Analyses of *Physcomitrella patens* Ankyrin Repeat Proteins by Computational Approach

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Received 7 March 2016; Revised 18 May 2016; Accepted 25 May 2016

1. Introduction

Ankyrin (ANK) repeats, composed of around 30–34 amino acids, are evolutionary conserved protein domains found to be involved in mediating protein-protein interactions [1]. In metazoans, the ANK repeat containing proteins have diversified functions in important processes like signal transduction, cell-cycle regulation, maintaining the integrity of cytoskeleton, transcriptional regulation, inflammatory response, development, and different types of cellular transport mechanisms [2]. Defect in ANK proteins has been found in a number of human diseases. For example, the ankyrin repeat domain 11 (ANKRD11) proteins interact with and also enhance the transcriptional activity of p53. In breast cancer cell lines, the expression level of ANKRD11 decreases compared to controls [3]. Ankyrin dysfunction has been linked with fatal human arrhythmias, such as the "ankyrin-B syndrome" in which there is an aberration of the human ankyrin-B gene (ANK2) [4].

The importance of ANK repeats can be underlined by their abundance in virtually all phyla. In photosynthetic organisms, these proteins have also been shown to be involved in a number of important physiological processes. Zhang and colleagues first reported on a light-dependent plant ANK protein which is involved in cell differentiation and development in *Arabidopsis* [5]. EMB506, a five-ANK repeat containing protein, has been shown to be essential for embryogenesis in *Arabidopsis* [6]. Another ANK protein, known as BOP1, is required for leaf morphogenesis [7]. XBAT32 and XBAT35 are linked with the regulation of ethylene biosynthesis [8, 9] and ethylene signaling [10], respectively. Several ANK proteins have been demonstrated to play role in responses to biotic and abiotic stresses in plants. The expression of rice *OsBIANK1* gene, encoding proteins containing ANK repeats, is altered in pathogen infected rice-seedlings compared to that of the controls which suggests its involvement in disease resistance response [11]. Furthermore, Yan and colleagues have shown that the *Arabidopsis* ANK
protein, AKR2, might be involved in regulating antioxidant metabolism during disease resistance and stress responses [12].

The recent advancement in genome sequencing has enabled the genome-wide identification and characterization of ANK proteins from several photosynthetic species like *Arabidopsis* [14], rice [15], and tomato [16]. The availability of the genome sequence of *Physcomitrella patens* [17] provided us with an excellent opportunity for a genome-wide analysis of this ANK family in bryophyte. Here, we report analyses of the ANK proteins of *P. patens* using first release of the moss genome annotation.

2. Methods

2.1. Data Retrieval and Identification of ANK Proteins. The publicly available protein sequences of *P. patens* were downloaded from the JGI Phytozome database (first release of the moss genome annotation) [18] and domain annotation of these proteins was done by InterProScan [19]. Then ANK proteins were screened by searching for the PF00023 domain using an in-house Perl script as described in a previous paper [20]. BLASTP was carried out with NCBI nonredundant protein database using the sequences retrieved from InterProScan as queries. After that, the candidate sequences were curated manually using available annotations in GenBank and existing literature. The molecular weights and isoelectric points were determined separately from online web server (http://www.bioinformatics.org/sms2/). Subcellular localization was predicted by the online web server of ProtComp 9.0 (http://www.softberry.com/berry.phtml?topic=protecomppl&group=help&subgroup=proloc).

2.2. Classification and Phylogenetic Analyses of the ANK Proteins. The proteins were classified into different subgroups based on the presence of additional conserved domains other than the ANK domain as described previously [15, 21]. Phylogenetic tree file was constructed by the online webserver, SATCHMO-JS [22]; and the tree was visualized by the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1 [23]. In addition, synonymous and nonsynonymous substitution pattern were determined as described previously [24].

3. Results and Discussion

Using our approach, we were able to identify a total of 54 proteins having at least one ANK repeat in *P. patens* (in the first released annotation of the moss genome). The identified sequences were further verified in a reiterative process through manual curation. The percentage of ANK proteins in *P. patens* (0.15%) is a bit lower compared to the other species from the tracheophyte lineage as listed in Figure 1(a).

The identified sequences from *P. patens* were designated as PpANK1, PpANK2, ...PpANK54, respectively, for analysis purpose during this study (Table 1). Figure 1(b) shows the distribution of the PpANKs according to the number of amino acids they contain within their primary sequence. The largest protein (PpANK8) had a length of 1,088 amino acids, while the shortest one (PpANK48) contained only 74 amino acids. The molecular weights (MW) and isoelectric points (PI) of the PpANK proteins deduced from their protein sequences are listed in Table 1. In addition, it was observed that these 54 PpANK proteins contained a total of 163 ANK repeats among themselves. The number of ANK repeats per protein in *P. patens* ranged in between 1 and 9, whereas the average number of repeats per protein was 3. The frequency of the proteins having different number of ANK repeats is shown in Figure 1(c). The highest number of repeats (9) was found in PpANK43 whereas PpANK4, PpANK18, PpANK22, PpANK33, and PpANK49 had just one ANK repeat motif each. In general, most ANK proteins have two to six repeats; and the largest known number of repeats is 34 that was found in a *Giardia lamblia* protein [25].

Based on their domain compositions, the predicted PpANK proteins were classified into nine subfamilies (Figure 2(a)). We have observed that a significant number of the PpANK proteins (21) had no other recognizable domain apart from the conserved ankyrin repeat and were classified as ANK-M. Proteins containing other known functional domains apart from the ANK domains were classified into the following subfamilies. Six proteins containing the RING finger domains were grouped as ANK-RF; three proteins containing the zinc-finger domain were designated as ANK-ZnF: BAR, PH and ArfGAP domain containing proteins were grouped as ANK-BPA (3 members). The ANK-BTB subfamily (3 members) had broad-complex, tramtrack, and bric-a-brac domains. Nine of the PpANK proteins having either serine/threonine or tyrosine kinase domain were classified as ANK-PK. Three proteins having the Acetyl-CoA binding domain were classified as ACBP. Two proteins having the GPCR-chapero-1 domain were classified as ANK-GPCR. This specific subfamily containing the GPCR domain has only been reported to be found in tomato and has not been reported in model plant species like *Arabidopsis* and rice [16] (Figure 2(a)). The rest of the PpANK proteins that contained other domains including CHROMO, IQ, TM, and RCCI were grouped as ANK-O. The structure of representative proteins from each subfamily is shown in Figure 2(b). There were no ANK proteins having the TPR domains (ANK-TPR) in *P. patens*, even though ANK proteins having these two domains are present in both *Arabidopsis* and rice [14, 15].

Next, we constructed a phylogenetic tree to compare between the members of different subfamilies of PpANKs. The tree file was generated from the Hidden Markov Model (HMM) based multiple sequence alignments of the sequences done by SATCHMO-JS and visualized by the Molecular...
Species | # of proteins | # of ANK proteins | % of ANK proteins | Source
---|---|---|---|---
P. patens | 35,938 | 54 | 0.15 | [17]
Oryza sativa | 37,544 | 175 | 0.47 | [15, 25]
Z. mays | 32,540 | 71 | 0.22 | [26, 27]
A. thaliana | 25,498 | 105 | 0.41 | [13, 14]
S. lycopersicum | 34,727 | 130 | 0.37 | [16]

Figure 1: (a) The total number of predicted ANK proteins identified by different groups in the four sequenced angiosperm genomes (Oryza sativa, Zea mays, Arabidopsis thaliana, and Solanum lycopersicum) along with their bryophyte counterpart P. patens. The total number of predicted proteins of each species is also provided. See source column for references. (b) Distribution of PpANK proteins according to their length. (c) Number of putative ANK repeats per protein shown graphically by bar diagram. The horizontal axis in the figure represents different numbers of ANK repeats while the vertical axis represents the frequency of the proteins corresponding to different number of repeats. A large percentage (41%) of the PpANKs have 3 repeats within their sequence, as seen in the graph. (d) The consensus sequence of the P. patens ankyrin repeat motif.

Evolutionary Genetics Analysis (MEGA) software version 4.1 [23]. Interestingly, in most of the cases, members of the same subfamily were clustered together in the phylogenetic tree (Figure 2(c)). We also analyzed the synonymous and nonsynonymous substitution patterns of the coding sequences of the genes encoding the ANK proteins in P. patens. The corresponding nucleotide sequences of the PpANK proteins were obtained from NCBI. Then we aligned the sequences using MEGA 4.1 and obtained the synonymous divergence (dS) and nonsynonymous divergence (dN) ratios. The ratio suggested positive selection for the genes of ANK superfamily of P. patens (Figure 2(d)). The codon based Z test indicated positive selection (data not shown) for most of the pairwise comparisons of the ANK genes. This further explains the fact that the ANK repeat encoding genes have acquired significant functional diversity by extensive domain shuffling or emerged multiple times independently, as a result of convergent evolution or parallel evolution or both [21].

In order to elucidate the function of a protein within a living cell, predicting the location where it resides in the cell is essential. In this study we have used ProtComp version 9.0 for predicting the subcellular localization of the PpANK proteins. The output revealed that the proteins are dispersed throughout the cells (Figure 3(a)). A large percentage (33%) of the PpANKs are located in the nucleus. Detailed information on the localization of each protein can be found in Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/9156735. We also tried to analyze if there is any relationship between the subfamilies of PpANKs with their respective subcellular localization. Interestingly, we have found that all the members of the ANK-BPA subfamily had similar localization pattern, that is, in the extracellular region (Additional File 1, Supplementary Table 1). For all the other subfamilies, we did not see any distinct pattern in their localization.

The PpANK sequences were also compared with the proteins in of NCBI nonredundant protein database which showed their homology with ANK proteins from diverse species ranging from bacteria to green algae to plants (Additional File 1, Supplementary Table 2). Not surprisingly, in many of the cases, the proteins having significant similarity with the corresponding PpANKs have functions either as protein binders or as kinases (Figure 3(b)). This further clarifies the fact that ANK proteins play significant role in protein-protein interaction and cellular signaling pathways.
| NCBI accession number | Our nomenclature | # of repeats | Length (A.A.s) | MW (KD)  | PI |
|-----------------------|------------------|--------------|----------------|---------|----|
| XP_001770032          | PpANK1           | 3            | 176            | 18.54   | 4.02 |
| XP_001770249          | PpANK2           | 4            | 211            | 22.88   | 9.52 |
| XP_001771297          | PpANK3           | 2            | 425            | 47.57   | 8.63 |
| XP_001774650          | PpANK4           | 1            | 418            | 47.15   | 7.01 |
| XP_001775781          | PpANK5           | 4            | 364            | 40.21   | 8.24 |
| XP_001756372          | PpANK6           | 3            | 144            | 15.3    | 4.43 |
| XP_001756378          | PpANK7           | 3            | 376            | 41.11   | 4.48 |
| XP_001777467          | PpANK8           | 2            | 1088           | 116.86  | 8.18 |
| XP_001779325          | PpANK9           | 5            | 425            | 45.81   | 6.71 |
| XP_001757508          | PpANK10          | 2            | 463            | 50.67   | 7.19 |
| XP_001782286          | PpANK11          | 3            | 350            | 38.41   | 4.15 |
| XP_001782585          | PpANK12          | 3            | 121            | 12.8    | 6.29 |
| XP_001759240          | PpANK13          | 2            | 440            | 48.17   | 6.39 |
| XP_001783731          | PpANK14          | 3            | 421            | 47.49   | 7.01 |
| XP_001784155          | PpANK15          | 3            | 442            | 49.87   | 7.19 |
| XP_001765240          | PpANK16          | 3            | 419            | 47.13   | 7.75 |
| XP_001765521          | PpANK17          | 3            | 167            | 18.4    | 8.75 |
| XP_001753203          | PpANK18          | 1            | 82             | 8.73    | 4.67 |
| XP_001766044          | PpANK19          | 3            | 342            | 37.48   | 4.19 |
| XP_001767452          | PpANK20          | 2            | 412            | 46.92   | 8.12 |
| XP_001771908          | PpANK21          | 2            | 438            | 48.19   | 8.08 |
| XP_001755743          | PpANK22          | 1            | 291            | 31.45   | 8.6  |
| XP_001784959          | PpANK23          | 6            | 247            | 27.98   | 6.27 |
| XP_001762570          | PpANK24          | 5            | 437            | 47.16   | 7.64 |
| XP_001764006          | PpANK25          | 2            | 337            | 37.53   | 4.44 |
| XP_001753287          | PpANK26          | 3            | 343            | 36.03   | 4.09 |
| XP_001774971          | PpANK27          | 3            | 378            | 41.73   | 5.74 |
| XP_001779754          | PpANK28          | 4            | 234            | 25.26   | 8.85 |
| XP_001759321          | PpANK29          | 3            | 608            | 68.4    | 6.69 |
| XP_001760427          | PpANK30          | 2            | 377            | 41.77   | 6.23 |
| XP_001765151          | PpANK31          | 4            | 625            | 69.45   | 5.03 |
| XP_001765275          | PpANK32          | 4            | 351            | 37.69   | 6.85 |
| XP_001767776          | PpANK33          | 1            | 752            | 82.78   | 9.39 |
| XP_001771059          | PpANK34          | 4            | 451            | 48.45   | 5.64 |
| XP_001780358          | PpANK35          | 3            | 634            | 71.25   | 8.42 |
| XP_001763413          | PpANK36          | 4            | 473            | 50.65   | 5.08 |
| XP_001773791          | PpANK37          | 3            | 271            | 30.45   | 4.58 |
| XP_001773863          | PpANK38          | 2            | 107            | 11.37   | 3.88 |
| XP_001774458          | PpANK39          | 2            | 731            | 82.73   | 7.11 |
| XP_001779036          | PpANK40          | 2            | 721            | 82.01   | 7.12 |
| XP_001759158          | PpANK41          | 2            | 752            | 84.75   | 8.64 |
| XP_001784362          | PpANK42          | 3            | 414            | 47.27   | 6.51 |
| XP_001765211          | PpANK43          | 9            | 795            | 90.39   | 7.16 |
| XP_001770763          | PpANK44          | 6            | 1060           | 114.9   | 8.03 |
| XP_001765353          | PpANK45          | 3            | 612            | 67.04   | 8.35 |
| XP_001754260          | PpANK46          | 5            | 402            | 43.17   | 7.72 |
| XP_001771412          | PpANK47          | 3            | 285            | 32.42   | 4.04 |
| XP_001755344          | PpANK48          | 2            | 74             | 7.65    | 4.34 |
| XP_001775223          | PpANK49          | 1            | 910            | 101.79  | 7.62 |
| XP_001758257          | PpANK50          | 3            | 380            | 42.3    | 8.66 |
| XP_001758416          | PpANK51          | 3            | 86             | 9.13    | 7.16 |
| XP_001782553          | PpANK52          | 3            | 111            | 11.72   | 6.67 |
| XP_001784176          | PpANK53          | 2            | 367            | 40.47   | 7.52 |
| XP_001760375          | PpANK54          | 3            | 130            | 14.03   | 10.41 |
| Subfamily | Pp | Os | Zm | At | Sl |
|-----------|----|----|----|----|----|
| ANK-M     | 21 | 73 | 30 | 18 | 26 |
| ANK-PK    | 9  | 4  | 4  | 7  | 9  |
| ANK-RF    | 6  | 9  | 9  | 5  | 7  |
| ANK-ZnF   | 3  | 7  | 3  | 6  | 8  |
| ANK-BTB   | 3  | 6  | 2  | 7  | 7  |
| ANK-BPA   | 3  | 3  | 2  | 4  | 4  |
| ANK-ACBP  | 3  | 0  | 0  | 2  | 0  |
| ANK-GPCR  | 2  | 0  | 0  | 4  |    |
| ANK-O     | 4  | 73 | 21 | 56 | 65 |
| Total     | 54 | 175| 71 | 105| 130|

(a)

(b)

Figure 2: Continued.
4. Conclusion

This study mainly focused on the sequences ANK proteins: their classification and phylogenetic analysis by using the first release of the moss genome annotation. We are aware that newer versions of the moss genome annotation are already available in Phytozome. As such the results shown here do not provide a complete overview of the whole repertoire of P. patens ankyrin proteins. Moreover, experimental verification and wet-lab functional studies of the genes encoding these
proteins are necessary to come to any definite conclusion about their biological function. Nevertheless, this may serve as a useful reference for more detailed functional analyses as well as for the selection of appropriate candidate genes for further studies and genetic manipulation of *P. patens* ankyrin proteins.

**Competing Interests**

The authors declare that they have no competing interests.

**Authors’ Contributions**

Niaz Mahmood conceptualized the study. Both Niaz Mahmood and Nahid Tamanna analyzed and interpreted the data; and Niaz Mahmood wrote the paper. The final version of the paper is approved by both of the authors.

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