| CaPIN5-4 | AATTGTGCAGACGCGCTG | TGACCAAAATTATCTCGTG | 196 |
Supplementary Materials: The following are available online at http://www.mdpi.com/2223-7747/9/9/1061/s1, Figure S1: Transmembrane topology analysis of coffee PIN proteins. Figure S2: Conserved domains of coffee PIN proteins. Table S1: Gene ID, accession, sequence length, molecular weight, pI and CELLO localization of Arabidopsis PINs. Table S2: Sequence identity, nonsynonymous (Ka), synonymous nucleotide substitutions (Ks) and the ratio (Ka/Ks) of the coffee PIN gene pairs. Table S3: Co-element prediction in upstream sequences of coffee PIN genes. Table S4: Ct values of CaPINs in different samples.

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References
1. Weijers, D.; Friml, J. SnapShot: Auxin signaling and transport. Cell 2009, 136, 1172. [CrossRef] [PubMed]
2. Adamowski, M.; Friml, J. PIN-dependent auxin transport: Action, regulation, and evolution. Plant Cell 2015, 27, 20–32. [CrossRef] [PubMed]
3. Finet, C.; Jaillais, Y. AUXOLOGY: When auxin meets plant evo-devo. Dev. Biol. 2012, 369, 19–31. [CrossRef] [PubMed]
4. Krecek, P.; Skupa, P.; Libus, J.; Naramoto, S.; Tejos, R.; Friml, J.; Zazay, J. The PIN-FORMED (PIN) protein family of auxin transporters. Genome Biol. 2009, 10, 249. [CrossRef]
5. Zhou, J.J.; Luo, J. The PIN-FORMED Auxin Efflux Carriers in Plants. Int. J. Mol. Sci. 2018, 19, 2759. [CrossRef]
6. Ding, Z.; Wang, B.; Moreno, I.; Duplakova, N.; Simon, S.; Carraro, N.; Reemmer, J.; Pencik, A.; Chen, X.; Tejos, R.; et al. ER-localized auxin transporter PIN8 regulates auxin homeostasis and male gametophyte development in Arabidopsis. Nat. Commun. 2012, 3, 941. [CrossRef]
7. Mravec, J.; Skupa, P.; Bailly, A.; Hoyerova, K.; Krecek, P.; Bielach, A.; Petrasek, J.; Zhang, J.; Gaykova, V.; Stierhof, Y.D. Subcellular homeostasis of phytohormone auxin is mediated by the ER localized PIN5 transporter. Nature 2009, 459, 1136–1140. [CrossRef]
8. Simon, S.; Skupa, P.; Viane, T.; Zwiewka, M.; Tejos, R.; Klima, P.; Carna, M.; Rolcik, J.; De Rycke, R.; Moreno, I.; et al. PIN6 auxin transporter at endoplasmic reticulum and plasma membrane mediates auxin homeostasis and organogenesis in Arabidopsis. New Phytol. 2016, 211, 65–74. [CrossRef]
9. Rakusová, H.; Abbas, M.; Han, H.; Song, S.; Robert, H.S.; Friml, J. Termination of shoot gravitropic responses by auxin feedback on PIN3 polarity. Curr. Biol. 2016, 26, 3026–3032. [CrossRef]
10. Rigo, G.; Ayadini, F.; Tietz, O.; Zsigmond, L.; Kovacs, H.; Páy, A.; Salchert, K.; Darula, Z.; Medzihradszky, K.F.; Szabados, L.; et al. Inactivation of plasma membrane-localized CDPK-RELATED KINASE5 decelerates PIN2 exocytosis and root gravitropic response in Arabidopsis. Plant Cell 2013, 25, 1592–1608. [CrossRef]
11. Rosquete, M.R.; Waidmann, S.; Kleine, V.J. PIN7 auxin carrier has a preferential role in terminating radial root expansion in Arabidopsis thaliana. Int. J. Mol. Sci. 2018, 19, 1238. [CrossRef] [PubMed]
12. Xi, W.; Gong, X.; Yang, Q.; Yu, H.; Liou, Y.C. PIN1At regulates PIN1 polar localization and root gravitropism. Nat. Commun. 2016, 7, 10430. [CrossRef] [PubMed]
13. Haga, K.; Sakai, T. PIN auxin efflux carriers are necessary for pulse-induced but not continuous light-induced phototropism in Arabidopsis. Plant Physiol. 2012, 160, 763–776. [CrossRef] [PubMed]
14. Zadnikova, P.; Petrasek, J.; Marhavy, P.; Raz, V.; Vandenbussche, F.; Ding, Z.; Schwarzerova, K.; Morita, M.T.; Tasaka, M.; Hejatko, J.; et al. Role of PIN-mediated auxin efflux in apical hook development of Arabidopsis thaliana. Development 2010, 137, 607–617. [CrossRef] [PubMed]
15. Okada, K.; Ueda, J.; Komaki, M.K.; Bell, C.J.; Shimura, Y. Requirement of the auxin polar transport system in early stages of Arabidopsis floral bud formation. Plant Cell 1991, 3, 677–684. [CrossRef]
Plants 2020, 9, 1061

16. Pahari, S.; Cormark, R.D.; Blackshaw, M.T.; Liu, C.; Erickson, J.L.; Schultz, E.A. Arabidopsis UNHINGED encodes a VPS51 homolog and reveals a role for the GARP complex in leaf shape and vein patterning. Development 2014, 141, 1894–1905. [CrossRef]

17. Rahman, A.; Takahashi, M.; Shibasaki, K.; Wu, S.; Inaba, T.; Tsurumi, S.; Baskin, T.I. Gravitropism of Arabidopsis thaliana roots requires the polarization of PIN2 toward the root tip in meristematic cortical cells. Plant Cell 2010, 22, 1762–1776. [CrossRef]

18. Vieten, A.; Vanneste, S.; Wisniewska, J.; Benková, E.; Benjamin, R.; Beeckman, T.; Luschnig, C.; Friml, J. Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development 2005, 132, 4521–4531. [CrossRef]

19. Chen, Q.; Liu, Y.; Maere, S.; Lee, E.; Van, I.G.; Xie, Z.; Xuan, W.; Lucas, J.; Vassileva, V.; Kitakura, S.; et al. A coherent transcriptional feed-forward motif model for mediating auxin-sensitive PIN3 expression during lateral root development. Nat. Commun. 2015, 6, 8821. [CrossRef]

20. Willige, B.C.; Chory, J. A current perspective on the role of AGCVIII kinases in PIN-mediated apical hook development. Front. Plant Sci. 2015, 6, 767. [CrossRef]

21. Friml, J.; Benková, E.; Bílou, I.; Wisniewska, J.; Hamann, T.; Ljung, K.; Woody, S.; Sandberg, G.; Scheres, B.; Jürgens, G.; et al. AtPIN4 mediates sink-driven auxin gradients and root patterning in Arabidopsis. Cell 2002, 108, 661–673. [CrossRef]

22. Cazzonelli, C.I.; Vanstraelen, M.; Simon, S.; Yin, K.; Carron-Arthur, A.; Nisar, N.; Tarle, G.; Cuttriss, A.J.; Searle, I.R.; Mathiesius, J.; et al. Role of the Arabidopsis PIN6 auxin transporter in auxin homeostasis and auxin-mediated development. PLoS ONE 2013, 8, e70069. [CrossRef] [PubMed]

23. Ditengou, F.A.; Gomes, D.; Nziengui, H.; Kochersperger, P.; Lasok, H.; Medeiros, V.; Paponov, I.V.; Nagy, S.K.; Nádai, T.V.; Mészáros, T.; et al. Characterization of auxin transporter PIN6 plasma membrane targeting reveals a function for PIN6 in plant bolting. New Phytol. 2017, 217, 1610–1624. [CrossRef] [PubMed]

24. Dal Bosco, C.; Dovzhenko, A.; Liu, X.; Woerner, N.; Rensch, T.; Eismann, M.; Eimer, S.; Hegermann, J.; Paponov, I.A.; Ruperti, B.; et al. The endoplasmic reticulum localized PIN8 is a pollen-specific auxin carrier involved in intracellular auxin homeostasis. Plant J. 2012, 71, 860–870. [CrossRef]

25. Lee, I. A showcase of future plant biology: Moving towards next-generation plant genetics assisted by genome sequencing and systems biology. Genome Biol. 2014, 15, 305. [CrossRef] [PubMed]

26. Forestan, C.; Farinati, S.; Varotto, S. The Maize PIN Gene Family of Auxin Transporters. Front. Plant Sci. 2012, 3, 16. [CrossRef] [PubMed]

27. He, P.; Zhao, P.; Wang, L.; Zhang, Y.; Wang, X.; Xiao, H.; Yu, J.; Xiao, G. The PIN gene family in cotton (Gossypium hirsutum): Genome-wide identification and gene expression analyses during root development and abiotic stress responses. BMC Genom. 2017, 18, 507. [CrossRef]

28. Liu, B.; Zhang, J.; Wang, L.; Li, J.; Zheng, H.; Chen, J.; Lu, M. A survey of Populus PIN-FORMED family genes reveals their diversified expression patterns. J. Exp. Bot. 2014, 65, 2437–2448. [CrossRef]

29. Qi, L.; Chen, L.; Wang, C.; Zhang, S.; Yang, Y.; Liu, J.; Li, D.; Song, J.; Wang, R. Characterization of the Auxin Efflux Transporter PIN Proteins in Pear. Plants 2020, 9, 349. [CrossRef]

30. Sáriko-Sawczenko, I.; Lotocka, B.; Czarnecka, W. Expression Analysis of PIN Genes in Root Tips and Nodules of Medicago truncatula. Int. J. Mol. Sci. 2016, 17, 1197. [CrossRef]

31. Shen, C.; Bai, Y.; Wang, S.; Zhang, S.; Wu, Y.; Chen, M.; Jiang, D.; Qi, Y. Expression profile of PIN, AUX/LAX and PGP auxin transporter gene families in Sorghum bicolor under phytohormone and abiotic stress. FEBS J. 2010, 277, 2954–2969. [CrossRef] [PubMed]

32. Wang, Y.; Chai, C.; Valliyodan, B.; Maupin, C.; Annen, B.; Nguyen, H.T. Genome-wide analysis and expression profiling of the PIN auxin transporter gene family in soybean (Glycine max). BMC Genom. 2015, 16, 951. [CrossRef] [PubMed]

33. Xie, X.; Qin, G.; Si, P.; Luo, Z.; Gao, J.; Chen, X.; Zhang, J.; Wei, P.; Xia, Q.; Lin, F.; et al. Analysis of Nicotiana tabacum PIN genes identifies NtPIN4 as a key regulator of axillary bud growth. Physiol. Plant. 2017, 160, 222–239. [CrossRef] [PubMed]

34. Yang, C.; Wang, D.; Zhang, C.; Kong, N.; Ma, H.; Chen, Q. Comparative Analysis of the PIN Auxin Transporter Gene Family in Different Plant Species: A Focus on Structural and Expression Profiling of PINs in Solanum tuberosum. Int. J. Mol. Sci. 2019, 20, 3270. [CrossRef] [PubMed]
35. Zhang, C.; Dong, W.; Huang, Z.A.; Cho, M.; Yu, Q.; Wu, C.; Yu, C. Genome-wide identification and expression analysis of the CaLAX and CaPIN gene families in pepper (Capsicum annuum L.) under various abiotic stresses and hormone treatments. *Genome* 2018, 61, 121–130. [CrossRef]

36. Yu, Q.; Guyot, R.; De Kochko, A.; Byers, A.; Navajas-Pérez, R.; Langston, B.J.; Dubreuil-Tranchant, C.; Paterson, A.H.; Poncet, V.; Nagai, C.; et al. Micro-collinearity and genome evolution in the vicinity of an ethylene receptor gene of cultivated diploid and allotetraploid coffee species (Coffea). *Plant J.* 2011, 67, 305–317. [CrossRef]

37. Thomas, B.C.; Pedersen, B.; Freeing, M. Following tetraploidy in an Arabidopsis ancestor, genes were removed preferentially from one homeolog leaving clusters enriched in dose-sensitive genes. *Genome Res.* 2006, 16, 934–946. [CrossRef]

38. Woodhouse, M.R.; Schnable, J.C.; Pedersen, B.S.; Lyons, E.; Lisch, D.; Subramaniam, S.; Freeing, M. Following tetraploidy in maize, a short deletion mechanism removed genes preferentially from one of the two homologs. *PLoS Biol.* 2010, 8, e1000409. [CrossRef]

39. Hunter, N. Meiotic Recombination: The Essence of Heredity. *Cold Spring Harb. Perspect. Biol.* 2015, 7, a016618. [CrossRef]

40. Zhao, B.; Cao, J.F.; Hu, G.J.; Chen, Z.W.; Wang, L.Y.; Shangguan, X.X.; Wang, L.J.; Mao, Y.B.; Zhang, T.Z.; Wendel, J.F.; et al. Core cis-element variation confers subgenome-biased expression of a transcription factor that functions in cotton fiber elongation. *New Phytol.* 2018, 218, 1061–1075. [CrossRef]

41. Liu, D.; Hu, R.; Palla, K.J.; Tuskan, G.A.; Yang, X. Advances and perspectives on the use of CRISPR/Cas9 systems in plant genomics research. *Curr. Opin. Plant Biol.* 2016, 30, 70–77. [CrossRef] [PubMed]

42. Tennesen, J.A.; Wei, N.; Straub, S.C.K.; Govindarajulu, R.; Liston, A.; Ashman, T.L. Repeated translocation of a gene cassette drives sex-chromosome turnover in strawberries. *PLoS Biol.* 2018, 16, e2000602. [CrossRef]

43. Zhao, Y. Essential Roles of Local Auxin Biosynthesis in Plant Development and in Adaptation to Environmental Changes. *Annu. Rev. Plant Biol.* 2018, 69, 417–435. [CrossRef] [PubMed]

44. Bacete, L.; Mélida, H.; Miedes, E.; Molina, A. Plant cell wall-mediated immunity: Cell wall changes trigger disease resistance responses. *Plant J.* 2018, 93, 614–636. [CrossRef]

45. Bottani, S.; Zabet, N.R.; Wendel, J.F.; Veitia, R.A. Gene Expression Dominance in Allopolyploids: Hypotheses and Models. *Trends Plant Sci.* 2018, 23, 393–402. [CrossRef]

46. NCBI. Available online: www.ncbi.nlm.nih.gov/genome/ (accessed on 7 July 2020).

47. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* 1990, 215, 403–410. [CrossRef]

48. ProtParam Tool. Available online: Web.expasy.org/protparam/ (accessed on 7 July 2020).

49. Yu, C.S.; Chen, Y.C.; Lu, C.H.; Hwang, J.K. Prediction of protein subcellular localization. *Proteins* 2006, 64, 643–651. [CrossRef]

50. Krogh, A.; Larsson, B.; Von Heijne, G.; Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J. Mol. Biol.* 2001, 305, 567–580. [CrossRef]

51. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* 2016, 33, 1870–1874. [CrossRef]

52. DNAMAN-Bioinformatics Solutions. Available online: www.lynnon.com (accessed on 7 July 2020).

53. Librado, P.; Rozas, J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 2009, 25, 1451–1452. [CrossRef]

54. Huang, X.; Wang, B.; Xi, J.; Zhang, Y.; He, C.; Zheng, J.; Gao, J.; Chen, H.; Zhang, S.; Wu, W.; et al. Transcriptome comparison reveals distinct selection patterns in domesticated and wild Agave species, the important CAM plants. *Int. J. Genom.* 2018, 2018, 5716518. [CrossRef] [PubMed]

55. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools—An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* 2020. [CrossRef]

56. Lescot, M.; Déhais, P.; Thijs, G.; Marchal, K.; Moreau, Y.; Peer, Y.V.D.; Rouzé, P.; Rombauts, S. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* 2002, 30, 325–327. [CrossRef] [PubMed]

57. Sequence Read Archive. Available online: www.ncbi.nlm.nih.gov/sra (accessed on 7 July 2020).

58. Li, B.; Dewey, C.N. RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinform.* 2011, 12, 323. [CrossRef]
59. Untergasser, A.; Cutcutache, I.; Koressaar, T.; Ye, J.; Faircloth, B.C.; Remm, M.; Rozen, S.G. Primer3—New capabilities and interfaces. *Nucleic Acids Res.* **2012**, *40*, e115. [CrossRef] [PubMed]

60. Huang, X.; Xiao, M.; Xi, J.; He, C.; Zheng, J.; Chen, H.; Gao, J.; Zhang, S.; Wu, W.; Liang, Y.; et al. De novo transcriptome assembly of Agave H11648 by Illumina sequencing and identification of cellulose synthase genes in Agave species. *Genes* **2019**, *10*, 103. [CrossRef] [PubMed]

61. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001**, *25*, 402–408. [CrossRef]