Research Article

Genetic Diversity Analysis of Major Intrinsic Proteins (MIPs) among Maize (*Zea mays* L.), Chickpea (*Cicer arietinum* L.) and Barrel Medic (*Medicago truncatula* L.) by using Phylogenetic Inferences

Muhammad Amir Maqbool[1]*, Muhammad Aslam[1], Waseem Akbar[2], Muhammad Waheed Anwar[3] and Ehtisham Shakeel Khokhar[1]

[1]Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan; [2]Maize and Millets Research Institute (MMRI), Yousafwala, Punjab, Pakistan;[3]Food and Agriculture Organization (FAO) of the United Nations, Islamabad, Pakistan.

Abstract | Major intrinsic proteins (MIPs) of chickpea (*Cicer arietinum* L.), barrel medic (*Medicago truncatula* L.) and maize (*Zea mays* L.) were targeted in current studies. Amino acid sequences of major intrinsic proteins of chickpea, barrel medic and maize were retrieved from NCBI database followed by BLASTP. Pairwise and multiple alignment of sequences was done by using ClustalX software, and phylogenetic trees were constructed by using MEGA5 software. *Medicago truncatula* found to have total 35 MIPs which further comprised of 10 *Mt*PIPs, 6 *Mt*TIPs, 17 *Mt*NIPs and 2 *Mt*SIPs. *Cicer arietinum* genome comprised of 41 MIPs which were consisted of 10 *Ca*PIPs, 12 *Ca*TIPs, 15 *Ca*NIPs and 4 *Ca*SIPs protein sequences. Genome of *Zea mays* was carrying 36 *Zm*MIPs which were categorized into 12 *Zm*PIPs, 13 *Zm*TIPs, 8 *Zm*NIPs and 3 *Zm*SIPs. Phylogenetic trees were constructed separately for every sub-families of barrel medic, chickpea and maize. PIPs were consisted of two main groups for all of three crops which were named as PIP-A and PIP-B. NIPs clustered into six subgroups which were named as NIP-1, NIP-2, NIP-3, NIP-4, NIP-5, and NIP6. TIPs and SIPs were scattered in four groups and entitled as TIP-1, SIP-1, TIP-2, SIP-2, TIP-3, SIP-3 and TIP-4 respectively. Collective phylogenetic tree was constructed for all of MIPs which showed that PIPs, TIPs, NIPs and SIPs of all of three crops clustered in respective groups and no outliers were present.

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***Correspondence** | Muhammad Amir Maqbool, Department of Plant Breeding and Genetics, University of Agriculture Faisalabad, Pakistan; **Email:** amirmaqbool2269@gmail.com
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Introduction

Aquaporin proteins are water channels which enables the water movement across the cellular membranes. Radial and across movement of water is very important for living, turgor maintenance, water homoeostasis, regulation of stomatal conductance, expansion of cellular volume, controlling water movement (Majeran et al., 2008) and CO_2 diffusion (Flexas et al., 2006). Aquaporins also have role in boron, ammonia, silicon, hydrogen peroxide, lactic acid, arsenic and urea transportation (Hove and Bhave, 2011).

Aquaporin proteins in plants are very divers and these are channels of major intrinsic proteins. Based on cellular localization and sequence homology, these

proteins are categorized into seven subfamilies; plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins (SIPs), NOD26-like intrinsic proteins (NIPs), hybrid intrinsic proteins (HIPs), GlpF-like intrinsic proteins (GIPs) and unclassified X intrinsic proteins (XIPs) (Danielson and Johanson, 2010). These subfamilies are further divided into groups depending on similarity of sequences.

MIPs mainly involve in transport of water, TIPs transport ammonia and urea, NIPs involve in glycerol transport, PIPs are involved in CO_2 diffusion across the leaf and SIPs are not studied in detail regarding substrate specificity (Jahn et al., 2004; Loque et al., 2005). Numerous physiological and cellular processes are indirectly affected by MIPs by playing role in osmolarity that described their promising potential in agriculture (Maurel et al, 2002).

MIPs also play role in plant reproduction (Kaldenhoff et al., 2008), plant cell osmoregulation (Kjellbom et al., 1999), cell elongation (Higuchi et al., 1998) and seed germination (Gao et al., 1999). These proteins are also influential in leaf movement, leaf physiology (Kaldenhoff et al., 2008), salinity tolerance (Peng et al., 2007), drought tolerance (Lian et al., 2004), fruit ripening (Mut et al., 2008), metal toxicity and nutrient transportation (Maurel, 2007). Cellular localization of aquaporin proteins has been studied by several researches and mentioned that PIPs and some NIPs are localized in plasma membranes (Takano et al., 2006), TIPs in tonoplast (Liu et al., 2003), SIPs in endoplasmic reticulum and other subcellular compartments (Maurel et al., 2008). Function of these proteins is modulated by co-translational, post-translational modifications (Santoni et al., 2006), and sub-cellular trafficking (Prak et al., 2008) or gating (Tornroth-Horsefield et al., 2006).

Chickpea belongs to subfamily papilionoid in legumes and is related to peas (*Pisum sativum*), clover (*Trifolium spp.*), alfalfa (*Medicago sativa*), lentil (*Lens culinaris*), Lotus japonicas, and model legume barrel medic (*Medicago truncatula*) more closely than Soybean (*Glycine max;* Varshney et al., 2013). Proteome conservation among chickpea predicted proteins, *Medicago truncatula* and *Arabidopsis thaliana* was evaluated by using BLASTP, and found that *M. truncatula* proteins are highly conserved (89.7% proteins) with chickpea predicted proteins than other crop species used in comparison. Maize is globally important cereal crop and going to lead in ranking in near future. We planned the comparative phylogenetic study of MIPs of *C. arietiunm, M. truncatula* and *Z. mays*. *M. truncatula* and *C. arietiunum* shares about 14106 genes between them out of 28,269 nonredundant chickpea genes (Varshney et al., 2013). There is no previous report available about the phylogenetic studies of MIPs of *C. arietinum and M. truncatula alongwith Z. mays*. Diversity analysis of the MIPs of chickpea, barrel medic and maize were the key objective of the current study.

Materials and Methods

Sequences for MIPs of *M. truncatula, C. arietinum* and *Z. mays* were retrieved from NCBI database. All available protein sequences for MIPs were retrieved by searching NCBI followed by subjecting them to Basic Local Alignment Search Tool (BLAST).

Unique sequences in each species were identified by using BLASTP and Pairwise alignment. Sequences were aligned after BLAST search and homology was identified by comparison of Peaks and Valleys. Peaks showed that sequences are homologous or conserved whereas, valleys showed that there are changed bases at that specific location. Region of 50 amino acids were not considered for comparison from both ends of aligned sequences. The sequence with even single amino acid change within comparable region was regarded as unique entity. Unique names were assigned to all sequences. The sequences that were considered unique but have same protein names were renamed and alphabetic series were used for renaming of proteins. ClustalX software were used for multiple and pairwise alignment of sequences. MEGA5 software was used for construction of phylogenetic trees. Phylogenetic trees for MIPs of three species were constructed separately. Highly similar sequences in tree were again subjected to pairwise alignment for reconfirmation of their uniqueness. Sequence data was saved in text format. After final selection, sequences were aligned, and files were saved in Phb, aln, and dnd format. Dnd file was imported in MEGA5 software for construction of phylogenetic trees based on Neighbor Joining (NJ) algorithm. This study was conducted in 2013 therefore, discoveries after that may not be the part of this study.

After separate construction of phylogenetic trees of three species (*M. truncatula, C. arietinum*, and *Z. mays*) their sequences were combined for cumulative alignment which was subjected to phylogenetic tree construction. Sequences of each MIP subfamily were separately arranged, aligned and subjected to construction of phylogenetic tree for each of MIP subfamilies (PIPs, TIPs, NIPs, and SIPs) separately. MIPs of *M. truncatula* were regarded as query for comparative analysis of MIPs across three species (*M. truncatula, C. arietinum*, and *Z. mays*). Sequence length and sequence percent similarity was recognized by using BLASTP. To find the conserved regions, sequences were separated in different pairwise combinations and subjected to pairwise alignment. In pairwise aligned sequences, the conservation is showed by peaks and valleys. Separate phylogenetic tree was constructed for every subfamily of three selected crop species and grouping within subfamilies was assigned.

Results and Discussion

MIPs in Medicago truncatula

Using NCBI search tool 80 aquaporin sequences of *M. truncatula* were retrieved. These sequences were further subjected to BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and pairwise alignment (ClustalX software) to identify unique sequences. Initially 23-MtPIPs, 9-MtTIPs, 33-MtNIPs, 4-MtSIPs, 4-MtMIPs and 7-MtAQPs (unidentified) were retrieved. Finally, 10-MtPIPs, 6-MtTIPs, 17-MtNIPs and 2-MtSIPs were found to be unique among all retrieved sequences. In *M. truncatula* 35 aquaporin proteins were unique. Some of the selected sequences carried the same protein name so, such sequences were renamed and alphabetical series was used for their renaming. Three-MtPIPs were renamed as MtPIP-type likeA, MtPIP-type likeB and MtPIP-type likeC. Two MtPIP2-7s were renamed as MtPIP-like2-7A and MtPIP-like2-7B whereas, seven MtNIP1-2 sequences were renamed as MtNIP1-2A, MtNIP1-2B, MtNIP1-2C, MtNIP1-2D, MtNIP1-2E, MtNIP1-2F and MtNIP1-2G. Two NIP-subfamily sequences were renamed as NIP-subfamA and NIP-subfamB. Renaming was mandatory for assigning every sequence a unique name to be processed by software for further analysis. Length of selected MIPs protein sequences of *M. truncatula* ranged from 193 to 331 amino acids (Figure 1).

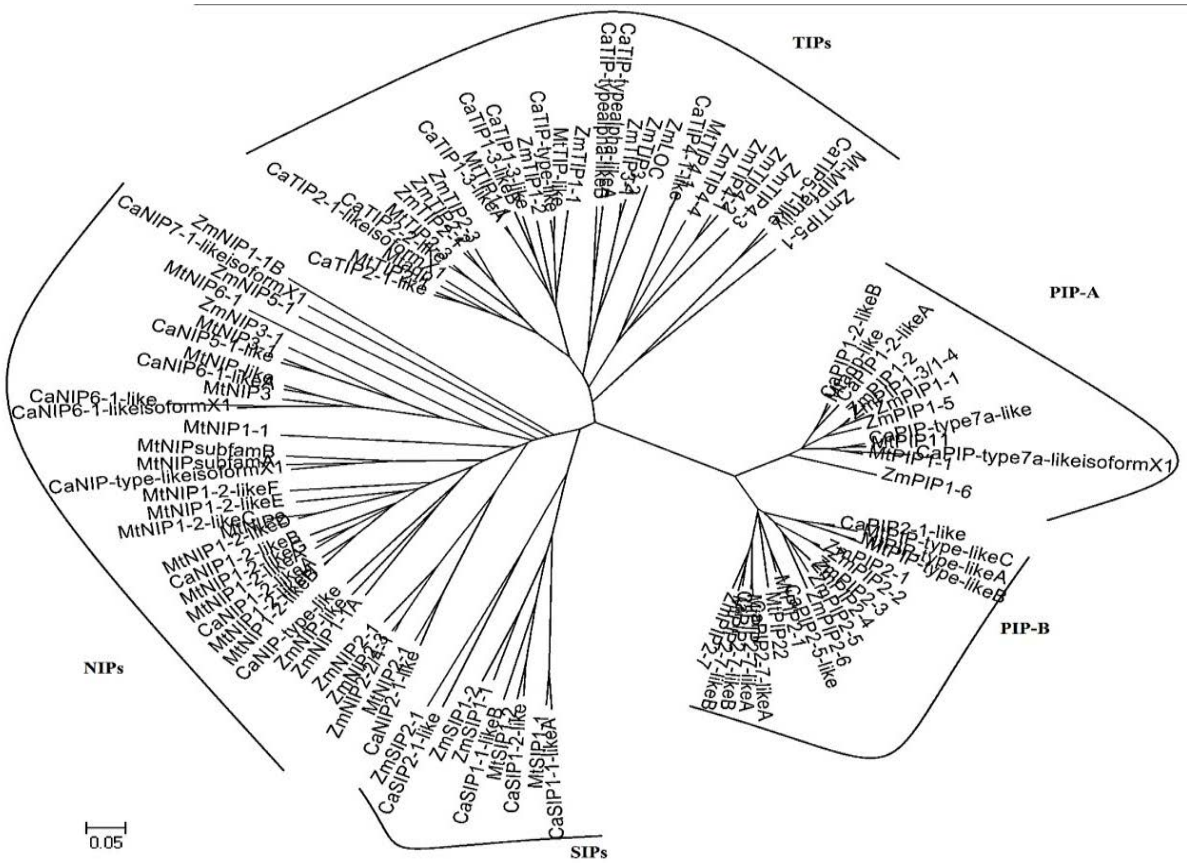

Figure 1: *Cumulative phylogenetic tree of major intrinsic proteins (MIPs) of C. arietinum, M. truncatula and Z. mays.*

MIPs in Cicer arietinum

NCBI-BLAST search enabled the retrieval of 41 MIPs of C. arietinum. Out of 41 MIPs, 10 were PIPs, 15 were NIPs, 12 were TIPs and 4 were SIPs. For selection of unique sequences same procedure was followed as done for M. truncatula. Thirty three MIPs were unique, and among them 8 were PIPs, 11 were TIPs, 10 were NIPs and 4 were SIPs (Figure 1). Renaming was also followed for sequences with same names as done in case of M. truncatula. Amino acid length of selected sequences ranged from 238 to 339.

MIPs in Z. mays

Total 36 aquaporin proteins have identified which belonging to four MIP subfamilies. Out of 36 MIPs, 12 were PIPs, 13 were TIPs, 8 were NIPs and 3 were SIPs. As ZmPIP1-3/1-4 were exactly similar to each other in length and alignment so, we used them as one entity. New sequences of current study were; ZmPIP2-5, ZmNIP-like and ZmLOC. Uniqueness of these newly reported sequences was also verified by BLASTP and pairwise alignment. There were certain differences in current study and study of Chaumont et al. (2001) who reported 31 MIPs. These differences are in amino acid length of proteins. Chaumont et al. (2001) reported 288, 290, 247, 294 and 295 amino acid length for PIP1-1, PIP2-5, TIP2-3, NIP2-1, and NIP2-2 respectively but in current study length of these proteins was 287, 285, 248, 295 and 294 respectively. In *Z. mays,* 31 aquaporin proteins belong to four subfamilies (PIPs, TIPs, NIPs and SIPs while Chaumont et al. (2001) subfamilies were comprised of following numbers; ZmPIPs (13), ZmTIPs (11), ZmNIPs (4) and ZmSIP (3). Variation in protein length was reported to be 243 to 302 amino acids. Sixty four to 100% similarities were found among PIPs (Chaumont et al., 2001). With addition of two more aquaporin proteins sequences in *Z. mays* total 33 aquaporin proteins were reported by Danielson and Johanson (2010). Newly reported proteins were ZmTIP3-2 (length: 266) and ZmNIP2-3 (length: 301). In current study, total 36 MIPs were identified which were comprised of 33 previously known and three were newly identified (Figure 1).

Cumulative Comparison of all MIPs subfamilies

Phylogenetic tree constructed by considering all MIP sequences of 4 subfamilies of *M. truncatula, C. arietinum and Z. mays.* Cumulative tree comprised of 4 main groups and PIP main group was further divided into 2 subgroups. These groups were named as; Group-1: TIPs, Group-2: PIPs (PIP-A and PIP-B), Group-3: SIPs, Group-4: NIPs. TIP and PIP groups consist of 31 and 30 members respectively. SIP group comprises of nine members and NIP group consisting of 34 members (Table 1; Figure 1).

Comparison of MIP subfamilies

PIPs: PIPs of *M. truncatula, C, arietinum* and *Z. mays* were compared for their similarity percentage and aligned in pairwise fashion to visualize the conserved regions across the species. Highest similarity of 98% was observed between MtPIP-type-likeB (*M. truncatula*) and CaPIP2-1-like (*C. arietinum*). In case *M. truncatula* and *Z. mays*, highest similarity percentage of 92% was existing between MtPIP-type-likeB and ZmPIP2-2. Comparative summary of PIPs of *M. truncatula, C. arietinum* and *Z. mays* was given in Figure 2 and Table 1 which showed that PIPs of *M. truncatula* and *C. arietinum* have relatively higher percentage of similarity between them comparative to PIPs of *M. truncatula* and *Z. mays*. PIPs of three species were categorized into two main groups by phylogenetic tree which named as PIP-A and PIP-B. These main groups are further divided into subgroups. PIP-A is divided into five subgroups and named as PIP-1a, PIP-2a, PIP-3a, PIP-4a, and PIP-5a. PIP-B is further divided into four subgroups which were named as PIP-1b, PIP-2b, PIP-3b, and PIP-4b (Table 1; Figure 2).

TIPs: Among TIP sequences of three crops greatest similarity (97%) was found between MtTIP1-1 and CaTIP1-3-like. Seventy seven percent similarities were found between ZmTIP2-3 and MtTIP2-3. Other TIP proteins of *Z. mays* have very low similarity with TIPs of *M. truncatula* that is represented by many valleys in pairwise alignment of TIPs. TIPs of *M. truncatula* and *C. arietinum* were evolutionary closer than TIPs of *Z. mays*. Phylogenetic tree divided the TIPs in 4 groups named as TIP-1, TIP-2, TIP-3 and TIP-4. TIP-1, TIP-2, TIP-3 and TIP-4 consists of 6, 8, 9 and 7 members respectively (Table 1; Figure 3).

NIPs: CaNIP6-1-like and MtNIP-like have highest similarity (91%) among NIPs. ZmLOC and MtNIP1-2-likeD have 68% sequence similarity that was highest among NIPs of *Z. mays* and *M. truncatula*. Similarity comparison showed that NIPs of *M. truncatula* and *C. arietinum* were closer with each other than *Z. mays*. Phylogenetic tree of NIPs categorized them in

Table 1: *Diversity of different major intrinsic proteins.*

Plasma membrane intrinsic proteins (PIPs)										
	PIP-A					**PIP-B**				
	PIP-1a	**PIP-2a**	**PIP-3a**	**PIP-4a**	**PIP-5a**	**PIP-1b**	**PIP-2b**	**PIP-3b**	**PIP-4b**	**Total**
MtPIPs	1	1	2	3	-	-	2	-	1	10
CaPIPs	-	1	2	1	-	-	2	-	2	8
ZmPIPs	-	-	1	-	6	1	-	4	-	12
Total	1	2	5	4	6	1	4	4	3	30
NOD26-like intrinsic proteins (NIPs)										
	Group NIP-1	Group NIP-2	Group NIP-3	Group NIP-4	Group NIP-5	Group NIP-6	Total			
MtNIPs	4	-	2	9	1	1	17			
CaNIPs	4	1	2	2	-	1	10			
ZmNIPs	1	2	2	-	-	3	8			
Total	9	3	6	11	1	4	35			
Tonoplast intrinsic proteins (TIPs)										
		TIP-1	TIP-2		TIP-3	TIP-4	Total			
MtTIPs		1	2		3	-	6			
CaTIPs		1	4		3	3	11			
ZmTIPs		4	2		3	4	13			
Total		6	8		9	7	30			
Small basic intrinsic proteins (SIPs)										
		SIP-1	SIP-2		SIP-3	SIP-4	Total			
MtSIPs		1	-		-	1	2			
CaSIPs		2	-		1	1	4			
ZmSIPs		-	2		1	-	3			
Total		3	2		2	2	9			
Summary of all MIPs										
		TIPs	PIPs			SIPs	NIPs	Total		
		-	PIP-A		PIP-B	-	-	-		
MtMIPs		6	3		7	2	17	35		
CaMIPs		11	4		4	4	10	33		
ZmMIPs		13	5		7	3	8	36		
Total		30	12		18	9	34	104		

6 groups which were named as NIP-1, NIP-2, NIP-3, NIP-4, NIP-5, and NIP6. NIP-1 comprised of 9 NIP proteins, of which 4, 4, and 1 belongs to *M. truncatula*, *C. arietinum* and *Z. mays* respectively. NIP-2 group consists of 3 NIP proteins, out of these 2 were from *Z. mays* and 1 from *C. arietinum* NIPs. NIP-3 group has six NIP proteins which consist of 2 accessions each from *M. truncatula*, *C. arietinum* and *Z. mays*. Group NIP-4 is largest group that consists of 11 NIPs proteins. Nine members of group four belong to *M. truncatula* and 2 members belong to *C. arietinum*. MtNIP1-1 is the only member of NIP-5 group. Out of total 5 members of NIP-6 group, 3 members were from *Z. mays* and 2 from *C. arietinum* (Table 1; Figure 4).

SIPs: MtSIP1-1 and CaSIP1-1-like were 90% similar that is highest percent value among SIPs of *M. truncatula* and *C. arietinum*. MtSIP1-2 and ZmSIP1-2 are 62% similar being highest percent value among SIPs of *M. truncatula* and *Z. mays*. Four groups are formed by phylogenetic analysis of SIP sequences of M. truncatula, C. arietinum and Z. mays. SIP-1, SIP-2, SIP-3 and SIP-4 group consists of 3, 2, 2, and 2 members their description in given in Table 1 (Figure5, 6).

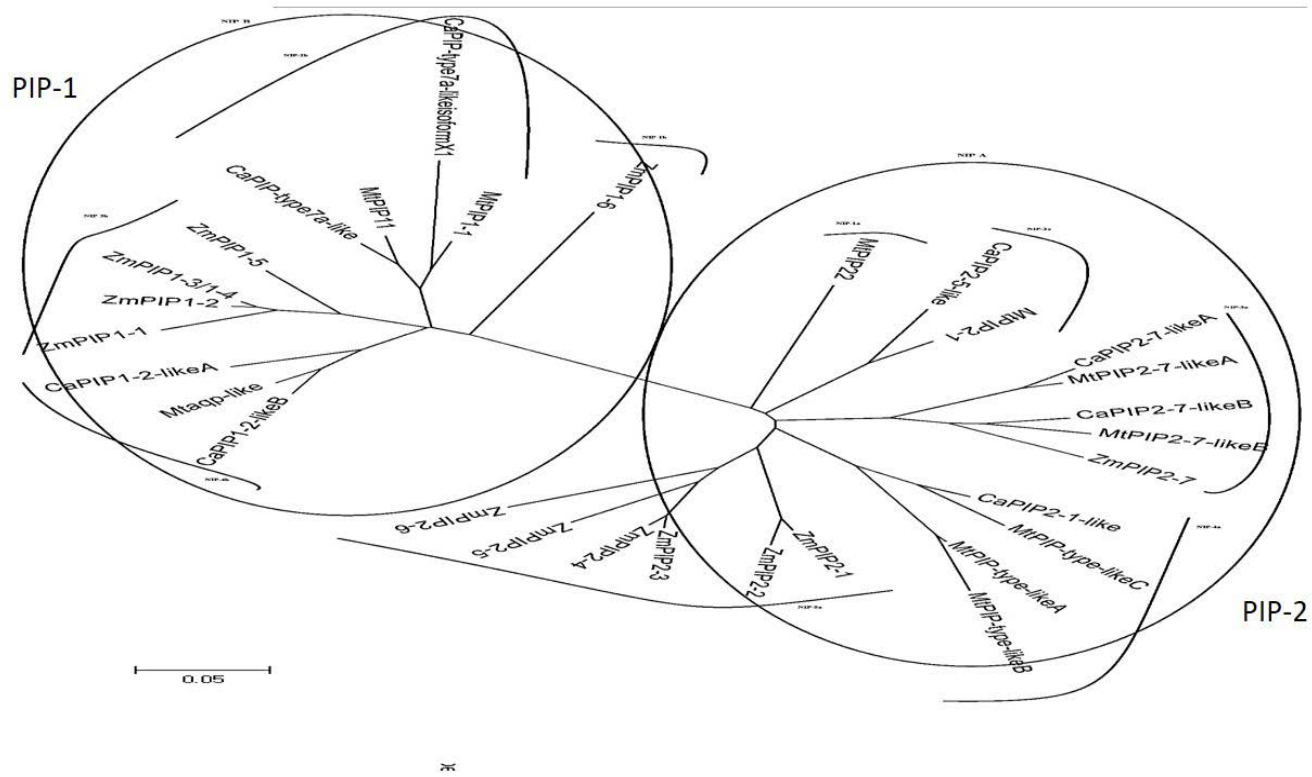

Figure 2: *Cumulative phylogenetic tree of PIPs proteins of C. arietinum, M. truncatula and Z. mays.*

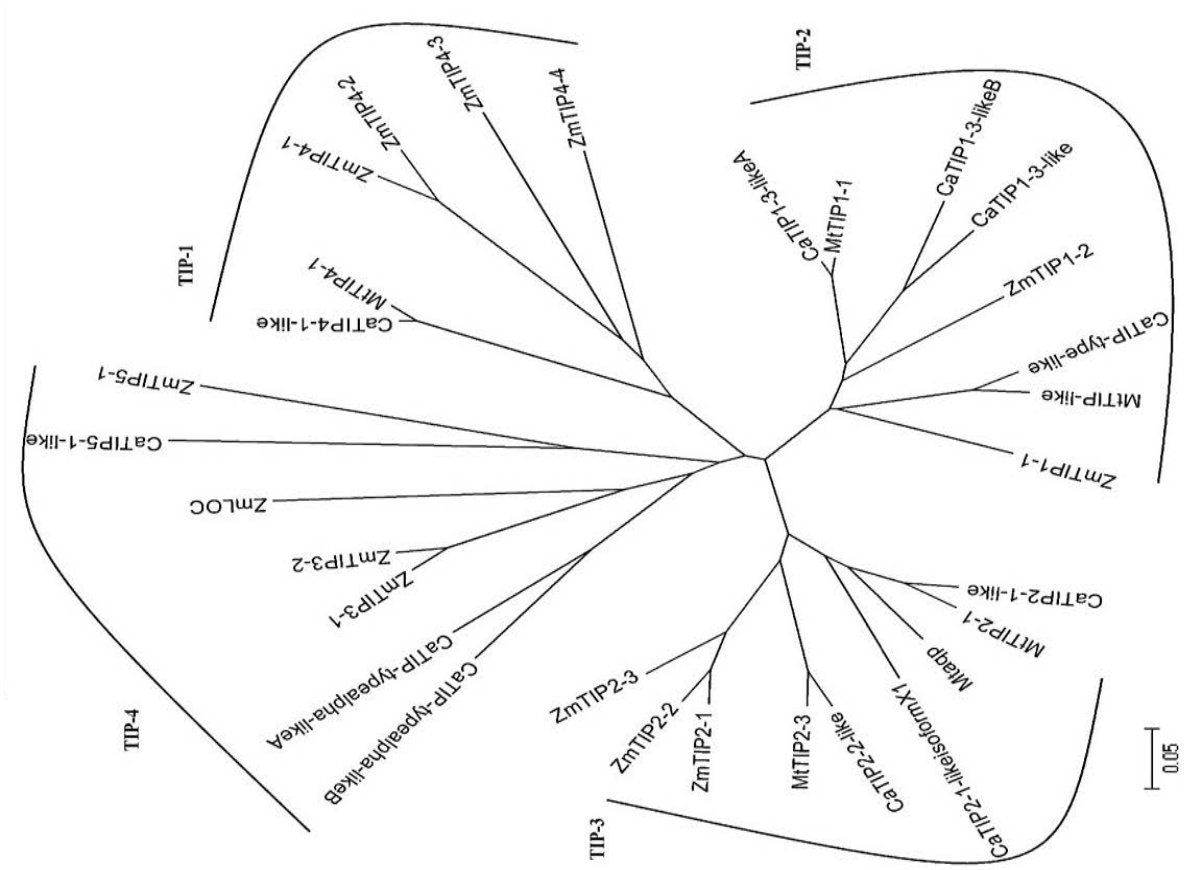

Figure 3: *Cumulative phylogenetic tree of TIPs proteins of C. arietinum, M. truncatula and Z. mays.*

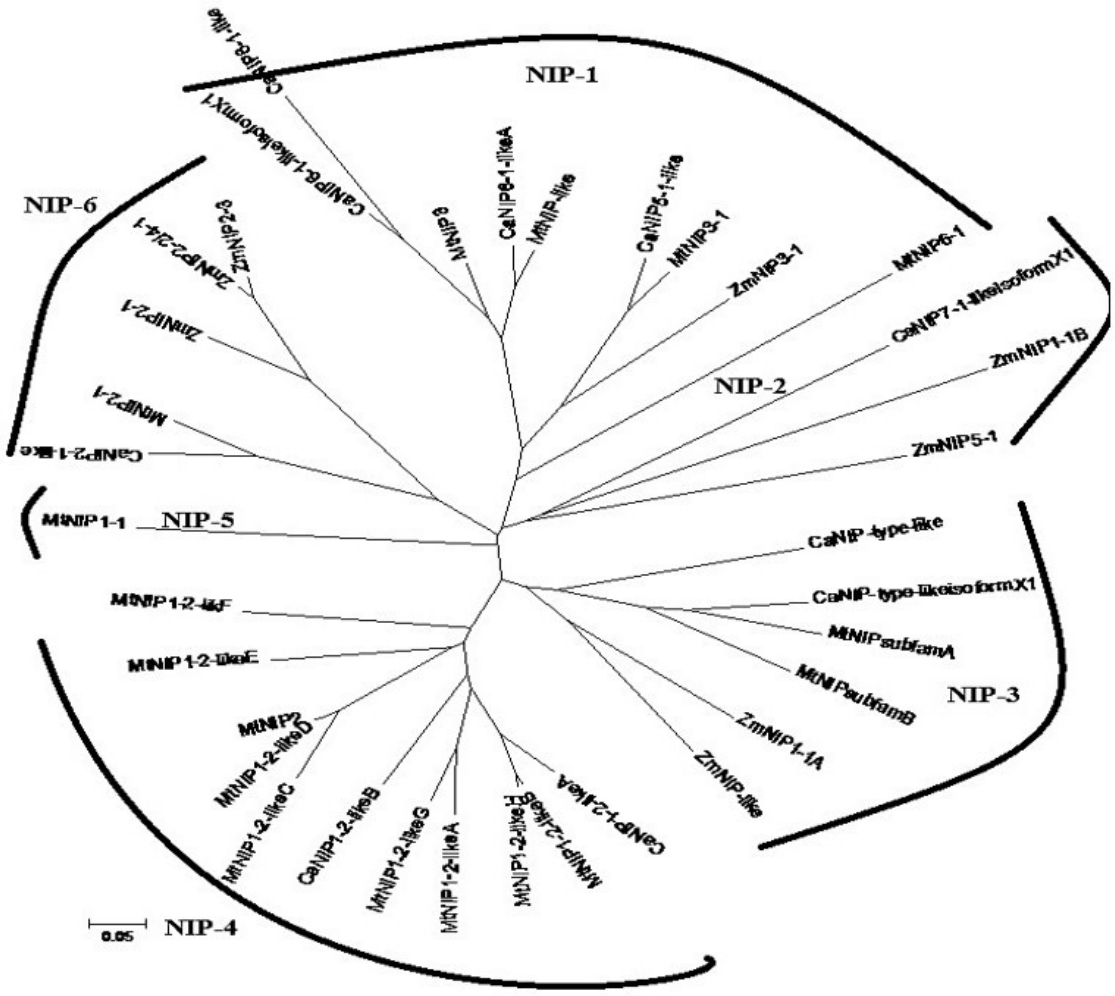

Figure 4: *Cumulative phylogenetic tree of NIPs proteins of C. arietinum, M. truncatula and Z. mays.*

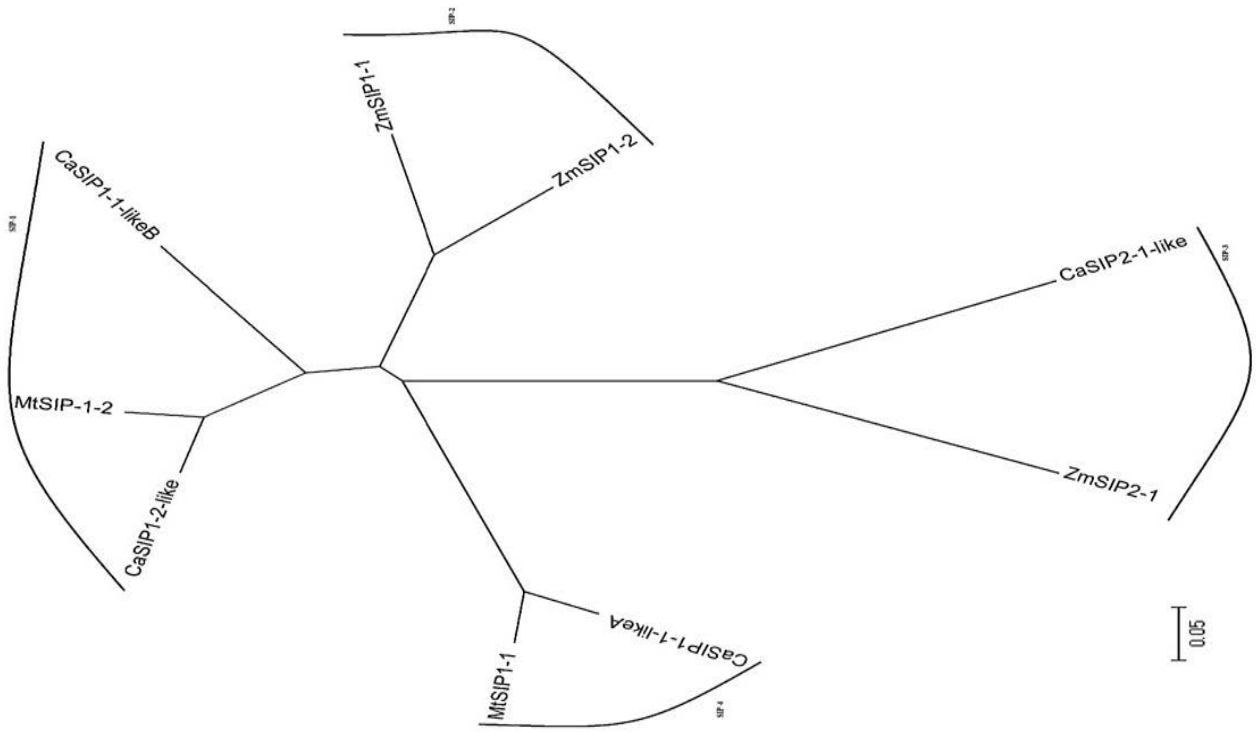

Figure 5: *Cumulative phylogenetic tree of SIPs proteins of C. arietinum, M. truncatula and Z. mays.*

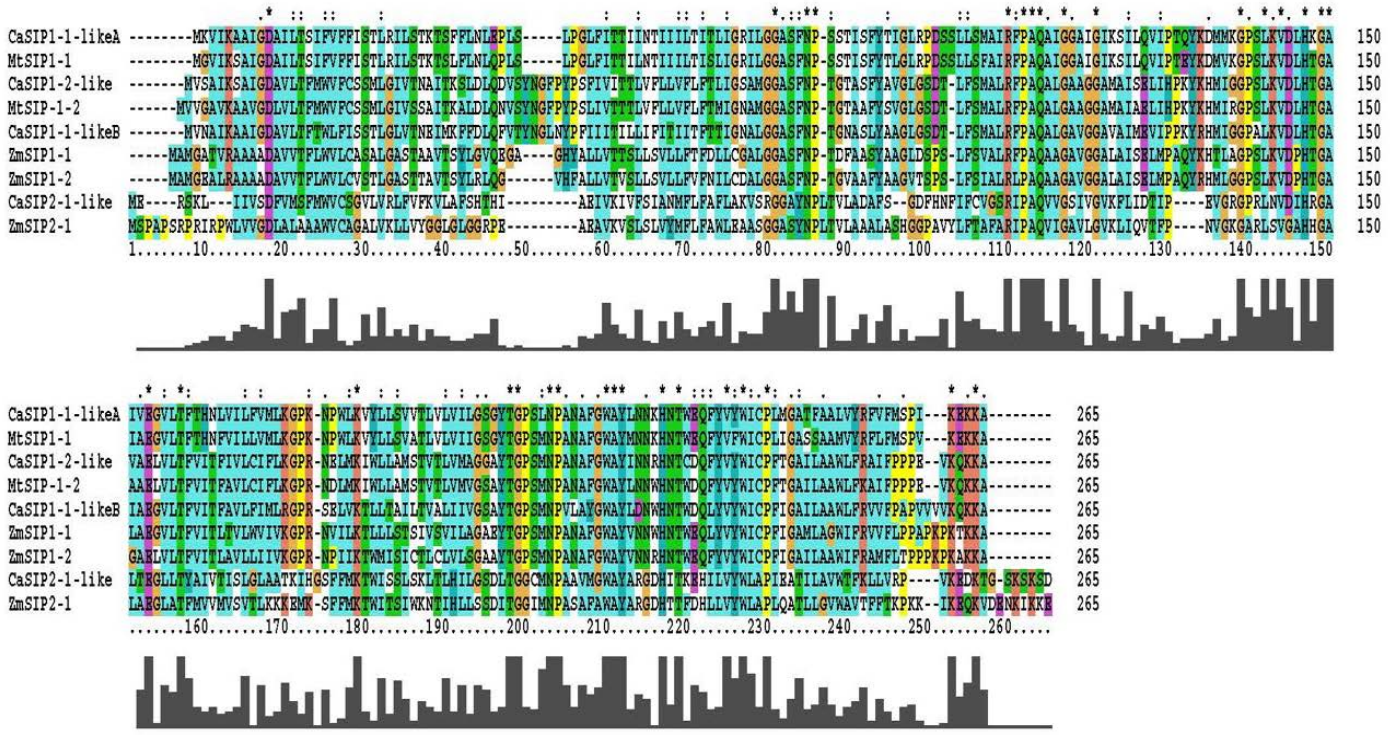

Figure 6: *Multiple sequence alignment of SIP subfamily of Cicer arietinum, Zea mays and Medicago trancatula.*

Substantial water absorption and evaporation during plant growth is linked with abundance of MIPs in plants. Diverse MIPs in plants are associated with differential expression during growth, development and multiple sub-cellular localization (Maurel et al., 2002). TIPs are present in vacuolar membranes or tonoplast and PIPs are present in plasma membranes. PIPs and TIPs perform considerably different physiological functions (Johanson et al., 2001). Gradient pressure of several bars is present across the plasma membrane which maintains the turgor, cell and tissue shape (Johanson et al., 2001). Under certain conditions, TIP and PIP functions are overlapping. NIPs plays distinct role in various plants. Water and metabolite flux is regulated by NIPs between roots and nitrogen fixing bacteria because these proteins are present in peribacteroid membranes of symbiotic root nodules (Guenther and Roberts, 2000). NIPs are present in higher number in *C. arietinum* and *M. truncatula* than *Z. mays* due to their symbiotic relationship with nitrogen fixing bacteria which is lacking in *Z. mays*. MIPs mainly express within vascular bundles and their surrounding cells, participate in xylem to and from phloem transportation. Stomatal guard cells, motor cells regulating leaf movement, elongating cells and seeds are also among the active sites for expression of MIPs in plants (Maurel et al., 2008).

Protein sequences of aquaporins have been identified previously in numerous crops including maize, rice and arabidopsis. Phylogenetic analysis revealed that there are 39 MIPs were present in rice which includes; 13 PIPs, 11 TIPs, 13 NIPs and 2 SIPs (Bansal and Sankararamakrishnan, 2007). In *O. sativa* 38 members of MIPs were reported by Mosa et al. (2012). In wheat there are almost 35 MIP members were reported (Ayadi et al., 2011). In *Populus* 55 MIPs were reported including; 17 TIPs, 15 PIPs, 6 SIPs, 11 NIPs, and 6 XIPs (Gupta and Sankararamakrishnan, 2009). Thirty five MIPs have been identified in *A. thaliana* which includes; 13 PIPs, 10 TIPs, 9 NIPs and 3 SIPs (Johanson et al., 2001). In *Phoenix dactylifera*, 33 members of MIPs have been identified, among them 11 are PdPIP, 8 are PdTIP, 9 are PdNIP and 5 are SIP encoding genes (Degu et al., 2013).

After extensive sorting of database for search of sequences we identified the 35 MIP sequences for maize. Out of these, 31 were already reported which included; 11 TIPs, 13 PIPs, 4 NIPs and 3 SIPs (Chaumont et al., 2001). Their report also stated that they were unable to obtain full length sequences for two TIPs, one PIPs and one NIPs. Our report had 13 TIPs that might include those two sequences for which they could not get full length sequences. Our report mentioned 12 PIPs because we considered ZmPIP1-3 and ZmPIP1-4 as one entity whereas, Chaumont et al. (2001) considered them as different accessions.

ZmPIP1-3 and ZmPIP1-4 were two different genes but their translational products are identical proteins. As we are dealing with protein sequences so, we regard them as one entity whereas, Chaumont et al. (2001) were dealing with gene sequences so they considered them as different accessions. SIPs number is unchanged but four NIPs were more than the report of Chaumont et al. (2001). It is perceived that these are not final MIPs for maize, there might be increase in number with further research activities and functional characterizations.

MIP sequences of these three selected crops are distributed in four subfamilies in almost similar fashion. Distribution of MIP sequences among four subfamilies provides a confident clue that we have retrieved most of MIP sequences of *Z. mays, M. truncatula* and *C. arietinum.* Functional specialization and localization is main reason for their different subfamilies and groups. Similarly subfamilies and groups were reported in maize and arabidopsis phylogenetic trees by Chaumont et al. (2001) and mentioned in explanation that functional and localization differences are main reasons for subfamilies and groups. We observed subfamilies and groups in *M. truncatula* and *C. arietinum* in combination with *Z. mays* which showed that functional and localization specificity also exists between *M. truncatula* and *C. arietinum*.

Among selected species two are dicot (*M. truncatula* and *C. arietinum*) and one is monocot (*Z. mays*). Grouping within subfamilies described different patterns of evolutionary changes among monocots and dicots. PIP-5a, PIP-3b, and SIP-2 groups consists of only Z. mays members whereas, most of groups share the *M. truncatula, C. arietinum* subfamily members. This pattern of distribution predicted the divergence of sequences prior to monocot and dicot diversification. Existence of these groups in monocots and dicots depicted that these are critical for water and solute relation across the species (Chaumont et al., 2001). Members of *Z. mays* are present on same branch in Zm-NIP-6, Zm-PIP-5a, Zm-PIP-3b, Zm-TIP-1, Zm-TIP-3 and SIP-2 groups in phylogenetic trees of their respective subfamilies. Whereas, *C. arietinum and M. truncatula* shares most of branches with each other in their phylogenetic tree of respective subfamily. Presence of proteins of same species on same tree indicated the DNA duplication occurred after mono and dicot diversification (Chaumont et al., 2001). Findings of our results concluded that higher DNA duplications are present for MIPs in *Z. mays* relative to *M. truncatula* and *C. arietinum* because their sequences are not present on same branch in most of cases. This also indicated that conserved sequences within species are also higher in *Z. mays* than *M. truncatula and C. arietinum*. Presence of MIPs sequences of *M. truncatula* and *C. arietinum* on same branch for most of cases also indicated that conserved sequences are more between them. Distinct branching of MIPs within subfamilies is indicator that homologous genes are evolved as a result of gene duplication (Danielson and Johanson, 2010).

Phylogenetic tree of subfamily for *M. truncatula, C. arietinum and Z. mays* formed two groups in case of PIP subfamily. This grouping of PIPs in our results is in agreement with results of several reports in different crop species. Two groups in PIP subfamily were also observed in maize, arabidopsis, flowering plants, *P. patens* (moss species), *P. trichocarpa*, *O. sativa* and *P. dactylifera* (Chaumont et al., 2001; Johanson et al., 2001; Bansal and Sankararamakrishnan, 2007; Danielson and Johanson, 2010; Gupta and Sankararamakrishnan, 2009; Degu et al., 2013). MIP sequences of ferns, gymnosperms, monocots and dicots were subjected to Phylogenetic analysis which showed that PIP proteins are comprised of two groups (PIP1 and PIP2) in their cumulative phylogenetic tree (Kjellbom et al., 1999; Zardoya, 2005). Lower level of pairwise divergence of PIPs is associated with slow evolution rate of these proteins (Zardoya, 2005). High similarity among PIPs of *A. thaliana* was associated with recent diversification of this subfamily. PIP genes were diversified independently after evolution of monocotyledonous and dicotyledonous groups (Chaumont et al., 2001). Results of our findings showed that these PIPs are more conserved relative to other subfamilies in leguminous and non-leguminous or monocots and dicots crops. Reason for PIPs organization in two groups was corroborated with identity of amino acid sequences and cluster organization in phylogenetic tree (Soto et al., 2012). High conservation of PIPs in plants is indicator that these are evolved earlier in evolutionary history of crop plants (Danielson and Johanson, 2010).

TIPs of *M. truncatula, C. arietinum* and *Z. mays* formed four (TIP-1 to TIP-4) groups which suggested that many types of vacuoles are present in legumes and cereals. Several types of vacuoles were also proposed in bryophytes and *A. thaliana* (Johanson et al.,

2001; Danielson and Johanson, 2010). HIPs and XIPs subfamilies are missing in higher plants might be due to the reason that TIP groups have taken over their functions. XIPs were reported to be present in some dicots but in our selected dicots (*C. arietinum and M. truncatula*) these were also missing whereas, these are missing in all monocots (Danielson and Johanson, 2010). Five subfamilies of MIPs are reported in *P. trichocarpa* (dicot tree) that also includes XIPs subfamily (Gupta and Sankararamakrishnan, 2009). Reason for highest MIPs in *P. trichocarpa* is the existence of XIPs subfamily and higher number of members in TIP and SIP subfamily. Seven subfamilies were reported in *P. patens* whereas, in our findings only four subfamilies were present. This might be due to the fact that some subfamilies were lost during evolution of higher plants and rest of subfamilies subjected to further diversification to form groups which taken over the functions of lost subfamilies (Danielson and Johanson, 2010).

NIPs are less conserved and more diverged in NIPs phylogenetic tree which suggested these are diverged in leguminous and cereals. Higher diversification of NIPs was reported in several higher plants and bryophytes which are in agreement with our findings (Danielson and Johanson, 2010). In higher plants GLPs (glycerol-uptake facilitators or aquaglyceroporins) are missing and glycerol transport is carried out by NIPs (Zardoya et al., 2002). PdNIPs of date palm are found to be more divergent than PIPs and TIPs (Degu et al., 2013).

SIPs are small in number and size. SIPs have resemblance with TIPs but different due to basic nature like PIPs. SIPs have two groups in *A. thaliana* (Johanson et al., 2001). PdSIPs are highly divergent in date-palm than other MIPs (Degu et al., 2013). SIPs reported to localize in membrane of endoplasmic reticulum (Ishikawa et al., 2005).

Conclusively stated that significant genetic diversity was observed among the MIPs of *M. truncatula, C. arietinum* and *Z. mays* which showed the evolutionary divergence among and within the species. Subfamilies of the MIPs also have similarities and differences across the species and within species which showed the functional conservations and evolutionary diversifications.

Author's Contribution

Muhammad Amir Maqbool: Retrieving the data from online databases, compilation of data, data analysis, manuscript preparation.
Muhammad Aslam: Data mining, result interpretation, manuscript preparation.
Waseem Akbar: Compilation and analysis of data, manuscript preparation.
Muhammad Waheed Anwar: Compilation of data and manuscript preparation.
Ehtisham Shakeel Khokhar: Technical inputs for interpretation of results, manuscript preparation, corrections and revisions.

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