



Article

Genome-Wide Identification and Expression of the PIN Auxin Efflux Carrier Gene Family in Watermelon (*Citrullus lanatus*)

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Abstract: As one of the world's most popular fruits, watermelon (*Citrullus lanatus*) is cultivated in more than 3 million hectares across the globe, with a yearly yield of more than 100 million tons. According to '97103' genome version 1, a previous study has shown that the watermelon genome consists of 11 PIN genes. However, the higher quality '97103' genome version 2 was recently assembled by using PacBio long reads with the benefit of fast development sequencing technology. Using this new assembly, we conducted a new genome-wide search for PIN genes in watermelon and compared it with cucumber and melon genomes. We identified nine, nine, and eight PINs in watermelon, cucumber, and melon, respectively. Phylogenetic analysis revealed a distinct evolutionary history of PIN proteins in watermelon, which is shown by the orphan *PIN6* in watermelon. We further conducted an expression analysis of the watermelon PIN genes in silico and by qRT-PCR. *ClapIN6* might not play an essential role during shoot regeneration, which is closely related to species-specific evolution. However, the up-regulated expression patterns of *ClapIN1-1* and *ClapIN1-3* indicated their important roles during shoot regeneration. The result of this research will benefit future studies to understand the regulating mechanisms of auxin in watermelon shoot regeneration.

Keywords: PIN family; auxin efflux carrier; phylogenetic analysis; expression; shoot regeneration watermelon; cucumber; melon

1. Introduction

As one of the most important hormones, auxin plays a vital role in the growth and development of plants [1]. Auxin is transported from its biosynthesis tissues to the sink organs where it acts through the phloem stream of photosynthetic assimilates in a fast manner [2]. Local auxin transport depends on polar auxin transport (PAT) between neighboring cells, which is slow but essential for generating auxin maxima in developing tissues [3]. Among the gene families related to auxin transport, the PIN-FORMED (PIN) family has been thoroughly characterized in *Arabidopsis*. [4]. Among the eight *Arabidopsis* PIN proteins, seven contribute to cell-to-cell PAT (*AtPIN1-4*, 7) and local auxin homeostasis in-cell (*AtPIN5*, 8) [4–6]. These functions are determined by their subcellular localizations. Besides, *AtPIN6* is located both at the plasma membrane and endoplasmic reticulum, which indicates its combined functions of cell-to-cell PAT and intracellular auxin homeostasis [7].

Arabidopsis PIN proteins are involved in regulating diverse growth and developmental processes, such as *AtPIN1*–3, 7 in gravitropic response [8–11], *AtPIN1*, 3, 4, 7 in phototropic responses [12,13], *AtPIN1*, 8 in flower development [14,15], *AtPIN3*, 4, 7 in apical hook development [13,16], *AtPIN2*–6 in root formation and development [6,7,17–19], and *AtPIN2*, 5, 7 in embryogenesis [1,6,20].

Watermelon (*Citrullus lanatus*) is an important fruit crop, which is cultivated in over 3 million hectares around the world, with a yearly production of over 100 million tons [21]. Watermelon contains a series of healthy nutritional compounds, such as lycopene, citrulline, arginine, and glutathione [22]. Consequently, it is of great importance to improve the fruit's quality traits [23]. Newly invented CRISPR/Cas9 mediated gene editing technology is an efficient method for genetic improvement of crops, which has long been successfully applied to watermelon [24,25]. However, there is limited knowledge of the mechanisms of shoot regeneration, which is a major impediment for genetic transformation of watermelon. Zhang et al. have clarified the importance of the WOX and SAUR gene families during shoot regeneration of watermelon [26,27]. In *Arabidopsis*, a complex gene regulatory network for shoot regeneration has been identified [28]. Among the genes involved, *AtPINs* play essential roles in callus formation and shoot initiation by regulating PAT [29,30]. However, it still remains unknown exactly how the PIN gene family regulates shoot regeneration in watermelon. Using the watermelon 97103 genome (version 1), a previous study has identified the PIN gene family and its expression patterns in response to auxin and extreme stress conditions [31]. Recently, a new high-quality 97103 genome (version 2), which has been assembled using PacBio long reads combined with BioNano optical and Hi-C maps, has been released [23]. In the present study, we have identified 9 PIN genes in the new genome. Two previous PIN genes were reassembled into a single gene. A phylogenetic analysis of PIN proteins was carried out to investigate the evolutionary pattern of PIN genes in *Arabidopsis*, watermelon, cucumber (*Cucumis sativus*), and melon (*Cucumis melo*). We further examined their expression patterns in watermelon tissues and during shoot regeneration. The results presented here will benefit future studies on auxin-mediated regulation mechanisms of shoot regeneration in watermelon.

2. Materials and Methods

2.1. Sequence Retrieval and Bioinformatic Analysis

All *Arabidopsis* PIN proteins were selected as the queries to search against the genomes of watermelon, cucumber, and melon in the Cucurbit Genomics Database (CuGenDB) with the TBLASTN method [3,32,33]. After removing the redundant sequences, PIN genes from the three species were named after their *Arabidopsis* homologs and chromosome positions. The details of these sequences are listed in Table 1. The ProtParam tool was used to predict the characteristics of proteins, including sequence lengths, molecular weights, and theoretical pI [34]. The CELLO software was selected for the prediction of subcellular localization [35]. Protein transmembrane topology was analyzed by the TMHMM Server [36]. A Neighbor-Joining (NJ) phylogenetic tree was constructed with the bootstrap value of 1000 trails in the MEGA7 software [37]. The conserved domains were analyzed using DNAMAN 7 software [38]. The TBtools software was used to extract the promoter (2Kb upstream sequence) and genomic sequences of the PIN genes [39]. The PlantCARE software was selected to identify putative *cis*-elements related to growth, development, and stress responses [40]. The images of chromosome position, *cis*-element, and intron/exon were displayed in TBtools [39].

Table 1. The IDs, CuGenDB accessions, sequence lengths, molecular weights, pI, and CELLO localizations of PIN genes in watermelon, cucumber, and melon.

Gene ID.	CuGenDB Accession		Chromosome Location	Coding Sequence (bp)	Predicted Protein (aa)	Molecular Weight	Theoretical pI	CELLO Localization
	97103_v2	97103_v1						
ClaPIN1-1	Cla97C04G072260.1	Cla012098	Chr04: 19082426–19084800(–)	1878	625	67109.39	8.22	PlasmaMembrane (4.260)
ClaPIN1-2	Cla97C04G077450.1	Cla018455	Chr04: 25019277–25022350(+)	1860	619	67585.84	9.13	PlasmaMembrane (4.643)
ClaPIN1-3	Cla97C06G123780.1	Cla018924	Chr06: 26050523–26053439(+)	1824	607	66418.71	9.19	PlasmaMembrane (4.680)
ClaPIN2	Cla97C06G123240.1	Cla018871	Chr06: 25556363–25559538(+)	1938	645	70749.52	9.28	PlasmaMembrane (4.073)
ClaPIN3-1	Cla97C01G006350.1	Cla003909	Chr01: 6303561–6306905(–)	1926	641	69902.79	7.12	PlasmaMembrane (4.561)
ClaPIN3-2	Cla97C07G134150.1	Cla011708– Cla011709	Chr07: 9679960–9684183(–)	1872	623	68190.85	8.67	PlasmaMembrane (4.735)
ClaPIN5	Cla97C11G217020.1	Cla010530	Chr11: 20331421–20339597(–)	1113	370	40169.63	7.61	PlasmaMembrane (4.639)
ClaPIN6	Na	Cla015026	Chr09: 9050290–9056689(+)	1449	482	53345.31	9.25	PlasmaMembrane (4.712)
ClaPIN8	Cla97C10G197710.1	Cla017028	Chr10: 27449992–27452031(–)	1071	356	38907.61	9.53	PlasmaMembrane (4.913)
CsaPIN1-1	CsaV3_1G004350.1		Chr01: 2730713–2733712(–)	1827	608	66630.87	9.09	PlasmaMembrane (4.798)
CsaPIN1-2	CsaV3_1G007160.1		Chr01: 4542181–4545627(–)	1857	618	67443.68	9.13	PlasmaMembrane (4.630)
CsaPIN1-3	CsaV3_4G029470.1		Chr04: 19013177–19015651(+)	1791	596	63704.54	8.77	PlasmaMembrane (4.476)
CsaPIN2	CsaV3_1G032010.1		Chr01: 19030317–19033521(+)	1938	645	70831.58	9.29	PlasmaMembrane (4.272)
CsaPIN3-1	CsaV3_5G013380.1		Chr05: 9982996–9987048(–)	1890	629	68479.02	8.5	PlasmaMembrane (4.767)
CsaPIN3-2	CsaV3_5G028620.1		Chr05: 23739663–23744022(–)	1911	636	69462.29	7.12	PlasmaMembrane (4.600)

Table 1. Cont.

Gene ID.	CuGenDB Accession		Chromosome Location	Coding Sequence (bp)	Predicted Protein (aa)	Molecular Weight	Theoretical pI	CELLO Localization
	97103_v2	97103_v1						
CsaPIN5-1	CsaV3_2G009610.1		Chr02: 6112179–6116586(+)	1116	371	40371.92	7.04	PlasmaMembrane (4.592)
CsaPIN5-2	CsaV3_2G009700.1		Chr02: 6189126–6193716(+)	1191	396	43043.53	7.57	PlasmaMembrane (4.916)
CsaPIN8	CsaV3_3G041710.1		Chr03: 34023847–34026847(+)	1071	356	38980.69	9.59	PlasmaMembrane (4.895)
CmePIN1-1	MELO3C017357.2.1		Chr02: 24004740–24008031(+)	1776	591	64524.14	8.5	PlasmaMembrane (4.654)
CmePIN1-2	MELO3C019102.2.1		Chr08: 13382844–13385789(+)	1836	611	65374.38	8.75	PlasmaMembrane (4.526)
CmePIN1-3	MELO3C002132.2.1		Chr12: 26099882–26104026(+)	1929	642	70303.64	9.01	PlasmaMembrane (4.183)
CmePIN2	MELO3C017414.2.1		Chr02: 23469145–23472527(+)	1872	623	68443.69	9.22	PlasmaMembrane (3.928)
CmePIN3-1	MELO3C005326.2.1		Chr09: 20480725–20484941(–)	1911	636	69499.35	7.15	PlasmaMembrane (4.624)
CmePIN3-2	MELO3C018353.2.1		Chr10: 18871332–18876126(+)	1887	628	68548.23	8.69	PlasmaMembrane (4.729)
CmePIN5	MELO3C008726.2.1		Chr05: 18006724–18012271(–)	1209	402	43622.16	6.23	PlasmaMembrane (4.858)
CmePIN8	MELO3C009892.2.1		Chr04: 28346209–28350422(–)	1038	345	37657.14	9.54	PlasmaMembrane (4.939)

2.2. Plant Materials and RNA Extraction

The diploid watermelon inbred line A7 was selected for shoot regeneration, as previously reported [26]. Decoated seeds were cultured on Murashige and Skoog (MS) medium after sterilizing with NaClO solution (10%) for 5 min. After 1-week-germination, cotyledons were collected and cut into segments (0.5 × 0.5 mm), which were incubated on MS medium (1.0 mg/L 6-BA and 1.0 mg/L NAA, Sigma-Aldrich, Saint Louis, MO, USA) at 25 ± 2 °C and a photoperiod of 16/8 h (light/dark). The samples were collected 0, 7, 14, 21, and 28 days after incubation (DAI). Samples at each time point were collected from three different plates as biological replicates, frozen in liquid nitrogen, and ground into a powder for RNA isolation. Total RNA extraction was carried out with a Tiangen RNA prep Pure Plant Kit (Tiangen Biomart, Beijing, China), following the manufacturer's instructions. All RNA samples were stored at −80 °C until expression analysis.

2.3. Expression Analysis

In silico expression data were calculated using the Cucurbit Expression Atlas Database of CuGenDB and the RPKM values were downloaded with the accession of each watermelon PIN gene [32]. For qRT-PCR expression analysis, RNA samples were used for cDNA synthesis with the GoScript Reverse Transcription System (Promega, Madison, WI, USA). The 20 µL mixture for qRT-PCR reaction contained 10 µL TransStart Tip Green qPCR Supermix (Transgen Biotech, Beijing, China), 0.5 µL gene-specific primers (10 µM), 0.4 µL Passive ReferenceDye (50×) (Transgen Biotech, Beijing, China), 1 µL cDNA template, and 7.6 µL ddH₂O. A QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) was used for qRT-PCR reaction. The thermal cycles were 94 °C for 30 s, 40 cycles of 94 °C for 5 s, and 60 °C for 30 s, followed with a dissociation stage. Three reactions were taken for each sample as the technical repeat. Specific primers for watermelon PIN genes were designed using the online tool Primer 3 (Table 2). [41]. Watermelon *GAPDH* gene was used as a reference gene [26]. The relative expression levels were calculated by the $\Delta\Delta Ct$ method [42].

Table 2. Primers of watermelon 9 PIN genes and *GAPDH* gene (as the endogenous control) used for qRT-PCR.

Gene ID.	Forward Primers	Reverse Primers	References
ClaPIN1-1	CTCTGTTTCTGACCTCTACC	TACGGCGAGGGCGGCAAGGA	
ClaPIN1-2	TCTTGCTGTTCTTGCCGTTT	GCTCAGAAATCAGCATTCTG	
ClaPIN1-3	TACATTATCAGACTTTTACC	GTCCAGATACCGAGCACCAC	
ClaPIN2	ACATATACGATGTATTCTCAG	GTGGAAGGAGAGTAATGG	
ClaPIN3-1	ATCATGGAAGGATCTTTACA	AAATCCCAAGAAAGAACAAC	[32]
ClaPIN3-2	ACTGTCTTGACGGCGGTCAT	AATTGTAAGAGCAACAAGCA	
ClaPIN5	CCATGGCCCCATTGTACTTC	AAGACGAGAACGATAACGAT	
ClaPIN6	AAGGTGATGTGTGCGATGGT	TTGCCGAGAAGAAAACAGC	
ClaPIN8	TTCGTTGTCTGATGTCTATC	TATCAGTTGCAGGGTCATTT	
GAPDH	TGGAAGAATCGGTAGGTTGG	CTGTCACTGTTTTGGCGTC	[27]

3. Results

3.1. Genome-Wide Identification and Phylogenetic Analysis of PINs in Cucurbitaceae Species

After the whole genome survey, we identified nine PINs in watermelon (*ClaPINs*), nine in cucumber (*CsaPINs*), and eight in melon (*CmePINs*) (Table 1). The CELLO results indicated that all the Cucurbitaceae PINs were located at the plasma membrane, implying their potential function in auxin transport. Most *ClaPINs* contained 4–5 transmembrane helices at N- or C-termini (Figure S1).

The PIN proteins of *Arabidopsis* and the three *Cucurbitaceae* species were aligned for phylogenetic analysis. These proteins were grouped into seven clusters (Figure 1A). One PIN gene from watermelon clustered together with *AtPIN6* from *Arabidopsis* in cluster I. Clusters IV, V, VI, and VII contained an equal number of PIN genes to the *Cucurbitaceae*

species. *Arabidopsis* PINs were also clustered in these groups. We further examined the conserved domains of all the PIN proteins (Figure S2). Most sequences contained two conserved domains at both the N and C-termini. The middle parts of these sequences were predicted to be located inside the transmembrane.

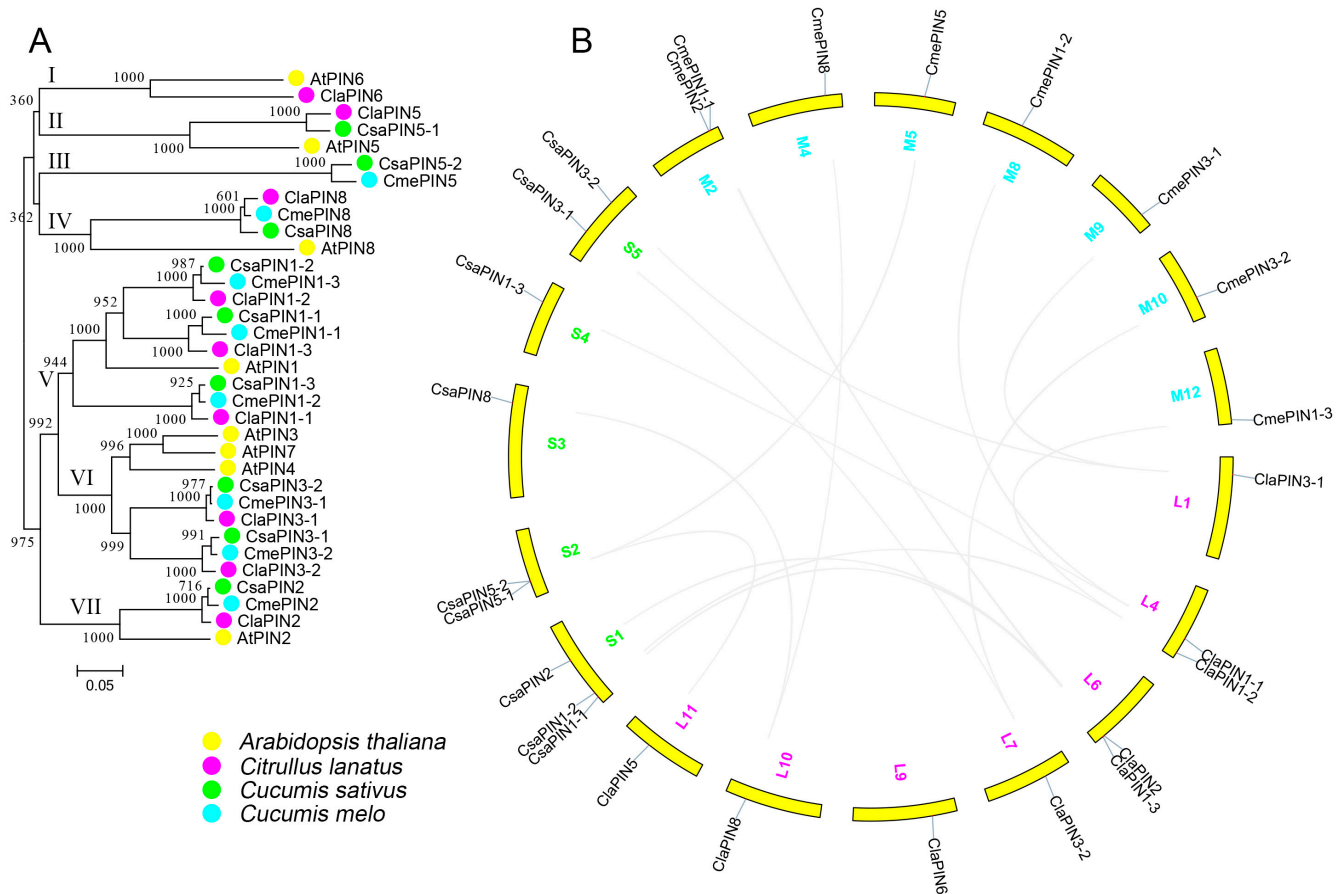


Figure 1. Phylogenetic (A) and chromosomal localization (B) of the *Cucurbitaceae* PIN family. The proteins from *Arabidopsis thaliana*, cucumber, melon and watermelon are shown in yellow, blue, green, and pink, respectively (A). The chromosome numbers are listed beside chromosomes (yellow) for watermelon (pink), cucumber (blue), and melon (green), respectively. The homologous gene pairs are connected with grey solid lines.

3.2. Cis-Element and Gene Structure Analysis of Watermelon PINs

The upstream sequences (2000 bp) of watermelon PINs were selected for scanning *cis*-regulatory elements (Table S1). We annotated 13 kinds of *cis*-elements that are related to salicylic acid, gibberellin, auxin, MeJA, light, low-temperature, defense/stress responses, drought, and anaerobic induction, regulation of zein metabolism, flavonoid biosynthesis, and circadian control (Figure 2A). Among these, the number of light responsive *cis*-element (78) was the most abundant (Table S1). The numbers of MeJA responsive, anaerobic induction responsive, and salicylic acid responsive elements were 16, 14, and 11, respectively. We further analyzed the introns of *ClaPINs* (Figure 2B). Most sequences contained 5 introns. There were 4 introns in *ClaPIN1-1* and 6 introns in both *ClaPIN1-3* and *ClaPIN2*.

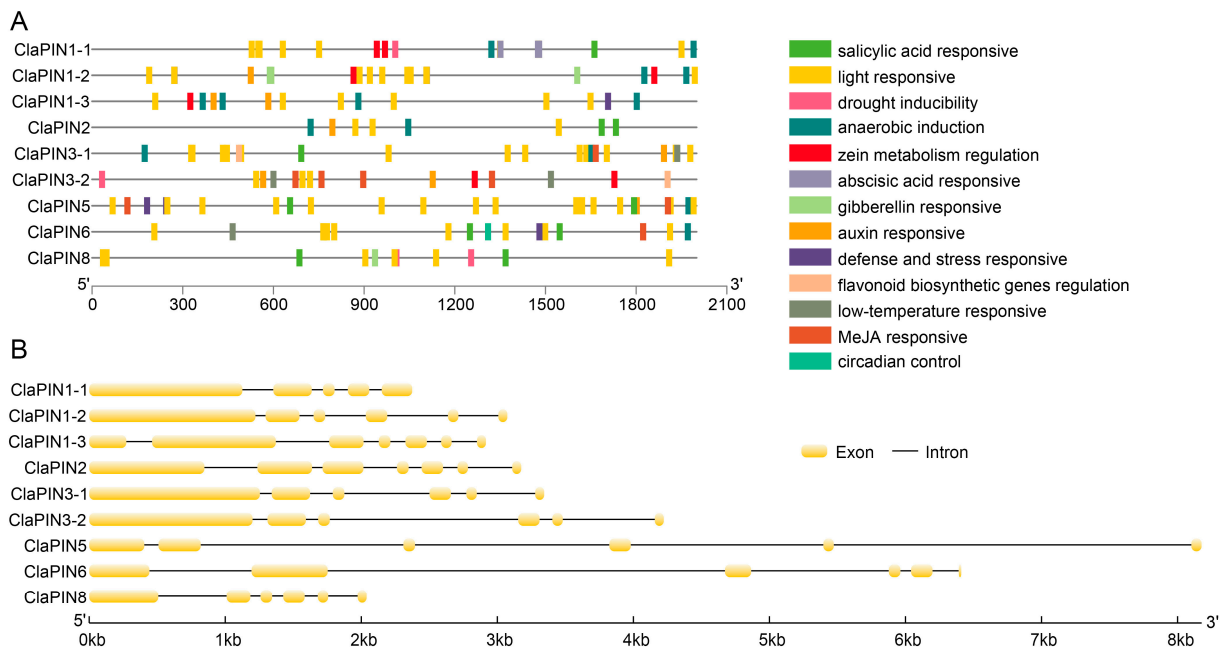


Figure 2. *Cis*-elements (A) and intron (B) structures of watermelon PIN family. The *cis*-element prediction was conducted with 2000 bp upstream sequences by the PlantCARE software [41]. The intron structure analysis was conducted with genomic and coding sequences of watermelon PIN family.

3.3. In Silico Expression of PINs in Watermelon Tissues

To estimate the expression patterns of *ClaPINs*, in silico analysis was carried out in watermelon tissues (Figure 3). Most *ClaPINs* were highly expressed in roots and leaves compared with flowers and seeds. *CaPIN1–2* showed high expression in both roots and leaves. *ClaPIN5* was highly expressed in seeds and leaves. *ClaPIN6*, meanwhile, showed relatively low expression in all tissues.

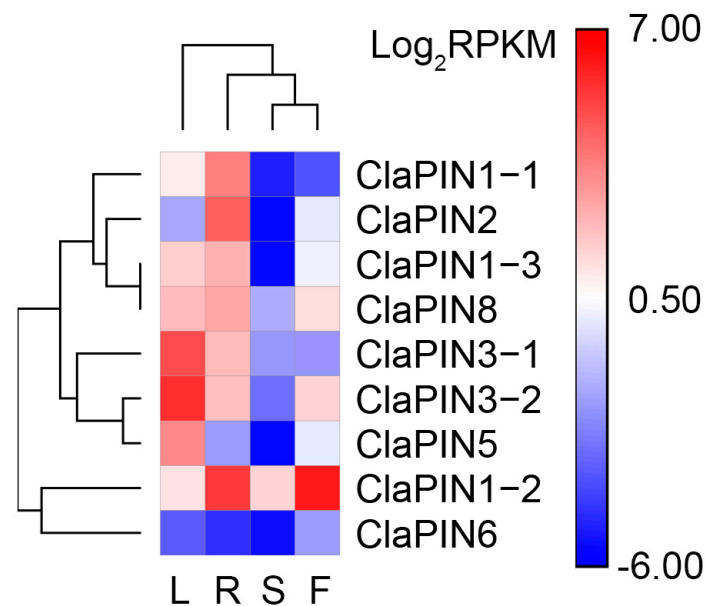


Figure 3. In silico expression of *ClaPINs* in watermelon tissues. The single capital letters represent root (R), leaf (L), flower (F), and seed (S).

3.4. Expression Profiles of Watermelon PINs during Shoot Regeneration

To further examine the expression patterns of *ClaPINs*, the expression profiles of *ClaPINs* were examined during shoot regeneration by qRT-PCR. The results indicated that nine *ClaPINs* followed four different types of expression patterns (Figure 4, Table S2). *ClaPIN1-1* and *ClaPIN1-3* were up-regulated at early stages of shoot regeneration and then down-regulated during the process. Five *ClaPINs* that include *ClaPIN1-2*, *ClaPIN2*, *ClaPIN3-1*, *ClaPIN3-2* and *ClaPIN8* showed the highest expression at the beginning of shoot generation, but had lower levels of expression during successive stages. While there was no significant change in expression levels of *ClaPIN6*, the expression of *ClaPIN5* was gradually down regulated during shoot regeneration.

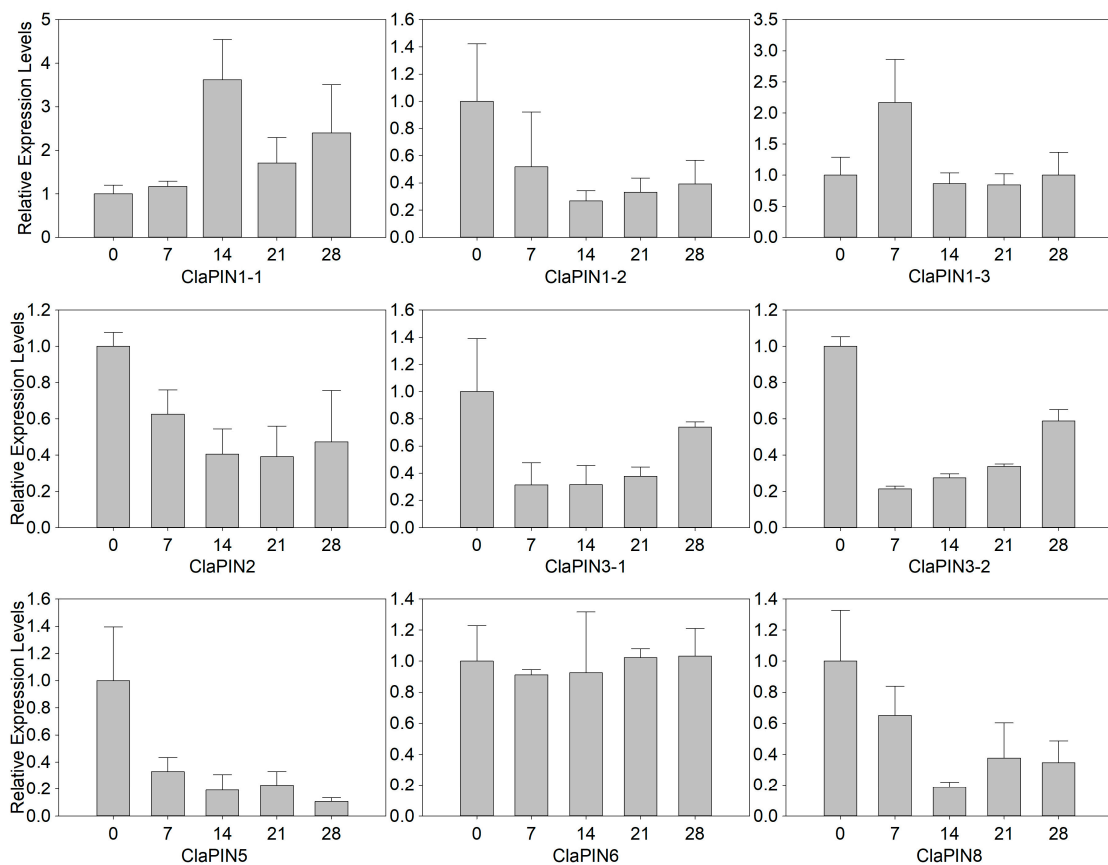


Figure 4. Expression pattern of *ClaPINs* during shoot regeneration by qRT-PCR. The numbers of the X-axis represent that the samples were collected at 0, 7, 14, 21, and 28 days after incubation.

4. Discussion

4.1. Identification of PINs in Cucurbitaceae Species

In the present study, PIN genes of watermelon (9 genes), cucumber (9 genes), and melon (8 genes) were partially characterized (Table 1). There might be evolutionary differences between watermelon and cucumber/melon genes. Previous studies revealed the absence of recent whole-genome duplications in cucumber and melon after the ancient eudicot triplication [43,44]. The evolution of PINs tends to be conserved between cucumber and melon. The phylogenetic results indicated that cucumber and melon shared the same number of PINs in each sub-cluster (Figure 1A). In this study, we carried out a new genome-wide characterization of the PIN gene family in watermelon using version 2 of the watermelon 97103 genome, which has been generated by using PacBio long reads, combined with BioNano optical and Hi-C chromatin interaction maps [23]. The results presented here are also different from the previous study [31]. Two of the PINs

(*Cla011708* and *Cla011709*) in genome version 1 were reassembled into a single gene as *ClaPIN3-2* in genome version 2 (Table 1). Moreover, despite the existence of its genomic sequence, *Cla015026* (*ClaPIN6*) was not annotated in genome version 2. Interestingly, only watermelon contained *PIN6* homologue, which also indicated the different evolutionary history of watermelon compared with cucumber or melon.

4.2. Putative Regulators in *ClaPINs*

The *in silico* expression analysis revealed a series of *ClaPINs* that are mainly expressed in roots (*ClaPIN1-1* and *ClaPIN2*) and leaves (*ClaPIN3-1*, *ClaPIN3-2* and *ClaPIN5*) (Figure 3). *ClaPIN1-2* was mainly and highly expressed in both roots and flowers. These results indicated the tissue specific expression of *PINs* in watermelon, which has also been reported in *Arabidopsis* [3,31]. There were four types of expression patterns for *ClaPINs* during the process of shoot regeneration (Figure 4). Among these, the up-regulation of *ClaPIN1-1* and *ClaPIN1-3* at a very early stage of shoot regeneration indicated their important functions in regulating auxin transport to generate auxin maxima, which is necessary for fast developing tissues like calli [29]. In contrast, several *ClaPINs* such as *ClaPIN1-2*, *ClaPIN2*, *ClaPIN3-1*, *ClaPIN3-2*, *ClaPIN5* and *ClaPIN8* were down-regulated during the callus stage but up-regulated during the shoot stage. They seemed to have functions in mature tissues after cell differentiation, which might be related to their tissue specific expression patterns. On the other hand, the expression of *ClaPIN6* was considerably low at all stages of shoot regeneration and did not show any significant differences among different stages suggesting that it may not play an essential role in shoot regeneration. The loss of *PIN6* in cucumber and melon genomes could partly explain the non-essential role of *ClaPIN6*, which also indicated a species-specific evolution of the *PIN* family in the *Cucurbitaceae* species. Besides, auxin responsive *cis*-elements were identified in six *ClaPINs*, including *ClaPIN1-2/3*, *ClaPIN2*, *ClaPIN3-1/2*, and *ClaPIN5* (Figure 2), which might contribute to their functions during shoot regeneration. There were other types of phytohormone responsive *cis*-elements identified in *ClaPIN1-1*, *ClaPIN6*, and *ClaPIN8*. This observation suggests that their potential roles might be regulated by auxin crosstalk with other phytohormones [45]. We could draw the preliminary conclusion that *ClaPIN1-1* and *ClaPIN1-3* are possible candidates for regulators of shoot regeneration. However, more work is still needed to reveal the functions of other *ClaPINs*.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agriculture11050447/s1>: Figure S1—The results of transmembrane topology analysis for watermelon *PIN* proteins. The blue and purple lines represent inside and outside the transmembrane, respectively; Figure S2—Conserved domains of watermelon *PIN* proteins; Table S1—*Cis*-element analysis in upstream sequences of watermelon *PIN* genes; Table S2—The raw data of qRT-PCR analysis.

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