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RESEARCH ARTICLE

Ammonium transporter genes in millets: insights into structure, function, evolutionary conservation, divergence, and phylogenetic analysis

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Abstract Millets, resilient and nutritionally rich crops, are increasingly recognized for their potential in sustainable agriculture. Ammonium transporter (AMTs) gene family significantly contribute to the absorption and transport of NH_4^+ form of nitrogen in plants. The information about the structure and function of ammonium transporter genes in millet species is lacking. The millet crops such as pearl millet, proso millet, finger millet, sorghum, foxtail millet and green foxtail millet exhibit genetic variation in AMTs, which can be harnessed to improve NUE. Thus, genomic sequences of the six millet species were used and a total of 53 *AMT* genes were identified. Further, comprehensive analysis of chromosomal distribution, transmembrane structure prediction, presence of exons and introns, domain and motif organization, phylogeny, and synteny analysis were carried out. The phylogenetic analysis illustrated that millet AMTs belong to two subfamilies AMT1 and AMT2 (AMT2/AMT3/AMT4). Ka/Ks analysis showed that segmental duplications have contributed considerably in the evolution of millet AMTs. Phylogenetic classification of members of *Poaceae* using the amino acid sequences of *AMT1.1* genes confirms the speciation patterns shown by *matK* gene sequence. Promoter analysis of millet AMTs showed presence of cis-elements related to light response, anaerobic induction, growth hormones, drought stress, biotic stress and several endogenous signals related to plant growth and development. This research provides insights into the structural and functional aspects of ammonium transporter genes in millets, and will serve as a foundation for utilizing AMTs for devising NUE strategies.

Keywords AMT · Millet · NUE · Evolution · Transmembrane domain · Cis-elements

Introduction

Nitrogen (N) is the most commonly required macronutrient for plant growth and development. It is an essential element for the synthesis of biomolecules such as nucleotides, amino acids, proteins, chlorophyll, and several others (Marcos de Leão et al. 2020). Nitrogen in the soil exists in three forms, which include organic nitrogen compounds, ammonium (NH_4^+), and nitrate (NO_3^-) ions (Williams and Miller 2001). Plants absorb nitrogen primarily in the ammonium and nitrate forms, and the organic nitrogen compounds need to be converted to these two forms before being taken by the plants. The use of nitrogen by plants involves absorption,

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assimilation, and remobilization during plant growth and development. In addition to N absorption from the soil, nitrogen use efficiency (NUE) depends on the assimilation of inorganic nitrogen from the soil, and the utilization of nitrogen during the life cycle of a crop plant (Masclaux-Daubresse et al. 2010; Xu et al. 2012). Plants have a preference for ammonium N form over nitrate nitrogen for uptake from the soil due to the direct assimilation of NH_4^+ into amino acids in plant cells, whereas, NO_3^- nitrogen has to be reduced to NH_4^+ before assimilation (Bloom et al. 1992; Jiang et al. 2019; Boschiero et al. 2019). The biological assimilation of nitrogen occurs either through the glutamine synthetase/glutamate synthase pathway (GS/GOGAT) or through glutamate dehydrogenase (GDH), resulting in the synthesis of glutamine which is the substrate for the synthesis of other amino acids via transamination reactions. Ammonium transport is tightly regulated during plant growth and development by the activities of high- and low-affinity ammonium transporters (Loque et al. 2006; Yuan et al. 2007; Kiba and Krapp 2016). Generally, high-affinity ammonium transport is preferred for NH_4^+ acquisition by plants due to the low ammonium concentration (< 1 mm) in the soil (Hao et al. 2020).

Ammonium transporters (AMTs) involved in the uptake of NH_4^+ have been identified in varied plant species (Couturier et al. 2007; Yuan et al. 2007; Tang et al. 2020). These AMTs are distributed in the plasma membranes of plant cells and form homo-or heterotrimers complexes for facilitating the passing of NH_4^+ ions or NH_3 through the pore (Shelden et al. 2001; Ludewig et al. 2003). The transport mechanism of plant AMTs could be an NH_4^+ uniporter, NH_4^+/H^+ symporter, or NH_3/H^+ co-transporter. Plant AMTs can be divided into the following two subfamilies: the AMT1 subfamily (AMT1 cluster) and the AMT2 subfamily (AMT2/3/4 cluster) (Huang et al. 2022).

The *AMT* genes were identified both in prokaryotic and eukaryotic organisms (Mcdonald and Ward 2016). The first ammonium transporter genes were identified in *Saccharomyces cerevisiae* and *Arabadopsis thaliana* (Marini et al. 1997; Ninnemann et al. 1994). Further, AMT family genes were characterized in several crop species namely; *Zea mays* (Gu et al. 2013), *Glycine max* (Kobae et al. 2010), *Arabidopsis thaliana* (Loqué et al. 2006; Yuan et al. 2007, 2009, 2013; Lanquar et al. 2009; Huang et al. 2015), *Lotus japonicas* (Guether et al. 2009; Wang et al. 2022), *Oryza sativa* (Ferreira et al. 2015; Li et al. 2016), *Medicago truncatula* (Breuillin-Sessoms et al. 2015), *Populus trichocarpa* (Wu et al. 2015), *Triticum aestivum* (Duan et al. 2016; Li et al. 2017), *Coffea canephora* (Santos et al. 2017), *Medicago truncatula* (Breuillin-Sessoms et al. 2015), Pinus (Castro-Rodriguez et al. 2016), *Solanum lycopersicum* (Filiz and Akbudak 2020), and *Malus domestica* (Huang et al. 2022).

Millets, a group of small-seeded grains, have gained recognition as a pivotal component in achieving global food security and contribute to agricultural sustainability. Millet species include pearl millet (*Pennisetum glaucum*), finger millet (*Eleusine coracana*), green foxtail millet (*Setaria viridis*), foxtail millet (*Setaria italica*), great millet (*Sorghum bicolor*), proso millet (*Panicum miliaceum*), kodo millet (*Paspalum scrobiculatum*), Japanese barnyard millet (*Echinocloa esculenta*), Indian barnyard millet (*Echinocloa frumentacea*), and little millet (*Panicum sumatrense*), among others (Goron and Raizada 2015). Millets are hardy, resilient crops that thrive in diverse agro-climatic conditions, making them an essential resource for enhancing agricultural sustainability. Their exceptional nutritional profile, including high levels of protein, fiber, and essential micronutrients, placed millets as key contributors to improving food security, especially in regions grappling with malnutrition and food scarcity (Ceasar 2023). Improving the NUE of the cereals is essential to enhance yields under low-nutrient soils and conserve the fertility of the soils (Baligar et al. 2001; Bariya and Ahish 2014; Naeem et al. 2017; Nieves-Cordones et al. 2020). Several investigations have been conducted during the last decade by the wider scientific community, employing various molecular genetic tools to study and improve the NUE of crop plants. These include the utilization of genome-wide association study (GWAS) approach, molecular marker-assisted breeding (MAB), nutrient transporter characterization and functional genomics approaches. These investigations have been reported for model crop plants to improve NUE (Hawkesford 2012; Avin-Wittenberg et al. 2018). However, genome-based and forward genetic research may not be easy for millets with limited genomic resources.

Complete annotated genome sequence information is lacking for many millet species, which limits the understanding of gene sequences involved

in determining the NUE traits in these crops. Nevertheless, the genome sequences of six millets, namely; *Setaria viridis, Setaria italica, Eleusine coracana, Sorghum bicolor, Pennisetum glaucum and Panicum miliaceum* are available for sequence analyses. These genome sequences provide a valuable resource to understand the structural and functional domains of genes coding for plant productivity, consequently enabling us to identify the *AMT* genes in the millet genome. Scanty reports for the *AMT* gene family characterization in millets are available in the literature, with limited coverage of genome sequence databases. Inadequate analysis of two AMTs in *S. bicolor* (Koegel et al. 2013), followed by an *EcAMT1* study with other nutrient transporters (Maharajan et al. 2022), and a brief report about *S. italica AMTs* (*SiAMT1.1* and *SiAMT1.3*) phylogeny and level of expression (Ahmad et al. 2018; Ceasar et al. 2023). Whereas, *S. viridis, P. glaucum and P. miliaceum* AMTs have not been taken into account for defining the structural and functional domains of this gene family.

After discussing all the above, in this study, six millet species are taken into consideration. We performed detailed analyses of the sequence characteristics, gene structures, chromosome distribution, motif compositions, and evolutionary relationships of millet *AMT* genes. In this context, the characterization of *AMT* genes from these six species and their comparative analysis to study and improve the NUE in millets may be helpful for further research.

Material and methods

Identification of *AMT* genes in different species

The genomic sequences, protein sequences, coding sequences (CDS) and genomic feature files (GFF) of six millet crops (*S. viridis, S. italica, E. corocana, S. bicolor, P. miliaceum, and P. glaucum*) obtained from Phytozome database (https://phytozome-next.jgi.doe.gov/ accessed on June1, 2023), National Genomics Data Center (NGDC) (https://ngdc.cncb.ac.cn/gwh/ accessed on June 1 2023) and the International Pearl Millet Genome Sequencing Consortium (IPMGSC) (https://cegsb.icrisat.org/ipmgsc/index.html accessed on June 1 2023). The Hidden Markov model (HMM) of all the conserved protein domain file Pfam-A.hmm was downloaded from InterPro (https://www.ebi.ac.uk/interpro/download/pfam/ accessed on 5 June, 2023). A simple HMM search of the TBtools software (Chen et al. 2020) was used to obtain ammonium transporters in different species. Pfam Id of ammonium transporter (Ammonium_transp—PF00909) was used for this study. Proteins with *e*-values of less than 5E-40 were included in further analyses. Different splicing variants of one gene and the incomplete genes were discarded. We searched for the ammonium-domain in all of the collected proteins using Interproscan (http://www.ebi.ac.uk/ Tools/pfa/iprscan/ accessed on June 6, 2023) and SMART software (Letunic et al. 2012).

Physicochemical properties and transmembrane structure analysis of AMT proteins

The theoretical molecular weight (kDa) and isoelectric point (pI) of millet *AMT*s were calculated using the ExPASy server (http://web.expasy.org/compute_pi/ accessed on June 6, 2023) (Gasteiger et al. 2003). The evaluation of the grand average of hydropathicity (GRAVY) of all identified proteins was measured through the GRAVY calculator (https://www.gravy-calculator.de/ accessed on June 6, 2023) (Gasteiger 2003). Predictions of subcellular localization of the concerned proteins were verified with the help of Plant-mPloc tool (http://www.csbio.sjtu.edu.cn/cgi-bin/PlantmPLoc.cgi/ accessed on June 6, 2023) (Chou and Shen 2010). The TMHMM server v. 2.0 (https://services.healthtech.dtu.dk/services/TMHMM-2.0/ accessed on June 6, 2023) (Krogh et al. 2001) was used for the prediction of transmembrane helices in AMT proteins. Individually, the physical locations of millet *AMTs* genes were obtained from the millet database (*S. viridis*, *S. italica, E. coracana*, *S. bicolor, P. miliaceum, and P. glaucum*) genome, and the map to locate genes on chromosomes of all six millet species was constructed through the PhenoGram (http://visualization.ritchielab.org/phenograms/plot accessed on June 10, 2023 (Wolfe et al. 2013).

Gene structure, conserved motif and conserved protein domain analyses of AMTs

The gene structures (CDS/exon/intron) of all the *AMT* genes were determined using the Gene Structure

Display Server (CSDS) (http://gsds.gao-lab. org/ accessed on June 8, 2023) (Hu et al. 2015). For these analyses, the predicted coding sequence (CDS) of *AMT* genes and their corresponding genomic DNA sequences were used. The MEME (Multiple Em for Motif Elicitation) online tool (Bailey and Elkan 1994; Bailey et al. 2009) was used to identify the conserved motifs in the promoter regions of *AMT* genes and AMT amino acid sequences (https://meme-suite.org/meme/tools/meme accessed on June 8, 2023). The conserved domains of AMT proteins were analysed by the NCBI-CD (National Center for Biotechnology Information- Conserved Domain) search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi accessed on June 8, 2023) (Marchler-Bauer and Bryant 2004). The TBtools software was used to integrate the phylogenetic tree, conserved motifs of and domains of millet AMT proteins (Chen et al. 2020).

Phylogenetic tree analysis of AMTs

The full-length amino acid sequences of AMTs from *Arabidopsis thaliana*, *Triticum aestivum*, *Oryza sativa, Zea mays, Brachypodium distachyon,* and *Hordium vulgare* were downloaded from the Phytozome database (Goodstein et al. 2012). AMT amino acid sequences of two bacteria, viz. *Escherichia coli* and *Nitrosomonas europaea* are also downloaded from NCBI (National Center for Biotechnology Information) (https://blast.ncbi.nlm.nih.gov/ accessed on June 6, 2023). To gain a deeper understanding of the taxonomical classification of the poaceae family, the chloroplast maturase K (matK) amino acid sequences were also downloaded. The amino acid sequences of AMTs as well as matK were aligned by MEGA-XI software (Koichiro et al. 2021), and a total of four phylogenetic trees were constructed by the maximum-likelihood method (ML). Bootstrap analysis was calculated for 1000 replicates. The evolutionary tree was visualized on the web-based tool Interactive Tree of Life (iTOL, https://itol.embl.de/ accessed on June 15, 2023) (Letunic and Bork 2021).

Ka/Ks analyses

The synonymous (*Ks*) and non-synonymous (*Ka*) substitution rates of the paralogs genes were further investigated by using the *Ka_Ks* calculator 2.0 (Zhang et al. 2006). A circular ideogram was made by Circos (Krzywinski et al. 2009) using TBTool software (Chen et al. 2020) to facilitate the display of relationships between paralogous pairs by the use of coloured lines. These encode the position, size, and orientation of related genomic elements in the Circos plots.

Synteny analysis of *AMT* genes

For visualization of protein sequence similarity between these six millet *AMT* genes, an online visualization tool named Circoletto (http://tools.bat.infspire.org/circoletto/ accessed on June 16, 2023) (Darzentas 2010) was used, which provides fast and informative overview of sequence similarity of search results. These results provide an essential first glimpse of the relationship between protein sequences.

Cis-element analysis of millet *AMT* promoter regions

The 2 kb upstream genomic DNA sequences of all six millet *AMT* genes were used for promoter analysis, and the cis-regulatory elements were predicted using the PlantCARE online website (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/ accessed on 18 June 2023) (Lescot et al. 2002). The data were visualised by TBtools (Chen et al. 2020).

Results

Identification of *AMT* genes in different species

After validation of AMTs by HMM search, a total of 53 AMT protein sequences (*S. viridis*-7; *S. italica*-9; *E. corocana* -12; *S. bicolor*-5; *P. glaucum* -8 and *P. miliaceum* -12) were identified from six millet genomes. The AMTs of these species were termed as per existing rules of nomenclature for gene symbols, such as *SvAMTs* (derived from *S. viridis*), *SiAMTs* (derived from *S. italica*), *EcAMTs* (derived from *E. corocana*), *SbAMTs* (derived from *S. bicolor*), *PgAMTs* (derived from *P. glaucum*) and *PmAMTs* (derived from *P. glaucum*) genes throughout the study.

Physicochemical properties and transmembrane structure analysis of AMT proteins

The physiochemical properties of AMT proteins have been established using parameters such as chromosome location, strand, protein length, molecular weight (MW), isoelectric point (pI), prediction of the hydrophobicity (GRAVY), subcellular location and their family. The lengths of the millet AMT proteins ranged from 304 (*SbAMT* 2.2b; Sobic.003G344700) to 632 (*EcAMT* 2.1; ELECO.r07.5BG0417460) amino acids, with molecular weight ranging from 32.07 kD (PmAMT4.1b; GWHPAAEZ055444*)* to 67.61 kD (EcAMT 2.1; ELECO.r07.5BG0417460*)* and theoretical pI values ranging from 5.37 (*SbAMT* 3.3; Sobic.004G173200) to 8.84 (*PmAMT1.3b*; GWHPAAEZ069937). Subcellular localization prediction showed that all millet AMTs were localized to the cell membrane with few exceptions such as, *SbAMT* 3.3 (Sobic.004G173200), *PmAMT1.3a* (GWHPAAEZ021947), *PmAMT3.2* (GWHPAAEZ054634), and *PmAMT3.3* (GWHPAAEZ070534) are located both in cell membrane as well as vacuole. And the *PgAMT1.1* (Pgl_GLEAN_10009225) is located in both cell membrane and mitochondrion whereas *PmAMT3.2* (GWHPAAEZ054634) found in cell membrane, vacuole and mitochondrion (Table 1). The grand average of hydropathy (GRAVY) value was calculated for all the millet AMT proteins.

In this study, the predictions of the hydrophobicity of the deduced amino acid sequences indicated that the GRAVY of all millet AMT proteins were above zero, this led to conclusion that these amino acids are polar in nature (Table 1). Further, distribution of all six millet AMTs on chromosomes was analysed. It was observed that *AMTs* of *S. viridis* were located on five chromosomes viz. Chr1, Chr3, Chr5, Chr7 and Chr9 (Online Resource: S1), AMTs of *S. italica* located on five scaffolds with number 1, 3, 5, 7, 9 (Online Resource: S2), AMTs of *E. corocana* placed on Chr 1A, Chr 1B, Chr 2A, Chr 3A, Chr 3B, Chr 4B and Chr 5B. (Online Resource: S3), *AMTs* of *S. bicolor* located on three chromosomes viz, *Chr1,* Chr3 and Chr4 (Online Resource: S4). *PgAMTs* were present on three chromosomes (Chr 1, Chr 3 and Chr 6) and on the scaffold 2474 (Fig. 1). Whereas, *PmAMTs* were located on six chromosomes viz. Chr 1, Chr 3, Chr 4, Chr 5, Chr 6 and Chr 12 (Online Resource: S5). Transmembrane domain analysis of all millet AMTs showed occurrence of conserved transmembrane domains (Fig. 2 and Online Resource: S6-S10). These transmembrane domains regulate membrane localization and transport activity of a protein. Generally, millet AMTs have 11–12 transmembrane domains, whereas, these *PgAMT1.1, PmAMT4.1b, SbAMT 2.2, SbAMT 3.3, PmAMT3.2, PgAMT1.2b, PmAMT2.2, PgAMT2.2, PmAMT1.3b* and *PgAMT1.2a* have transmembrane domains varying from 6 to 10) which may be due to the small size of their protein sequences (Table 1).

Gene structure, conserved motif and conserved protein domain analyses of AMTs

Structural analysis of the AMT proteins of six millet species were carried out expending the conserved domains and motifs based on the evolutionary relationships (Fig. 3). Gene structures of millet AMT proteins were predicted by using their CDS and genomic sequences. The graphical representation derived using GSDS showed that the AMT1 superfamily has less number of introns whereas in AMT2 the presence of introns is very common (Fig. 3). The domain analysis prediction showed that, the ammonium transporter (Ammonium_transp; PFam ID: PF00909, InterPro ID: IPR001905) structural domains are present in all the query proteins. This Ammonium_transp domains have found to relate the cl03012 protein superfamily and are mainly associated with transporting NH_4^+ across the membrane. In MEME server, the number of motif finder parameter was set to 20, so that upto 20 putative conserved motifs were found from each of the query protein sequences. Throughout the motif analysis it was found that, the subfamilies AMT1 and AMT2 had variable motif compositions. Also, proteins in the same subgroup showed identical motif components. Some of the motifs usually range from 1 to 5 were found present in AMTs of all the species this indicated that there are characteristic motifs of ammonium transporters.

Noticeably, presence of a small motif can cause differences in subgroups and which may give an idea about the evolution of AMTs. In *S. viridis* AMT proteins, four motifs (motif-3, 6, 7 and 9) were found commonly present in all the AMT proteins of this

Table 1 Physicochemical properties of millet AMT proteins

Gene	Gene identifier	Chromosome location	Strand	Protein (aa)	MW (KDa)	pI	GRAVY	TM	Subcellular prediction	Category
SvAMT 3.2	Sevir.9G019200	Chr_09:998,603..1001096	Reverse	480	51.01	7.58	0.582	11	Cell membrane	AMT 2
SvAMT 1.1	Sevir.7G171200	Chr_07:23,574,218..23576366	Forward	495	52.3	7.62	0.49	11	Cell membrane	AMT 1
SvAMT 1.2	Sevir.1G242300	Chr_01:30,890,338..30892125	Forward	491	51.78	7.65	0.534	12	Cell membrane	AMT 1
SvAMT 3	Sevir.1G193600	Chr_01:26,681,321..26685481	Forward	479	51.9	6.24	0.572	11	Cell membrane	AMT 2
SvAMT 3.1	Sevir.5G401300	Chr_05:41,285,195..41288490	Forward	489	52.91	6.25	0.42	11	Cell membrane	AMT 2
SvAMT 2.2	Sevir.5G374500	Chr_05:39,451,115..39453016	Reverse	504	53.82	7.08	0.499	11	Cell membrane	AMT 2
SvAMT 2.1	Sevir.3G215200	Chr_03:16,122,574..16125844	Reverse	489	51.7	8.41	0.534	11	Cell membrane	AMT 2
SiAMT 2.2	Seita.5G368900	scaff old_5:40,571,965..40573763	Reverse	504	53.82	7.08	0.499	11	Cell membrane	AMT 2
SiAMT 2.3	Seita.5G368800	scaff old_5:40,566,668..40568911	Reverse	489	51.79	8.82	0.516	11	Cell membrane	AMT 2
SiAMT 3.1	Seita.5G395800	scaff old_5:42,463,248..42466526	Forward	489	52.91	6.25	0.42	11	Cell membrane	AMT 2
SiAMT 4.1	Seita.9G091900	scaffold_9:5,543,609..5545092	Reverse	465	49.46	6.65	0.592	11	Cell membrane	AMT 2
SiAMT 3.2	Seita.9G019500	scaffold_9:1,021,605..1024466	Reverse	487	51.79	7.59	0.538	11	Cell membrane	AMT 2
SiAMT 3.3	Seita.1G189700	scaff old_1:27,222,972..27226374	Forward	479	51.91	6.24	0.562	11	Cell membrane	AMT 2
SiAMT 1.2	Seita.1G237300	scaff old_1:31,515,483..31517415	Forward	491	51.78	7.65	0.534	12	Cell membrane	AMT 1
SiAMT 1.1	Seita.7G162400	scaff old_7:24,600,123..24602275	Forward	495	52.3	7.62	0.49	11	Cell membrane	AMT 1
SiAMT 2.1	Seita.3G209900	scaff old_3:16,480,176..16483336	Reverse	489	51.73	8.41	0.54	11	Cell membrane	AMT 2
EcAMT 1.2	ELECO.r07.2AG0137750	2A:51,671,690..51673175	Forward	494	52.27	6.21	0.486	12	Cell membrane	AMT 1
EcAMT 4.1	ELECO.r07.3BG0261410	3B:5,459,818..5461296	Reverse	461	49.27	6.38	0.602	10	Cell membrane	AMT 2
EcAMT 3.2a	ELECO.r07.3BG0254210	3B:1,002,670..1005397	Reverse	486	51.48	6.29	0.561	11	Cell membrane	AMT 2
EcAMT 2.1	ELECO.r07.5BG0417460	5B:5,299,385..5304851	Reverse	632	67.61	9.57	0.275	11	Cell membrane	AMT 2
EcAMT 2.2a	ELECO.r07.1AG0038750	1A:51,435,994..51437924	Reverse	491	52.33	7.21	0.471	11	Cell membrane	AMT 2
EcAMT 2.2b	ELECO.r07.1AG0038760	1A:51,438,679..51440419	Reverse	504	53.77	6.71	0.49	11	Cell membrane	AMT 2
EcAMT 3.1a	ELECO.r07.1AG0041350	1A:53,000,182..53002554	Forward	489	53	6.96	0.442	11	Cell membrane	AMT 2
EcAMT 1.1a	ELECO.r07.4BG0342310	4B:8,731,647..8733144	Reverse	498	52.42	8.09	0.506	11	Cell membrane	AMT 1
EcAMT 4.2	ELECO.r07.3AG0216120	3A:6,014,435..6015927	Reverse	466	49.62	6.12	0.643	11	Cell membrane	AMT 2
EcAMT 2.2c	ELECO.r07.1BG0088780	1B:66,982,724..66984427	Reverse	504	53.79	6.71	0.483	11	Cell membrane	AMT 2
EcAMT 2.2d	ELECO.r07.1BG0088770	1B:66,980,046..66981963	Reverse	470	49.65	7.77	0.527	11	Cell membrane	AMT 2

Table 1 (continued)

Gene	Gene identifier	Chromosome location	Strand	Protein (aa)	MW (KDa)	pI	GRAVY	TM	Subcellular prediction	Category
EcAMT 3.1b	ELECO.r07.1BG0091350	1B:68,446,299..68449194	Forward	487	52.77	6.93	0.451	11	Cell membrane	AMT 2
SbAMT 3.1	Sobic.003G370400	Chr03:68,640,432..68644118	Forward	488	53.18	6.62	0.459	11	Cell membrane	AMT 2
SbAMT 2.2	Sobic.003G344700	Chr03:66,599,389..66602632	Reverse	304	35.84	5.82	0.533	8	Cell membrane	AMT 2
SbAMT 1.2	Sobic.004G217800	Chr04:56,726,026..56727822	Forward	489	51.78	7.13	0.523	11	Cell membrane	AMT 1
SbAMT 3.3	Sobic.004G173200	Chr04:52,589,694..52592427	Forward	341	36.38	5.37	0.629	8	Cell membrane, Vacuole	AMT 2
SbAMT 4.1	Sobic.001G089400	Chr01:6,937,980..6939958	Reverse	465	49.76	6.05	0.623	11	Cell membrane	AMT 2
PmAMT3.1	GWHPAAEZ067017	Chr5: 49,161,984–49,162,932, 49,164,192–49,164,709	Forward	488	52.62	6.72	0.417	11	Cell membrane	AMT 2
PmAMT2.1	GWHPAAEZ051598	Chr3: 15,959,560–15960116, 15,960,747–15961032, 15,961,793–15,962,419	Reverse	489	51.6	8.4	0.556	11	Cell membrane	AMT 2
PmAMT3.3	GWHPAAEZ070534	Chr6: 14,647,264–14,647,772, 14,648,952–14,649,237, 14,649,312–14,649,956	Reverse	479	51.96	6.1	0.536	11	Cell membrane, Vacuole	AMT 2
PmAMT2.3	GWHPAAEZ066648	Chr5: 47,283,907–47284463, 47,284,687–47,284,972, 47,285,234–47,285,884	Reverse	497	52.56	7.15	0.52	11	Cell membrane	AMT 2
PmAMT4.1a	GWHPAAEZ001864	Chr1: 5,294,262–5,295,743	Reverse	493	52.63	7.55	0.625	11	Cell membrane	AMT 2
PmAMT2.2	GWHPAAEZ066649	Chr5: 47,286,672–47,287,228, 47,287,342–47,287,627, 47,287,923–47,288,366	Reverse	428	45.67	7.58	0.46	9	Cell membrane	AMT 2
PmAMT1.2a	GWHPAAEZ021948	Chr12: 33,432,823–33,434,292	Forward	489	51.84	8.13	0.492	11	Cell membrane	AMT 2
PmAMT1.2b	GWHPAAEZ069936	Chr6: 10,702,560–10704026	Reverse	488	51.56	8.12	0.51	11	Cell membrane	AMT 2
PmAMT3.2	GWHPAAEZ054634	Chr4: 1,013,732–1014028, 1,014,144–1014791	Reverse	314	33.68	8.49	0.615	8	Cell membrane, Mitochondrion, Vacuole	AMT 2
PmAMT1.3a	GWHPAAEZ021947	Chr12: 33,427,550–33428986	Forward	478	50.72	6.44	0.519	11	Cell membrane, Vacuole	AMT 1
PmAMT4.1b	GWHPAAEZ055444	Chr4: 4,860,552–4861379, 4,861,936–4,862,034	Reverse	308	32.09	5.42	0.446	7	Cell membrane	AMT 2
PmAMT1.3b	GWHPAAEZ069937	Chr6: 10,707,555–10708796	Reverse	413	44.18	8.84	0.563	10	Cell membrane	AMT 1
PgAMT2.1	Pgl_GLEAN_10026660	chr1:157,402,877:157,405,839	Reverse	492	51.8	7.1	0.555	11	Cell membrane	AMT 2
PgAMT3.3	Pgl_GLEAN_10007710	chr3:37,114,320:37,116,955	Forward	479	51.85	5.97	0.564	11	Cell membrane	AMT 2
PgAMT3.2	Pgl_GLEAN_10030271	scaffold2474:562,144:564,806	Forward	480	51.25	6.29	0.539	11	Cell membrane	AMT 2
PgAMT2.3	Pgl_GLEAN_10012021	chr6:102,755,180:102,757,058	Forward	445	47.04	7.15	0.513	11	Cell membrane	AMT 2
PgAMT2.2	Pgl_GLEAN_10012022	chr6:102,752,566:102,754,178	Forward	454	48.56	6.87	0.318	9	Cell membrane	AMT 2

Table 1 (continued)

Gene	Gene identifier	Chromosome location	Strand	Protein (aa)	MW (KDa)	pI	GRAVY	TM	Subcellular prediction	Category
PgAMT1.2a	Pgl_GLEAN_10027285	chr3:92,191,961:92,193,431	Forward	474	50.24	6.54	0.495	10	Cell membrane	AMT 1
PgAMT1.2b	Pgl_GLEAN_10027258	chr3:92,531,232:92,532,569	Forward	392	41.34	6.7	0.569	8	Cell membrane	AMT 1
PgAMT1.1	Pgl_GLEAN_10009225	chr3:51,968,953:51,970,373	Reverse	334	35.55	6.64	0.466	6	Cell membrane, Mitochondrion	AMT 1

species (Online Resource: S11). In *S. italica* AMT proteins, the five motifs (motif-5, 6, 8, 9 and 11) were found common in all AMTs (Online Resource: S12). Whereas in *E. corocana,* only single motif (motif-1) was found common in both the AMT1 and AMT2 families (Online Resource: S13). Similarly, in *P. miliaceum*, a single motif (motif-4) was observed common in all the AMTs (Online Resource: S15). Furthermore, in *S. bicolor* three motifs (motif-1, 2 and 19) are common in all the AMT proteins, (Online Resource: S14). Motif analysis of *P. glaucum* AMTs showed four common motifs in both the protein subfamily (AMT 1 and AMT 2) (Fig. 4).

Phylogenetic tree analysis of AMTs

A total of 53 millet AMT protein sequences have been identified using sequence search and alignment and used to understand the evolutionary relationships among *AMT* genes (Fig. 5). The common feature among AMTs of all the millet crops showed that they belong to two subfamilies i.e. AMT1 and AMT2 (AMT2/AMT3/AMT4). The major evolutionary conservation among the transporters exhibited that all millet AMTs stemmed from two major AMT transporter groups necessitates confirmation and further exploration of the evolutionary relationships with the AMTs from other species. The AMT protein sequences of twelve plant species (*Setaria viridis*, *Setaria italica*, *Eleusine corocana*, *Sorghum bicolor*, *Pennisetum glaucum, Panicum miliaceum, Arabidopsis thaliana*, *Oriza sativa*, *Zea mays*, *Hordium vulgare, Triticum aestivum* and *Brachypodium distachyon)* and two bacteria namely, *Escherichia coli* and *Nitrosomonas europaea* (ammonia oxidizing bacterium) were used to construct phylogenetic tree (Fig. 6). The phylogenetic tree structure clearly demonstrated the association of AMTs of Poaceae family which include all millets and other cereal species. The AMTs of *Poaceae* family have some degree of similarity with *AMT* genes from *Arabadopsis thaliana,* however, formed a different clade and clearly indicated divergence of the monocot transporters from the dicot ammonium transporters. The exclusion of bacterial group AMTs (*E. coli* and *N. europaea)* as an outgroup further, confirmed the *AMT* relationships and divergences in the phylogenetic grouping.

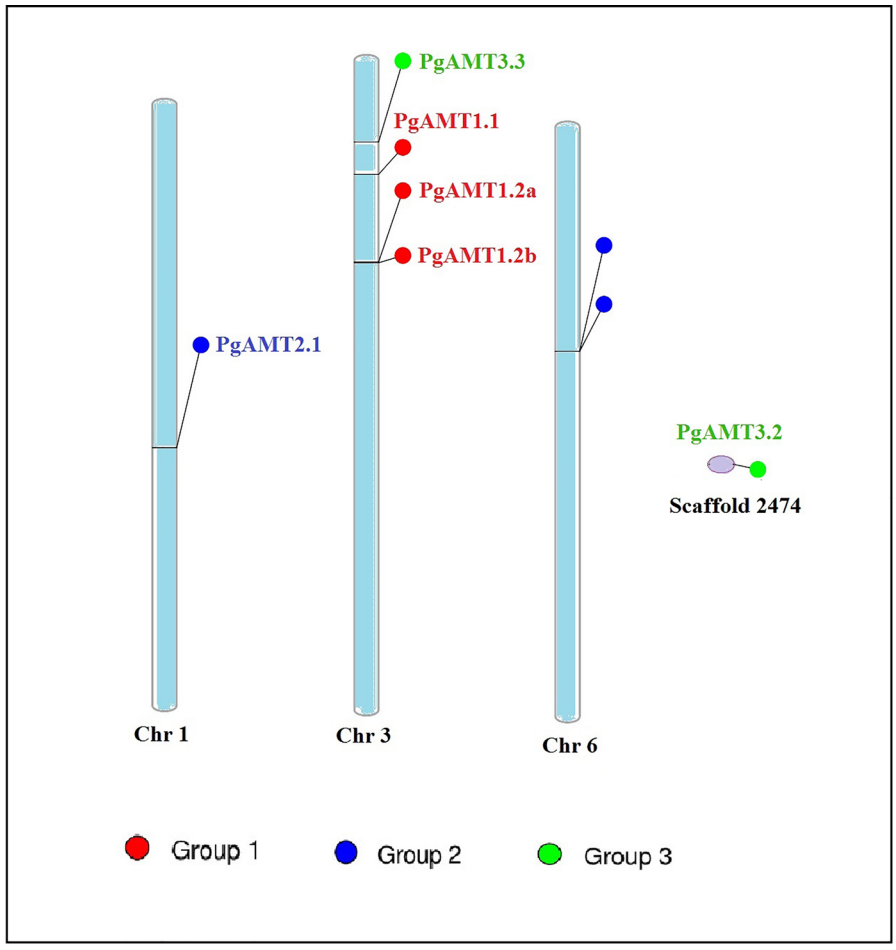

Fig. 1 Representative figure chromosomes of *Pennisetum glaucum* showing distribution of *PgAMT* genes. The chromosome number is listed below each chromosome while the numbers on the left represent location of the *PgAMT* genes. (AMTs chromosomal locations for other five millet species are given in Online Resource: S1-S5)

Ka/Ks analyses

Nucleotide substitutions in the coding regions may or may not result into amino acid change in the protein. One of the parameters i.e. Ka/Ks ratio which is the measure of the number of nonsynonymous substitutions per nonsynonymous site (Ka) and the number of synonymous substitutions per synonymous site (Ks). This Ka/Ks ration is the measure of selection pressure a gene has experienced during evolution. In the millet AMT family, analysis of selection types of duplicate gene pairs in the *SvAMTs*, *SiAMTs*, *EcAMTs, SbAMTs, PgAMTs* and *PmAMTs* genes were carried out using the Ka/Ks ratio. Majority of duplicated gene pairs has Ka/Ks ration less than one hence, less nonsynonymous substitutions taken place and the most of millet AMT genes have undergone negative selection. Majority of AMT genes are the resultant products of purifying selection during evolution (Table 2). However, the gene pair *SbAMT1.2* and *SbAMT4.1* showed that it had undergone neutral selection (Ka/Ks = 1). Schematic representations of the chromosomal distribution and inter chromosomal relationships was studied for all the six millet species by making circos plot of each species separately. These graphical representations showed gene duplication events in circular format. Among the 7 *SvAMTs* genes, three segmental duplication pairs (*SvAMT1.2/SvAMT1.1*, *SvAMT2.2/SvAMT2.1* and *SvAMT3.1/SvAMT3.2* were identified (Online Resource: S16). In *S. italica*, out of 9 *SiAMTs* genes only one tandem repeat pair (*SiAMT2.3/SiAMT2.2*) was found and other 3 pairs (*SiAMT4.1/SiAMT2.1, SiAMT3.2/SiAMT3.1 and SiAMT1.1/SiAMT1.2*) showed segment duplication (Online Resource: S17). Further, the 12 *EcAMTs,* 5 segment duplication pairs (*EcAMT3.1b/EcAMT3.1a, EcAMT2.2c/EcAMT2.2b, EcAMT2.2d /EcAMT2.2a, EcAMT1.1a/EcAMT1.2a*

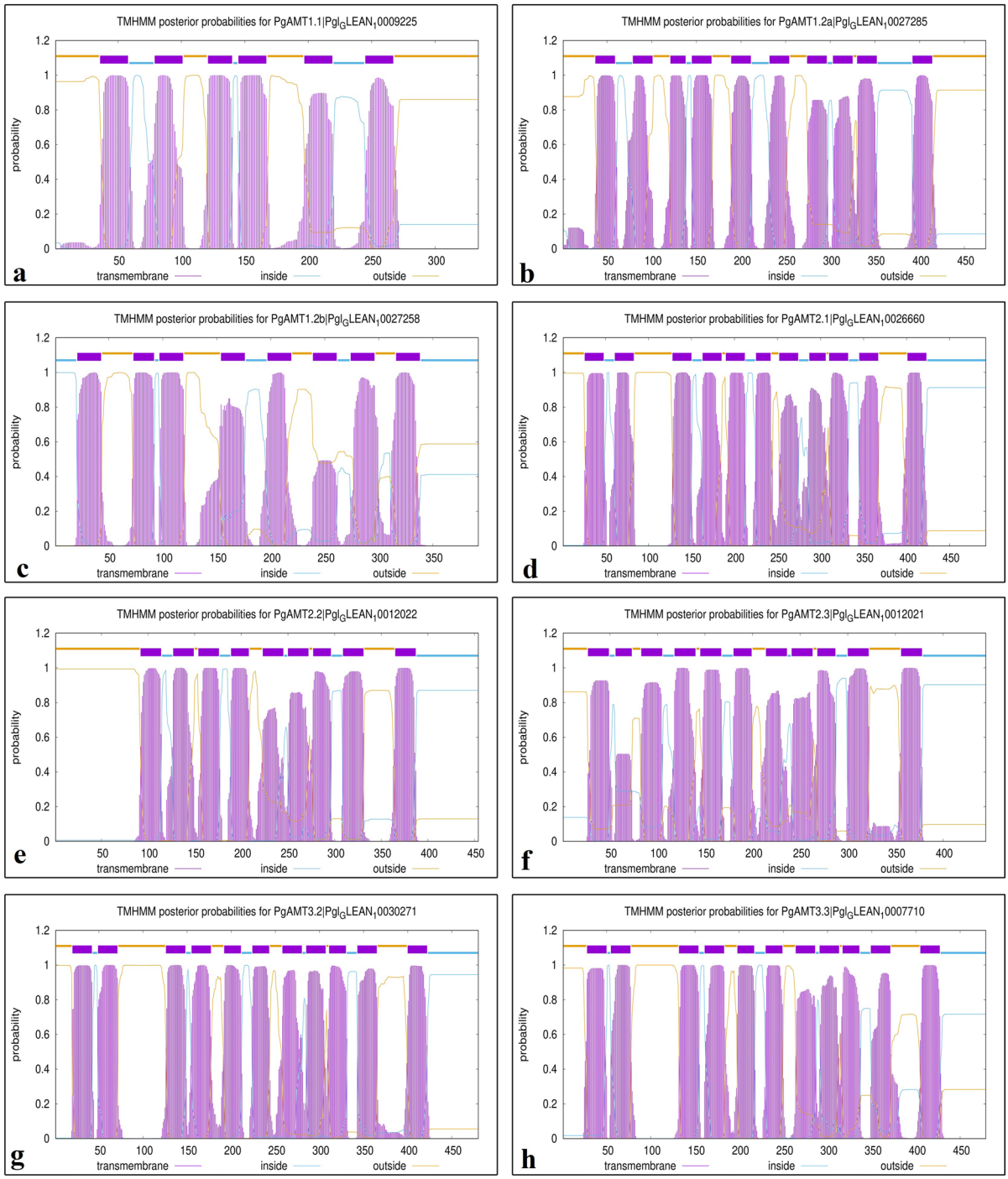

Fig. 2 Representative figure of transmembrane structure prediction AMT proteins of *Pennisetum glaucum.* *Orange line represents outside, purple line indicates on transmembrane and blue line represents inside transmembrane position. (Transmembrane structure prediction of AMTs of other five millet species are given in Online Resource: S6-S10)

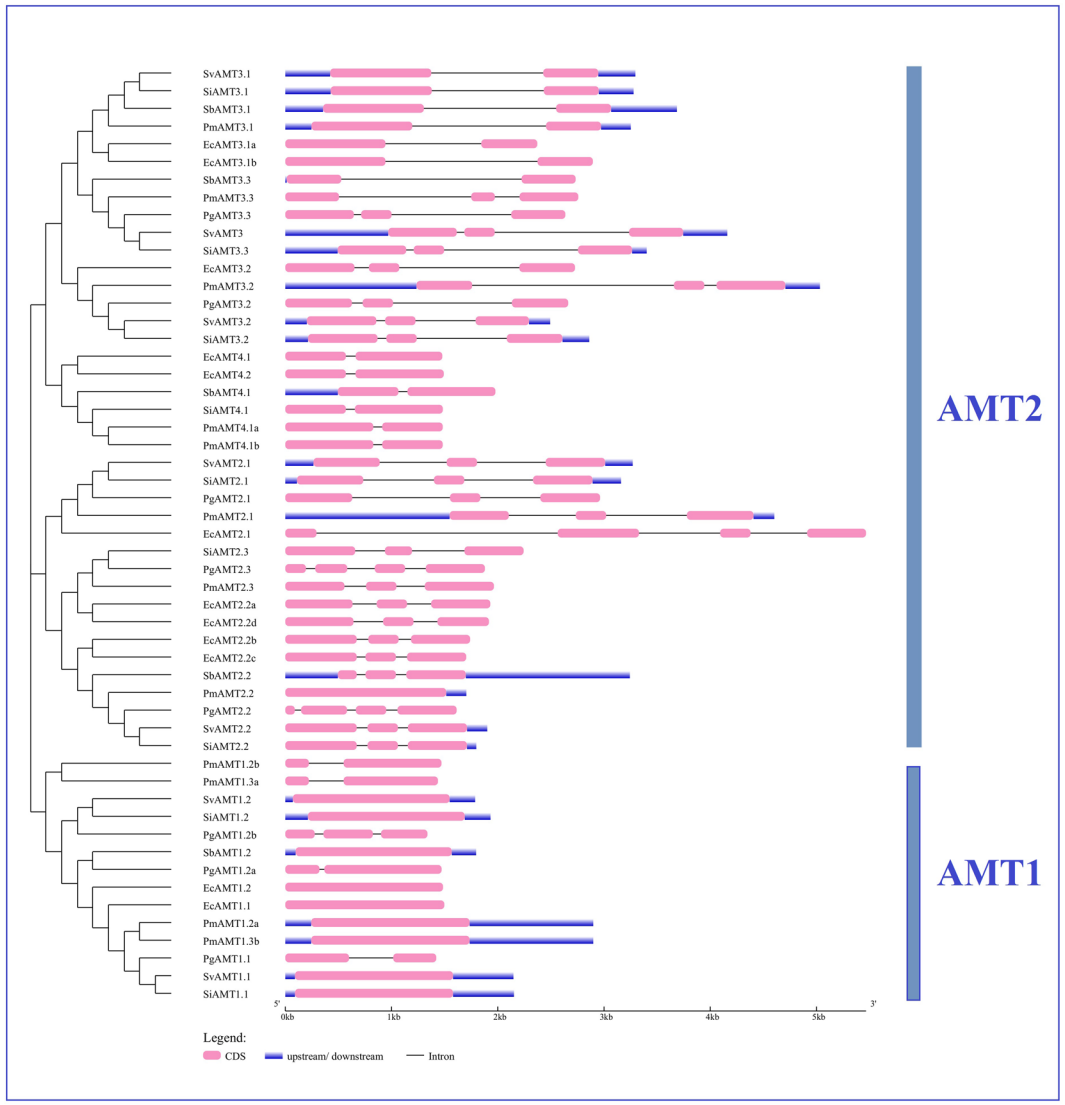

Fig. 3 Representation of the millet *AMT* genes structure showing exons, upstream/downstream regions of the gene and introns with the pink box, blue and black lines, respectively. Scale bar indicates number of nucleic acids (bp)

and *EcAMT4.2/EcAMT4.1*) were identified (Online Resource: S18). In *S. bicolor*, two segmental duplication pairs were observed out of 5 *SbAMTs* genes (Online Resource: S19). In *P. glaucum*, out of 8 genes, one *PgAMT* pair (*PgAMT1.2b/PgAMT1.2a*) found as tandem while other two pairs (*PgAMT3.2/PgAMT3.3* and *PgAMT2.2/PgAMT2.1*) showed segmental duplications (Fig. 7). Whereas, in case of *P. miliaceum,* all the five pairs (*PmAMT4.1b/PmAMT4.1a, PmAMT2.1/PmAMT2.3, PmAMT1.3a/PmAMT1.3b, PmAMT1.2b/PmAMT1.2a* and *PmAMT3.3/PmAMT3.1*) among twelve genes showed segmental duplications (Online Resource: S20). The frequency of occurrence of segmental duplication events in the AMTs of these millet species suggest that these duplications plays bigger role in evolution of these genomes. Several research reports mentioned the significant role of tandem and segmental duplication events in gene family expansion and evolution of their genomes (Canon et al. 2004; Panchy et al. 2016; Kuo et al. 2019).

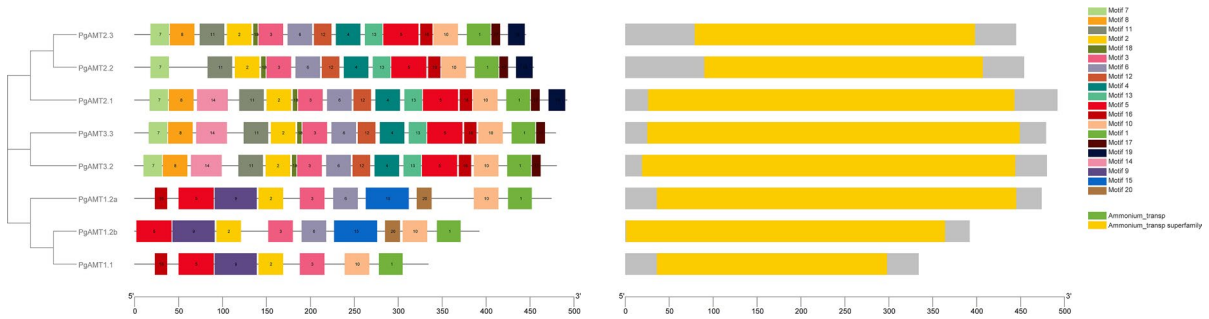

Fig. 4 Illustration of conserved protein motifs and conserved domain of AMTs in *Pennisetum glaucum.* An unrooted phylogenetic tree represents AMT1 and AMT2 subfamilies with their respective motifs are represented using different colours and conserved domains are shown by yellow boxes. (Conserved protein motifs and conserved domain analysis of AMTs of other five millet species are given in Online Resource: S11-S15)

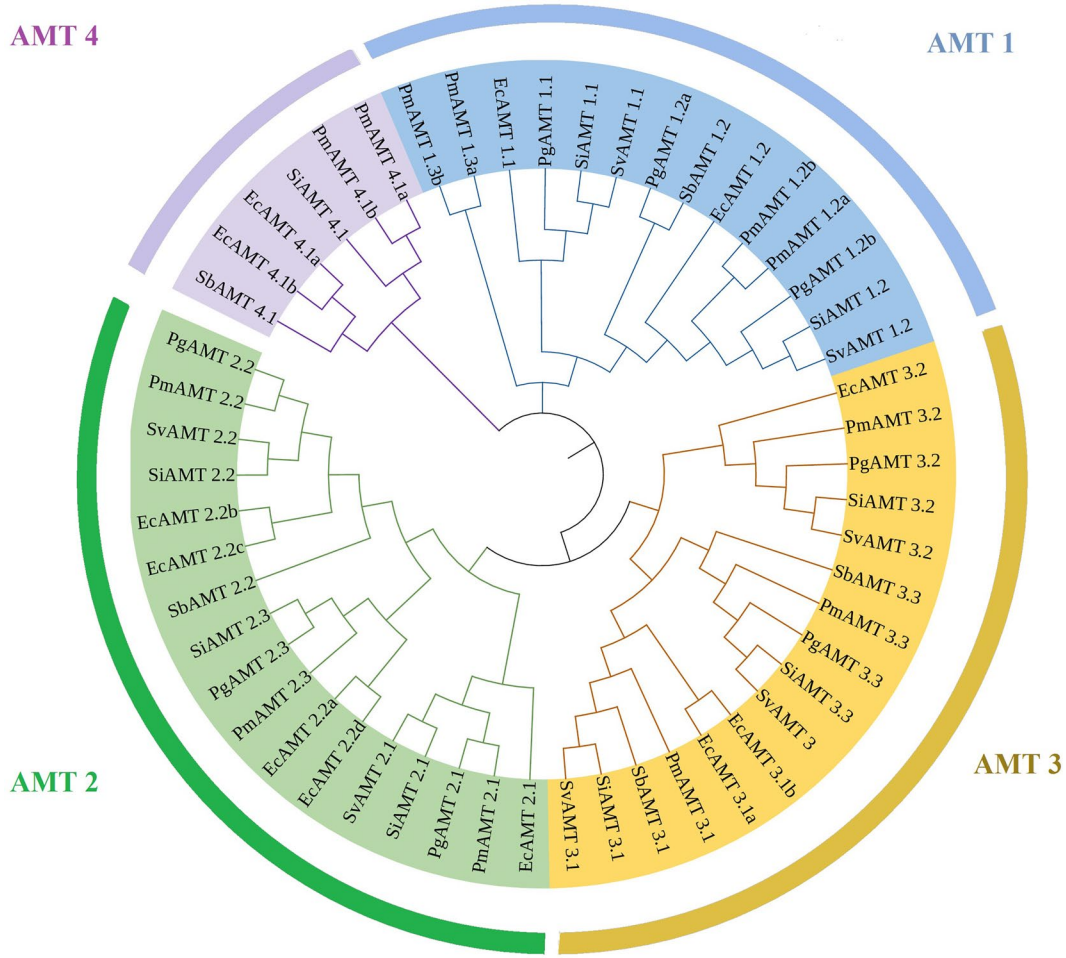

Fig. 5 Phylogenetic tree analysis of AMTs from six millet species *Setaria viridis* (Sv), *Setaria italica* (Si), *Eleusine coracana* (Ec), and *Sorghum bicolor* (Sb), *Pennisetum glaucum* (Pg) *and Panicum miliacium* (Pm). *Different colours of circles represent different clusters

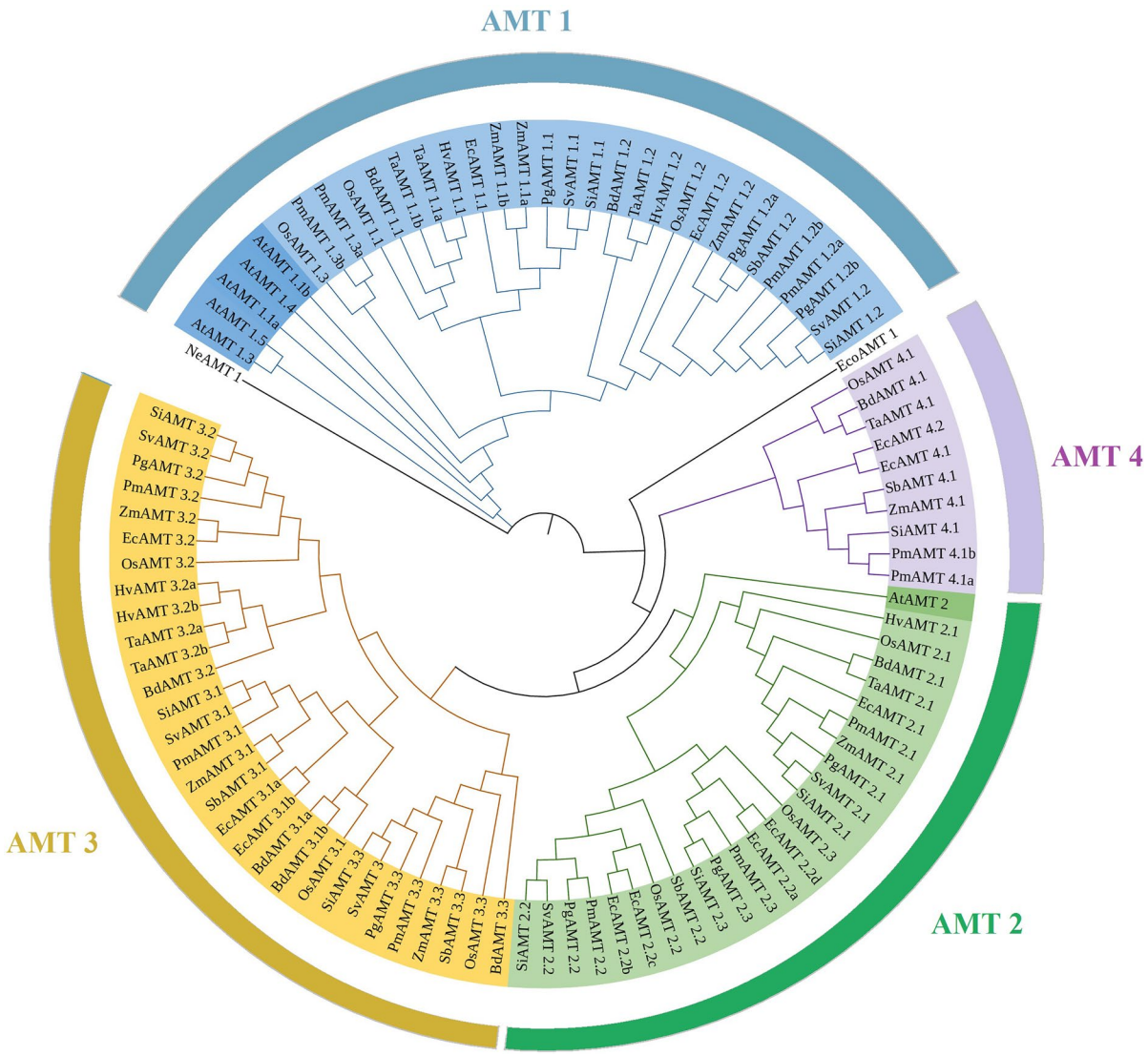

Fig. 6 Phylogenetic tree analysis of AMTs of *Setaria viridis* (Sv), *Setaria italica* (Si), *Eleusine corocana* (Ec), *Sorghum bicolour* (Sb), *Arabidopsis thaliana* (At), *Oriza sativa* (Os), *Zea mays* (Zm), *Hordium vulgare* (Hv), *Triticum aestivum* (Ta) and *Brachypodium distachyon* (Bd). Two bacterial AMT namely, *Escherichia coli* (Eco) and *Nitrosomona seuropaea* (Ne) showing out grouping. *Different colors of circles represent different clusters

Synteny analysis of *AMT* genes

In this study, synteny analysis between six millet AMT proteins (*S. viridis*, *S. italica*, *E. corocana, S. bicolor, P. glaucum* and *P. miliaceum*) with other species of poaceae family (*O. sativa*, *Z. mays*, *H. vulgare, T. aestivum* and *B. distachyon)* was performed by circoletto tool to understand the evolutionary history of genomes. In this analysis, amino acid sequences of AMTs of one species were used as query and while the rest of all the AMT sequences used as comparative files. 'E-value' and 'score/max' ratio parameter was considered to produce the colour bands and the colour of the bands indicate the sequence similarities (blue ≤ 0.25, green ≤ 0.50, orange ≤ 0.75, and red > 0.75). Based on the synteny analyses, maximum high synteny blocks (maximum red coloured bands > 0.75) were identified between AMTs of millets and other poaceae members/species. Considering these maximum

Table 2 Ka/Ks ratios and estimated divergence time for paralogous *AMT* genes in millets

Paralogous pairs	Chromosomal location	Duplication event	Ka	Ks	Ka/Ks	Selection
Setaria viridis						
SvAMT1.2/SvAMT1.1	Chr 1/Chr 7	Segmental	0.05283	0.1924	0.27461	Purifying
SvAMT2.2/SvAMT2.1	Chr 5/Chr 3	Segmental	0.05191	0.22515	0.23055	Purifying
SvAMT3.1/SvAMT3.2	Chr 5/Chr 9	Segmental	0.08041	0.1868	0.43046	Purifying
Setaria italica						
SiAMT2.3/SiAMT2.2	scaffold 5/scaffold 5	Tandem	0.04863	0.14225	0.34188	Purifying
SiAMT4.1/SiAMT2.1	scaffold 9/scaffold 3	Segmental	0.13879	0.30335	0.45753	Purifying
SiAMT3.2/SiAMT3.1	scaffold 9/scaffold 5	Segmental	0.07653	0.17625	0.43421	Purifying
SiAMT1.1/SiAMT1.2	scaffold 7/scaffold 1	Segmental	0.04995	0.1767	0.2827	Purifying
Eleusine corocana						
EcAMT3.1b/EcAMT3.1a	Chr 1B/Chr 1A	Segmental	0.00084	0.04375	0.01927	Purifying
EcAMT2.2c/EcAMT2.2b	Chr 1B/Chr 1A	Segmental	0.00042	0.04607	0.00908	Purifying
EcAMT2.2d /EcAMT2.2a	Chr 1B/Chr 1A	Segmental	0.00784	0.06567	0.11942	Purifying
EcAMT1.1a/EcAMT1.2a	Chr 4B/Chr 2A	Segmental	0.06475	0.2043	0.31691	Purifying
EcAMT4.2/EcAMT4.1	Chr 3A/Chr 3B		0.00471	0.04019	0.11715	Purifying
Sorghum bicolor						
SbAMT1.2 /SbAMT4.1	Chr 4/Chr 1	Segmental	0.3701	0.34905	1.06031	Neutral
SbAMT3.3/SbAMT3.1	Chr 4/Chr 3	Segmental	0.06694	0.31795	0.21053	Purifying
Pennisetum glaucum						
PgAMT1.2b/PgAMT1.2a	Chr 3/Chr 3	Tandem	0.02125	0.1033	0.20574	Purifying
PgAMT3.2/PgAMT3.3	scaffold 2474/Chr 3	Segmental	0.08944	0.4701	0.19025	Purifying
PgAMT2.2/PgAMT2.1	Chr 6/Chr 1	Segmental	0.07949	0.23305	0.34109	Purifying
Panicum miliaceum						
PmAMT4.1b/PmAMT4.1a	Chr4/Chr1	Segmental	0.01625	0.03797	0.42791	Purifying
PmAMT2.1/PmAMT2.3	Chr3/Chr5	Segmental	0.04889	0.19785	0.24711	Purifying
PmAMT1.3a/PmAMT1.3b	Chr12/Chr6	Segmental	0.02618	0.0572	0.45762	Purifying
PmAMT1.2b/PmAMT1.2a	Chr6/Chr12	Segmental	0.0038	0.03853	0.09858	Purifying
PmAMT3.3/PmAMT3.1	Chr6/Chr5	Segmental	0.09066	0.5224	0.17354	Purifying

red coloured synteny blocks, it can be concluded that, the *AMT* genes are more conserved in terms of evolutionary and genomic architecture in poaceae family.

Based on best score match parameter in circoletto tool, the best matched AMT sequences of different species showed synteny blocks. The AMTs of the species *S. viridis* and *S. italica* showed best synteny association (Online Resource: S21, S22). In case of *E. corocana* AMTs, 12 best score synteny blocks were identified between *E. corocana* AMTs and *S. viridis*, *S. italica* and *S. bicolor* AMTs (Online Resource: S23), while in case of *S. bicolor* 5 synteny blocks with best score was found between *S. bicolor* and *Zea mays, S. viridis*, *S. italica* (Online Resource: S24). In *P. glaucum,* eight best score synteny blocks were found in between *P. glaucum* and *S. viridis*, *S. italica, S. bicolor* AMTs (Fig. 8). Twelve best score synteny blocks were found to be associated with *P. miliaceum* AMTs and *P. glaucum* and *S. viridis*, *S. italica* (Online Resource: S25). Genomic dynamicity and evolutionary improvement along mobile elements in the genome of these six studied millet species were determined in these syntenic circles.

Cis-element analysis of millet *AMT* promoter regions

To study the expression characteristics and potential functions of millet *AMT* genes, 2000 bp upstream sequences of start codons of the *AMT* genes of the six studied species were obtained as promoter sequences and used to analyse their

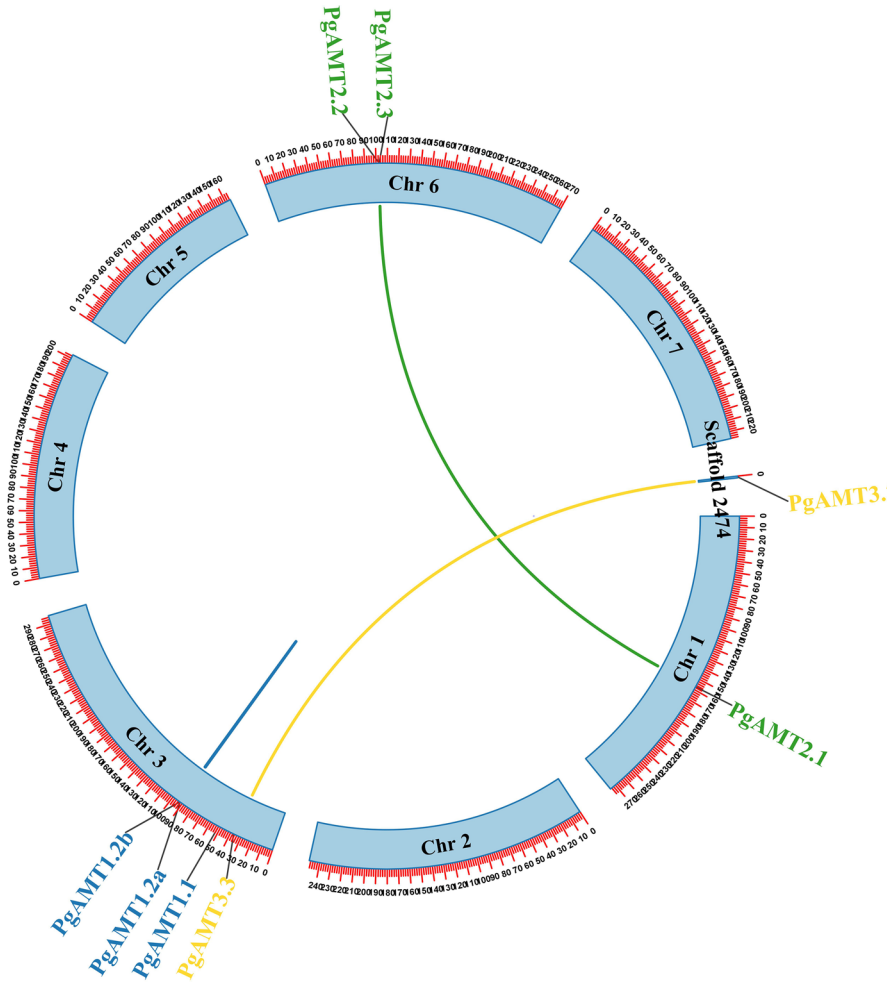

Fig. 7 Schematic representations of the chromosomal distribution and inter-chromosomal relationships among *AMT* genes of *Pennisetum glaucum*. *Duplication events occurred in *AMT* gene family of *P. glaucum* are represented by blue, green, and yellow lines. *Chromosomes are represented in sky blue colors with the chromosomal number indicated inside each chromosome. (Visualization of chromosomal distribution and inter chromosomal relationships of other five millet species are given in the Online Resource: S16-S20)

cis-acting elements. The comprehensive results showed that millet *AMT* promoters have numerous cis-elements that respond to endogenous signals related to plant growth and development (viz. zein metabolism, circadian control, endosperm and meristem expression, root-seed-palisade mesophyll cells regulations), growth hormones (mainly auxin, gibberellin, abscisic acid, salicylic acid and methyl jasmonate), and environmental stresses (e.g. light response elements, low temperature stress-related elements, defense and stress, wound, anaerobic induction, anoxic specific induction and drought stress) (Fig. 9, Online Resource: S26-S30, Table. S1). All the millet *AMT* promoters have cis-elements responsive to light, suggesting an essential role of these *AMT* genes in plant growth and metabolism. From the data, it was evident that each gene promoter contains response element (s) to different phytohormone (s) with varied numbers ranging from 1 to 20, indicating that these *AMT* genes are under the regulation of hormone (s) and are involved in the hormone-mediated biological processes. Cis-elements involved in regulation of anaerobic induction are also common in all the millet *AMT* promoters, suggesting their possible role in plant growth and metabolism in anaerobic conditions. Almost all the promoter sequences have binding site for MYB-transcription factors related to many biological processes, such as plant growth and development, primary and secondary metabolic reactions, different physiological activity and responses to environmental stresses. Cis-elements related to drought- inducibility are also present adequate amount in almost all the promoters.

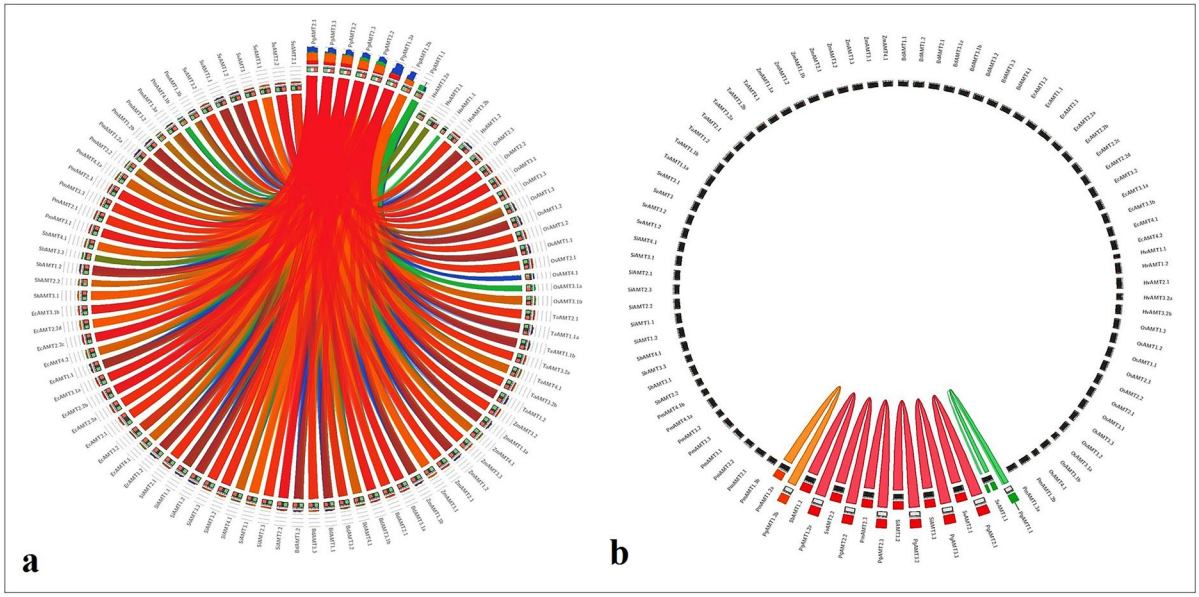

Fig. 8 Visualization of the sequence similarity of *AMT* genes between *Pennisetum glaucum* with other millet AMTs as well as AMTs of different species of poaceae family (*Oryza sativa*, *Zea mays*, *Hordeum vulgare, Triticum aestivum* and *B. distachyon)*. **a** Representation of synteny of PgAMTs and other AMTs. **b** Synteny blocks in 'best score' matching parameter of circoletto showing best matches between AMTs of P. *glaucum* and AMTs belongs to same tribe (*S. italica, S. viridis and P. miliaceum*). (Visualization of synteny analysis of other five millets are given in Online Resource: S21-S25)

Discussion

In plants, the *Arabidopsis thaliana AtAMT* gene was first recognized as an ammonium transporter (Ninnemann et al. 1994; Sohlenkampet al. 2000). Further analysis in *Arabidopsis thaliana,* proved that these *AMTs* also act as ammonium sensors that can sense the signal for cell–cell communication during plant growth and promote root to shoot ammonium translocation (Giehl et al. 2017). Genetic and molecular analysis in rice *AMTs* also proved that it acts in cell–cell communication and enhance the crown root formation in plants (Luo et al. 2022). In poaceae, several AMT homologues have been reported to play important roles in ammonium transport, such as *Triticum aestivum* (Li et al. 2017; Jiang et al. 2019), *Oriza sativa* (Li et al. 2009; Su-mei et al. 2012), *Zea mays* (Gu et al. 2013; Dechorgnat et al. 2019), *Hordium vugare* (Han et al. 2016) and *Saccharum spontaneum* (Wu et al 2021). In some millets, these *AMT* genes were also identified and are predicted to be associated with plant growth and development via ammonium transport (Maharajan et al. 2022; Ceasar et al. 2023). In *Sorghum bicolor*, induction of *AMTs* by arbuscular mycorrhizal fungi was studied which enhances the ammonium transport in plant parts (Koegel et al. 2013). The results suggested that, this AMT gene family has been involved in many biological processes in *poaceae* family. Millets are highly nutritious cereal crops and realizing their potential as nutraceutical food, much emphasis is given to improvement of these crops. Understanding the genomic loci involved in response, uptake and utilization of the nitrogen, a major nutrient in millet growth and production has utmost significance. There are two transporters involved in nitrogen uptake, the NRTs and AMTs in crop plants. Extensive research on *in-silico* analysis of NRTs has been carried out in millets. However, information about AMTs in millet crops is scanty. Hence, we performed an *in-silico* characterization of millet *AMT* genes that belong to two subfamilies viz. AMT1 and AMT2 (AMT2/AMT3/AMT4). Generally, the approximate length of members *AMT* gene family are between 400–450 amino acids and the structure can range from 45 to 50 kDa (Ninnemann et al. 1994; Blakey et al. 2002). The present study involved AMTs of six millet species and the length of the amino acids ranged

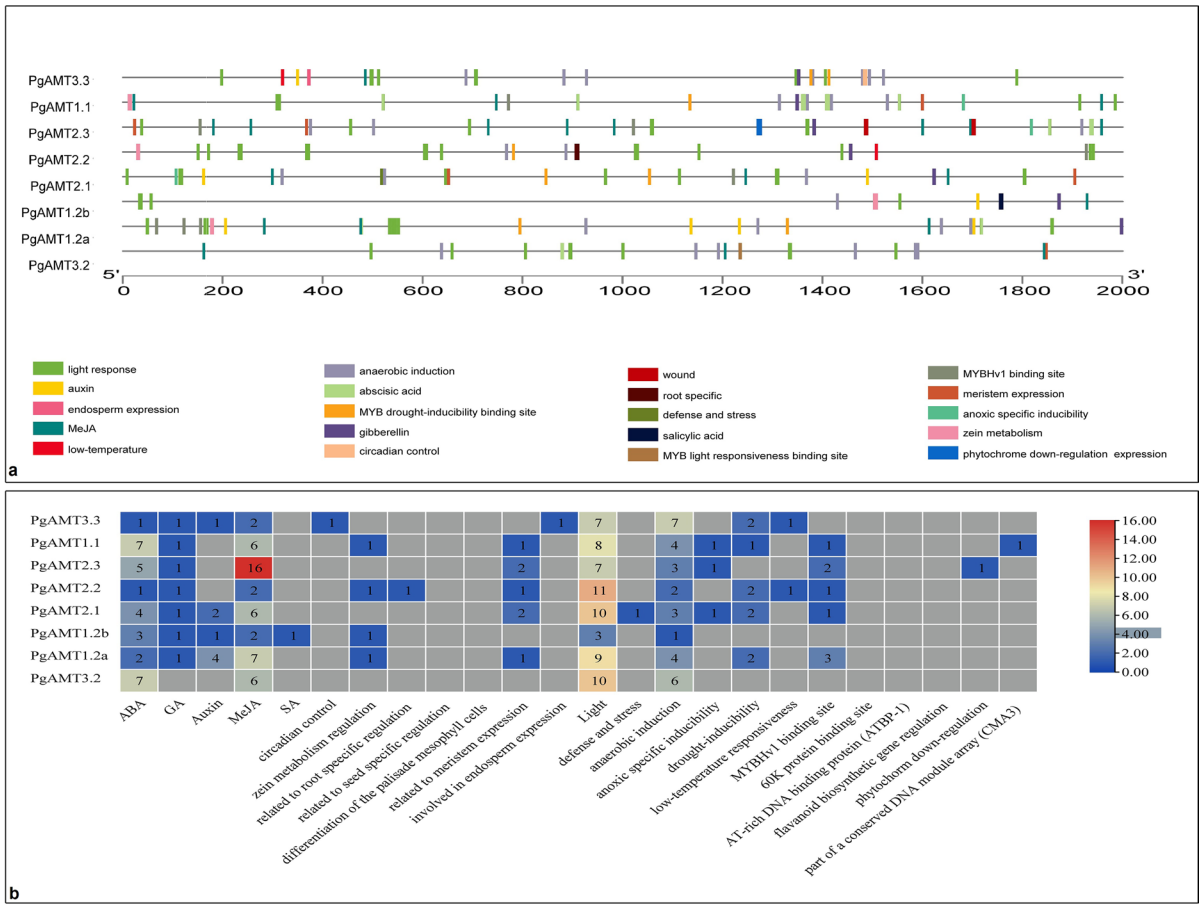

Fig. 9 Representation of promoter cis-element analysis of *AMT* genes in *P. glaucum*. **a** Promoter position information. The different colored markers indicate different predicted cis-acting elements. **b** Promoter number analysis. The color scale to the right of the heat map represents the number of promoters. (Promoter cis-element analysis of *AMT* genes of other five millets are given in Online Resource: S26-S30)

from 304 to 632, and molecular weights ranging from 32.07 to 67.61 kDa are in consensus with earlier research.

Structural analysis AMT genes of millet revealed that the two subfamilies AMT1 and AMT2 exhibit divergent exon–intron patterns (Fig. 3). The structure of *AMT* genes of millet are highly conserved, among all the studied millets. AMT1 of *P. glaucum* (*PgAMT1.2b*, *PgAMT1.2a*, *PgAMT1.1*) and *P. miliaceum* (*PmAMT1.2b*, *PmAMT1.3a*) have introns in it, others are intronless. Similar research in *MdAMT1 of* Apple and *GmAMT1* of Soybean reported absence of introns in AMT1 sub family (Huang et al. 2022; Yang et al. 2023). In *Populus*, *Lotus japonicus*, chilli pepper, most *AMT1* genes have no introns in it, with the exception of *LjAMT1.1, PtAMT1.7* and *CaAMT1.1* that have one intron (Wu et al. 2015; Wang et al. 2022; Fang et al 2023). Millet *AMT2* genes contain introns (ranges from 1 to 3), exons, and UTRs. The lengths of the UTRs, exons and introns vary among these *AMT2* genes. Introns are usually involved in the regulation of gene expression and/or RNA stability (Shaul 2017). Mutations in critical regions in gene structure, including upstream region and coding sequence site may alter the expression patterns of members of gene family under evolution events (Heidari et al. 2022; Yaghobi and Heidari 2023). The lack of introns in the AMT1 subfamily genes suggests that the expression of these genes is essentially regulated at the transcriptional level. Large variations in the length and number of introns in different AMT2 subfamily genes indicate that these

genes may undergo more complicated regulation, such as mRNA transport, alternative splicing, or chromatin assembly, which have been reported previously (Zhao et al. 2014; Jo and Choi 2015).

The phylogenetic analysis for ammonium transporters genes of six millets (Fig. 5) revealed that *S. viridis, S. italica, P. glaucum* and *P. miliaceum* shares a close relationship after alignment of retrieved proteins sequences of all the AMTs. This could be due to taxonomic commonality for instance, these four species *(S. viridis, S. italica, P. glaucum* and *P. miliaceum*) belong to the same tribe *Paniceae* (Li and Bruntnell 2011). Further, combined phylogenetic analysis using AMT proteins of all cereals (millets, rice, wheat, maize, barley and brachypodium), arabidopsis and bacterial AMTs (*E. coli* and *N. europaea)* evidently identified close association among six millets species for two ammonium transporter subfamilies. The maize transporter (*ZmAMTs*) were found closely related with millet AMTs as *Zea mays* (maize) is a member of *Andropogoneae*, which is a sister tribe to millet family, the *Paniceae* (Li and Brutnell 2011). The phylogenetic analysis of all millet AMTs and the combined phylogenetics involving AMTs of rice, wheat, maize, arabadopsis AMTs and bacterial AMTs (*E. coli* and *N. europaea)* clustered into conspicuous two subgroups of AMTs and similar findings of AMTs grouping has been reported in several investigations carried on other crops such as, soybean (Yang et al. 2023), poplus (Wu et al. 2015) and cassava (Xia et al. 2023).

In synteny analysis, the high score synteny blocks (red > 0.75) reinforce the idea that, *AMT* genes of *poaceae* are conserved in this family (Fig. 8, Online Resource: S21-S25). Five studied millets (*S. viridis, S. italica, P. glaucum, P. miliaceum* and *S. bicolor*) belongs to the subfamily *panicoideae* showed the best score synteny blocks frequently, imparting knowledge about the conservation of *AMT* genes in this subfamily. Again, *AMTs* of *S. viridis, S. italica, P. glaucum* and *P. miliaceum* exhibit maximum best scores synteny blocks as they belong to the same tribe *paniceae*. Furthermore, best score synteny blocks were found between *S. bicolor* and *Z. mays*, which again supporting the concept that AMTs are also conserved in tribes, as those two belongs to the identical tribe *andropogoneae*. In best match synteny analysis of *E. corocana* synteny blocks were also appeared between *E. corocana* and other millet AMTs, but the frequency is low. *E. corocana* belongs to *chloridoideae* subfamily, which is a close relative of subfamily *panicoideae*, and this may suggest that, there are resemblance of AMT genes between two closely related sister subfamilies. No best score synteny blocks were found between millet AMTs and other members of poaceae viz. *O. sativa* (subfamily: *oryzoideae*), *B. distachyon, T. aestivum* and *H. vulgare* (subfamily: pooideae) considered for this study, as they shared distant relationship from *panicoideae* subfamily. The phylogenetic tree generated by chloroplast matK genes of all the species of poaceae family in this study gives a depiction of taxonomic classification of poaceae family (Fig. 10), (Sorenget al. 2015, 2017, 2022). Interestingly, the phylogenetic tree constructed using *AMT1.1* gene of all the previously studied *Poaceae* family crops reflected precisely the same pattern as proposed in their taxonomic classification (Fig. 10). This suggests that, in the course of evolution, *AMT* genes were also evolved by means of gene flow, natural selection, mutation or genetic drift.

A promoter is a region of DNA upstream of a gene where relevant proteins viz. RNA polymerase and transcription factors have to bind and initiate transcription of that gene (Hernandez-Garcia and Finer 2014). The level of transcriptional activation in eukaryotes is coordinated by upstream cis-acting elements in the regulation of gene expression, which are key links in plant environmental responses. Plant gene promoters contain a variety of important cis-acting elements that are involved in regulating the expression of corresponding downstream genes at the transcriptional level, thereby enabling plants to resist environmental stresses (Li et al. 2020). Cis-acting regulatory element analysis of millet *AMTs* promoter regions revealed a great abundance of light responsive elements, which implies that *AMT* gene expression is closely associated with photosynthesis and might be diurnally regulated. In research with *Arabidopsis* AMTs, *AtAMT1.3* exhibited a typical diurnal pattern of change in expression; absorption of ammonium increased significantly towards the end of the day's light, and decreased as light intensity decreased (Gazzarrini et al. 1999). Additionally, two tomato AMTs (*LeAMT1.2* and *LeAMT1.3*) also demonstrated rhythmic regulation

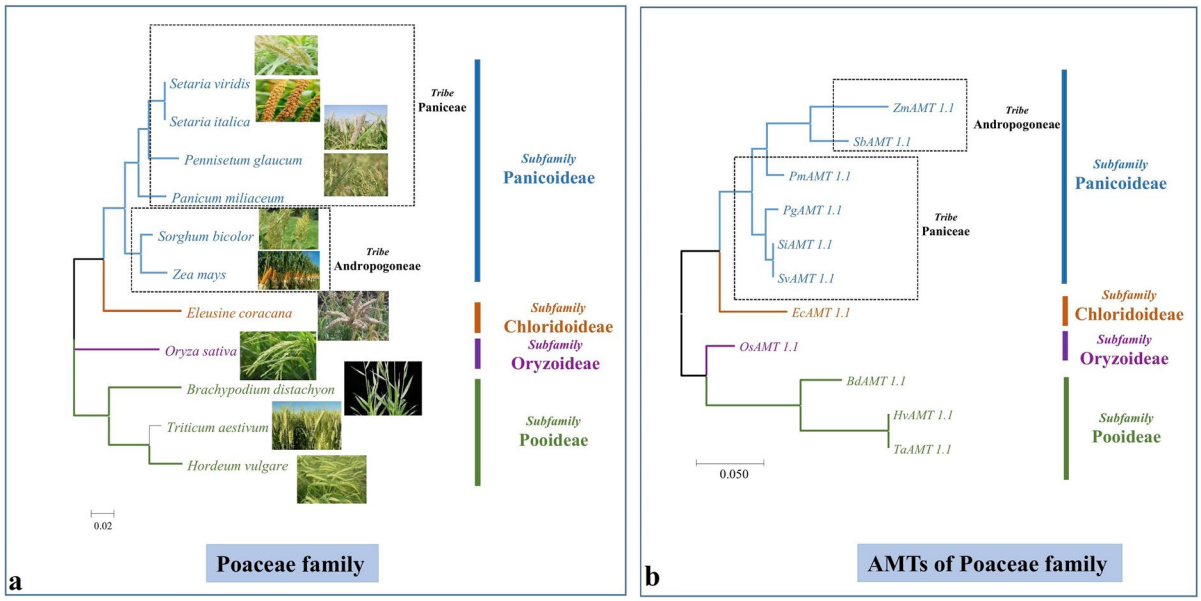

Fig. 10 Phylogenetic classification of *Poaceae* family by using *matK* (**A**) and *AMT1.1* (**B**) gene sequences

(Von Wirén et al. 2000). Further, all the *AMT* genes share *cis*-elements responsive to anaerobic conditions. This has functional application in rice where AMTs has been widely studied for their role to uptake and utilize ammonium form of nitrogen in anaerobic conditions (Konishi and Feng. 2021). Cis-element analysis of *AMT* genes in majority of millet species showed involvement of at least one cis element in host defense response to the various biotic stresses in this study. It has been revealed in wheat and rice that ammonium transporters 1.1, 1.3, and 2.3 are associated with defense response to pathogens (Wu et al. 2022; Li et al. 2017; Jiang et al. 2019). Similarly evidences in support of role of AMTs in plant–microbe symbiosis e.g. *LjAMT2.1* and *LjAMT2.2* of *Lotus japonicus* and *MtAMT2.3* of *Medicago truncatula* could be involved in ammonium transport from the host plants to nitrogen-fixing rhizobia and arbuscular mycorrhizae (Simon-Rosin et al. 2003; Guether et al. 2009; Breuillin-Sessoms et al. 2015). In addition, these *AMT* genes are under the control of different phytohormone (s) during the development and their response varies under diverse environmental conditions, thereby co-ordinately regulating ammonium uptake and metabolism.

Conclusion

The ammonium transporter gene (*AMT*) family plays a key role in the acquisition and transport of NH4 + forms of nitrogen in plants. This study identified a total of 53 *AMT* genes in the genomic sequences of the six millet species and classified them into two subfamilies, AMT1 and AMT2 (AMT2/AMT3/AMT4), based on phylogenetic analysis. The expansion of millet AMTs is the outcome of segmental and tandem duplication events in evolution. Syntenic conservation was observed in the structure and function of ammonium transporters in members of Poaceae. Promoter analysis of millet AMTs showed the presence of cis-elements regulating light response, anaerobic induction, growth hormones, drought stress, biotic stress, and several endogenous signals related to plant growth and development. This study provides in-depth information about the ammonium transporter gene family in millets, which would assist in improving nitrogen use efficiency through genomic manipulation of the expression patterns of these transporters.

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Data availability All data generated or analysed during this study are available from the corresponding author upon request.

Declarations

Competing interests The authors have no conflict of interest.

Human and animal rights This research does not involve any human participants and/or animals.

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