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Genome-wide identification and characterisation of ammonium transporter gene family in barley

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Abstract: Nitrogen (N) is an essential macronutrient for the growth and development of plants, but excessive use of nitrogen fertiliser in agriculture can result in environmental pollution. As a preferred nitrogen form, ammonium (NH_4^+) is absorbed from the soil by the plants through ammonium transporters (AMTs). Therefore, it is important to explore AMTs to improve the efficiency of plant N utilisation. Here, we performed a comprehensive genome-wide analysis to identify and characterise the *AMT* genes in barley (*HvAMTs*), which is a very important cereal crop. A total of seven *AMT* genes were identified in barley and further divided into two subfamilies (*AMT1 and AMT2*) based on phylogenetic analysis. All *HvAMT* genes were distributed on five chromosomes with only one tandem duplication. *HvAMTs* might play an important role in plant growth, development, and various stress responses, as indicated by *cis*-regulatory elements, miRNAs, and protein interaction analysis. Further, we analysed the expression pattern of *HvAMTs* in various developmental plant tissues, which indicated that *AMT1* subfamily members might play a major role in the uptake of NH_4^+ from the soil through the roots in barley. Altogether, these findings might be helpful to improve the barley crop with improved nitrogen use efficiency, which is not only of great significance to the crop but also for land and water as it will reduce N fertiliser pollution in the surrounding ecosystem.

Keywords: ammonium transporter (AMT), bioinformatics, gene expression, barley (*Hordeum vulgare*), nitrogen use efficiency

INTRODUCTION

Nitrogen (N) is an essential macronutrient for the growth and development of plants. Nitrogen is an inseparable component of complex compounds such as nucleic acids, proteins and enzymes, which are crucial for plant growth (Lv *et al.*, 2021). The production of crops can be significantly improved by the application of N fertilisers (Good, Shrawat and Muench, 2004). Despite its benefits, excessive use of N fertilisers causes environmental pollution. While carbon pollution gets all the headlines for its role in climate change, N pollution is arguably a more challenging problem. Nitrogen pollution can impact air pollution, eutrophication, biodiversity loss, stratospheric ozone depletion, and climate change (Kanter *et al.*, 2020). These impacts, directly and indirectly, contribute to several human health concerns. In Europe alone, the environmental and human health costs of N pollution were estimated to be EUR 35–230 billion per year being greater than the economic benefits of using N (Sutton *et al.*, 2011; Vries de, 2021). A similar conclusion was drawn in a study about the impact of ammonia emission reductions on $PM_{2.5}$ concentrations and the impact on avoided premature mortality in the EU, costing ca EUR 4.3 billion (Giannakis *et al.*, 2019). Taking that into consideration, refining the nitrogen use efficiency (NUE) is immensely important for crops, as improved efficiency will cause a reduction in pollution with N fertiliser in the surrounding ecosystem. Therefore, it is important to improve plant NUE (Zhang *et al.*, 2018), for which comprehending completely the uptake, transport, and metabolism of N in plants is a necessity.

In plants, N assimilation takes place in the form of inorganic and organic nitrogen (Chantranupong, Wolfson and Sabatini,

2015). Among inorganic nitrogen compounds, the most common are ammonium (NH_4^+) and nitrate (NO_3^-). The NH_4^+ concentration (1–25 μM) is often lower than that of nitrate (100 μM to 70 mM) in the soil (Dechorgnat *et al*., 2011). Ammonium is the preferred N source by plants as the energy required to absorb and process NH_4^+ is lower than that required for NO_3^- uptake (Wirén von *et al*., 2000a; Masumoto *et al*., 2010). There are two types of NH_4^+ absorption systems in plants, low-affinity transport system (LATS) and high-affinity transport system (HATS) (Wang *et al*., 1994; Kronzucker, Siddiqi and Glass, 1996). The HATS is utilised as the primary pathway when available NH_4^+ concentrations are in the submillimolar range and exhibit saturation kinetics with the Michaelis-Menten constant (K_m) value less than 100 μM (Owen and Jones, 2001), while the LATS is used when NH_4^+ concentrations are in the millimolar range (Couturier *et al*., 2007). The studies on HATS have gained more attention for improving plants' NH_4^+ uptake due to the low concentration of ammonium in the soil. In plants, ammonium transporters (AMTs) function for HATS absorption of ammonium (Loque *et al*., 2006; Ludewig, Neuhäuser and Dynowski, 2007), and thereby AMTs play an important role in N metabolism. AMTs are a class of membrane transporter proteins, which mediate the ammonium transport through the plasma membrane of root cells (Couturier *et al*., 2007) and fluxes in plants (Ranathunge *et al*., 2014). In addition, many pieces of evidence suggest that AMTs among various plant organs might be important for nitrogen metabolism and nutrition in leaves and roots (Pearson, Finnemann and Schjoerring, 2002; Sonoda *et al*., 2003b; Ranathunge *et al*., 2014). Considerable pieces of evidence indicate that AMTs from plant roots play a role in the uptake of NH_4^+ at low N levels (<1 mM), and belong to the major HATS transport system (Glass *et al*., 2002). The first *AMT* gene in plants was identified in *Arabidopsis thaliana* by functional complementation of a yeast mutant defective in HATS NH_4^+ uptake (Ninnemann, Jauniaux and Frommer, 1994). Afterwards, a number of *AMTs* were identified in other plant species including *A. thaliana*, *O. sativa*, *S. lycopersicum*, *C. canephora* (Li *et al*., 2016a; Santos *et al*., 2017; Sun *et al*., 2019; Hao *et al*., 2020b). Although the studies on *AMT* gene family have been reported in some plants, the *AMT* gene family in barley is yet to be studied.

Barley (*Hordeum vulgare* L.) is an important staple crop, and it shows great adaptation to harsh environmental conditions. It was one of the first domesticated crops and is the fourth most productive cereal crop after maize, rice, and wheat (FAO, no date). Barley has a large genetic diversity and it is grown under a large array of environmental and soil conditions with areas ranging from high latitudes and altitudes to desert regions (Ryan and Sommer, 2012; Muñoz-Amatriaín *et al*., 2014; Dawson *et al*., 2015). Due to the quite big genetic diversity of barley and its adaptability and hardiness to unfavourable environmental conditions, this crop is valuable for agroecological transition and the requirement for the reduction of N fertilisers usage. A few studies have been reported for barley related to N assimilation (Comadira *et al*., 2015; Tanaka and Nakano, 2019; Decouard *et al*., 2022). Nitrogen deficiency in barley seedlings was found to induce molecular and metabolic adjustments that trigger aphid resistance (Comadira *et al*., 2015). Decouard *et al*. (2022) showed that the *HvNRT2.10* gene is one of the key physiological markers for adaptation to low availability of N in the early stages of development of North African barley. In the cropping season of 2016–2017, the application of N could be utilised for the production of late-emerging tillers as the result of restricted sink capacity, while in the 2017–2018 cropping season, it could be used to effectively increase grain yield (Tanaka and Nakano, 2019). However, these processes have not yet been investigated thoroughly.

In the last decade, a huge amount of nucleotide sequencing data has been produced and utilised for screening the potential genes associated with specific traits in crop plants. Additionally, in past years accessibility to a number of new reference genomes for crop plants significantly increased, which eased genetic research and allowed for the discovery of new evidence supporting the structure and function of various genes (Xia *et al*., 2020). By utilising this data, here we identified the *AMT* gene family in the barley genome and analysed their phylogenetic relationships, evolution, gene structures, conserved motifs, and expression profiles in various developmental tissues. Overall, this study lays the groundwork for a new understanding of the *AMT* gene family in barley. These findings might be utilised to improve the barley crop with improved nitrogen use efficiency, which is of great significance not only to the crop but also to the surrounding ecosystem for reducing nitrogen fertiliser pollution.

MATERIALS AND METHODS

IDENTIFICATION OF *AMT* GENES IN BARLEY AND PHYSIOCHEMICAL CHARACTERISATION OF PROTEINS

The sequence of conserved Ammonium_transp domain of *AtAMT1.2*, obtained from Arabidopsis database (TAIR, 2022), was used as a basic local alignment search tool: protein (BLASTP) query search in *H. vulgare* genome deposited in Phytozome database (Goodstein *et al*., 2012) to identify the *AMT* gene family in barley. The identified sequences were further verified by BLAST at National Center for Biotechnology Information (NCBI) with threshold *E*-value $1e^{-10}$. The *AMT* genes were named *HvAMT1.1* through *HvAMT3.2* as per the sequence homology obtained by phylogenetic analysis. Similarly, the *AMT* genes were identified in the barley pan-genome database (Jayakodi *et al*., 2020). The physicochemical parameters of the identified AMT proteins in barley were computed using the ProtParam tool at the ExPASy resource portal (Duvaud *et al*., 2021). Furthermore, the Plant-mPLoc webtool (Chou and Shen, 2010) was used to predict the subcellular localisations and TMHMM ServerV.2.0 software (Krogh *et al*., 2001) was used for the prediction of putative transmembrane regions.

PHYLOGENETIC ANALYSIS OF *AMT* GENES OF BARLEY

The protein sequences of AMTs from various plants, including *Arabidopsis*, *O. sativa*, *S. lycopersicum*, *N. tabacum* L. and *P. trichocarpa*, in addition to those of HvAMTs identified in this study, were used for phylogenetic analysis. The ClustalW program with default settings was used for multiple sequence alignments of the protein sequences and the phylogenetic tree was generated by the MEGA-11 program (Tamura, Stecher and Kumar, 2021) using the neighbor-joining (NJ) method with Poisson correction and 1000 bootstrap values. The phylogenetic tree was visualised on the iTOLv6 webtool (Letunic and Bork,

2021). Evolutionary analyses were conducted in MEGA-11 (Tamura, Stecher and Kumar, 2021) and the evolutionary distances were computed using the p-distance method. All ambiguous positions were removed for each sequence pair (pairwise deletion option).

CONSERVED MOTIFS, GENE STRUCTURE, AND CHROMOSOMAL LOCATIONS

The conserved motifs in the protein sequences of *HvAMT* genes were predicted by the MEME suite 5.3.3 (Bailey *et al*., 2009) based on "zero or one occurrence per sequence (ZOOPS)". The HMMER database by the Inter-Pro scan program (Jones *et al*., 2014) was used for the hidden Markov model analysis. The gene structures of *HvAMT* genes were determined according to the genomic and coding sequences (CDS) by Gene Structure Display Server 2.0 software (Hu *et al*., 2015). Chromosomal distributions of *HvAMT* genes were determined using the genome annotation (GFF3/GTF) files of barley available at Ensembl Plants database (Cunningham *et al*., 2022). The figures of exon/intron organisation, conserved protein motifs and chromosomal mapping were drawn by TBtools (Chen *et al*., 2018).

GENE DUPLICATION AND EVOLUTIONARY RATE CALCULATIONS

Gene duplication analysis was carried out by using the Multiple Collinearity Scan toolkit (MCScanX) with the default settings (Wang *et al*., 2012), and the duplicated genes were visualised by TBtools software (Chen *et al*., 2018). The ratio of non-synonymous to synonymous substitution (*Ka*/*Ks*) was calculated by TBtools software. The divergence time (*T*) was estimated as $T = Ks/(2{\cdot}6.5{\cdot}10^{-9}){\cdot}10^{-6}$ million years ago (mya) for monocots (Wolfe, Sharp and Li, 1989; Lynch and Conery, 2000; Cui *et al*., 2019; Ju *et al*., 2019), based on a rate of $6.5{\cdot}10^{-9}$ substitutions per site per year.

CIS-ACTING REGULATORY ELEMENTS, miRNA TARGET SITES PREDICTION AND PROTEIN INTERACTION ANALYSIS

The sequences of the 1500 bp upstream region of the start codons (ATG) of *AMT* genes were downloaded from the barley genome database, and were analysed by the PlantCARE online tool (Lescot *et al*., 2002) for *cis*-acting regulatory elements analysis (Rombauts *et al*., 1999). The coding sequences of barley *AMT* genes were analysed by the psRNATarget server for miRNA target site prediction (Dai, Zhuang and Zhao, 2018). The protein–protein interaction analysis was carried out on the STRING webtool (Szklarczyk *et al*., 2021), and clustering was done as per *k*-means clustering.

IN SILICO EXPRESSION ANALYSIS OF *HvAMT* GENES

The expression analysis of *HvAMT* genes in various developmental tissues (i.e. elongation zone, maturation zone, roots, seminal root, seedling, root tip, rachis, blade (lamina), lemma, spikelet, spike, internode, leaf, shoot, palea, lodicule, caryopsis, microspore, shoot apex, scutellum, microspore culture, embryo, aleurone layer and endosperm transfer cell) of barley plant was carried out using publically available databases, Affymetrix Barley Genome Array and mRNA-Seq Gene Level *Hordeum vulgare* (ref: Morex V3) on Genevestigator v3 tool (Grennan, 2006; Hruz *et al*., 2008). The heat map of gene expression data (log2FC) thus obtained was generated by using the TBtools software (Chen *et al*., 2018).

RESULTS AND DISCUSSION

IDENTIFICATION OF *AMT* GENES IN BARLEY AND PHYSIOCHEMICAL CHARACTERISATION OF PROTEINS

We identified a total of seven *HvAMT* genes in barley using a BLASTP query for the conserved sequence of Ammonium_transp domain in Barley Genomics Database. Detailed information of these identified genes is shown in Table 1. Our results are consistent with other genome-wide identification of *AMT* genes in different plant species (Tab. 2). To better understand the significance of *HvAMT*s in barley domestication and adaptive evolution, we evaluated and summarised the changes in 20 barley pan-genome accessions between wild accession B1K-04-12 and other cultivated types, using Morex as a representative. In terms of gene quantity, wild accession B1K-04-12 contains six numbers of *AMT*s, whereas all cultivated materials have an average of seven (Tab. 3). The syntenic relationships of *AMT* genes between wild barley B1K-04-12 and cultivated barley are displayed in Figure S1. The majority of homologous gene pairs undergo purification selection (*Ka*/*Ks* < 1), a very small portion go through neutral selection, and a few homologous gene pairs go through positive selection, according to the calculation of the *Ka*/*Ks* value of homologous *HvAMT* gene pairs in wild and cultivated materials. This phenomenon is most pronounced in the *AMT* homologous gene pairs of Morex and B1K-04-12, where just one gene pair has a *Ka*/*Ks* value that is positively selected, while the rest of all are negatively selected, with no neutral selection (Tab. S1). Our results are consistent with the previous genome-wide studies in the barley pan-genome (Wu *et al*., 2022), and this suggests that the *AMT* gene family is highly conserved in barley evolution. The *HvAMT* encode proteins ranging from 503 (HvAMT1.2) to 471 amino acids (HvAMT3.2), with molecular weight from 53,415.26 Da (HvAMT3.1a) to 50,054.57 Da (HvAMT3.2). The molecular weight of proteins is a much more conserved feature than their isoelectric point (*pI*) (Nandi *et al*., 2005). The calculated values of *pI* ranged from 6.23 (HvAMT3.2) to 8.37 (HvAMT2.1) (Tab. 1). The GRAVY (grand average of hydropathy) values varied from 0.413 (HvAMT3.1b) to 0.660 (HvAMT3.2). Moreover, all the HvAMTs contained 11 transmembrane domains (TMDs), except HvAMT1.2 with 10 TMDs. The amino acid length, molecular weights and *pI* values of *HvAMTs* are consistent with those reported in other plants species, although in *P. trichocarpa*, *M. domestica* and *O. sativa* authors have identified 16, 15 and 12 genes encoding AMTs, respectively (Li, Li and Shi, 2012; Wu *et al*., 2015; Huang *et al*., 2022). All of the HvAMTs were predicted to be localised in the cell membrane, as most of the studied AMTs so far (Ludewig, Wirén von and Frommer, 2002; Ludewig *et al*., 2003; Loque *et al*., 2006; Yuan *et al*., 2007). However, studies on *Arabidopsis* mutants showed a more complex localisation pattern of AtAMT1.1, suggesting its presence also in nuclear peripheral and intimal systems (Bu, Takano and Liu, 2019).

Table 1. Details of identified *HvAMT* genes and proteins in barley

Gene name	Gene ID	CDS (bp)	Protein (aa)	Molecular weight (Da)	Theoretical *pI*	GRAVY	Subcellular localisation	TMD number
HvAMT1.2	HORVU.MOREX.r3.6HG0595580.1	1509	503	52719.68	8.08	0.528	cell membrane	10
HvAMT1.1	HORVU.MOREX.r3.2HG0180820.1	1485	495	52315.30	7.17	0.515	cell membrane	11
HvAMT2.1	HORVU.MOREX.r3.1HG0072140.1	1449	483	51291.08	8.37	0.552	cell membrane	11
HvAMT3.1b	HORVU.MOREX.r3.3HG0299130.1	1476	492	53253.05	8.13	0.413	cell membrane	11
HvAMT3.1a	HORVU.MOREX.r3.3HG0299120.1	1479	493	53415.26	8.09	0.426	cell membrane	11
HvAMT3.2	HORVU.MOREX.r3.5HG0530810.1	1413	471	50054.57	6.23	0.660	cell membrane	11
HvAMT2.3	HORVU.MOREX.r3.3HG0303830.1	1485	495	52369.25	8.27	0.569	cell membrane	11

Explanations: *pI* = isoelectric point, GRAVY = grand average of hydropathicity index, TMD = transmembrane domains.
Source: own study.

Table 2. Number of ammonium transporters (AMTs) identified in different plant species

Plant species	Number of AMTs identified	References
Malus domestica	15	(Huang *et al*., 2022)
Zea mays	8	(Xu *et al*., 2022)
Populus trichocarpa	16	(Wu *et al*., 2015)
Camellia sinensis	16	(Wang *et al*., 2022)
Saccharum spontaneum	6	(Wu *et al*., 2021)
Coffea canephora	8	(Santos *et al*., 2017)
Oryza sativa	12	(Sonoda *et al*., 2003b; Li *et al*., 2009)
Arabidopsis thaliana	6	(Gazzarrini *et al*., 1999)
Lycopersicon esculentum	3	(Wirén von *et al*., 2000b)
Triticum aestivum	23	(Li *et al*., 2017)
Nicotiana tabacum	9	(Liu *et al*., 2018)
Pyrus betulifolia	1	(Li *et al*., 2015)
Lotus japonicus	5	(Guether *et al*., 2009)
Sorghum bicolor	8	(Koegel *et al*., 2013)
Manihot esculenta	6	(Xia *et al*., 2022)
Brassica alboglabra	2	(Song *et al*., 2017)

Source: own elaboration based on literature.

Table 3. The number of *HvAMT* genes in each accession of barley pan-genome

Accessions	Number of ammonium transporters
Akashinriki	7
B1K-04-12	6
Barke	7
Golden Promise	7
Hockett	7
HOR3081	6
HOR3365	7
HOR7552	7
HOR8148	7
HOR9043	7
HOR10350	7
HOR13821	6
HOR13942	7
HOR21599	7
Igri	6
Morex	7
OUN333	7
RGT_Planet	7
ZDM01467	6
ZDM02064	7

Source: own study.

The protein isoelectric point (*pI*) is a key player in the contribution of this molecule in maintaining the cell's homeostasis. This *pI* value represents the pH at which the protein is electrically neutral and carries no charge (Tokmakov, Kurotani and Sato, 2021). The pH regulates in diverse modes the activity of AMTs. For example, the transporting properties of TaAMT1;1 and PvAMT1;1 depend on pH value, exhibiting an acid-stimulated regulatory mode (Søgaard *et al*., 2009; Ortiz-Ramirez *et al*., 2011), whereas the transport activities of other AMTs such as LeAMT1;1 (Ludewig, Wirén von and Frommer, 2002), LeAMT1;2 (Ludewig *et al*., 2003), AtAMT1;1 (Wood *et al*., 2006; Loqué *et al*., 2009) and OsAMT1;1 (Yang *et al*., 2015) are pH-independent. All the HvAMT proteins had positive GRAVY values, which indicates that the protein is polar.

PHYLOGENETIC ANALYSIS OF BARLEY *AMT* GENES

For the evaluation of the evolutionary relationships between *AMT* orthologous, we have created a phylogenetic tree of HvAMT proteins, which was compared to that of other plant species. Based on the homology of AMT proteins, the results revealed that AMTs are highly conserved proteins in these plant species and phylogenetic relationships show that plant AMTs can be clustered into two clades namely the AMT1 and the AMT2 subfamilies as was described previously (Loqué and Wirén von, 2004; McDonald and Ward, 2016; Hao *et al*., 2020a). Two of the identified transporters, namely HvAMT1.1 and HvAMT1.2, clustered to the AMT1 subfamily with other AMT1 proteins from the analysed species (Fig. 1). The remaining five HvAMTs were most similar to AMT2 proteins and clustered within this subfamily of transporters (Fig. 1). Within the AMT1 subfamily, the barley transporter HvAMT1.1 clustered with its homologs from maize (ZmAMT1.1b, 85.69% homology) and rice (OsAMT1.1, 92.58% homology), while HvAMT1.2 with AMT from the wheat (TaAMT1.1, 98.61% homology). In the AMT2 subfamily, HvAMT2.1 and HvAMT2.3 were clustered together with their rice homologs OsAMT2.1 (89.19% homology) and OsAMT2.3 (84.68% homology), respectively. The HvAMT3.2 protein shared high homology with TaAMT2.1 (97.23% homology), and there was one sister pair of HvAMT3.1a and HvAMT3.1b which were most closely related to OsAMT3.1 (87.42 and 84.58% homology, respectively), ZmAMT3.1 (86.23 and 83.57% homology, respectively) and SbAMT3.1 (85.25 and 83.37% homology, respectively).

The members of both clades vary in a number of characteristics (Neuhauser, Dynowski and Ludewig, 2009; McDonald, Dietrich and Lutzoni, 2012; Wittgenstein von *et al*., 2014; Li *et al*., 2016b; Giehl *et al*., 2017). First of all, protein characteristics and gene structures are more complicated in *AMT2* than in *AMT1*, since *AMT2* genes hold several introns (Castro-Rodríguez *et al*., 2016), while *AMT1* have none (Becker *et al*., 2002; Yuan *et al*., 2007), except for *AMT1;2* from *Lotus japonicus* (Salvemini *et al*., 2001). The AMT2 subfamily is also more closely related to the prokaryotic AMTs rather than to plant AMT1s (Loqué and Wirén von, 2004; McDonald, Dietrich and Lutzoni, 2012). In addition, there is only 20 to 25% identity among AMT2 families, while plant AMT1s share 65 to 95% identity (Pantoja, 2012). Second of all, except for maintaining ammonium transport, AMT2;1 can mediate the electroneutral ammonia transport (Guether *et al*., 2009; Neuhauser, Dynowski and Ludewig, 2009). Thus, theoretically, AMT2;1 transports ammonium from the cytosol into the apoplast more efficiently than AMT1-type transporters (Giehl *et al*., 2017). Lastly, members of the AMT2 family play a role in the translocation of ammonium from root to shoot in *A. thaliana* (Giehl *et al*., 2017). Although AMT2;1 in *Arabidopsis* is a functional ammonium transporter (Neuhauser, Dynowski and Ludewig, 2009), members of the AMT2 subfamily from different species typically exhibit different characteristics and appear to have different physiological roles and transport mechanisms (Li *et al*., 2016b). The existence of several *AMT* genes in *H. vulgare* indicates the importance of

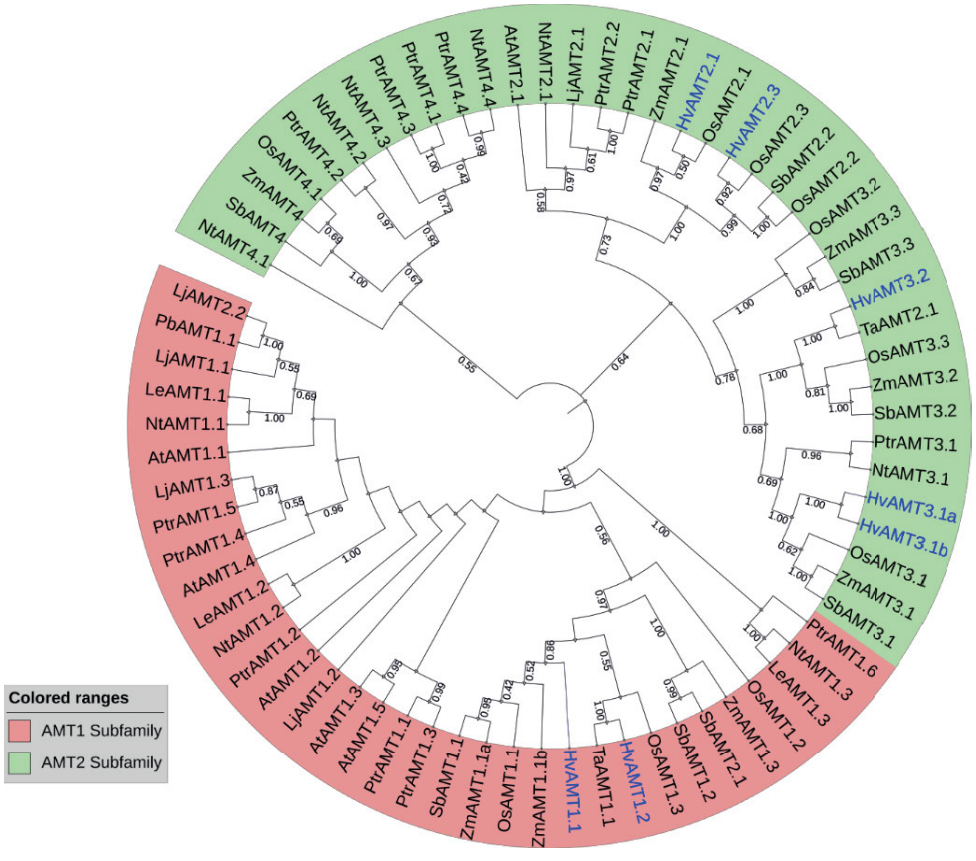

Fig. 1. Phylogenetic tree of AMT proteins from barley and other plants: *A. thaliana* (*At*), *O. sativa* (*Os*), *L. esculentum* (*Le*), *N. tabacum* (*Nt*), *P. trichocarpa* (*Ptr*), *L. japonicas* (*Lj*), *Z. mays* (*Zm*), *P. betulifolia* (*Pb*), *T. aestivum* (*Ta*), *S. bicolor* (*Sb*), *H. vulgare* (*Hv*); blue colour = HvAMT proteins identified in this study; source: own study

NH_4^+ as a mineral nutrient in this species, indicating that NH_4^+ transport is a tightly regulated process (Li *et al*., 2016b) suggested that transporters of the AMT1 subfamily are responsible for the large capacity of high-affinity transport systems (HATS) NH_4^+ uptake in plants.

CONSERVED MOTIFS, GENE STRUCTURE, AND CHROMOSOMAL LOCATIONS

The MEME Suite is a great software tool allowing for the discovery and analysis of sequence motifs such as DNA binding sites or protein interaction domains. We identified 10 conserved motifs in HvAMT proteins with the usage of the MEME tool (Fig. S2), and the motif sequences and annotations were predicted by Pfam. The conserved motifs ranged from 21 to 50 amino acids. Out of ten predicted conserved motifs, seven putative AMT domains were identified (Tab. 4). Among the subgroups, the identified motifs varied, however, members of the same phylogenetic group exhibited similar patterns of motif distribution. All ten motifs were conserved in the HvAMT1 subfamily, while five motifs (motifs 1, 3, 6, 8, and 9) were highly conserved in all HvAMT proteins (Fig. S2a). In the AMT1 subfamily, the proteins had motif 7 at N-terminus, whereas HvAMT proteins of the AMT2 subfamily had motif 9. All the HvAMTs (except HvAMT1.2) ended with motif 2. The Pfam domain analysis showed that all the HvAMTs, regardless of the phylogenetic division, had the highly conserved Ammonium_transp (pfam00909) domain (Fig. S2b). It has been reported that alternative combinations of introns and exons lead to changes in gene function. In order to unravel the structural heterogeneity of *HvAMT* genes, we analysed the distribution of exon-intron combinations. As demonstrated in Figure S2c, the *AMT1* subfamily genes contain no introns. The genes from the *AMT2* subfamily ranged in the number of introns from zero (*HvAMT3.1a* and *HvAMT3.1b*) to two (*HvAMT2.1* and *HvAMT2.3*). *HvAMT3.2* gene had one intron (Fig. S2c). Seven *HvAMT* genes were mapped and designated to the five chromosomes of *H. vulgare* (Fig. S3). Chromosomes 4H and 7H contained no *HvAMT* genes. Chromosome 3H contained three genes (*HvAMT2.3*, *HvAMT3.1a*, and *HvAMT3.1b*), while the rest of the four chromosomes contained one gene each. Chromosome 1H contained the *HvAMT2.1* gene, chromosome 5H contained the *HvAMT3.2* gene, and *AMT1* subfamily genes, *HvAMT1.1* and *HvAMT1.2* were located on chromosomes 2H and 6H.

Here, we retrieved seven *AMTs* from the barley genome and assigned them to two subfamilies. Studies conducted so far on e.g. rice (*O. sativa*), poplar (*Populus tremula* × *alba*), or peach (*Prunus persica*) (Sonoda *et al*., 2003b; Couturier *et al*., 2007; Tang *et al*., 2020) indicate that *AMTs* can be further divided into four subclades. The AMT1 and AMT2 subfamilies comprise 11 putative transmembrane domains (TMDs) and one AMT signature motif (Couturier *et al*., 2007; McDonald, Dietrich and Lutzoni, 2012) which was also found to be present in all identified HvAMTs. Additionally, it was thought so far that *AMT* genes from the same family manifest similar exon/intron structure, with the *AMT1* subfamily possessing no introns except for *LjAMT1;1* and *PtrAMT1;7*, which have an intron in their open reading frames (ORFs) (Salvemini *et al*., 2001; Wu *et al*., 2015) and *AMT2* having a number of introns in their sequence (Couturier *et al*., 2007; McDonald, Dietrich and Lutzoni, 2012). In *H. vulgare* two *AMT2s*, namely *HvAMT3.1a* and *HvAMT3.1b*, contained only one exon and no introns in their open reading frames (Fig. S2), which seems to be a novel finding, since *AMT2s* are thought to possess two to four introns (Couturier *et al*., 2007; McDonald, Dietrich and Lutzoni, 2012; Huang *et al*., 2022). Based on the chromosome mapping analyses, we observed the opposite pattern of localisation of the two *HvAMT1* genes compared to a study performed earlier by Han *et al*. (2016), which demonstrated that *HvAMT1.1* is mapped on chromosome 6H and *HvAMT1*.2 on chromosome 2H. While in this study we mapped them on chromosomes 2H and 6H, respectively (Fig. S3).

GENE DUPLICATION AND EVOLUTIONARY RATE CALCULATIONS

One of the main forces driving the evolution and expansion of the gene family, and the establishment of new protein functions in plants is considered the gene duplication events (tandem/segmental) (Cannon *et al*., 2004). Here, we observed no segmental gene duplications in the seven *HvAMTs* genes, but only one pair of tandem duplication gene pair (*HvAMT3.1a* and *HvAMT3.1b*) on chromosome 3H was identified (Fig. S3). The presence of the

Table 4. Putative ten conserved motifs in ammonium transporter (AMT) proteins in barley

Motif	Width	Protein sequences	Pfam domain
1	41	FLFQWGVIDYSGGYVIHLSSGIAGFTAAYWVGPRIKKDRER	Ammonium_transp
2	49	WNVVVTSIICLVVRLIVPLRMPEEZLAIGDDAVHGEEAYALWGDGEKYD	Ammonium_transp
3	50	VLYGSIVKKKWAVNSAFMALYAFAAVWLCWVLWGFNMAFGEKLLPFWGKA	Ammonium_transp
4	41	NTNICAATSLLVWTCLDVIFFKKPSVIGAVQGMITGLVCIT	Ammonium_transp
5	41	WAAIVMGVLAGSIPWFTMMVLHKRSKLLQKVDDTLGVFHTH	Ammonium_transp
6	50	TLVLFQCVFAAITLILLAGSLLGRMNIKAWMIFTPLWLTFSYTVGAFSLW	Ammonium_transp
7	29	VPEWLNKGDNAWQLTAATLVGJQSMPGLV	nd
8	29	FPPNNILLMLAGAGLLWMGWAGFNGGAPY	Ammonium_transp
9	21	FATPELCSMFAPVTNSRGAFY	nd
10	27	QAVLPASAHLFADGSLETPWIEPFYPM	nd

Source: own study.

genes at the same location on chromosome suggests a common origin, from which they might have evolved through a series of duplication events (Rehman *et al*., 2020). The *HvAMT* genes which have no duplication seem to have originated from different ancestors. To understand the evolutionary dynamics the calculation of nonsynonymous (Ka) and synonymous (Ks) substitution rates is an important factor. The Ka/Ks ratio is used in genetics to indicate the selective pressure acting on a gene encoding the protein. In order to better understand the relationship between natural selection and duplication events, we calculated the Ka/Ks ratio of duplicated gene pair (*HvAMT3.1a/HvAMT3.1b*). The result was less than one (0.1953), which indicates that the *HvAMT* family underwent purification selection after gene duplication (Hurst, 2002). These findings are consistent with other evolutionary studies in barley (Andersen *et al*., 2016; Habachi-Houimli *et al*., 2018). We also estimated the divergence periods for tandem duplicated *HvAMT* gene pair in barley. The calculated time of divergence was 9.7131 million years ago (mya), which indicates that it is a newly duplicated gene pair assessed to have originated after the divergence of the *Hordeum* genus (12–13 mya) (Gaut, 2002; Nevo, 2013).

CIS-ACTING REGULATORY ELEMENTS, miRNA TARGET SITES PREDICTION AND PROTEIN INTERACTION ANALYSIS

The gene promoter regions characterisation is a key factor in understanding the potential transcriptional regulatory mechanisms. Upstream of the translation start site, the 1500 bp sequences of *HvAMTs* were analysed to detect the *cis*-acting regulatory elements (CREs). We found a total of 582 putative CREs (Fig. S4). The distinguished CREs were further separated into four groups: hormone response (110), stress response (126), light response (63), and growth and development (251), while 32 components were uncharacterised. These results suggest that various factors regulate the expression of *AMTs* in barley. The growth and development groups comprised 12 elements among which the most abundant were CAAT-box and TATA-box, 106 and 94, respectively, commonly shared by most of the *HvAMT* genes (Fig. S4). It is believed that these elements determine the transcription efficiency (Porto *et al*., 2014), as CAAT-box is a common CRE in the regions of promoter and enhancer, and TATA-box is a core promoter around –30 of the transcription start site. Also, the light-responsive elements were comprised of 12 different types of members; mostly the G-box was the most abundant. Comparatively, to the hormone response group, ten types of elements were assigned. These are TCA-element involved in the salicylic acid response, ABRE in the abscisic acid (ABA) response, TGA-element in the auxin response and CGTCA-motif and TGACG-motif in the jasmonic acid (MeJA) response. Additionally, stress-responsive elements (18) comprised STRE, LTR (low-temperature response), MBS for drought inducibility, WUN-motif for wound response, and ARE and GC-motif for anaerobic induction. These results show that the above-mentioned genes might play significant roles in response to a variety of stresses in barley. *AMT* genes from other plants have been found to respond to environmental stresses, phytohormones, and light (Husted and Schjoerring, 1996; Wirén von *et al*., 2000a). Considering the CREs diversity in *HvAMT* genes, the AMT and assimilation of N in metabolic pathways of the cell seems to be conducted in a quite complex gene expression reprogramming.

Further, we explored the potential control mechanisms regulating *HvAMT* gene expression by analysing the microRNA target sites in the coding sequences of *HvAMT* genes. MicroRNAs (miRNAs) are small non-coding RNA molecules that can possess a significant regulatory role in gene expression. It happens through targeting mRNAs for cleavage or translational repression (Bartel, 2004; Zhang *et al*., 2020; Zhang *et al*., 2022). In this study, we identified a total number of three *H. vulgare* miRNAs (hvu-miR) comprising target sites in two *HvAMT* genes (Tab. S2). In the *AMT1* subfamily, both *HvAMT1*.1 and *HvAMT1*.2 genes had the target sites for hvu-miR6176. In the *AMT2* subfamily, only one gene *HvAMT3*.2 had a target site for hvu-miR6186. The important factor engaged in target recognition, which is the accessibility of the mRNA target site to small RNA, has been identified. To represent the target accessibility, the energy required to unpair the secondary structure around the target site (unpaired energy – *UPE*) was also calculated by RNAup (Mückstein *et al*., 2006). Our results indicated that the *UPE* differed from 13.56 (hvu-miR6176) to 22.17 (hvu-miR6186). These *UPE* values indicate a better miRNA-target binding (Alptekin, Akpinar and Budak, 2016). In plants, miRNAs have been implicated in various stress responses, both biotic (bacterial and viral pathogenesis) and abiotic (oxidative, mineral nutrient deficiency, drought, salinity, temperature, and cold) (Sunkar, Li and Jagadeeswaran, 2012; Dang, Ziemann and Bhave, 2014; Kumar, 2014; Aslam *et al*., 2020; Chen *et al*., 2022; Zhang *et al*., 2022). Many reports have shown the role of miRNAs in regulating the responses of barley under different stress conditions (Kantar, Unver and Budak, 2010; Hackenberg *et al*., 2012; Sunkar, Li and Jagadeeswaran, 2012; Wu *et al*., 2018). Thus, it would be of great interest to explore the functions of *HvAMT* genes in response to various biotic and abiotic stress processes in future studies.

In addition, we constructed a regulatory network of protein–protein interaction (PPI), using a search tool designed to retrieve the interacting genes (STRING), for the barley AMTs, which showed considerable interactive networks with the other proteins. Out of a total of seven HvAMT proteins, five showed protein–protein interactions (Fig. 2), while HvAMT1.2 and HvAMT2.1 did not show any interaction. The interactions between proteins were further analysed using *k*-means clustering. Proteins were grouped in three clusters (Tab. S3), Cluster 1 consisted of ten members including HvAMT1.1, HvAMT3.1a, and HvAMT3.1b, cluster 2 consisted of six proteins including HvAMT2.3 and HvAMT3.2, and cluster 3 contained nine proteins while did not have any HvAMT member. HvAMT1.1 showed interaction with A0A287N574 (TPT domain-containing protein), A0A287WZA9 (TPT domain-containing protein), and A0A287G4Q6 (Ammonium_transp domain-containing protein) along with two uncharacterised proteins. HvAMT3.2 gene showed interactions with A0A287KK40 (AA_permease_C domain-containing protein) and A0A287RIH1 (glutamate receptor). HvAMT2.3 showed interaction with A0A287RIH1 (glutamate receptor). Previous reports showed that many of these proteins are involved in the development and various stress responses in plants (El-Araby, Nassar and Shaaban, 2006; Peng *et al*., 2014; Flores-Tornero *et al*., 2017; Cheng *et al*., 2018; Chen *et al*., 2021). For example, the TPT domain-containing protein belongs to the triose phosphate/phosphate translocator involved in photosynthetic acclimation, a light response resulting in increased

tolerance to high-intensity light (Häusler *et al*., 2014). Glutamate receptors, which probably act as a non-selective cation channel, belong to the glutamate-gated ion channel family, which is involved in light-signal transduction in plants (Lam *et al*., 1998). Therefore, it seems that AMTs in barley might play important roles in plant physiology and stress response by interacting with other proteins.

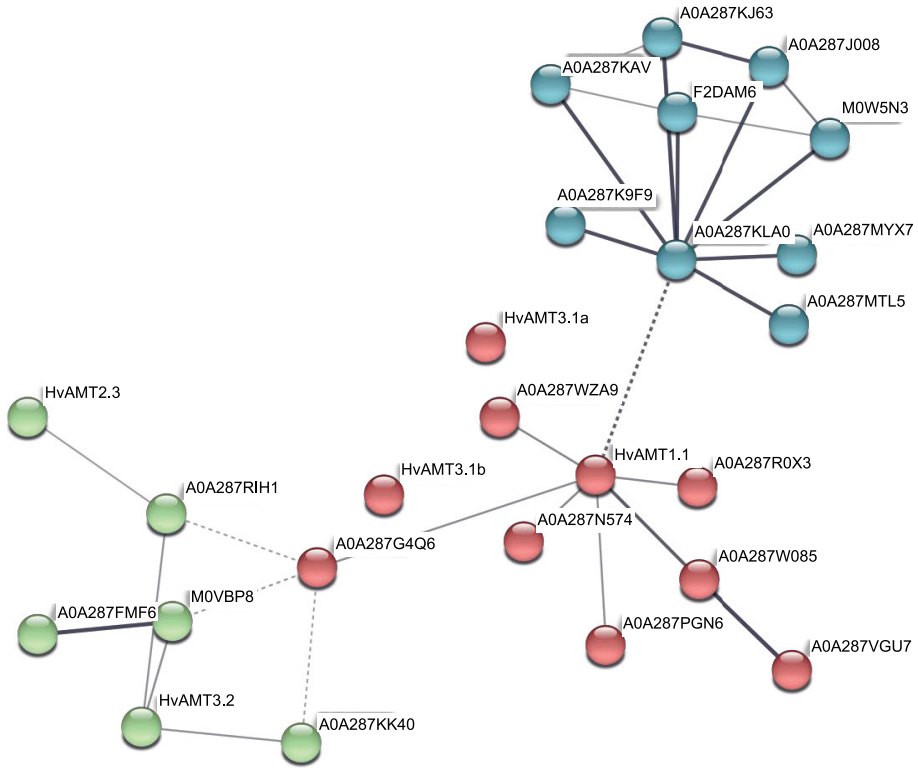

Fig. 2. Protein–protein interaction network of HvAMT proteins in barley; nodes (circles) = proteins, straight lines = interactions between different proteins, line thickness = the strength of data support, nodes in red colour = cluster 1, nodes in green colour = cluster 2, nodes in blue colour = cluster 3; A0A287G4Q = Ammonium_transp domain-containing protein, A0A287N574 = TPT domain-containing protein, A0A287W085 = G_PROTEIN_RECEP_F2_4 domain-containing protein, A0A287WZA9 = TPT domain-containing protein, A0A287FMF6 = proline dehydrogenase, A0A287KK40 = AA_permease_C domain-containing protein, A0A287RIH1 = glutamate receptor, A0A287J008 = protein kinase domain-containing protein, A0A287K9F9 = protein kinase domain-containing protein, A0A287KAV2 = protein kinase domain-containing protein, A0A287KJ63 = protein kinase domain-containing protein, A0A287KLA0 = protein kinase domain-containing protein, A0A287MTL5 = protein kinase domain-containing protein, A0A287MYX7 = protein kinase domain-containing protein, M0W5N3 = protein kinase domain-containing protein; source: own study

IN SILICO EXPRESSION ANALYSIS OF *HvAMT* GENES

Tissue and developmental-specific expression analysis of genes provide valuable clues about their important roles in the growth and development of plants. We analysed the expression of *HvAMT* genes in different tissues using the publically available data. As for the genes from the *AMT1* subfamily in barley, *HvAMT1*.1 was mainly expressed in roots, leaf, stem, blade (lamina), and microspore, while *HvAMT1.2* showed high expression in roots only (Fig. 3). These results are consistent with the previous studies on *AMT1* genes. *AMT1s* are activated to take up NH_4^+ directly from the soil (Ninnemann, Jauniaux and Frommer, 1994; Yuan *et al*., 2007). Furthermore, *AMT1s* show a distinctive role in the development of roots (Lima *et al*., 2010). It has been shown in *Arabidopsis*, that these *AMT1s* are transcribed in all main organs, with a higher expression level in roots (Ninnemann, Jauniaux and Frommer, 1994). In *Arabidopsis*, six genes encoding AMT can be distinguished, and among them, five belong to the *AMT1* subfamily (*AtAMT1*.1–*AMT1.5*) and one (*AMT2*) belongs to *AMT2s*. Specifically, *AtAMT1*.2, *AtAMT1*.3, *AtAMT1*.5, and *AtAMT2* are expressed mainly in roots, *AtAMT1*.4 in pollen, and *AtAMT1*.1 is expressed in roots, stems, as well as leaves. Studies on *Arabidopsis* mutants showed that three *AtAMT1* (*AtAMT1*.1–*AtAMT1*.3) contributed to approx. 90% of NH_4^+ uptake (Loque *et al*., 2006; Yuan *et al*., 2007). In *O. sativa*, *AMT* genes are more abundant showing more diverse patterns of expression and functions compared to those found in *Arabidopsis* (Sonoda *et al*., 2003a; Li, Li and Shi, 2012; Li, C. *et al*., 2016). The *OsAMT1;1*, *OsAMT1;2*, and *OsAMT1;3* seem to be the most important functional *AMT1s*. *OsAMT1;1* is continuously expressed in roots and stems, while *OsAMT1;2* and *OsAMT1;3* are limited to roots. Taken together with the previous studies, *AMT1* subfamily members might play a major role in the uptake of NH_4^+ from the soil through the roots in barley.

The expression of *AMT2* genes in barley was found to be lower than the *AMT1* genes (Fig. 3). The gene *HvAMT2.1* was found to be highly expressed in roots, leaves, and microspore culture, while *HvAMT3.2* showed high expression in the seminal root, root tip, and leaf. The rest of the *HvAMT2* genes in barley did not show any considerable expression in any tissue. This might be because *AMT2s*, as pH-dependent genes, are involved in AMT from root to xylem together with *AMT1s* (Giehl *et al*., 2017)

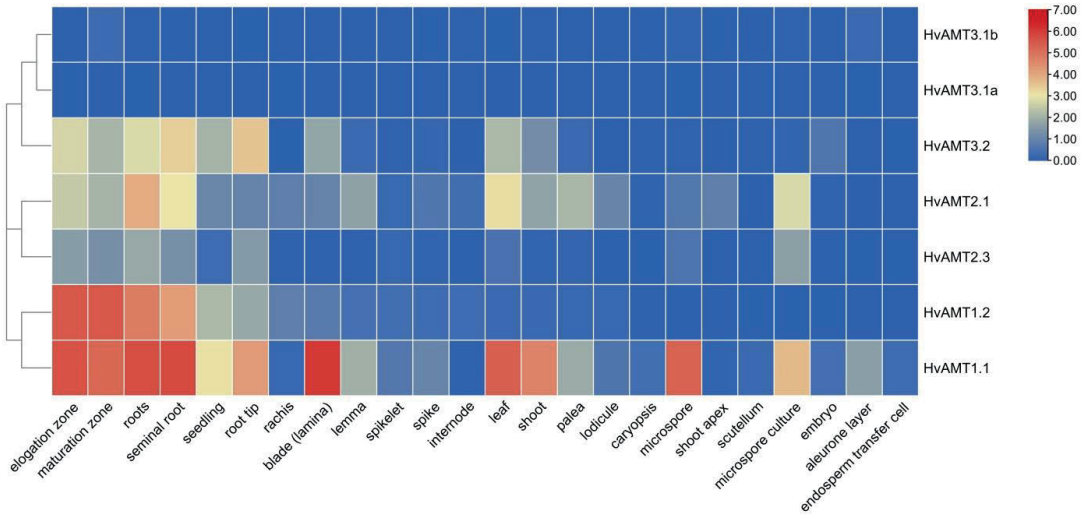

Fig. 3. Expression patterns of *HvAMT* genes in various developmental tissues of barley; colour scale = the log2FC values; source: own study based on data from databases on Genevestigator v3 tool

and are expressed at relatively low levels in different plant organs including roots, branches and leaves (Neuhauser, Dynowski and Ludewig, 2009). Because *AMT2s*, in contrast to *AMT1s*, are not able to transfer 14 C-methylamine, their physiological roles have not been well characterised so far (Sohlenkamp *et al.*, 2002). Even though, it was previously shown that *AMT2s*, similarly to *AMT1s*, possess high-affinity transport system (HATS) to NH_4^+ (Wirén von and Merrick, 2004), later it was demonstrated that they are transporters with low-affinity transport system (LATS) and act at high NH_4^+ concentrations in *Arabidopsis* and other plants (Sohlenkamp *et al.*, 2000; Sohlenkamp *et al.*, 2002). Altogether the gene expression results indicate that the genes of the *AMT1* subfamily might be of interest in improving the nitrogen use efficiency (NUE) in barley.

Overall, this study lays the groundwork for a new understanding of the *AMT* gene family in barley. Our analyses further increased our understanding of the genetics and function of the *AMT* genes and helped to identify candidate genes that may be utilised to improve the barley crop with improved NUE which is not only of great significance to the crop but also for land and water as it will reduce N fertiliser pollution in the surrounding ecosystem.

CONCLUSIONS

In this study, we identified seven *HvAMT* genes in the barley genome, which based on a phylogeny with other plant's *AMT* genes can be classified into two separate subfamilies. Genes assigned to the same subgroup possessed similar properties and structures. We identified one pair of tandem duplicated *HvAMT* genes and found that *HvAMT* genes stayed quite conserved throughout their evolution. These genes might be involved in various processes during plant growth and development, and stress response, as revealed by the gene regulatory elements and protein–protein interaction analysis. The gene expression patterns showed that *HvAMT1* subfamily genes might play a major role in the uptake of NH_4^+ from the soil by roots. This study provides useful information for further research on efficient nitrogen utilisation in barley and functional investigation of the AMT mechanism of action in plants. Our findings might be useful to improve nitrogen use efficiency (NUE) in barley which is of great significance to both the crop and the surrounding ecosystem.

SUPPLEMENTARY MATERIAL

Supplementary material to this article can be found online at https://www.jwld.pl/files/Supplementary_material_Tanwar.pdf

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