The Journal of Animal & Plant Sciences, 29(1): 2019, Page: 232-244
ISSN: 1018-7081

CLONING AND CHARACTERIZATION OF AUXIN EFFLUX CARRIER GENES *ECPIN1C* AND *ECPIN1D* FROM FINGER MILLET *ELEUSINECORACANA* SUBSP. *CORACANA*

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ABSTRACT

Auxin is one of the most important plant hormone and its transport from the tip of the plant towards the root tip is mediated by auxin transporters. There are four different types of auxin transporters reported so far in plant kingdom. They are auxin influx carrier (AUX/LAX), auxin efflux carrier (PINs), ATP-binding cassette transporters (ABCBs), and PIN-Likes (PILS). Auxin efflux carrier mediates long as well as short distance auxin transport and plays critical role in root development in plants. Lack of genome sequence data in the crop plant *Eleusine coracana* hampers the advanced study in root development. Therefore, we cloned two auxin efflux carrier genes *EcPIN1c* and *EcPIN1d* from the orphan plant *E. coracana*. In-silico analysis revealed *EcPIN1c* and *EcPIN1d* were encoded for 1677 bp and 1716 bp, respectively. EcPIN1c and ECPIN1d were found to be localized to the plasma membrane. Multiple sequence alignment revealed presence of several conserved motifs in EcPIN1c and EcPIN1d. Functional motif analysis revealed the presence of thirty-three functional motif in EcPIN1c and thirty-one in EcPIN1d protein. Phylogenetic study showed EcPIN1c and EcPIN1d grouped with the PIN1 genes of *Oryza sativa*, suggesting these genes are monocot specific. Expression analysis showed significant up-regulation of *EcPIN1c* genes in leaf and root tissue at different developmental stages, suggesting their important role in plant development.

Key words: Auxin, influx carrier, efflux carrier, PIN, transmembrane domain, myristoylation, phosphorylation.

INTRODUCTION

An extensive and effective cellular communication is required between the tissues and cells in order to harmonize the growth and development of the plant (Gururani *et al*. 2015; Mohanta *et al*. 2015a; Singh *et al*. 2015). The coordinated growth and development of plant is mediated by highly responsible signaling molecule, commonly known as "auxin". Due to the non-polar characteristics of the auxin, it remains in the anioinic (IAA^-) form in the extracellular environment due to its low pH (5-5.5) level (Zazímalová *et al*. 2010).Because of the hydrophobic nature of the plasma membrane, IAA^-cannot pass the membrane from outside of the cell to the inside due to the presence of free carboxyl group (Zazímalová *et al*. 2010). Therefore, only IAA^+ molecule enters the cell. The pH of the cytosol remains approximately within 7 to 7.5. At such alkaline pH, the auxin molecules remain in IAA^- (anioinic) form inside the cell. Continuous accumulations of IAA^- inside the cell make it a small anion chamber. To overcome this problem, it required to efflux out the anionic IAA^- molecule outside of the cell. To conduct this process, it requires sophisticated transporter channel molecules. One of the channel molecules that conduct the transport of auxin is commonly known as auxin efflux carrier (PIN) (Friml *et al*. 2002; Křeček *et al*. 2009; Petrášek and Friml 2009; Mohanta and Mohanta 2013; Mohanta *et al*. 2014b). Considerable progress has been made in the field of PIN molecule in the plant kingdom. However, in the era of next-generation sequencing, the genome sequence of one of the most important crop plant *Eleusine coracana* is lacking.

The finger millet plant *Eleusine coracana* is a self-pollinated allotetraploid variety having 2n = 4x=36 number of chromosome in its genome(Mehta *et al*. 2016; Sood *et al*. 2016). It is an annual plant and grown as a cereal crop in the arid and semi-arid area of India and central Africa and belongs to the tribe Chlorideae of family Poaceae. There are nine species of genus *Eleusine* distributed in Asia, Africa and South America. The allotetraploid species *E. coracana* exhibits morphological similarity with *E. coracana* subsp. Africana that possess 2n = 36 and *E. indica* that possess 2n = 18 chromosome. *E. indica* is the maternal diploid genome donor of *E. caracana* subsp. *coracana* as well as *E. coracana* subsp. *Africana* (Sood *et al*. 2016). The millet *E. coracan* is used as an important staple food and it is an economically important nutraceutical crop (Kumar *et al*. 2016). This plant can able to adapt dehydration related stress conditions and posse's enormous genetic significance to grow in adverse environmental conditions. Adaptability to adverse stress conditions has made this plant an important model organism for researchers across the

globe. However, the dehydration related stress condition is highly depends upon the root system of the plant. Therefore, it is highly important to focus the research in root growth and development in *E. coracana*. The auxin efflux carrier protein plays proactive and critical role in development of root system in plants. Therefore, we cloned two auxin efflux carrier genes from *E. coracana* and reported here which can be very helpful for further advanced research.

MATERIALS AND METHODS

Plant material and growth conditions: The surface sterilized seeds of *E. coracana* were sowed in autoclaved and sterilized compost soil followed by the supply of sprinkle water at regular intervals. The sterilization of *E. coracana* seeds were done using 1% (v/v) sodium hypochlorite solution for 5 minutes followed by cleaning with 70% (v/v) ethanol for a period of 1 minute as described previously(Mohanta and Bae 2017). The seeds were cleaned with autoclaved double distilled water for thrice. Later, the seeds were put in the pots filled with compost soil and allowed them to germinate and grow in the green house. The pots were supplied with around 700 µmol m^{-2} S^{-1} light intensity for 16 hours per day. The germinated pots were allowed to grow, and leaf and root tissues of the seedlings were harvested at 7^{th}, 14^{th}, 21^{st}, and 28^{th} days, respectively. The harvested tissue samples (roots and leaves) were immediately frozen using liquid nitrogen and kept at -80° C for further analysis. All the experiments were conducted in triplicates.

Extraction of total RNA and in-vitro cDNA synthesis: To extract the total RNA, tissue samples of *E. coracana* were removed from the -80° C freezer and grinded to powder in the presence of liquid nitrogen. Trizol reagent was added to the grinded tissue samples followed by centrifugation and washing with 70% ethanol to extract the total RNA.DNAse enzyme was added to the extracted RNA sample to remove the presence of any residual DNA. The isolated RNA of leaf and root samples were used for in-vitro cDNA synthesis as described previously(Mohanta and Bae 2017). The RevertAid first strand cDNA synthesis kit was used to synthesize the first strand of the cDNA. The reaction mixture for the synthesis of cDNA was as follows; 2 µg of total RNA was mixed with 1 µl of oligo dT primers in the presence of 9 µl of nuclease free distilled water followed by incubation at 65° C for 5 minutes to remove the GC-rich secondary structure of RNA. The samples were immediately transferred to the ice followed by the addition of 4 µl of 5X reaction buffer, 1 µl Ribolock RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTP mix, and 1 µl of RevertAid reverse transcriptase (200 U/µl). The final volume of the reaction was made to 20 µl and subjected to cDNA synthesis by incubating the sample at 42° C for 60 minutes followed by termination of reaction at 70° C for 5 minutes. The synthesized cDNA was diluted 10 times with nuclease free water and stored at -80° C for further use. A PCR reaction was conducted with *actin* gene to validate the synthesis of cDNA. The PCR reaction of actin gene was checked in agarose gel electrophoresis.

Primer designing& cloning: Due to the lack of genome sequence data of *E. coracana*, the orthologous gene sequence of *OsPIN1c*(LOC_OsOs11g04190) and *OsPIN1d*(LOC_Os12g04000) was taken from the rice genome annotation database to design the primers for *EcPIN1c* and *EcPIN1d*. The forward and reverse primers for *EcPIN1c* and *EcpIN1d* genes were designed manually. The forward and reverse primer of *EcpIN1c* were 5'-ATGATCACGGTGGTGGACCT-3' and 5'-GAGCCCCAGCAGTATGTAGT-3', respectively whereas the forward and reverse primers of *EcPIN1d* were 5'-ATGGAGCAGTTCCCGGACAC-3' and 5'-GAGCCCCAGCAGTATGTAGT-3', respectively. Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) software was used to design the primers for qRT-PCR analysis. The full-length coding CDS sequences of *EcPIN1c* and *EcPIN1d* were used to design the primers for qRT-PCR analysis. The forward and reverse primers of *EcPIN1c* for qRT-PCR analysis were 5'-TCATCGTCCTCGCCCTCCTC-3'and 5'-TGAGGCTGCCGGAGTCGGCG-3', respectively whereas the forward and reverse primers of *EcPIN1d* were 5'-CCGAGCAGTTCCCGGACACC-3' and 5'-TCAGTGTGGTTGAAGCTGGA-3', respectively. To clone the full-length gene of *EcPIN1c* and *EcPIN1d*, 20 µl PCR reaction mixtures was prepared for each gene. The PCR reaction mixture contained 1 µl of first strand cDNA supplied with 4 µl of high fidelity 5x phusion buffer, 1 µl (10 µM) of each of forward and reverse primer, 0.5 µl of dNTPs (10 mM), 2 µl DMSO solution, 0.1 µl of high-fidelity DNA polymerase and 10.4 µl of nuclease free water. The thermal profile of the polymerase chain reaction (PCR) was followed as described previously(Mohanta and Bae 2017). In short, the thermal profile was followed by initial denaturation at 95° C for 5 minutes, followed by the second step of 35 cycle denaturation at 95° C for 30 s, annealing of primers at 58° C for 30 s, and polymerization at 72° C for 2 minutes. The final chain extension of PCR reaction was kept at 72° C for 5 minutes. Upon completion of PCR reaction, the PCR tubes were removed from the thermal cycler and loaded and separated in agarose gel electrophoresis. The agarose gel was visualized under UV-irradiation and resulted bands were isolated and eluted for cloning. The eluted PCR product of *EcPIN1c* and *EcPIN1d* were cloned in pGEMT vector. The cloned *pGEMT:EcPIN1c* and *pGEMT:EcPIN1d* genes were subsequently sequenced to find out the full length CDS of *EcPIN1c* and *EcPIN1d* gene, respectively.

Bioinformatics analysis: The sequences resulted from the clone of *pGEMT:EcPIN1c* and *pGEMT:EcPIN1d* were translated to the protein sequences using the online server ExPASy translation tool (http://web.expasy.org/translate/). Besides this, the sequences of *EcPIN1c* and *EcPIN1d* were further re-checked for its translation in Emboss Transeq server of EMBL(http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The isoelectric point and molecular mass of EcPIN1c and EcPIN1d were calculated using the protein calculator version 3.5 (http://protcalc.sourceforge.net/)and the presence of transmembrane domains was studied using TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/)(Kahsay *et al*. 2005).The presence of motifs in EcPIN1c and EcPIN1d were studied using MEME suit software (http://meme-suite.org/).Molecular structure of EcPIN1c and EcPIN1d was modeled using Swiss model workspace (http://swissmodel.expasy.org/workspace/) (Arnold *et al*. 2006).

Construction of phylogenetic tree: To construct the phylogenetic tree, the protein sequences of PIN protein of *Oryza sativa* and *Arabidopsis thaliana* were downloaded from the rice genome annotation database and "The Arabidopsis information portal" (TAIR). Along with the PIN proteins of *E. coracana*, the PIN proteins of *A. thaliana* and *O. sativa* were aligned to obtain a clustal file using the online server MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). The clustal file was later converted MEGA file format using MEGA6 software (Tamura *et al*. 2013). The aligned MEGA file of PIN proteins was uploaded to MEGA6 software to construct the phylogenetic tree using maximum likelihood statistical method and Jones-Taylor-Thornton model/method with 1000 boot strap replicates as described previously (Kanchiswamy *et al*. 2013; Mohanta *et al*. 2014a; Mohanta *et al*. 2015a; Mohanta *et al*. 2015b; Mohanta *et al*. 2015c).

Quantitative real time PCR (qRT-PCR): The expression levels of *EcPIN1c* and *EcPIN1d*genes were studied using the quantitative real time PCR (qRT-PCR) system Mx3000P (Stratagene, Santa Clara, CA, USA). The real time PCR reaction was performed in 20 µl reaction mixture. The reaction mixture contained 12.5 µl of SYBR green/ROX master mix (Fermentas, USA), 1 µl of template first strand cDNA, 1 µl of 10 µM forward and reverse primers and 4.5 µl of nuclease free water. The *actin* gene of *E. coracana* was used as an internal reference gene to normalize the expression of the gene of interest. All experiments were conducted with three biological replicates and each biological replicate had three technical replicates as well. The thermal profile of qRT-PCR reaction was started with the initial denaturation at 95^{o} C for 10 minutes followed by 40 cycles of second step that contained 95^{o} C for 30 seconds, primer annealing at 60^{o} C for 30 seconds, polymerization at 72^{o} C for 30 seconds. The expression profiles of *EcPIN1c* and *EcPIN1d* were calculated using the $2^{-\Delta\Delta Ct}$ method(Schmittgen and Livak 2008).

RESULTS AND DISCUSSION

The orphan plant finger millet has several agronomic characteristics that make it an indispensable crop in tribal, hilly, arid and semi-arid area of Africa and India. The rapid development of next generation sequencing technology led to dissect the complete genome sequence of crop plant maize, rice and wheat. Due to the bigger genome size, the genome sequencing of *E. coracana* has not been succeeded yet. However, it should not be the factor to stop the advanced research for the highly important crop plant. Due to its characteristic stress tolerant properties, the plant can able to grow in arid, and semi-arid area with minimum requirement of water. It absorbs ground water for its growth and development and hence it requires a strong root system to further strengthen its growth potential. The role of auxin efflux carrier is eminent in plant root development(Friml *et al*. 2002; Blilou *et al*. 2005; Rebouillat *et al*. 2009). However, due to the lack of genome sequence data, it is very difficult to conduct the advanced molecular work for root development in *E. coracana*. Therefore, we decided to clone a few auxin efflux carrier genes from *E. coracana*. The auxin efflux carrier gene sequences of *O. sativa* were used as orthologous query genes to clone the auxin efflux carrier of *E. coracana*. During our study, we cloned two auxin efflux carrier genes from *E. coracana* and named them as *EcPIN1c* and *EcPIN1d*. The nucleotide sequences of both the genes were translated to the protein sequences using protein translation tool Emboss Transeq server of EMBL. The nucleotide and protein sequences of both the genes were subjected to BLASTP and BLASTN analysis in NCBI database. The BLASTP result of EcPIN1c showed 90% and EcPIN1d showed 99% similarities with the auxin efflux carrier proteins of *O. sativa*. The result confirmed that the cloned genes belonged to the *EcPIN* genes. The nucleotide sequences of *EcPIN1c* and *EcPIN1d* were submitted to the NCBI database and the accession number are MF135231 (*EcPIN1c*) and MF135232 (*EcPIN1d*).

The sequencing report revealed that the cloned *EcPIN1c* was 1677 bp and *EcPIN1d* was 1716 bp long and was predicted to possess 558 and 572 amino acids respectively (Supplementary Figure 1 &Supplementary Figure 2). Pair wise nucleotide sequence alignment between *EcPIN1c* and *EcPIN1d* revealed the presence of 66.2% similarity between them whereas, the pair wise sequence alignment between EcPIN1c and EcPIN1d protein revealed the presence of 60.6% similarity. The molecular mass of EcPIN1c and EcPIN1d was found to be 59.292 and 62.022 kDa, respectively whereas the

isoelectric point (pI) at pH 7.0 was found to be 7.61 and 8.85, respectively. The estimated charge of EcPIN1c and EcPIN1d at pH 7.0 was found to be 3.4 and 8.0, respectively (Supplementary Table 1). The charges of EcPIN1c and EcPIN1d decreased upon the increase in pH. However, there was a surprising difference of charge between EcPIN1c (3.4) and EcPIN1d (8) at pH 7 (Supplementary Table 1). The variations of charge atpH7 were might be due to the difference of side chain amino acids and free amino acids. The sulphur and free oxygen atom provide negative charges to the proteins. There are 24 sulphur and 763 oxygen atoms in EcPIN1c and 30 sulphur and 795 oxygen atoms in EcPIN1d, respectively (Supplementary Table 1).

Multiple sequence alignment was conducted to find out the conserved motifs in EcPIN proteins. Therefore, EcPIN proteins were aligned with the PIN proteins of *O. sativa.* Sequence alignment of long domain PIN proteins of *O. sativa* with EcPINs showed the presence of highly conserved consensus sequences at N- and C-terminal domain (Supplementary Figure 3). The major conserved motifs of long transmembrane domain PIN proteins were Y-H-V-x-T-A-x-V-P-L-Y-V-A-M-x-L-A-Y-x-S-V-x-W-W-R-I-F-x-P-D-Q-C-S-G-I-N-R-F-V-A-L-F-A-V-P-L-L-S-F-H-F-I-S-T-N-N-P-Y-x-M-N-L-R-F-x-A-A-D-T-L-Q-K-L-x-V-L-A-M-L,L-S-x-R-G-S-L-E-W-x-I-T-L-F-S-L-S-T-L-P-N-T-L-V-M-G-I-P-L-L-K-G-M-Y S-G-S-M-V-Q-I-V-V-L-Q-C-I-I-W-Y-T-L-M-L-F-L-F-E-Y-R-G-A-R, L-V-M-E-Q-F-P-x-T-A, V-D-x-D-V-V-S-L, D/E-V-x-E-D-G-x_3-V-T-V-R-x-S-x_2-S-R-S-D/E, E-I-Y-S-L-Q-S-S-R-N-P-T-P-R-G-S-S-F-N-H-x-D/E-F-x_3-V-G, D-L-H-M-F-V-W-S-S-S-A-S-P-V-S-D/E, D-D/E-F-S-F-G-N, A-M-P-P-S-V-M-T-R-L-I-L-I-M-V-W-R-K-L-I-R-N-P-N-T-Y-S-S-L-x-G-x-I-W-S-L-V-x-F-R-W, E-M-P-A-I-I-x_2-S-I-S-I-L-S-D-A-G-L-G-M-A-M-F-S-L-G-L-F-M-A-L-Q-P-x-I-I/M-A-C-G-N, and F-A-M-A-V-R-F-L-x-G-P-A-V-M-A-A-A-S-x-A-V-G-L-R-G-x-L-L-H-V-A-I-V-Q-A-A-L-P-Q-G-I-V-P-F-V-F-A-K-E-Y-x-V-H-P-x-I-L-S-T-A-V-I-F-G-M-L-I-A-L-P-I-T-L-V-Y-Y-I-L-L-G-L. Conserved two to three amino acid repeat sequences were found in PIN proteins, suggesting their possible role in auxin transport. The repetitive motifs were A-A-D, A-A/G-A, A-A-A and A-A-L; S-S-R, S-S-A, S-S-F, S-S-L; M-V-Q, M-V-W; P-N-T-L, P-N-T-Y, A-M-P, A-M-F; and S-L-S, S-L-G (Supplementary Figure 3).

Transmembrane domain analysis was conducted to confirm whether EcPIN1c and EcPIN1d protein posse's membrane spanning transmembrane domain. It was found that both EcPIN1c and EcPIN1d posses membrane spanning transmembrane helices. Both proteins were found to posses nine transmembrane helices (Supplementary Figure 4). Previous study revealed the presence of similar number of transmembrane helices in EcPIN1a and EcPIN1b(Mohanta and Bae 2017). Analysis has revealed that the N-terminal domain of EcPIN1c was present outside of the plasma membrane whereas the N-terminal domain of EcPIN1d present towards the cytosol. The three-dimensional molecular structure of a protein can helps to reveal the exact mechanism and binding site as well as efficacy of a molecule with the protein. Due to the lack of molecular structure of PIN protein, it is yet to determine the molecular mechanism of auxin transport by these proteins. Therefore, we modeled the molecular structure of EcPIN1c (Figure 1) and EcPIN1d (Figure 2) protein using Phyre^2 server (Kelley and Sternberg 2009). The modeled molecular structure of EcPIN proteins was resembled to the molecular structure of sodium/bile acid/cation/ABC transporter with more than 98% confidence. The Ramachandran plot of EcPIN1c and EcPIN1d were fall in the favorable region, suggesting error free model of EcPIN1c and EcPIN1d (Figure 1 &Figure 2).

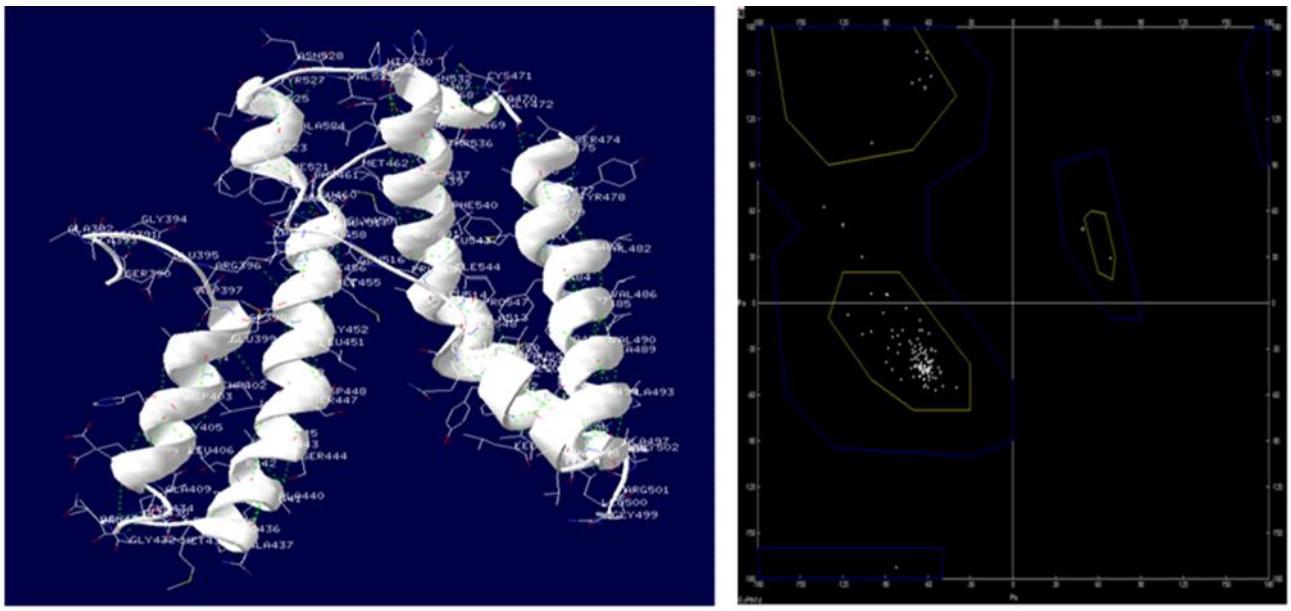
Functional motif analysis was carried out for EcPIN1c and EcPIN1d protein using the motif scan software of ExPASy bioinformatics portal. It revealed the presence of at least thirty-three functional motifs in EcPIN1c whereas thirty-one in EcPIN1d protein (Supplementary Table 2). From thirty tree motifs of EcPIN1c, three belonged to membrane transport; eight belonged to protein kinase phosphorylation site (PKC), three belonged to casein kinase 2 (CK2) site, two belonged to amidation, three belonged to Asn glycosylation, and fourteen belonged to myristoylation sites. Similarly, EcPIN1d contained one membrane transport, one CAMP phosphorylation site, nine PKC phospho sites, five CK2 sites, one amidation site, three Asn glycosylation sites, and eleven myristoylation sites. In a comparative study, it was found that EcPIN1c contained eight PKC phosphorylation sites whereas EcPIN1d contained nine; EcPIN1c contained three CK2 phospho sites whereas EcPIN1d contained five; EcPIN1c contained two amidation sites whereas EcPIN1d contained only one; EcPIN1c contained fourteen myristoylation sites whereas EcPIN1d contained only eleven. However, both proteins were found to contain three Asn glycosylation sites. The presence of these functional motifs clearly indicated that these motifs are most probably plays critical role in the function of PIN proteins. Most specifically, the presence of PKC and CK2 phosphorylation site in PIN protein indicates possible phosphorylation of PINs by PKC and CK2 kinase. Sub-cellular localization of proteins and their targeting requires signaling sequences(Mohanta *et al*. 2015d). The presence of several myristoylation sites in EcPIN protein reflects that PIN proteins requires myristoylation event to localize in the plasma membrane in a polar manner(Campos-Soriano *et al*. 2011; Moriya *et al*. 2013; Asai *et al*. 2013). The glycans serves variety of functional roles in membrane bound proteins and hence presence of

glycosylation site in EcPIN proteins might be crucial for their functional significance.

A phylogenetic tree was constructed to understand the evolutionary relationship of EcPIN proteins with the PIN proteins of *A. thaliana* and *O. sativa*. Phylogenetic analysis of EcPIN1c and EcPIN1d proteins with AtPINs and OsPINs showed the presence of three phylogenetically distinct groups. All the PIN1 proteins were found to fall in group A (fuschia) whereas PIN3, PIN4 and PIN7 were found in group B (green) and PIN2, PIN5, PIN6 and PIN7 were found in group C (red) (Figure3). This confirms that the PIN proteins EcPIN1c and EcPIN1d cloned from *E. coracana* belonged to PIN1 group. The EcPIN1c and EcPIN1d were grouped with the PIN proteins of *O. sativa*. This suggests that EcPIN proteins are monocot specific and evolved from the common ancestor of monocot plant lineages.

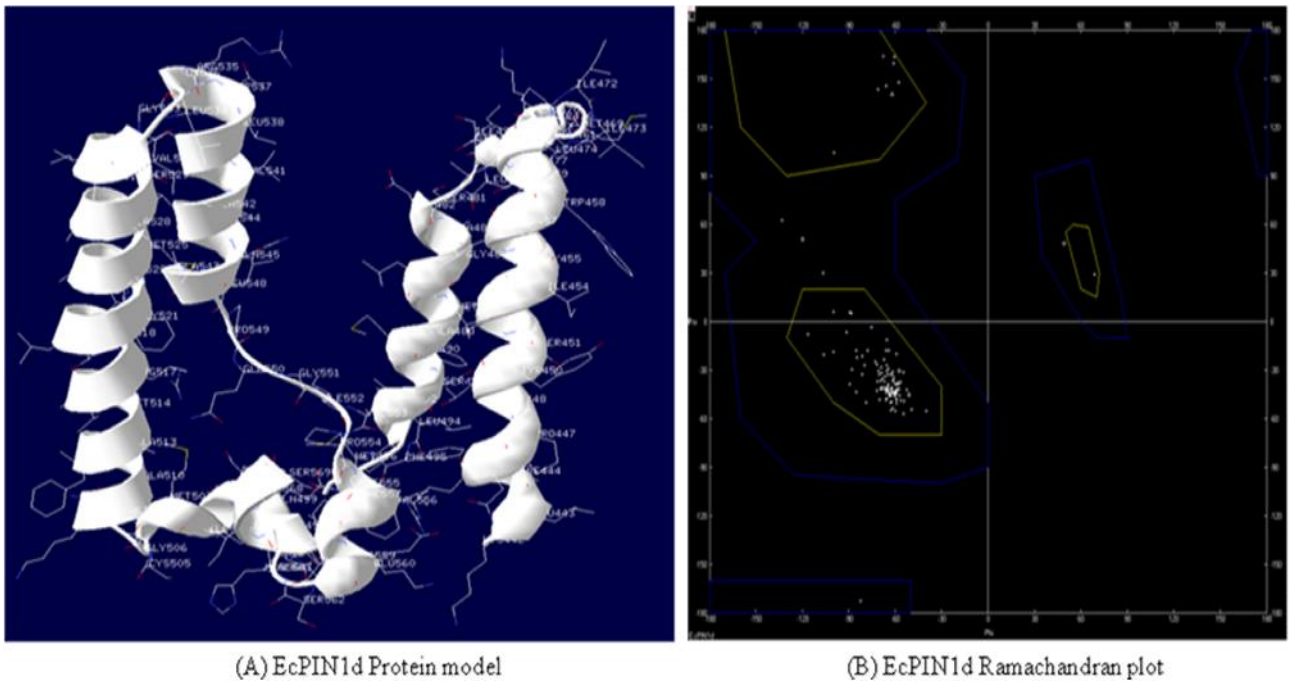
Expression analysis of *EcPIN1c* and *EcPIN1d* was conducted to understand their transcript level at different developmental stages to understand their role in regulation of plant growth and development. The expression profile of *EcPIN1c* was found to be up-regulated in both the leaf and root tissues in all the studied time points. In leaf tissues, *EcPIN1c* was found to be up-regulated 3.83 folds at 7 days' time period followed by up-regulation of 3.5 folds in 21 days and 15.56 folds up-regulated at 28 days (Figure 4). It was slightly less up-regulated at 14 days time period. Like leaf tissues, the expression of *EcPIN1c* was found to be up-regulated in root tissues as well. Maximum up-regulation was found at 14 days (6.91 fold) and 21 days (3.65 fold) time period. Compared to *EcPIN1c*, the expression of *EcPIN1d*in leaf tissues was found to be down regulated at 7, 14, and 21 days time period (Figure 4). However, up-regulation of *EcPIN1d* was observed at 28 days time period. In root tissues, the expression of *EcPIN1d* was found to be dynamic. At seven days time period, it was found to be down regulated whereas up-regulated at 14 days time period. Similarly, it was again found to be down regulated at 21 days whereas up-regulated at 28 days time period (Figure 4). However, *EcPIN1d* was found to be down regulated at 7- and 21-days' time period whereas found to be up-regulated at 28 days time period in leaf and root tissues (Figure 4).

In multicellular organisms, comprehensive communication between the cells and tissues is very important to coordinate the growth and development. Plants have its inherent signaling molecule which is known as auxin that mediates short-and long-distance communication. Since long days, the directional (polar) movement of auxin is well elucidated(Reinhardt *et al*. 2003; Zažímalová *et al*. 2007; Bandyopadhyay *et al*. 2007; Viaene *et al*. 2013). This polar auxin transport is tightly regulated. The polar auxin transport occurs in cell-to-cell manner at 5-20 mm/h (Michniewicz *et al*. 2007). The polar auxin transport is responsible for long distance (whole plant) and short-distance (within specific tissues) auxin distribution (Petrášek and Friml 2009; Zazímalová *et al*. 2010; Raven 2013). These polar transports of auxin molecule are carried out by auxin efflux carrier, which are commonly known as PIN proteins (Kleine-Vehn *et al*. 2008; Křeček *et al*. 2009). The major auxin flow can be traced from the apical tissue of the plant towards the root tip. The downward auxin flow can be observed in vascular cambium and xylem parenchyma (Burg and Burg 1967; Petrášek and Friml 2009). However, it is also found in peripheral cells of the plant(Boonsirichai *et al*. 2003). This suggests that auxin can be distributed laterally in stem as well. Once the auxin reaches to the root tip (acropetal) through vascular tissue, it is redirected upwards (basipetal) the root elongation zone and recycled back to vascular tissue(Rashotte *et al*. 2000; Zazímalová *et al*. 2010). Wang *et al*., (2009) reported tissue specific expression of *OsPIN* genes and found that *OsPIN1b* was highly expressed in stem, leaves and young panicles (Wang *et al*. 2009). Low expression of *OsPIN1c* was observed in young panicles. Further, *OsPIN1b*, and *OsPIN1c* were predominantly expressed in the stele whereas *OsPIN1b* and *OsPIN1c* were detected in meristems(Wang *et al*. 2009). Mutational analysis revealed that *AtPIN1* is responsible for embryogenesis, organogenesis, and photo tropism (Paponov *et al*. 2005). *A. thalianapin1* mutant resulted in PIN like deformed inflorescence. This signifies the importance of *PIN* genes and the cloning of *EcPIN* genes will be very valuable for conducting further research to understand the root development in *E. coracana*.



(A) EcPIN1c Protein model (B) EcPIN1c Ramachandran plot

Figure 1: Molecular modeling of EcPIN1c protein. (A) Molecular model of EcPIN1c protein, (B) Ramachandran plot of EcPIN1c protein..



(A) EcPIN1d Protein model (B) EcPIN1d Ramachandran plot

Figure 2: Molecular modeling of EcPIN1d protein. (A) Molecular model of EcPIN1d protein, (B) Ramachandran plot of EcPIN1d protein.

Figure 3: Phylogenetic tree of EcPIN1c and EcPIN1d proteins with the PIN proteins of *A. thaliana* and *O. sativa*. The phylogenetic tree grouped into three major groups where all PIN1 proteins were clustered together. EcPIN1c and EcPIN1d proteins were grouped with the PIN proteins of *O. sativa*. This suggests that EcPIN1c and EcPIN1d proteins are monocot specific.

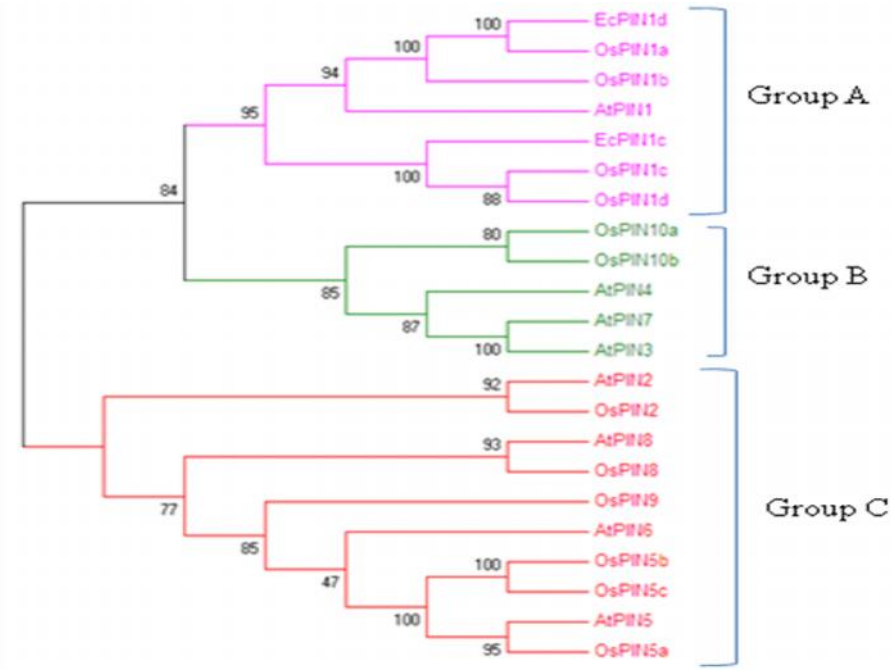
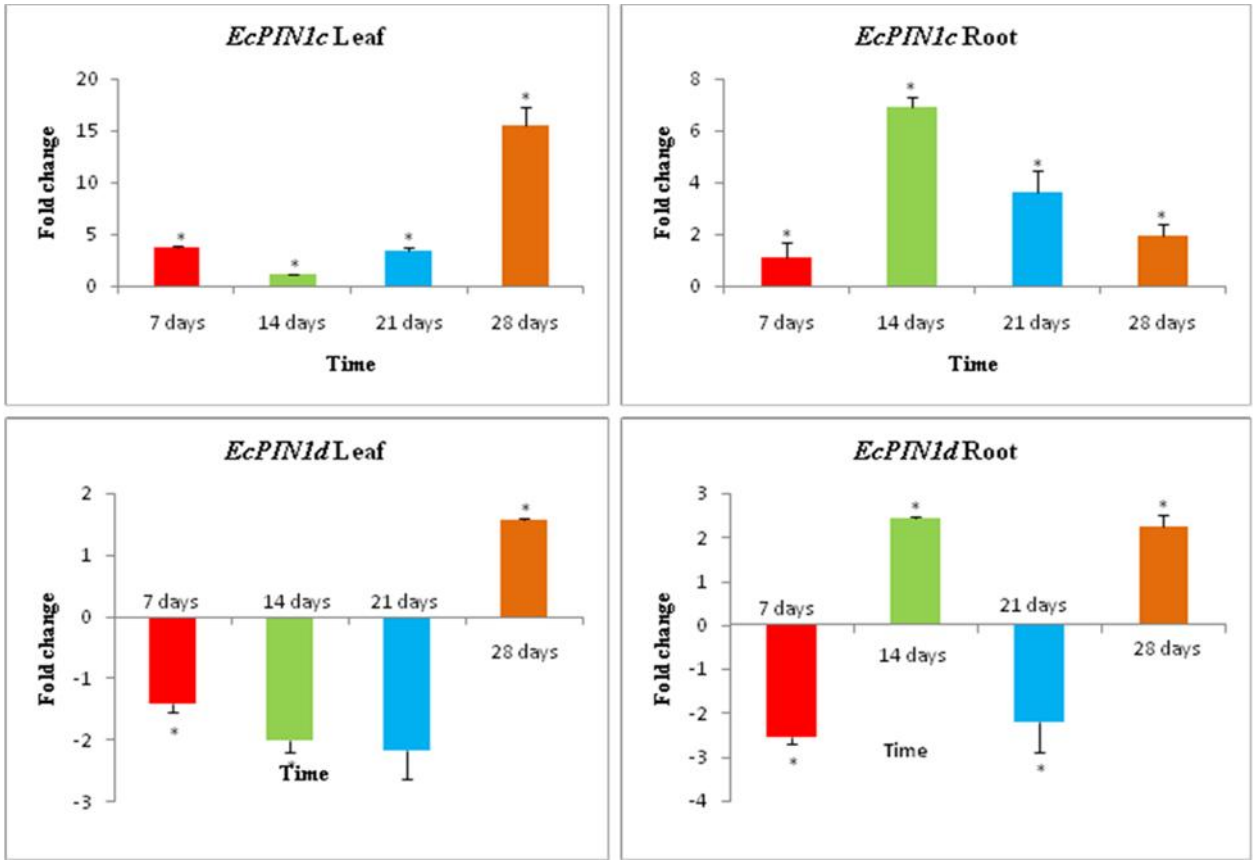


Figure 4: Differential expression of *EcPIN1c* and *EcPIN1d* genes at different developmental stages. Analysis showed, EcPIN1c gene in leafs and roots was up-regulated at all the time point where as the expression pattern of EcPIN1d was dynamic. Asterisk in the graph indicates statistically significant differences (*≤0.05). Statistical analysis was conducted using unpaired *t*-test.



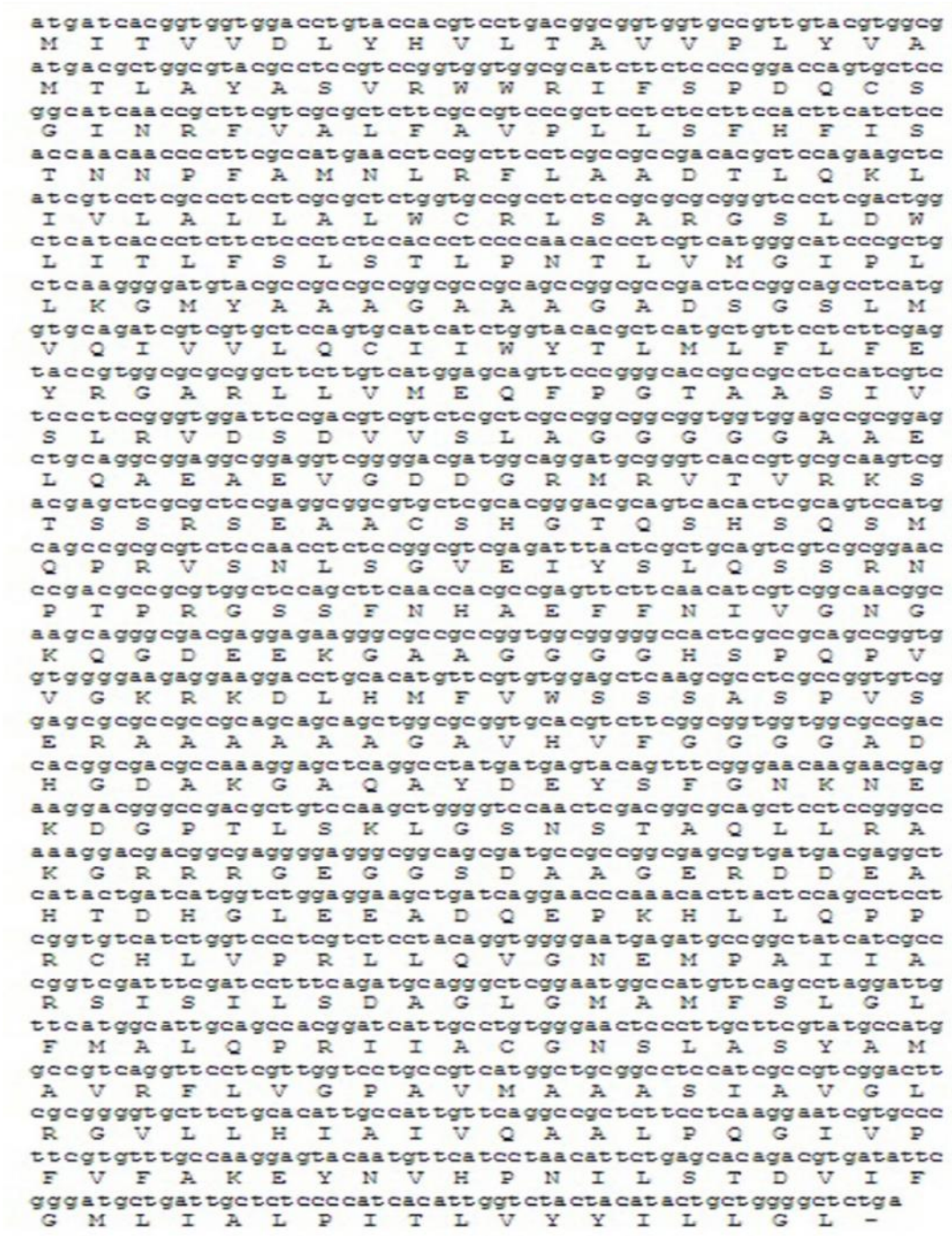
Supplementary Table 1. Table showing molecular details and charges of EcPIN1c and EcPIN1d at different pH in *E. coracana*.

Atoms	EcPIN1c	EcPIN1d
Carbon	2650	2789
Hydrogen	4200	4373
Nitrogen	732	745
Oxygen	763	795
Sulphur	24	30
pH	**Charge**	
4.00	45.8	47.3
4.5	33.9	34.9
5.0	23.2	23.8
5.5	16.8	17.5
6.0	12.4	13.9
6.5	7.7	10.8
7.0	3.4	8.0
7.5	0.5	6.1
8.0	-1.7	4.5
8.5	-4.4	2.2
9.0	-7.9	-1.2
9.5	-13.0	-7.3
10.0	-20.6	-17.2

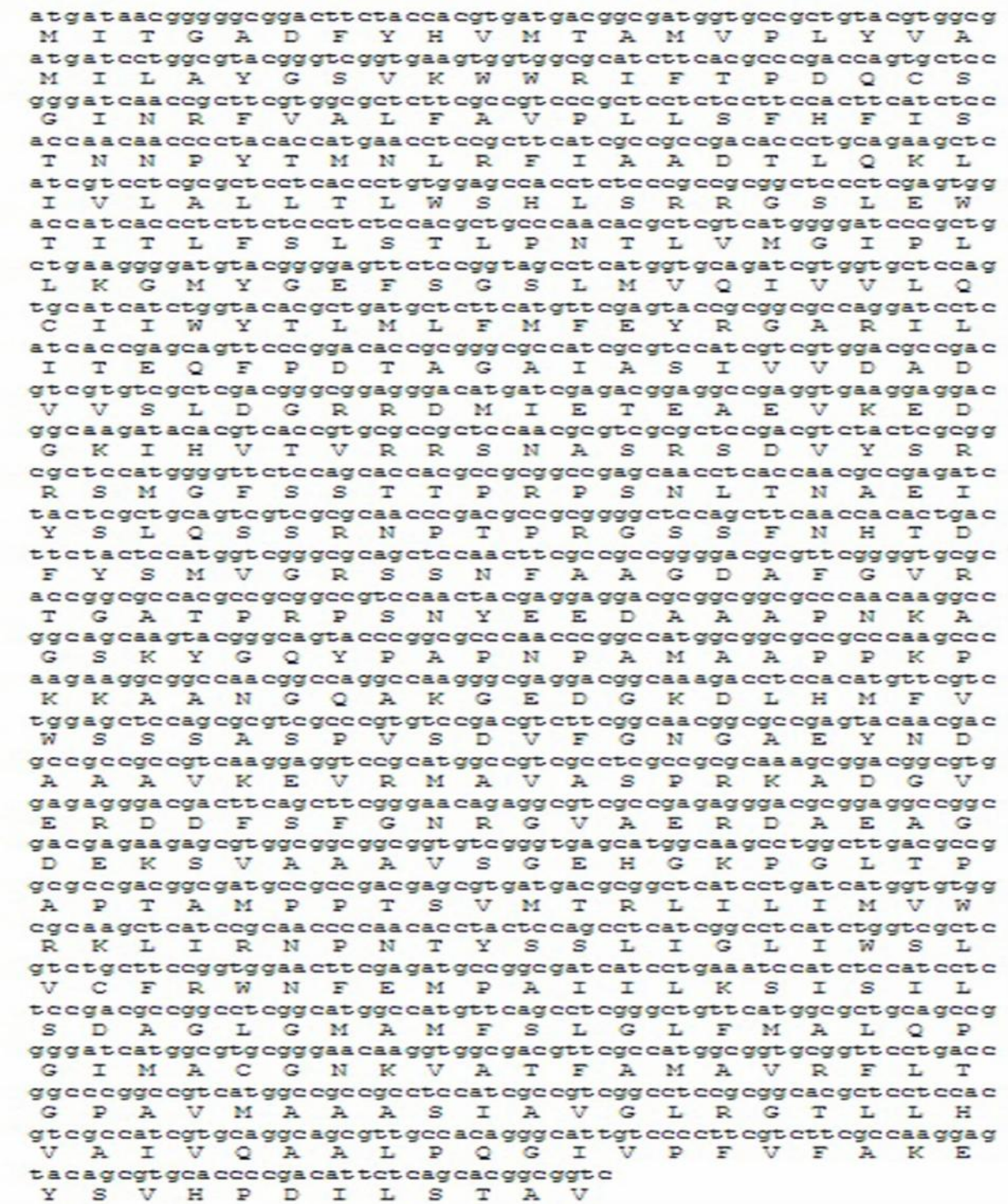
Supplementary Table 2. Putative functional motifs of EcPIN1c and EcPIN1d proteins of *E. coracana*.

EcPIN1c		EcPIN1d	
Position	**Domain**	**Position**	**Domain**
9-555	Membrane transport	9-572	Membrane transport
434-553	Membrane transport	94-97	CAMP phospho site
9-415	Membrane transport		
320-322	PKC phospho site	373-375	PKC phospho site
262-264	PKC phospho site	284-286	PKC phospho site
257-259	PKC phospho site	250-252	PKC phospho site
222-224	PKC phospho site	245-247	PKC phospho site
216-218	PKC phospho site	229-231	PKC phospho site
181-183	PKC phospho site	219-221	PKC phospho site
93-95	PKC phospho site	206-208	PKC phospho site
27-29	PKC phospho site	93-95	PKC phospho site
		27-29	PKC phospho site
3-6	CK2 phospho site	3-6	CK2 phospho site
223-226	CK2 phospho site	193-196	CK2 phospho site
248-251	CK2 phospho site	213-216	CK2 phospho site
		236-239	CK2 phospho site
		288-291	CK2 phospho site
301-304	Amidation	185-188	Amidation
381-384	Amidation		
246-249	Asn glycosylation	211-214	Asn glycosylation
372-375	Asn glycosylation	234-237	Asn glycosylation
218-221	Asn glycosylation	257-260	Asn glycosylation
123-128	Myristoylation	170-175	Myristoylation
129-134	Myristoylation	253-258	Myristoylation
174-179	Myristoylation	278-283	Myristoylation
193-198	Myristoylation	301-306	Myristoylation
265-270	Myristoylation	326-331	Myristoylation
288-293	Myristoylation	355-360	Myristoylation

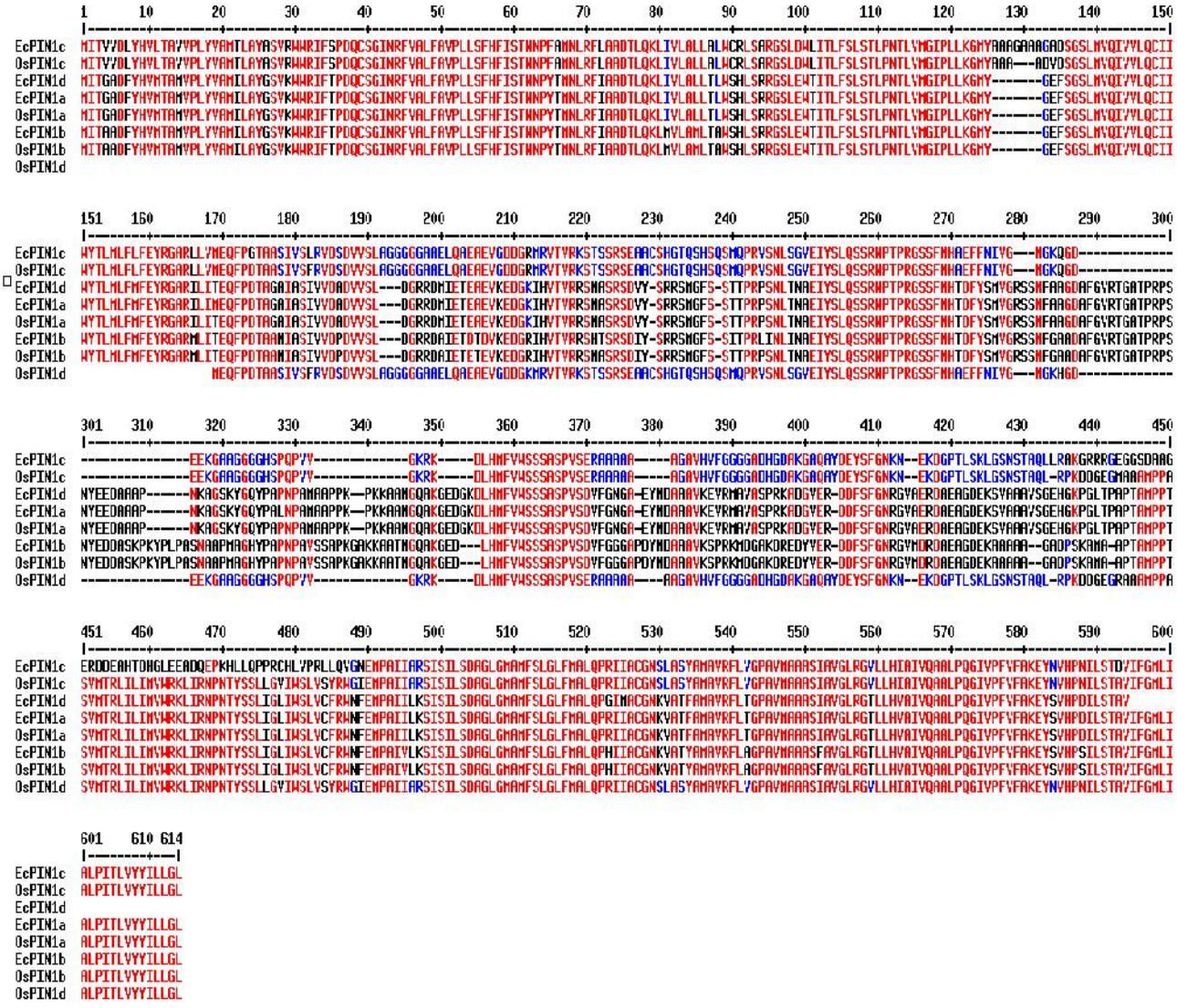
335-340	Myristoylation	455-460	Myristoylation
370-375	Myristoylation	484-489	Myristoylation
388-393	Myristoylation	493-498	Myristoylation
405-410	Myristoylation	501-506	Myristoylation
450-455	Myristoylation	533-538	Myristoylation
459-464	Myristoylation		
472-477	Myristoylation		
541-546	Myristoylation		



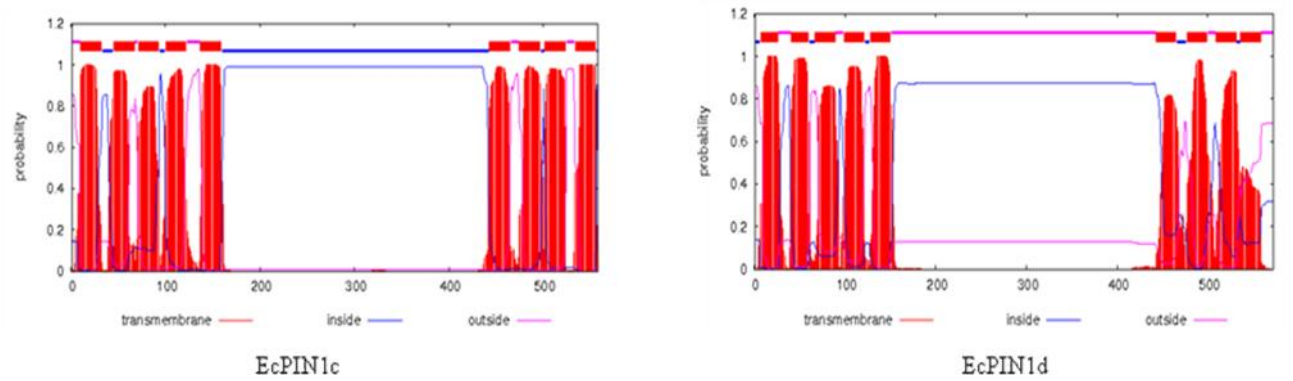
Supplementary Figure 1: Cloned nucleotide sequence of *EcPIN1c* gene. It contained 1677 nucleotides that encoded for 558 amino acids.



Supplementary Figure 2: Cloned nucleotide sequence of *EcPIN1d* gene. It contained 1716 nucleotides that encoded for 572 amino acids.



Supplementary Figure 3: Multiple sequence alignment of EcPIN1c and EcPIN1d proteins with the long domain PIN proteins of *O. sativa*. Alignment showed the presence of several conserved motif between PIN proteins of *O. sativa* and *E. coracana*.



Supplementary Figure 4: TMHMM analysis for the presence of transmembrane domains in EcPIN1c and EcPIN1d proteins. Analyses revealed presence of nine membrane bound transmembrane helices in EcPIN1c and EcPIN1d.

Conclusions: Cloning and characterization auxin efflux carrier genes *EcPIN1c* and *EcPIN1d* were done in the finger millet plant *E. coracana*. Expression analysis revealed up-regulation of EcPIN1c genes in leaf and root tissues. *EcPIN1d*gene of leaf tissues was down regulated at 7, 14 and 21 days and up-regulated at 28 days time period whereas dynamic regulation was observed in root tissues. The PIN proteins were found to possess two to three amino acids repetitive sequences and deciphering their role will be very crucial to understand the auxin transport and signaling mechanism in plant.

Competing of Interest: Author declares there is no competing of interested towards the publication of this manuscript.

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