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Full Length Article

Genome-wide Identification and Expression Analysis of the *PIN* Gene Family during Abiotic Stress in Tomato (*Solanum lycopersium*)

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Abstract

It is widely believed that the *PIN* gene family is the most important auxin efflux carrier in plants since the separation of pin-formed mutant from *Arabidopsis thaliana* in 1991. Auxin transport and accumulation can be indirectly measured by the expression level of *PIN* genes. In recent years, members of the *PIN* gene family have been cloned in different species. Ten *PIN* genes (*SlPINs*) were identified in the genome data of tomato using the *Arabidopsis* PIN protein family for reference sequences. Phylogenetic analysis showed that 10 members were divided into "short" and "long" PINs. Chromosomal distribution map revealed that the *SlPIN* genes were unevenly distributed in eight chromosomes. We also studied the expression of 9 *SlPIN* genes under cold, drought and salinity by qRT-PCR, (*SlPIN2* was not expressed in tomato leaves). The obtained levels of expression of the same genes were different under different stresses. Some genes were up-regulated under some stresses but were down-regulated under other stresses. The relative expression of *SlPIN6* clearly changed under cold and drought stress. The results here would provide the theoretical basis for the molecular cloning and resistance breeding of *PIN* genes in tomato. © 2018 Friends Science Publishers

Keywords: Abiotic stresses; Auxin efflux carriers; *PIN*; Relative expression; Tomato

Introduction

In early 1880, it was found that there is a transport signal substance – auxin. Auxin is the only hormone that undergoes polar transport in plants and is an indispensable regulator of plant growth and development. Auxin is synthesized in many plant tissues *via* several different pathways, including the stem apex, young leaves, and flowers as well as during the growth of lateral root vascular tissue (Zhao, 2010), and then transported to the other tissue parts of the plant. The concentration gradient of auxin is typically established by the different sites of synthesis and methods of transportation. Auxin is involved in plant apical dominance, tropism, vascular differentiation, floral tissue differentiation, photomorphogenesis, embryo formation, root development and plant responses to internal and external stimuli (Dubrovsky *et al*., 2008; Xi *et al*., 2016). The interaction of auxin, gibberellins and cytokinins promote the growth of plants by promoting the growth of cells (Fleet and Sun, 2005; Růžička *et al*., 2009). Different auxin concentrations in different tissues are caused by polar auxin transport (PAT). The PAT study using coleoptile as a test material improved that auxin is transported from the morphological top to the morphological bottom of the plant, and this transportation direction is not affected by gravity. There is auxin carrier protein at the bottom of each cell membrane, but not on the top of the cell membrane (Yu and Cui, 2009). Multiple classes of auxin transport proteins participate in cell-to-cell auxin transport. Auxin is transported by the efflux carriers pin-formed (PIN) and Multidrug-Resistant/P-glycoprotein (MDR/PGP), and the influx carriers auxin resistant 1/like aux1 (AUX/LAX). The PIN protein has a speed limit function during the output process of auxin, and sensitivity to auxin output inhibitory factor. Recently, some members of the *PIN* genefamily are localized to the endoplasmic reticulum and participated in the regulation of intracellular auxin homeostasis, the plasma membrane (Barbez *et al*., 2012; Feraru *et al*., 2012). Other members are located in the plasma membrane and involved in various tropic responses and apical shoot establishment in *Arabidopsis*.

There have been some studies on auxin transport genes in monocotyledons and dicotyledonous plants. Many *PIN* genes have been reported in detail in dicotyledonous plants, but there are also a few *PIN* genes whose research is rare in monocotyledons. *PIN1* was one of the most studied and earliest members of the *PIN* gene family (Goto, 1987). Gälweiler *et al*. (1998) cloned the *PIN1* gene as an auxin transporter from *Arabidopsis thaliana* by transposon tagging. The *PIN* gene has been predicted in potato, Me*dicago truncatula* (Schnabel and Frugoli, 2004), maize (Carraro *et al*., 2006), *Brassica juncea* (Ni *et al*., 2002) and *Mangifera indica* (Li *et al*., 2012). In higher plants, all PIN proteins are polar distributed on one side or another side of the cell, which is consistent with auxin transport. PIN proteins are

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characterized as cell specific and tissue specific. Different members of the PIN family are expressed in different plant parts, and the function of PIN proteins changes with a change in polarity position. The subcellular localization of *PIN* genes also has the characteristics of dynamic change and not only is affected by the growth and development of plants but also is restricted by external environmental conditions. Presently the information of PIN-dependent polar auxin transport in plants mainly comes from the extensive investigation of the *PIN* gene family in rice (*Oryza sativa*) (Ma and Jie, 2010) and *Arabidopsis*. In *Arabidopsis*, the PIN family comprises of 8 members and is divided into two groups. AtPIN1-AtPIN4, AtPIN6 and AtPIN7 belonged to the typical long PINs. These long PIN proteins are located in the plasma membrane, and have a relatively long central hydrophilic loop, which share high sequence similarity especially in the hydrophobic domains of both N- and C-termini (Roumeliotis *et al*., 2013). These proteins are primarily responsible for the transport of auxin to the outside of cell and unevenly distributed on the cell membrane and participated in various tropic responses (Robert *et al*., 2013). In contrast to the long PINs, the short PINs include AtPIN5 and AtPIN8, which lack a central loop domain. They are localized in the endoplasmic reticulum and mediated the auxin communication of the cytoplasm and endoplasmic reticulum. They also participate in the homeostasis of auxin and compartmental localization by working together with members of the PIN auxin efflux carriers (Mravec *et al*., 2009; Ding *et al*., 2012; Cazzonelli *et al*., 2013). In addition, the PIN gene has been studied to be involved in abiotic stress responses, including those that involve dehydration, salt and drought (Shen *et al*., 2010).

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops and is also a model plant for molecular biology research in *Solanaceae* plant. Tomato has high economic and medicinal value. In 2012, the tomato genome sequencing project was completed (Consortium 2012), which laid the foundation for the whole-genome bioinformatics analysis of the *PIN* gene family, and studies on the bioinformatics analysis of the *PIN* gene in tomato have been reported (Zhao *et al*., 2017). Recently, the expression of three *PIN* genes was examined in tomato vegetative and reproductive organs. Nishio *et al*. (2010) focused on *PIN* gene expression patterns and auxin distribution patterns during early fruit development. The tissue-specific expression and the spatial and temporal expression patterns of auxin accumulation with respect to the tomato *PIN* gene family have also been reported; these studies have especially emphasized the process of fruit growth (Pattison and Catalá, 2012). However, no studies have analysed the effects of abiotic stresses on the tomato PIN family. In sorghum, the expression of 5 *SbPIN* genes highly increased under ABA, salt and drought treatments, whereas the rest of the 6 *SbPIN* genes were almost inhibited. Here, we present a genome-wide of the tomato *PIN* gene family and its expression profiling under abiotic stresses such as salt, cold and drought. This work identified the tomato PINs associated with abiotic stresses responses. Some of these PINs would be candidate genes for further functional studies.

Materials and Methods

Identification of *PIN* Gene Family Members in Tomato

Protein sequences of the tomato *PIN* gene were obtained from the SOL Genomics Network (SGN, http://solgenomics.net/search/loci). According to analysing the conserved PIN domains (PF03547), all candidate protein sequences of tomato were further confirmed using the software programs HMMER (http://www.ebi.ac.uk/Tools/hummer/search/hmmscan) and Pfam (http://pfam.janelia.org). Essential on exons and chromosome locations of tomato PIN members were obtained from the SGN sequence database. Theoretical pI, molecular weight and other physicochemical properties of PIN amino acids were calculated using the online program ProtParam (http://expasy.org/tools/protparam/).

Prediction of the Secondary Structure of Tomato PIN Members

Tomato PIN signal peptides were predicted using the SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) online tool. The secondary structure of tomato PIN members was forecast utilizing SOPMA online software (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html).

Phylogenetic Tree and Multiple Sequence Alignment

The sequence alignment of tomato, sorghum, rice and *Arabidopsis* was performed by the software ClustalX (Sun *et al*., 2015). The results of the sequence alignments were used to construct an unrooted phylogenetic tree using the neighbour joining method with a bootstrap analysis of 1000 replicates by MEGA 5.2 program (Tamura *et al*., 2011; Chai and Subudhi, 2016). The multiple sequence alignment was performed using the DNAMAN program.

Conserved Motifs and Chromosomal Mapping

Conserved motifs of the tomato PIN members were statistically identified by the MEME program (http://meme.nbcr.net/meme/) according to the searched amino acid sequence. Each *SlPIN* gene chromosome localization were determined based on the protein sequence from SGN. The *SlPIN* genome location was drawn from top to bottom by the MapInspect software.

Plant Growth Conditions and Quantitative RT-PCR (qRT-PCR) Analysis

The experiment was performed in the horticultural station of

Northeast Agricultural University (Harbin, China), during 2016-2017. Tomato seeds (*S. lycopersicum cv.* Moneymaker) were grown in a greenhouse. Unified managements of watering, pest and disease controls were used during the whole growth period (Liu *et al*., 2015). For abiotic stress treatments, five- or six-leaf seedlings were treated with salinity (100 m*M* NaCl), cold (5°C) and drought (10% PEG6000). The leaves of seedlings were saved at 0, 2, 4, 8, 12 and 24 h after stimulation of the salt and cold treatments, and three biological replicates were performed for every treatment. For drought treatment, the young leaves were collected separately at 0, 4, 6, 8 and 12 h after treatment. The samples were frozen immediately in liquid nitrogen after collection and kept at -80°C, and each sample was repeated three times.

Total RNA was extracted with using Trizol reagent (Invitrogen) according to the manufacturer's guidances. First-strand cDNA was synthesized from 3 mg of total RNA from each sample using the TranScript® One-Step gDNA removal kit and cDNA Synthesis SuperMix reverse transcriptase. Quantitative real-time PCR performed in an iQ 5 system using SYBR® Green I. Primer pairs (Table 1) for individual gene families were designed using Primer Premier 5.0 program. Among them, SlPIN1, 5, and 6 primer pairs have been published (Pattison and Catalá 2012). The results of RNA were checked using agarose gel electrophoresis and analysed by AlphaEaseFC image (Zhang *et al*., 2014a), and images were collected under UV light. The qPCR reaction mixture contained SYBR® Green Master Mix (10 μL), each primer (0.5 μL), ROX Reference Dye one (0.5 μL), cDNA template (1 μL) and sterile distilled water up to a total volume of 20 μL. The thermal conditions were as follows: 95°C for 10 min and 40 cycles of 95°C for 5 s, 56°C for 15 s, and 72°C for 30 s. Slaction was employed as an internal control, and qRT-PCR (quantitative reverse transcription-polymerase chain reaction) data were analysed in accordance with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Results

Identification of *SlPIN* Gene Family in Tomato

Based on gene uniqueness, a total of ten *SlPIN* genes were identified and analysed, these genes are listed in Table 2. The 10 *PIN* genes were orderly named from *SlPIN1* to *SlPIN10* in the later work. The longest gene for encoding amino acids is *SlPIN4*, 653 amino acids (aa). The shortest gene for encoding amino acids is *SlPIN10*, 347 aa, with an average length of 527 aa. The molecular weights of SlPIN proteins ranged from 38411.53 (SlPIN10) Da to 71197.03 (SlPIN4) Da, and the variation range isoelectric points was 6.76 to 9.19. The majority of amino acids were basic amino acids; only SlPIN3, 4, and 10 proteins were acidic amino acids. The gene structure was highly conserved for most *SlPINs*. Six of 10 *SlPINs* contain 6 exons. These conserved exon structures of *PIN* genes were also found in other land plant species (Křeček *et al*., 2009; Forestan *et al*., 2012; Wang *et al*., 2015). In addition, the structural change of the N-terminus was stable, and the carboxyl terminal of the PIN protein changed greatly. Analysis of the instability coefficient of the coding protein showed that SlPIN2 and SlPIN4 proteins are instability proteins (instability coefficient>40); the other 8 SlPIN proteins are stable proteins. It was found that SlPIN5, 8 and 10 were hydrophobic proteins, as their grand averages of hydropathicity were more than 100. SignalP online tool reveal all of the identified SlPIN proteins that had no signal peptide were non-secreted proteins.

The Secondary Structure of the PIN Protein Family Members in Tomato

Similar to other plant PINs proteins, SlPIN proteins have a typical conserved transmembrane domain, and the 10 SlPIN proteins are composed of two hydrophobic regions connected by a central hydrophilic loop at both ends of the protein. At the N-terminus, all SlPINs have 4 to 5 transmembrane helices, except for SlPIN7and SlPIN8. The C-terminus end has 3 to 4 transmembrane helices (Zhao *et al*., 2017). The 10 SlPIN proteins consisted of an alpha helix, turn, coil and beta sheet, and the ratio of the turn is smaller (Table 3). There are significant similarities (Coil>Alpha helix>Beta sheet>Turn) in the secondary structure of the analysed proteins except for that of SlPIN9 (Coil>Beta sheet>Alpha helix>Turn). SlPIN8 and SlPIN10 have the same structure characteristics (Alpha helix> Coil>Beta sheet>Turn). The secondary structure of proteins determines the folding and spatial configurations to some extent. It is of great significance to understand the structure and function of proteins by analysing and predicting their secondary structure.

Phylogenetic Analysis of *SlPIN* Genes

To investigate evolutionary relationships among PINs in tomato and other species, 41 PIN protein sequences from tomato, *Arabidopsis*, rice and sorghum were comprehensive aligned by ClustalX and the MEGA 5.2 Software (Fig. 1). According to the difference of the length central hydrophilic loop, the SlPIN proteins were divided into two groups (Křeček *et al*., 2009; Shen *et al*., 2010): long PINs (SlPIN1, 2, 3, 4, 7 and 9) and short PINs (SlPIN5, 6, 8 and 10). The fact that SlPIN7 and SlPIN9 are very similar in sequence level and are located on the same branch indicates that they come from a common ancestor. SlPIN3, SlPIN4 and SlPIN5, SlPIN10 are also clustered on the same branch respectively. Meanwhile, SlPIN1 and AtPIN1, SlPIN8 and AtPIN8, SlPIN6 and AtPIN6 are clustered on the same branch respectively. In addition, the number of PIN genes in tomato, *Arabidopsis*, rice and sorghum was almost similar.

Analysis of PIN Gene Structure

The multiple sequence alignment indicated that the

Table 1: Primers for qRT-PCR

Primer name	Sequence (5'-3')	Primer name	Sequence (5'-3')
SlPIN1-F	GCTGCAGGCTGGTCTAGATT	SlPIN1-R	AACAATGGCAACAAAGCACA
SlPIN3-F	GTGGGAACACTGTGGCTACT	SlPIN3-R	TGCATTGGCCTAATACATCTCTA
SlPIN4-F	TGCTTCAATTGCTGTTGGGC	SlPIN4-R	TTGAACCAACAATTTTAATGCAACA
SlPIN5-F	ACATTGAGCTGGCATTTTGG	SlPIN5-R	TCCACTACCAGCCTTTGACA
SlPIN6-F	GCAGCTCTTCCCCAAGGAAT	SlPIN6-R	GCGAAGACAAATGGAACGAT
SlPIN7-F	CATCAGCGGTCCAGCAGTCA	SlPIN7-R	TGTTTCCGAAGGGTCCTCAGTT
SlPIN8-F	CAGCCCTTCCCCAAGGAATC	SlPIN8-R	CCCTGCAATCAGAATGAAACCA
SlPIN9-F	TCTTTTAGGTGGAATGTTCAAATGC	SlPIN9-R	CGACAATGCCATGAACAAACC
SlPIN10-F	AGAGCATGTTTGGCTCAGCTT	SlPIN10-R	ACCCCTACCCCAGCTTTTAAC
Slaction-F	GAAATAGCATAAGATGGCAGACG	Slaction-R	ATACCCACCATCACACCAGTAT

Table 2: The distribution of PIN gene family members on the scaffolds of the genome and physico-chemical analysis in tomato

Gene name	Locus name	Amino acids (No.)	Exons (No.)	Molecular weight (Da)	Theoretical Pl	Instability index (II)	Grand average hydropathicity (GRAVY)	Aliphatic index (AI)
SlPIN1	Solyc03g118740.2.1	611	6	66938	9.09	38.93	0.038	87.15
SlPIN2	Solyc07g006900.1.1	631	7	68565.38	9.19	44.16	0.180	97.34
SlPIN3	Solyc04g007690.2.1	613	6	67612.20	6.76	37.33	0.151	93.83
SlPIN4	Solyc05g008060.2.1	653	6	71197.03	6.98	40.74	0.073	89.62
SlPIN5	Solyc01g068410.2.1	358	5	39673.22	8.80	34.09	0.732	120.67
SlPIN6	Solyc06g059730.1.1	521	7	56876.39	8.61	34.08	0.379	105.71
SlPIN7	Solyc10g080880.1.1	586	6	63858.08	8.93	34.49	0.240	97.03
SlPIN8	Solyc02g087660.2.1	357	6	38966.79	8.89	35.40	0.778	136.30
SlPIN9	Solyc10g078370.1.1	594	6	64304.33	9.16	33.22	0.195	95.54
SlPIN10	Solyc04g056620.1.1	347	5	38411.53	6.99	33.37	0.698	111.01

Table 3: The secondary structure of PIN protein family members in tomato

Gene	Alpha helix	turn	coil	Beta sheet
SlPIN1	193 (31.59%)	70 (11.46%)	222 (36.33%)	126 (20.62%)
SlPIN2	204 (32.33%)	72 (11.41%)	219 (34.71%)	136 (21.55%)
SlPIN3	167 (27.24%)	63 (10.28%)	234 (38.17%)	149 (24.31%)
SlPIN4	177 (27.11%)	69 (10.57%)	259 (39.66%)	148 (22.66%)
SlPIN5	224 (31.11%)	89 (12.36%)	227 (31.53%)	180 (25.00%)
SlPIN6	158 (30.38%)	56 (10.77%)	171 (32.88%)	135 (25.96%)
SlPIN7	183 (31.23%)	73 (12.46%)	206 (35.15%)	124 (21.16%)
SlPIN8	143 (40.06%)	32 (8.96%)	94 (26.33%)	88 (24.65%)
SlPIN9	147 (24.75%)	69 (11.62%)	223 (37.54%)	155 (26.09%)
SlPIN10	159 (45.82%)	26 (7.49%)	84 (24.21%)	78 (22.48%)

sequences of transmembrane helices in SlPIN proteins were highly conserved at both ends of the protein and that the central hydrophilic loop was high heterogeneity (Fig. 2). Similar to the AtPIN (Paponov *et al*., 2005), OsPIN (Wang *et al*., 2009) and SbPIN (Shen *et al*., 2010) proteins, the central hydrophilic loop consists of C1, C2, C3 structural domains and two variable regions (V1, V2) for long PINs. The hydrophilic region of the short PINs group has only one constant C1 region and a variable V1 region. The length of the central hydrophilic loop is approximately 350 amino acids for members of the long PINs. However, the length of the short PINs members is 47–243 amino acids. The conserved structure NPNXY is found between the hydrophilic loop and the C-terminal hydrophobic domain of all SlPIN proteins (except SlPIN8). The last one amino acid of SlPIN8 is histidine rather than tyrosine. NPXXY plays an important role in clathrin dependent endocytosis (Yu and Cui, 2009). The difference in length between the proteins is the result of the difference in length of the hydrophilic region located between the transmembrane helice domains present at C- and N-terminal of the protein. Most SlPINs contain two phosphorylation active sites that are marked in the figure by black triangles. These sites can be phosphorylated by serine/threonine protein kinases (Křeček *et al*., 2009).

Motifs Analysis and Chromosome Mapping of the *SlPINs*

To further study the multiformity of *SlPINs*, conserved motifs in *PIN* genes were identified by MEME software. Based on the distribution of the 8 predicted motifs, the tomato *PIN* genes were also categorized into two groups based on the distribution of the 8 predicted motifs, which was consistent with the phylogenetic analysis. The essential information of the logo and sequence of each motif are shown in Fig. 3 and Table 4. We found that the tomato *PIN* gene family members not only have a typical conserved

Table 4: The conserved motif of PIN protein family from tomato

Motif	Width	Best possible match
1	50	PLYVAMILAYGSVKWWKIFSPDQCSGINRFVALFAVPLLSFHFIASNNPY
2	49	LLHVAIVQAALPQGIVPFVFAKEYNVHPDILSTAVIFGMLIALPITLVYY
3	49	RKLIRNPNTYSSLJGLIWSLISFRWNVQMPKIIEKSISILSDAGLGMAMF
4	50	GSLEWSITLFSLSTLPNTLVMGIPLLKAMYGDYSGSLMVQIVVLQCIIWY
5	41	FMALQPKIIACGKSVATFAMAVRFLTGPAVMAAASIAVGLR
6	42	DTAGSIVSFKVDSDVISLDGREPLETDAEIGDDGKLHVTVRK
7	41	MTPRPSNLTGAEIYSLQSSRNPTPRGSNFNHTDFYSMVGGK
8	29	MNYRFIAADTLQKVIVLFVLAIWANVSKR

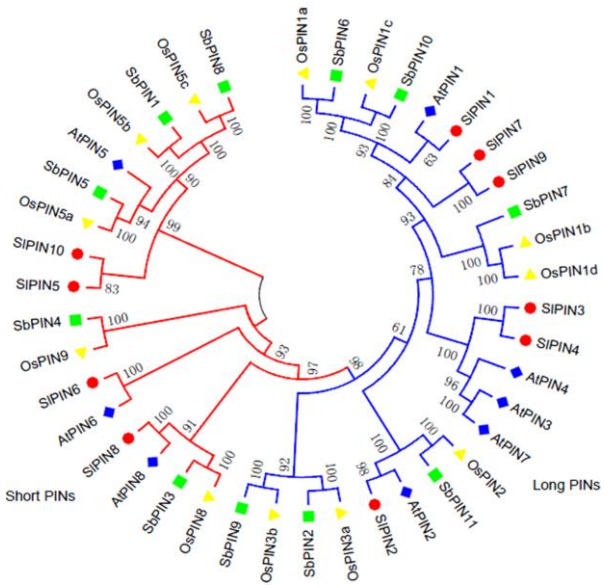

Fig. 1: Phylogenetic relationships of PIN auxin transporters from tomato (Sl), *A. thaliana* (At), Rice (Os) and Sorghum (Sb). Triangles, squares, circles and rhombi represent the PINs of Rice, Sorghum, *A. thaliana* and tomato, respectively

motif but also have some relatively conserved motifs that consist of 29-50 amino acid residues. We also found that different clades shared similar motifs: 8 motifs were distributed in the long PINs, and 6 motifs were in the short group. Motifs 1, 2, 3, and 4 encoded PIN domains in all the studied genes. The internalizational NPXXY structure is located within motif 3. The N-terminus of the SlPIN protein family has a conserved motif 1. Motif 2 was present in the C-terminus of all members, and the rest motifs were unevenly distributed in two groups.

Chromosome map positions of tomato PINs that were identified are reported in Fig. 4. Our results showed that 10 SlPIN genes were unevenly distributed on eight chromosomes in the whole tomato genome. The number of SlPIN genes per chromosome ranged from zero to two, with zero genes on chromosomes 8, 9, 11 and 12; one gene each on chromosomes 1, 2, 3, 5, 6 and 7; and two genes on chromosomes 4 and 10. In tandem replication, the distances of adjacent genes on the same chromosome were less than 100 kb, thus, tandem replication did not occur.

Expression of *SlPIN* Genes under Abiotic Stresses Treatment

To understand the expression patterns of *SlPIN* genes under different stresses conditions, the expression profiles of 9 selected *SlPIN* genes (excluding *SlPIN2*) were studied in response to drought, salt and cold treatments by qRT-PCR experiments based on tissue-specific expression patterns of *SlPIN* genes. A histogram representation for transcript expression fold changes in response to abiotic stresses is shown in Fig. 4. The expression level of the *PIN* genes was affected under one or more treatments. More SlPINs were up-regulated in response to drought compared with cold and salt stress.

Gene expression was slightly induced in tomato plants treated with salt (Fig. 5 a). Expression of *SlPIN3* decreased over time. *SlPIN1*, 5, and 7 decreased first but then increased. The expression of *SlPIN1* and 7 reached the minimum value at 4 h, and *SlPIN8* reached the minimum value at 8 h. *SlPIN4* significantly decreased compared to the high expression at the normal 0-h point. The change patterns of *SlPIN6* and 10 were consistent, as both reached their maximum at the 12-h point. The relative expressions of partial *PIN* genes were remarked by cold stress (Fig. 5b). The expression of *SlPIN7*, 8 and 10 increased only at 8 h; nevertheless, their expression was low at the other time points. The relative expression of *PIN* genes *SlPIN3* and 6 increased rapidly at the 2 h post-stimulus point and showed a tendency of increasing first and then decreasing. *SlPIN1* was up-regulated compared to the low expression at the normal 0 h point, whereas the *SlPIN* genes were moderately induced. Under drought stress, all *PIN* genes possessed different expression patterns (Fig. 5c). The expression of *SlPIN1*, 6, 8 and 9 was up-regulated at first but then decreased after 8 h. *SlPIN10* was also up-regulated over time but decreased at the 12 h point. The expression of *SlPIN4* and 5 increased at 4, 6 and 12 h. The expression of *SlPIN7* increased at the 4 h point, and then held steady, with moderate changes.

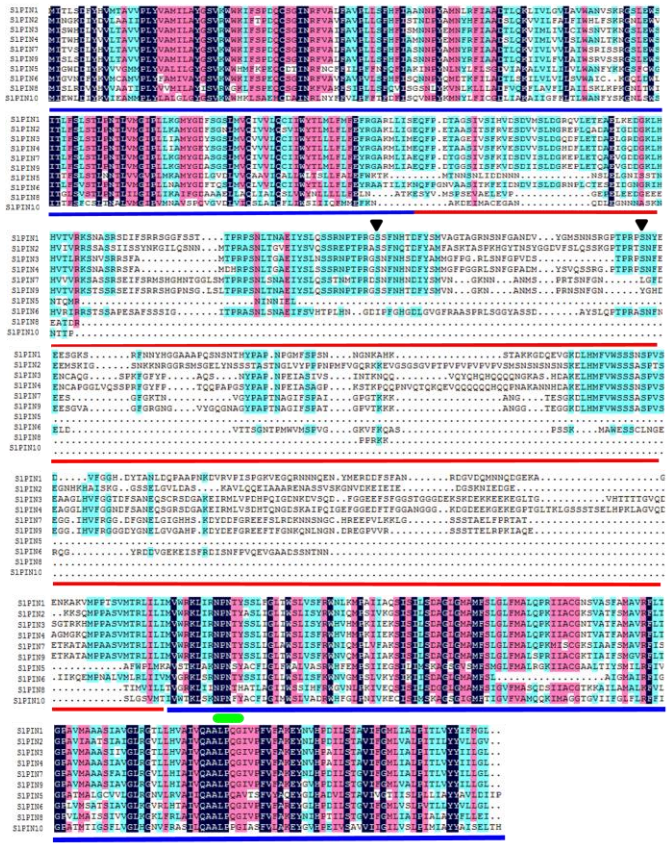

Fig. 2: Multiple sequence alignment of the SlPIN gene family. Two hydrophobic domains in the SlPIN proteins are underlined with solid blue boxes, while the hydrophilic loop region is underlined with a solid red box. The predicted transmembrane helix-formed regions in the primary structure of SlPIN proteins are marked. The possible phosphorylation sites are marked with triangles. The NPXXY structure is represented with a green ellipse

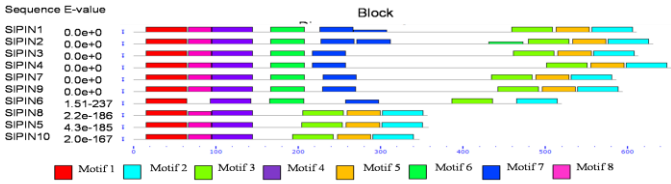

Fig. 3: Schematic distribution of conserved motifs in the SlPIN proteins. The distribution of conserved motifs in tomato identified using the MEME program is shown. Eight conserved motifs are shaded in different colours. Details of the individual motifs are shown in Supplementary Table 4. Among them, motif 3 contains the conserved structure NPXXY

Discussion

Based on the current tomato genome sequence and the SGN database, we identified 10 *PIN* genes and synthetically analysed the SlPIN family of the tomato genome. Our results showed that 10 SlPIN proteins are stable proteins, most of which are composed of basic amino acids, and are non-secreted proteins. However, the protein length of SlPIN3 and 6 and the exon numbers of SlPIN2 were different from those reported in previous studies (Zhao *et*

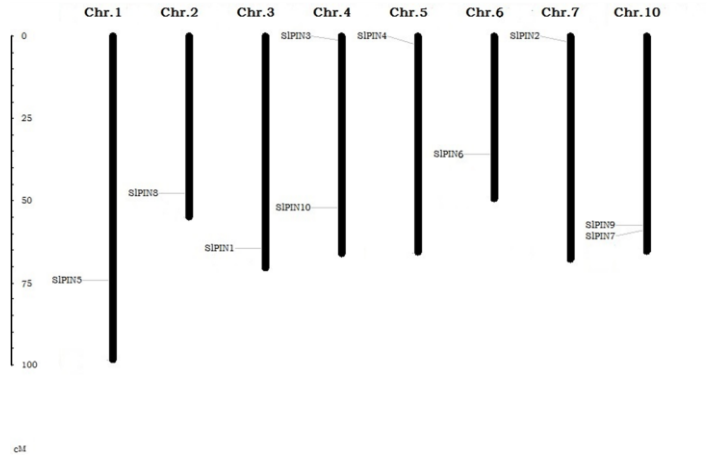

Fig. 4: Chromosomal distributions of the identified tomato PIN genes. Chromosomal positions of the PIN genes in tomato were mapped on the basis of the SGN tomato genome database. There were no PIN genes distributed on tomato chromosomes 8, 9, 11, or 12. The scale is according to physical position (Mb)

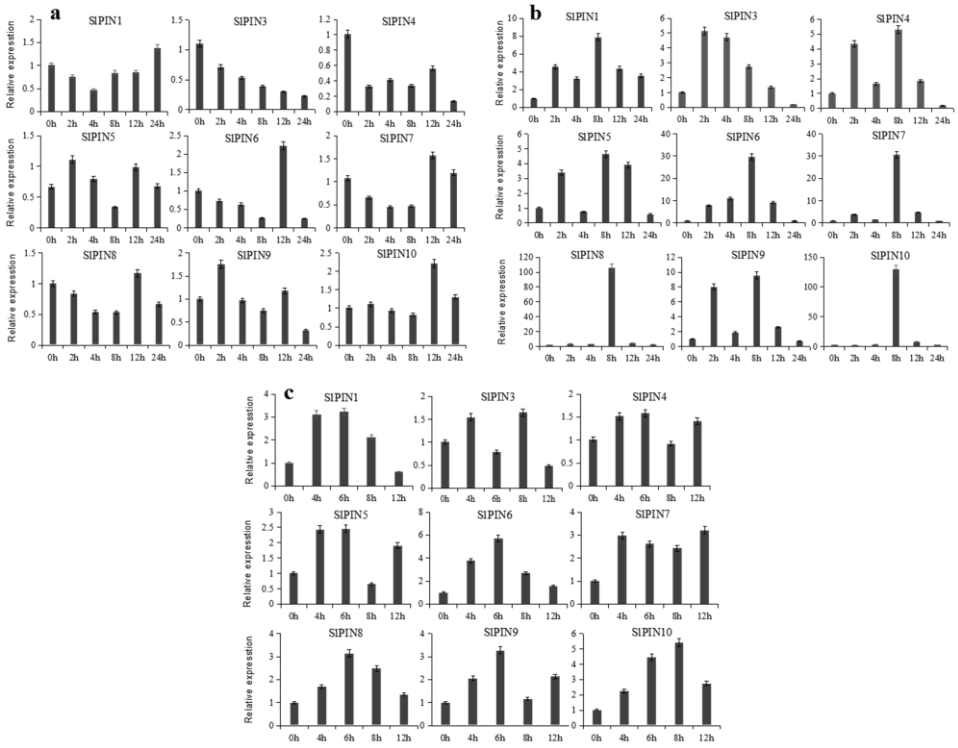

Fig. 5: Expression profiles of SlPIN genes in response to different abiotic stresses. The relative expression of SlPIN genes was analysed using qRT-PCR. The Y axis is the scale of the relative expression level; the X axis is the time course of cold stress treatment. The statistical bars indicate the relative expression of SlPIN genes in different treatments. (a) shows the expression levels of SlPIN genes under salt treatment. (b) shows the expression levels of SlPIN genes under cold treatment. (c) shows the expression levels of SlPIN genes under drought treatment

al., 2017), which may be due to differences in information collected by different databases. The secondary structure of SlPIN proteins was highly similar. It is speculated that the *SlPIN* genes have a similar function. The function of genes can be inferred according to the phylogenetic relationships among homologues from different species, thus *Arabidopsis*, sorghum and rice PIN protein families were selected as

reference sequences. Compared with rice and sorghum, the evolutionary relationship between tomato and *A. thaliana* was relatively closer, which may be because they are dicotyledons. The cluster analysis of proteins based on similarity between *AtPIN* and *SlPIN* genes sequence indicates a similar function in the species-dependent developmental processes.

In agreement with the very recent study of Mounet (Mounet *et al*., 2012). *AtPIN1* is located in root and shoot vascular tissues and embryos. This gene is involved in the regulation of the development of various organs that are involved with roots and tillers. It is speculated that *SlPIN1* may take on these functions. At present, the functions of *AtPIN6* and *AtPIN8* are not very clear; many experiments are needed to further verify and analyse these functions. Subsequent studies can refer to the expression and function of *Arabidopsis* PIN proteins and carry out the related research of tomato PIN proteins. Motif analysis showed that motifs 1, 2, 3 and 4 were present in all the members of *SlPIN*. In addition, different groups shared similar motifs, indicating that these conserved motifs might play vital roles in specific functions. Alignment of amino acid sequences of SlPIN proteins showed that the N-terminal and C-terminal regions were highly conserved, divided by the hydrophilic loop. The PIN protein hydrophilic loop can modulate intracellular auxin homeostasis, which is dependent on cell type and developmental stage (Ganguly *et al*., 2014; Yue *et al*., 2015). The relatively high amino acid identity between PIN proteins indicated that all the *PIN* genes evolved from a common ancestral sequence. Both the conserved domain and variable domain of the SlPINs may determine the specific function of these genes. These bioinformatic analyses are useful for studying the biological functions of genes.

Auxin transport plays important roles in plant growth and development by controlling a large number of auxin-responsive genes (AUX/LAX, ABCB, PIN and PILS) (Zhang *et al.*, 2014b). It has been studied that various abiotic treatments can alter auxin polarity distribution by modulating PIN protein (Friml, 2010). At the same time, PIN families are regulated under various abiotic stresses, including dehydration drought, low temperature, high temperature, salinity, and hormones in the leaves (Ranawake *et al*., 2012) and roots (Yue *et al*., 2015). However, there are few investigations of tomato PIN genes under abiotic stress. Therefore, we investigated the change of relative expression of *SlPIN* genes under abiotic stress using the above analysis as a foundation. The relative expression of *SlPIN4* decreased rapidly at the 2 h point after salt treatment, which indicated that *SlPIN4* might be involved in the mechanism of salt tolerance in tomato. Only the expression of *SlPIN3* decreased gradually under salt stress treatment without any rebound in this study, but the range of overall change of this gene was small. It has recently been reported that salt stress causes severe yield loss in salt-affected areas (Guan *et al*., 2014) and promotes auxin accumulation in the developing primordia of *Arabidopsis*. Low temperature has different effects on plant growth, photosynthesis, dry matter accumulation and carbon metabolism. The results suggested that the relative expression of most the *SlPIN* genes peaked at 8 h after cold treatment, except for *SlPIN3*. *SlPIN3* and *SlPIN6* were greatly affected by cold stress, which implied that these *SlPIN* genes play a part in the mechanism that helps tomato plants tolerate cold stress. Kyohei Shibasaki (Shibasaki *et al*., 2009) also showed that cold stress influences the polar transport of auxin through selectively inhibiting the intracellular trafficking of proteins, including auxin influx carriers and efflux carriers, and indicated that the AtPIN3 proteins might be responsible for the inhibited auxin polar transport under cold stress conditions. The expression levels of *SlPIN7*, 8 and 10 were low at the any time points, expect for 8 h point. Their more distinguishingly fluctuations were complex and should be considered as a basic information for further investigation. In maize, the expression of most *ZmPIN* genes was up-regulated by drought in the shoots but was down-regulated in the roots. Generally in a tissue-specific manner, many soybean *PIN* genes were responsive to drought conditions at the transcriptional level, at different degrees of stress. Our data showed that the relative expression level of all *SlPIN* genes was enhanced compared with that of untreated plants under drought conditions. In particular, the expression of *SlPIN1* and *SlPIN6* varied greatly under drought stress. It is suggested that tomato responds to drought stress through complicated network, which necessitates the mediated regulation of most *SlPINs*. It is important that the relative expression of *SlPIN6* significantly increased under cold and drought stress, as *SbPIN6* was inhibited by salt and drought treatments**.** These results show that *SlPIN6* were obvious relative to cold and drought. The molecular mechanism of *SlPIN6* will be studied further. Candidate genes would provide a useful reference for further functional investigation of *PIN* gene family in *Solanaceae* crops.

Conclusion

In this study, we identified total 10 *PIN* genes in the genome of tomato and classified them into two groups ("short" and "long" PINs), according to the known *Arabidopsis thaliana*, rice and sorghum PIN protein family as reference sequences, and the results of the motif analysis are in agreement with the phylogenetic tree analysis results. There are significant similarities in the secondary structure of the 10 PIN members. Alignment of amino acid sequences showed that the tomato PIN domain was highly conserved. Chromosome map positions showed that 10 *SlPIN* genes were unevenly distributed on eight chromosomes. In addition, the expression levels of SlPIN family members were affected by salt, drought and cold stresses to different degrees. More *SlPIN* genes were involved in response to drought and cold than to salt stress. They might participate in the response to

environmental stresses. These results provide fundamental information for further exploration of *PIN* genes in tomato.

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