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Characterization and Expression of Ammonium Transporter in Peach (*Prunus persica*) and Regulation Analysis in Response to External Ammonium Supply

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Abstract: As the preferred nitrogen (N) source, ammonium (NH_4^+) contributes to plant growth and development and fruit quality. In plants, NH_4^+ uptake is facilitated by a family of NH_4^+ transporters (AMT). However, the molecular mechanisms and functional characteristics of the AMT genes in peach have not been mentioned yet. In this present study, excess NH_4^+ stress severely hindered shoot growth and root elongation, accompanied with reduced mineral accumulation, decreased leaf chlorophyll concentration, and stunned photosynthetic performance. In addition, we identified 14 putative AMT genes in peach (*PpeAMT*). Expression analysis showed that *PpeAMT* genes were differently expressed in peach leaves, stems and roots, and were distinctly regulated by external NH_4^+ supplies. Putative *cis*-elements involved in abiotic stress adaption, Ca^{2+} response, light and circadian rhythms regulation, and seed development were observed in the promoters of the *PpeAMT* family genes. Phosphorylation analysis of residues within the C-terminal of PpeAMT proteins revealed many conserved phosphorylation residues in both the AMT1 and AMT2 subfamily members, which could potentially play roles in controlling the NH_4^+ transport activities. This study provides gene resources to study the biological function of AMT proteins in peach, and reveals molecular basis for NH_4^+ uptake and N nutrition mechanisms of fruit trees.

Keywords: *Prunus persica*; AMT transporters; regulation by NH_4^+ supply; *cis*-elements; phosphorylation site analysis

1 Introduction

Nitrogen (N) is one of the most important nutrients that contributes to plant growth [1,2], flower budding [3] and fruit quality [4–6]. As the primary source of N, ammonium (NH_4^+) is the preferential form absorbed by both perennial and annual plant species, growing either in soil or in water, especially under N-deficient conditions [7,8]. However, with the increasing soil N input and atmospheric deposition, plants have to deal

with NH_4^+ stress from sources below and above the ground. Notably, excessive energy consumption was associated with rapid and futile NH_4^+ transport across the plasma membranes of roots of sensitive barley, concomitant with elevated NH_4^+ accumulation in both roots and shoots [9–12].

To meet typical growth demands, an optimal amount of NH_4^+ should be absorbed effectively from the soil via the roots. In particular, NH_4^+ is uptaken by NH_4^+ transporters (AMT) through the plasma membrane of root cells and present in the cytoplasm and plastids, which have been largely studied in angiosperms [2,12,13], and in basal land plant liverwort [14].

In plants, the first AMT family gene was identified in *Arabidopsis* by functional complementation of a yeast mutant deficient in NH_4^+ uptake systems [15]. Subsequent studies of AMT transporters have been identified in diverse plant species, including *Arabidopsis thaliana* (*A. thaliana*) [16–18], *Lotus japonicus* (*L. japonicus*) [19,20], *Lycopersicon esculentum* (*L. esculentum*) [21–23], *Populus trichocarpa* (*P. trichocarpa*) [24], *Oryza sativa* (*O. sativa*) [25–28], *Triticum aestivum* (*T. aestivum*) [29] and *Alternanthera philoxeroides* (*A. philoxeroides*) [30], which have been characterized in yeast mutant or in *Xenopus* oocytes. The plant AMT family transporters are divided into two subfamilies, AMT1 and AMT2 [2,12,13], and the AMT2 family can be further divided into three subclades in rice [31] and poplar [24]. Members of the AMT1 subfamily have no introns, except for LjAMT1;1 [19], whereas AMT2 subfamily members contain introns in their gene sequences. In particular, phosphorylation of AMT proteins is a recognized means, by which NH_4^+ activities may be regulated [32,33]. In *Arabidopsis*, the phosphorylation of threonine (Thr) residue in the C-terminal tail region of AtAMT1.1 (Thr^{460}), AtAMT1.2 (Thr^{472}) and AtAMT1.3 (Thr^{464}) led to the loss of NH_4^+ transport activity in response to increasing external NH_4^+ supplies [18,32–35], providing a novel regulatory mechanism that NH_4^+ transport can be rapidly shut-off under high NH_4^+ supply condition.

However, molecular basis towards N nutrition in fruit trees are largely rare, and just observed in citrus [36] and pear [37]. As one of the most economically important fruit crops, peach (*Prunus persica*) is one of the most genetically well-characterized species of the *Rosaceae* family [38,39]. In particular, NH_4^+ is closely related to peach fruit quality and yield [4]. In this study, we initially isolated and characterized 14 putative AMT family genes from peach (entitled as *PpeAMT*), and analyzed the expression profiles and regulatory response to external NH_4^+ supplies. Moreover, we performed *cis*-elements and phosphorylation analysis of *PpeAMT* family members. Our findings directly provide molecular basis of NH_4^+ uptake in peach, which might improve utilization and decrease wastage of N fertilizer in fruit tree cultivation.

2 Materials and Methods

2.1 Plant Material and Growth Condition

'Hanlumi' peach seedlings growing at the Key Laboratory of Molecular Module-Based Breeding of High Yield and Abiotic Resistant Plants in Universities of Shandong in Yantai, China were used throughout this study. Seeds were germinated in soil in a green house. Seedlings with similar growth status were transferred into 1/2 MS liquid solution ([40], containing approximately 1 mM NH_4Cl), cultivated in a climate-controlled growth cabinet that maintained under 28°C/23°C and 12/12 h light/dark, with 60% relative humidity.

For the NH_4^+ deficiency treatments, NH_4^+ was omitted from the MS medium. For the NH_4^+ excess treatments, germinated seedlings were grown in 1/2 MS solution containing 20 mM NH_4Cl (pH5.8). Seedlings were exposed to treatment for 72 h (for qRT-PCR determination) or 14 d (for physiological analysis).

2.2 Physiological Analyses

After being exposed to different NH_4^+ level treatments for 14 days, seedlings were collected and rinsed in distilled water, and then weighed to obtain the fresh weight. The roots were scanned with an Epson Rhizo

scanner (RHIZO 2004b), and the total root length and surface area were acquired with Epson WinRHIZO software. Plant chlorophyll quantification was carried out using the method as described by Song et al. [41], and total chlorophyll concentration was expressed as milligrams per gram fresh weight. Tissue NH_4^+ content was analyzed by the o-phthalaldehyde (OPA) method using a high-performance liquid chromatography (HPLC) system (Waters Corp., Milford, MA, USA), as previously described [42]. For mineral analysis, the seedlings were dried and digested using the HNO_3–$HCLO_4$ method, and subjected to ICP-AES (IRIS Advantage, Thermo Electron, Waltham, MA, USA). Photosynthetic analysis was carried out on a portable photosynthetic system LI-6400 (Li-COR,Lincoln, NE, USA) to determine stomatal conductance and net photosynthetic rate, at the terminal leaflet of fully grown second leaf, as described by Song et al. [41].

2.3 Isolation and Localization of Predicted AMT Genes in Peach

Protein sequences of AMT genes of *P. trichocarpa* [24] were obtained at Phytozome Poplar Genome Database (http://www.phytozome.net). These sequences were used as query to BLAST peach genome to identify putative homologues in peach. Nucleic acid sequences and amino acid sequences of the identified putative *PpeAMT* genes were obtained at Phytozome peach genome database. To confirm these predicted *PpeAMT* members, protein sequences were then searched for PpeAMT domains by using InterProScan 4.8 (http://www.ebi.ac.uk/Tools/pfa/iprscan/) and CD search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) web servers. Locus name, location, scaffold distribution of all the obtained *PpeAMT* genes are listed in Tab. 1. The exon and intron structure was analyzed using the online Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/index.php). Transmembrane domains of PpeAMT proteins were analyzed by TMpredict online program (http://ch.embnet.org/software/TMPRED_form.html). Expressed sequence tag (EST) data of *PpeAMT* genes were obtained from the dbEST database on National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) via BLASTN alignment between the corresponding full-length coding sequence (CDS) of candidate *PpeAMT* genes and the NCBI EST database.

Table 1: Information of *PpeAMT* family genes in peach

Gene	Locus name	Gene location	Scaffold distribution	ORF (bp)	Amino acids	Transmembrane regions
PpeAMT1;1	ppa004542m	3655374-3657084	1	1515	504	11 (N-terminus outside)
PpeAMT1;2	ppa004450m	28182013-28183551	2	1530	509	11 (N-terminus inside)
PpeAMT1;3	ppa005341m	176876-178278	6	1401	466	10 (N-terminus outside)
PpeAMT1;4	ppa004613m	18874579-18876081	8	1503	500	10 (N-terminus outside)
PpeAMT1;5	ppa015730m	43101574-43103076	1	1503	500	10 (N-terminus outside)
PpeAMT2;1	ppa022420m	17889600-17891484	7	1410	469	10 (N-terminus outside)
PpeAMT3;1	ppa008980m	28342145-28345100	4	939	312	7 (N-terminus outside)
PpeAMT3;2	ppa020093m	28437353-28439602	4	684	227	5 (N-terminus inside)
PpeAMT3;3	ppa019792m	28462529-28465742	4	789	262	4 (N-terminus outside)
PpeAMT3;4	ppa022674m	28279751-28284237	4	1065	354	11 (N-terminus outside)
PpeAMT3;5	ppa004749m	28131401-28135102	4	1482	467	11 (N-terminus outside)
PpeAMT4;1	ppa004845m	24446309-24449047	6	1470	471	11 (N-terminus outside)
PpeAMT4;2	ppa027111m	38371072-38372953	1	1443	480	11 (N-terminus outside)
PpeAMT4;3	ppa005235m	19984263-19986069	8	1416	471	11 (N-terminus outside)

2.4 Phylogenetic Analysis of Predicted AMT Genes in Peach

Full-length amino acid sequences were aligned by CLUSTALW and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 4.1. Phylogenetic analyses were conducted according to the neighborjoining (NJ) method implemented in MEGA, with the pairwise deletion option for handling alignment gaps, and with the Poisson correction model for distance computation. Branch lengths indicate the corresponding phylogenetic distances. Sequence logos of AMT1 and AMT2 subfamilies were shown on the right, generated using WEBLOGO (http://weblogo.berkeley.edu/logo.cgi).

2.5 Cis-Elements Analysis of Predicted AMT Genes in Peach

To investigate *cis*-elements in promoter region of *PpeAMT* genes, 2 kb of individual genomic DNA sequences upstream of the initiation codon (ATG) were downloaded from Phytozome Grape Genome Database. *cis*-elements were predicted via the help of PLACE online server (http://www.dna.affrc.go.jp/PLACE/).

2.6 Phosphorylation Analysis of Residues within the C-terminal Cytoplasmic Tail

We scanned the sequences of the PpeAMT proteins using the NetPros program in order to identify potential phosphorylation (http://www.cbs.dtu.dk/services/NetPhos/), and integrated it with the alignment of AtAMT1;1 sequence to look for homologies of the known phosphorylation sites.

2.7 RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from leaves, stems, or roots using Plant RNAKit (OMEGA) and treated with gDNA Eraser to remove genomic DNA contamination. The first strand cDNA was synthesized using PrimeScript™ RT reagent Kit (TaKaRa). Specific primers of *PpeAMT* genes and control gene *Ubiquitin* in peach were designed using NCBI/Primer-BLAST on-line server. Primer sequences were listed in Tab. 2. Quantitative real-time 2RT-PCR (qRT-PCR) was carried out on 7500 Real Time PCR System (Applied Biosystems, USA). PCR condition for thermal cycling was as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s. The relative expression levels of the target genes were presented after normalization to the internal control from three independent biological repeats.

2.8 Functional Complementation of PpeAMT1;1 in Yeast Mutant 31019b

The recombinant plasmid pYES2-AMT1;1 was constructed by cloning the CDS region of *PpeAMT1;1* gene into pYES2 vector [30], using the forward primer of 5'-GAGAGGTACCATGGCGACGTG-GGCTACCTTAGA-3' (introduced *Kpn* I site that underlined), and reverse primer of 5'-GAGAGCGGCCGCCTAAACGGAAGTGGGTGTGG-3' (underlined with *Not* I site). The yeast strain 31019b (*MATa mepl△ mep2△::LEU2 mep3△::KanMX2 ura3*, [15,24,30]) was transformed with pYES2 harboring the CDS of PpeAMT1;1. Yeast complementation tests were carried out as desceibed previously [15,30]. Yeast strain 31019b was transformed with pYES2 or pYES2-*AMT1;1*. Yeast cell growth was determined in YNB liquid medium supplemented with 0.02, 0.2 or 2 mM NH_4^+, respectively, as the sole N source at pH value of 5.8. Pictures were taken 3 days after incubation at 30°C. The growth of transformed yeast cells in YNB liquid medium was checked by determining absorbance at 600 nm [30].

2.9 Statistical Analysis

Data were statistically analyzed using Student's *t*-test in the SPSS 13.0 software (SPSS Chicago, IL, USA). Details are described in figure legends. Graphs were produced using Origin 8.0 software.

3 Results

3.1 Biological Response of Peach Seedlings in Response to NH_4^+ Supply

To investigate the biological response of peach seedlings in response to external NH_4^+ supplies, peach seedlings were exposed to different normal NH_4^+ status (1 mM NH_4Cl), NH_4^+ depletion (0 mM), and excess

Table 2: Primer sequences used in this work

Gene	Sequence (5' to 3')	Amplicon (bp)
PpeAMT1;1	F: GTCGTCTCCCACTGGTTCTG	240
	R: GAACGTGCCCAAGACTACCA	
PpeAMT1;2	F: TGCACTGCTGCTCTGACTAC	202
	R: TTAGCTTCTCCGCCACCTTG	
PpeAMT1;3	F: TTAGCTTCTCCGCCACCTTG	165
	R: AGTCGAACCGGCTAATGTGG	
PpeAMT1;4	F: AGGTAGGTCTGTGGCTTTGC	250
	R: GTTCCAATGACCGGAGAGCA	
PpeAMT1;5	F: GAGGTGGAAAGCTCTTGGCT	202
	R: GCCGGATCGTTGCATTCTTC	
PpeAMT2;1	F: TATTGCTTGGGGGCTCCTTG	232
	R: TGCCTGTCATGTGATTGCCT	
PpeAMT3;1	F: CGGGGCTTTTCGCTGAGCCTG	228
	R: CTGCGTCATCCCCAATCAGT	
PpeAMT3;2	F: CCTTCCGTCATTGGAGCTGT	238
	R: CGTTAAAACGCCGCCAAGAA	
PpeAMT3;3	F: TCTGAGGTCGTTTGGCCTTC	238
	R: GGACAGCTCCAATGACGGAA	
PpeAMT3;4	F: TGTCGGAGCTTTCAGCTTGT	224
	R: TGAAACCTGCCCATCCCATC	
PpeAMT3;5	F: GATGGGATGGGCAGGTTTCA	159
	R: ACAGCTCCAATGACGGAAGG	
PpeAMT4;1	F: TCACCGCAGCTTATTGGGTT	250
	R: ATCACTGATGGCTTGCCGAA	
PpeAMT4;2	F: CGTTCGAACAAGGACAGGGA	194
	R: TCAAGCAGGAGCCAAGTGAG	
PpeAMT4;3	F: TCCTCACCGGCTTCTTTTCC	209
	R: AGCGCCCCAAGAGGAAGCAA	
Ubiquitin	F: AGGCTAAGATCCAAGACAAAGA	145
	R: CCACGAAGACGAAGCACTAAG	

NH_4^+ stress (20 mM). Results showed that NH_4^+ depletion caused mild phenotype to peach seedling (Fig. 1a). Fresh weight of leaves and stems, roots was reduced approximately 24%, 22%, and 16%, respectively (Figs. 1a and 1b). Correspondingly, total root length, total root surface area, and total leaf chlorophyll concentration was obviously decreased (Figs. 1c and 1d). While excess NH_4^+ stress severely hindered shoot growth and root elongation, as evidenced by dramatically reduced fresh weight, smaller and chlorotic leaves, decreased leaf chlorophyll concentration, decreased total root length and surface

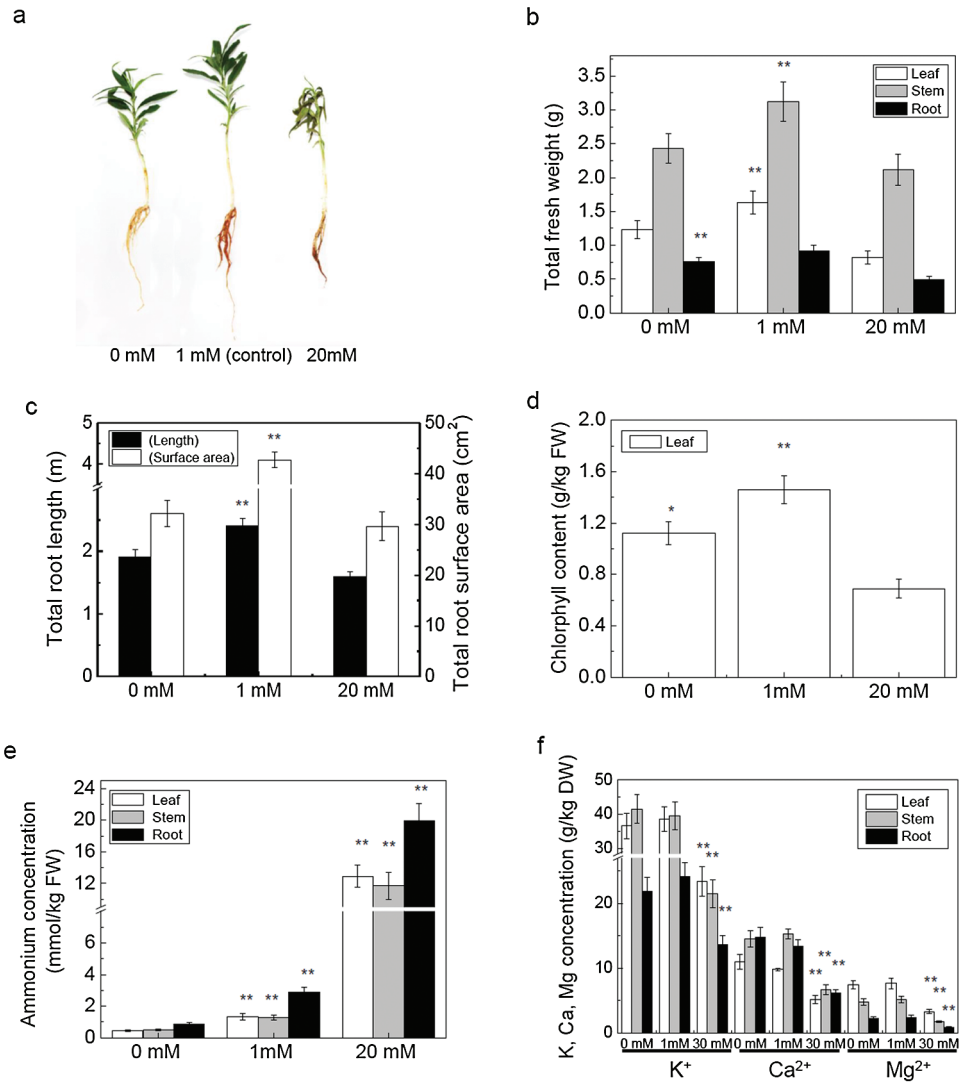

Figure 1: Biological response of peach seedlings in response to NH_4^+ supply. Seedlings were grown in 1/2 MS solution supplied with 0, 1, and 20 mM NH_4Cl for 14 d before examination. (a) Phenotype. (b) Total fresh weight. (c) Total root length and surface area. (d) Leaf chlorophyll concentration. (e) Tissue NH_4^+ concentration. (f) Tissue mineral concentration. Values are presented as means ± SE. *Asterisks* indicate statistical differences between plants under different NH_4Cl treatment ($0.01 < *P < 0.05$, $**P < 0.01$, independent-samples *t* test)

area (Figs. 1a–1d). Particularly, excess NH_4^+ stress also significantly reduced the stomatal conductance and net photosynthetic rate (Tab. 3), indicating a stunned photosynthetic performance.

Determination of the tissue NH_4^+ concentration showed that NH_4^+ were accumulated more in roots than in leaves and stems under each treatment, whereas mineral cations, i.e., potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) was concentrated more in shoots than in roots (Fig. 1e). In particular, NH_4^+ depletion caused a significant decrease in tissue NH_4^+ concentration (Fig. 1e), whereas had little effect to K^+, Ca^{2+}, and Mg^{2+} accumulation in all tested organs, including leaves, stems, and roots, respectively (Fig. 1f). Notably, excessive NH_4^+ treatment dramatically enhanced tissue NH_4^+ concentration under excessive NH_4^+ stress, associated with a reduction in tissue K^+, Ca^{2+}, and Mg^{2+} concentration (Fig. 1e).

Table 3: Photosynthesis analysis under drought stress[a]

Treatment	Control (1 mM)	0 mM	30 mM
P_N (μmol·m^{-2}·s^{-1})	9.45 ± 0.21	8.67 ± 0.17	5.93 ± 0.14**
g_s (mmol·m^{-2}·s^{-1})	0.24 ± 0.02	0.23 ± 0.02	0.12 ± 0.00**

Note: [a] Seedlings were exposed to treatment of normal NH_4^+ (1 mM NH_4Cl), NH_4^+ depletion (0 mM), and excess NH_4^+ stress (30 mM) for 10 days before examination. Data are given as the means ± SE from 3 independent experiments. Asterisks indicate statistical differences between treatments (**$P < 0.01$, independent samples t test).

3.2 Identification of Predicted AMT Genes in Peach

To gain further insights into the molecular basis underlying the uptake and transport of NH_4^+ in peach, we isolated 14 AMT family genes from peach seedlings, named as *PpeAMT* (Tab. 1). InterProScan results indicated that all *PpeAMT* genes were predicted to encode proteins containing single AMT domain (data not shown). Gene name, locus ID, gene location and open reading fram (ORF) length were shown in Tab. 1. Gene structure analysis showed that peach AMT1 subfamily members have no introns, whereas AMT2 subfamily members contain 2 to 4 introns with different intron phases, varied distinctly in lengh (Fig. 2). In addition, transmembrane prediction revealed that all PpeAMT family proteins possess 10 to 11 TM domains, with the exception of *PpeAMT3;1-3;3* that has 7, 5, and 4 TM domains, respectively (Tab. 1). Interestingly, PpeAMT1;2 and PpeAMT3;2 had N-terminus inside the cell membrane, while the other members had N-terminus outside the cell membrane (Tab. 1).

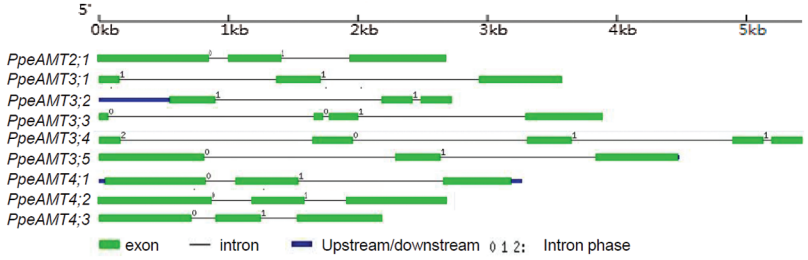

Figure 2: Gene structure of *PpeAMT* family genes. Exons and introns are depicted by filled green boxes and single lines, respectively. Intron phases 0, 1 and 2 are indicated by numbers 0, 1 and 2 in the figure. UTRs are displayed by thick blue lines at the two ends

3.3 Phylogenetic Analysis of Predicted PpeAMT Proteins

The phylogenetic tree was constructed based on the alignment of the AMT amino acid sequences between peach and other reported plants, including poplar, *Arabidopsis*, rice, tomato, wheat, and *Lotus*. As shown in Fig. 3, the AMT family members of peach were classified into 2 subfamilies, entitled as AMT1 (5 members) and AMT2 (9 members), and the sequence logos of AMT1 and AMT2 subfamilies showed on the right. Notably, PpeAMT1;1, PpeAMT1;2, and PpeAMT1;3, was strictly clustered with LjAMT1;1, LjAMT1;2, and PtrAMT1;6 in AMT1 subfamily, respectively. In AMT2, PpeAMT3;1-3;5 are closely aggregated, and together closely clustered with PtrAMT3;1 and PtrAMT3;1 of poplar. Similarly, PpeAMT4;1 and PpeAMT 4;3 are closely aggregated, and together was closely clustered with PtrAMT4;1 of poplar. PpeAMT4;2 was closely clustered with PtrAMT4;2 of poplar. In particular, PpeAMT2;1 was solely clustered with AMT2 and AMT3 subclades of other plants (Fig. 3).

3.4 Expression Profiles of PpeAMT Family Genes

EST data revealed that all *PpeAMT* genes had corresponding ESTs, except *PpeAMT3;2* and *PpeAMT4;2*. Database expression profiles showed that *PpeAMT* family genes were expressed in different

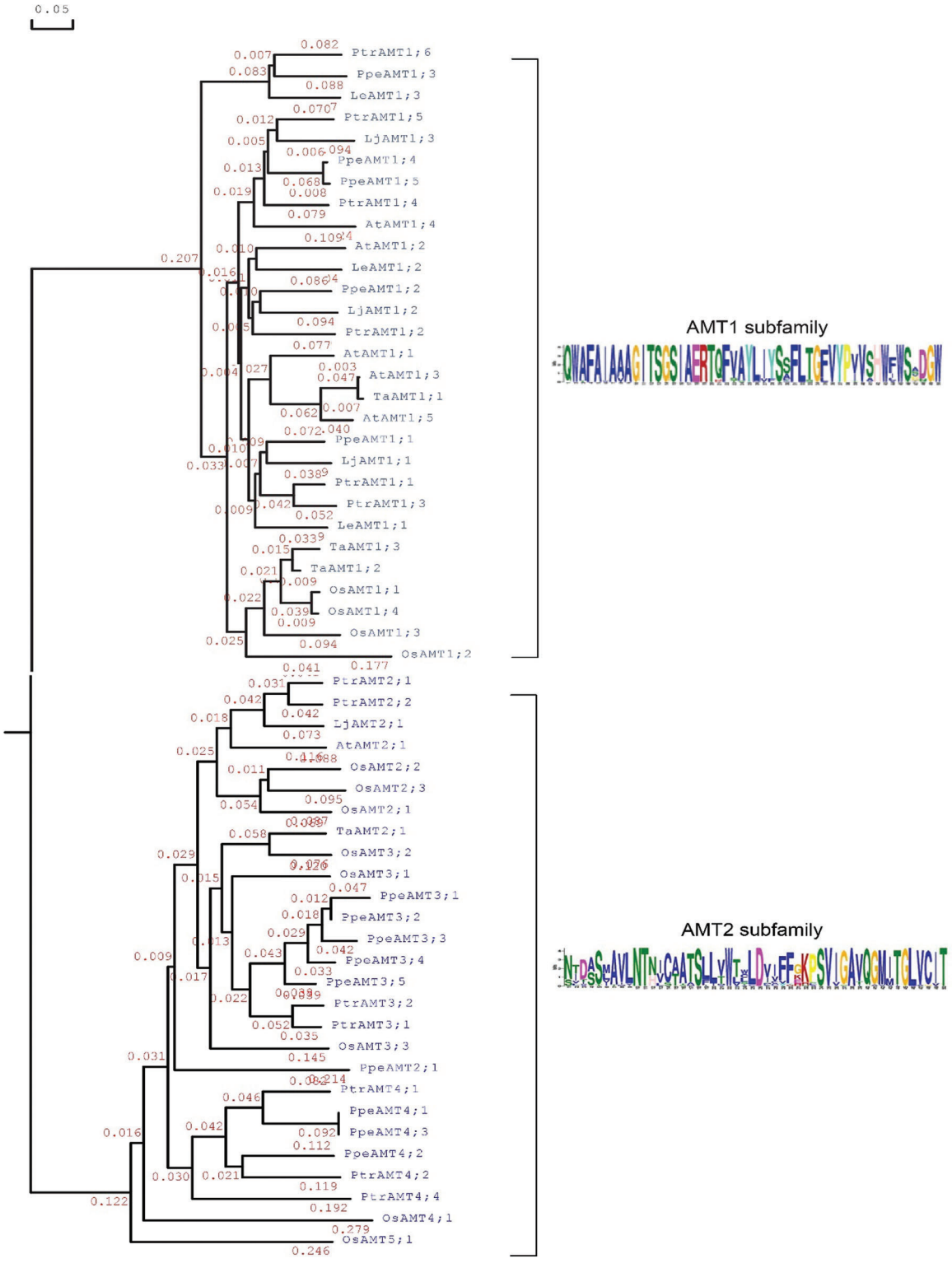

Figure 3: Phylogenetic tree of the AMT family in peach and other plants. The analysis was performed as described in the Material and Methods section. Branch lengths (drawn in the horizontal dimension only) are proportional to phylogenetic distances. Sequence logos of AMT1 and AMT2 subfamilies showed on the right. The locus names or accession numbers used for phylogenetic tree construction were listed in the Tab. A1

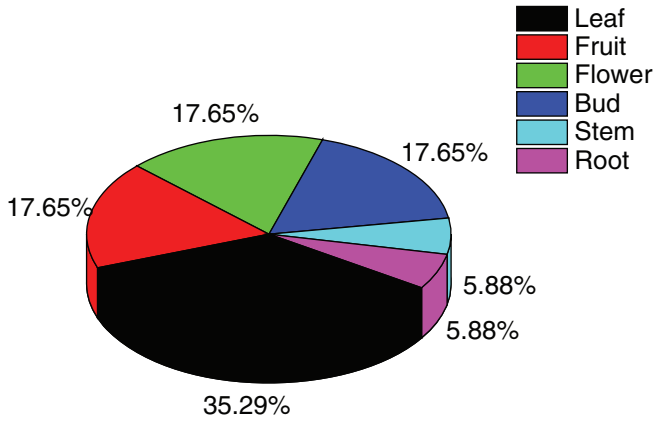

Figure 4: Database expression profiles of *PpeAMT* genes. EST data were downloaded from NCBI online EST database

organs or tissues in peach, and the highest percentage was observed in leaf (35.29%), followed by fruit (17.65%), flowers (17.65%), bud (17.65%), stem (5.88%) and root (5.88%) (Fig. 4).

To verify the location and expression profiles of putative *PpeAMT* genes, we carried out qRT-PCR determination. Results showed that all *PpeAMT* genes were expressed in tested organs, including leaves, stems and roots (Fig. 5a). The most high expressed gene was *PpeAMT1;1*, followed by *PpeAMT1;4*, *PpeAMT1;2* and *PpeAMT3;3*, while expression of *PpeAMT1;3*, *PpeAMT1;5*, *PpeAMT3;1* and *PpeAMT3;2* were extremely low (Fig. 5a). In particular, *PpeAMT3;3* was evenly distributed throughout the entire plant. Expression of *PpeAMT1;2*, and *AMT3;4* were significantly higher in roots than in shoots, whereas *PpeAMT1;1*, *PpeAMT1;3*, *PpeAMT1;4*, and *AMT2;1* were higher in leaves (Fig. 5a). Notably, *PpeAMT4;1*, *PpeAMT4;2*, and *AMT4;3* were largely localized to the stems (Fig. 5a), implying that *PpeAMT4* subfamily members might contribute to NH_4^+ transport from roots to shoots during normal growth conditions.

3.5 Regulation of PpeAMT Genes in Response to NH_4^+ Supply

The transcript levels of AtAMTs were regulated by the available N supply [43]. In this present study, NH_4^+ deficiency mainly affected *PpeAMT* genes in roots (Fig. 5b). Five genes of *PpeAMT1;1*, *PpeAMT1;5*, *PpeAMT2;1*, *PpeAMT3;2*, and *AMT4;3* were mildly up-regualted in roots. Three genes of *PpeAMT1;2*, *PpeAMT1;3*, and *AMT3;1* were increased by 3–5 fold. *PpeAMT1;5* and *AMT3;2* were more sensitive to NH_4^+ depletion, whose expression levels was increased throughout the entire plant. In addition, expression of *PpeAMT1;1* in stems and *PpeAMT3;2* in leaves were also simultaneously enhanced under NH_4^+ deficiency (Fig. 5b). In contrast, the other 6 genes, including *PpeAMT1;4*, *PpeAMT3;3*, *PpeAMT3;5*, *PpeAMT4;1*, and *AMT4;2*, were not significantly affected by NH_4^+ depletion.

Excess NH_4^+ stress differently regulated the *PpeAMT* genes in individual tested organs (Fig. 5c). In particular, expression of *PpeAMT1;3*, *PpeAMT3;1*, *PpeAMT3;2*, *PpeAMT3;4*, and *AMT4;1* were decreased, whereas *PpeAMT1;1* and *PpeAMT4;3* in roots and *PpeAMT1;5* and *PpeAMT3;5* in all tested organs were obviously increased. Notably, expression of *PpeAMT2;1* was changed little in response to excess NH_4^+ stress (Fig. 5c).

3.6 Putative Cis-Element Analysis

Promoter regions of *PpeAMT* family genes were obtained from Phytozome Grape Genome Database, via cloning 2 kp range genomic DNA sequences upstream of translation start site of AMT family genes,

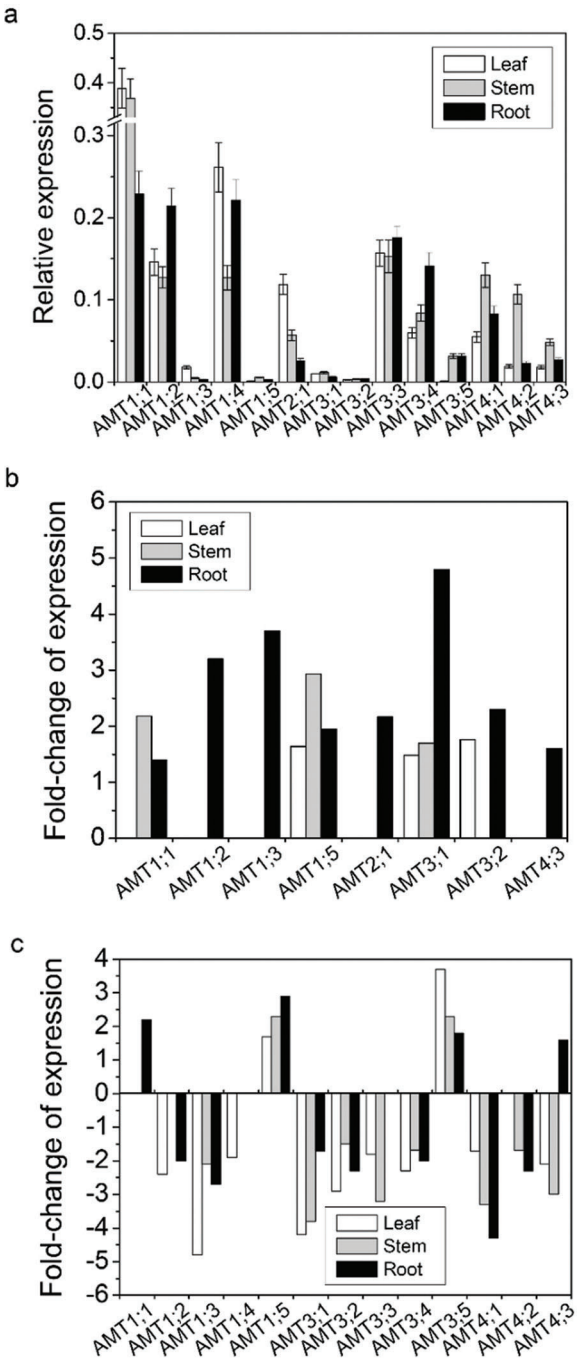

Figure 5: Expression profiles of *PpeAMT* genes in response to NH_4^+ supply. Seedlings were grown in 1/2 MS solution supplied with 0 (NH_4^+ depletion), 1 (control conditions), and 20 mM (excess NH_4^+) NH_4Cl for 72 h before qRT-PCR examination. Expression values are given as a ratio relative to the values of actin. (a) Relative expression under control conditions. (b) Fold-change of expression under NH_4^+ depletion. (c) Fold-change of expression NH_4^+ excess stress. Data are the means ± SE, obtained from three independent experiments

respectively. By searching the PLACE database, approximately 60 putative *cis*-elements more than 8 bp length were identified (data not shown). In particular, most of these *cis*-elements were found in *PpeAMT* promoter regions that involved in abiotic stress responsive, Ca^{2+}-responsive, light and circadian rhythms regulation and seed development. In detail, CIACADIANLELHC (for circadian regulation), INRNTPSADB (for light regulation) and SEF4MOTIFGM7S (for protein storage) were found in all *PpeAMT* genes, whereas ABRERATCAL (for Ca^{2+}-responsive) in 6 *PpeAMT* genes, ACGTATERD1 (for dehydration responsive) in all *PpeAMT* genes except *PpeAMT1;3*, ANAERO1CONSENSUS (for anaerobic responsive) in all *PpeAMT* members except *PpeAMT1;5* and *PpeAMT3;1*, BOXLCOREDCPAL (for wound responsive) in 9 *PpeAMT* genes, DPBFCOREDCDC3 (for protein storage) in all *PpeAMT* genes except *PpeAMT1;1* and *PpeAMT1;5*, PRECONSCRHSP70A (for dehydration responsive) in 11 *PpeAMT* genes, RYREPEATGMGY2 (for protein storage) in 7 *PpeAMT* genes, SEBFCONSSTPR10A (for dehydration responsive) in 9 *PpeAMT* genes.

3.7 Phosphorylation Analysis of Residues within the C-Terminal Cytoplasmic Tail

Potential phosphorylation residues within the C-terminal cytoplasmic tail of the PpeAMT proteins were perdicted using the NetPros program. In *Arabidopsis*, 6 phosphorylation sites (T^{460}, S^{475}, S^{488}, S^{490}, S^{492} and T^{496}) were located within the C-terminal tail of AtAMT1.1 [33,35,44]. In particular, all five AMT1 proteins of peach contain a homologue of AtAMT1.1 T^{460} (Fig. 6), implying that a similar regulation by phosphorylation in *Arabidopsis* may take place in peach. In addition, another three more highly conserved potential phosphorylation sites within the C-terminal region were also identified: one homologous to AtAMT1.1 S^{449} in OsAMT1, one homologous to AtAMT1.1 T^{477} in OsAMT2, and one homologous to AtAMT1.1 S^{483} in OsAMT2, which were indicated by boxes (Fig. 6). Nonetheless, these residues are worthy of being studied of phosphorylation *in vivo* in peach. Although the C-terminal regions of the PpeAMT proteins are quite varied in length, all of them contain the highly conserved glycine residue (Fig. 6).

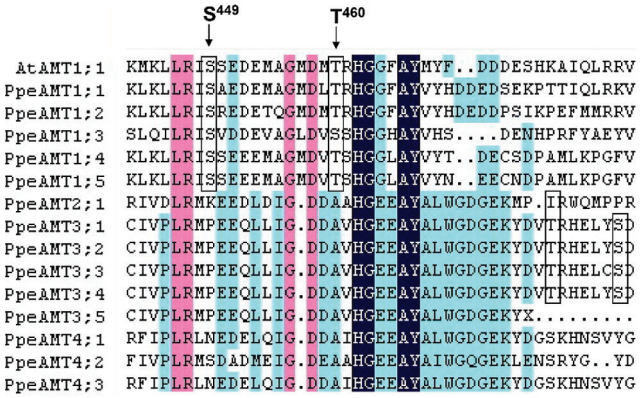

Figure 6: Conserved phosphorylation sites within the C-terminal region of PpeAMT proteins. Potential phosphorylation sites were identified using the NetPhos program and sites that are conserved within phylogenetic groups are indicated by boxes

3.8 Functional Determination of PpeAMT1;1 in Yeast Mutant

The most high expressed gene was *PpeAMT1;1*, especially in the shoots, which was quite sensitive to external NH_4^+ supplies. We further carried out functional determination of *PpeAMT1;1* via complementation of the yeast mutant 31019b [14–16]. Yeast cells harboring pYES2 or pYES2- *PpeAMT1;1* grew well on YNB medium that containing 2 mM Arg as the sole N source, but yeast cells harboring pYES2 could not grow well

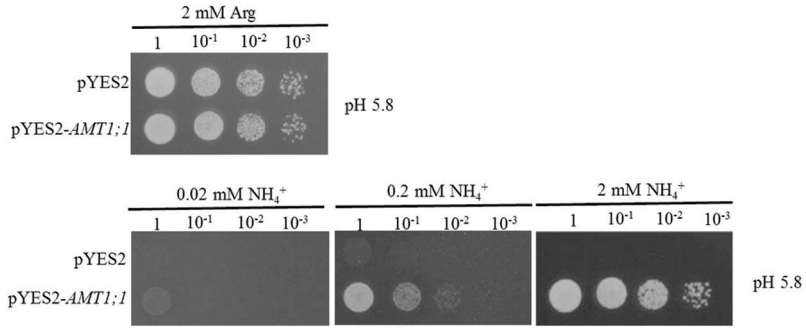

Figure 7: Functional determination of PpeAMT1;1-mediated NH_4^+ uptake in yeast. Complementation of yeast mutant 31019b by PpeAMT1;1. Yeast cells grown on YNB medium supplemented with 2 mM Arg as the sole N source was used as positive control. Yeast strains 31019b transformed with pYES2 or pYES2-*AMT1;1* were grown on YNB medium, supplemented with different concentrations of NH_4Cl. Final diluted concentration are indicated by 1, 10^{-1}, 10^{-2}, and 10^{-3}

on YNB medium containing with 0.02, 0.2 or 2 mM NH_4Cl under pH 5.8, while yeast cells harboring pYES2- *PpeAMT1;1* grew normally on YNB medium supplemented under 0.2 and 2 mM NH_4Cl (Fig. 7). These findings imply that PpeAMT1;1 is involved in NH_4^+ in yeast.

4 Discussion

NH_4^+ is the dominant form of N transport for lower energetic cost in assimilation [2,12,13,43], while excess NH_4^+ is toxic to plant growth and development [9–11]. In this present study, excess NH_4^+ stress definitely inhibited the growth peach seedlings (Fig. 1). In particular, the reduced total leaf chlorophyll concentration may partially explain the smaller and chlorotic leaves under excess NH_4^+ stress. Notably, excess NH_4^+ stress brought a clear reduction in K^+ content (Fig. 1f), which might be an inevitable reason for its consequent suppression in K^+-dependent metabolic processes that further suppressed the growth of peach seedlings. Ca^{2+} is widely known to regulate important physiological pathways in plant, i.e., flowering and vegetative organogenesis [45–47], and NH_4^+ stress has been frequently reported to promote early flowering and a shortened life cycle [10,48]. According to our findings, a significant decrease in tissue Ca^{2+} concentration was also observed under excess NH_4^+ stress (Fig. 1f). Favorably, N supply may be expected to play an important role in flower formation and development, which needs further verification. Moreover, photosynthesis is always affected as part of exposure to high external NH_4^+ [10,49]. Correspondingly, tissue Mg^{2+} level in peach seedlings was significantly reduced, especially in shoots under excess NH_4^+ stress, which was in keeping with the decreased total leaf chlorophyll concentration, stomatal conductance, and net photosynthetic rate (Figs. 1d and 1f).

Genome analysis showed that 6, 12 and 13 *AMT* genes was observed in *Arabidopsis*, rice and poplar, respectively [17,24,31], respectively. Similar to perennial woody plant poplar [24], 14 *AMT* genes were identified in the peach genome, and assigned to the AMT1 subfamily (5 genes) or to the AMT2 subfamily (9 genes). Interestingly, peach possesses a much greater number of *AMT2* genes than *Arabidopsis* (1 gene) and more *AMT1* genes than rice (3 genes), indicating that perennial woody plants may need more NH_4^+ transporters than annual plant species during the whole plant life. Comparison of the number of *AMT* genes in different species might suggest that plant species from different environments or with different life style organize NH_4^+ transport with a fairly unequal number of NH_4^+ transporters. Multiple forms of NH_4^+ transporters in higher plants allow a greater regulatory flexibility and organelle-, cell-, tissue- or organ- specialization, and enable cells to take up NH_4^+ over a wide range of concentrations [20]. Expression features of *PpeAMT* genes showed complex tissue specificities and

fine regulations by NH_4^+ supply (Fig. 5), which might be related to the peculiar physiological functions of PpeAMT transporters in peach.

Transcript levels of AMT1 genes were closely related to the N nutrition status of the plant. Starvation of NH_4^+ largely enhanced the transcript levels of most *PpeAMT* genes in individually tested organs (Fig. 5b), may it be a clue of 'starvation response' for peach fruit trees to grow under suboptimal N-source conditions, thus, to adapt to the internal metabolic mechanisms. Such findings have been exclusively mentioned in plants [2,23,29,31]. Although excess NH_4^+ stress reduced expression of most of the *PpeAMT* genes in different organs, expression of *PpeAMT1;1* and *PpeAMT4;3* in roots, *PpeAMT1;5* and *PpeAMT3;5* in all tested organs were obviously increased (Fig. 5c). In particular, expression of *PpeAMT2;1* changed little under excess NH_4^+ stress. We suppose that when exposed to sufficient NH_4^+ supply, such genes might be involved in that maintaining 'luxury uptake' of external- NH_4^+ or transporting excess NH_4^+ to where it does not bother plant metabolism, which possiblely secures plant growth from death under heavy NH_4^+ stress. Nonetheless, these findings may partically explain the increased NH_4^+ accumulation in peach seedlings or stored under excessive NH_4^+ supply (Fig. 1e). It is worthy mentioning that *PpeAMT3;1* gene was a unique gene, tightly regulated by iron levels throughout the entire plant, which was reduced by NH_4^+ deficiency (especially in roots) and increased by NH_4^+ excess (particularly in shoots). Interestingly, *PpeAMT1;5* gene was a peculiar gene, whose expression was up-regulated by either NH_4^+ deficiency or NH_4^+ excess in all tested organs, compared to normal conditions (Fig. 5c). Probably, these two genes might possess peculiar functions during peach seedlings development in response to environmental NH_4^+ supplies.

Heterologous expression studies using AMT defective yeast mutant or *Xenopus* oocytes indicate that AMT1 family genes encode high affinity transporters [2,12,18,27]. These findings together suggest that plant AMT1 family proteins play a key role in high affinity NH_4^+ uptake from soil. In addition, phylogenetic analysis showed that the PpeAMT1 members were closely clustered with the plant AMT1 members, including *Arobidopsis*, poplar, tomato, and lotus. Mightly, these findings suggest that the function and mechanism of AMT1 subfamily members in peach are similar to those in *Arobidopsis*, poplar, tomato or lotus. In this present study, PpeAMT1;1 could utilize the external NH_4^+ in yeast (Fig. 7). Nonetheless, PpeAMT1;1 might be an functional AMT1 transporter responsible for NH_4^+ uptake in peach, especially during the shoots N nutrition and balance.

Frankly, *cis*-elements studies could provide key foundation for further functional research of the AMT family in peach. In this work, a series of putative *cis*-elements were found that involved in abiotic stress responsive, Ca^{2+}-responsive, light and circadian rhythms regulation, and seed development. Among these putative *cis*-elements, the CIACADIANLELHC (for circadian regulation) and INRNTPSADB (for light regulation) were distributed in all the *PpeAMT* family genes, implies that *PpeAMT* genes are prone to be regulated by circadian rhythm or light. The ACGTATERD1 (for dehydration responsive) was found in all *PpeAMT* members except *PpeAMT1;3*, suggesting that *PpeAMT* genes are likely to be regulated by drought. Moreover, there were other several types of abiotic stress responsive *cis*-elements, i.e., dehydration responsive, anaerobic responsive, and wound responsive. In particular, an widely distributed *cis*-element ABRERATCAL, famous for Ca^{2+}-responsive element, was detected in 6 of the 14 *PpeAMT* family genes. As a secondary messenger, Ca^{2+} was responded to several environmental stresses and could activate or deactivate a gene by regulating its *cis*-elements in plants [45–47]. Nonetheless, *cis*-elements studies in this work contributes to further functional regulation studies of *PpeAMT* genes.

In *Arabidopsis*, phosphorylation sites in a number of plasmamembrane proteins have been identified, including AtAMT1.1 [32–35,44]. In particular, T^{460} lies within the first short helix and its phosphorylation in AtAMT1.1 could conceivably change the conformation of the C-terminal region, thereby in some way regulating the protein's activity [18,30,32,44]. Though the C-terminal regions of the

PpeAMT proteins are quite varied in length, all of them contain the highly conserved glycine residue (Fig. 6). Moreover, the co-expression of wild-type transporter and its specific mutation in yeast or *Xenopus* oocytes inhibited NH_4^+ transport [34,50]. These observations suggest that in plant AMT proteins, conformational change or coupling between monomers may play a role either in the normal activity of the protein and/or in the regulation of NH_4^+ uptake [18,32,34,50]. Nonetheless, several phosphorylation residues have been detected in this present study, and further studies of phosphorylation *in vivo* in peach are worthy of our attention.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix

Table A1: Locus names of plant AMT proteins used for phylogenetic tree construction

Protein	Locus names	Protein	Locus ID
PtrAMT1;1	Potri.010G063500	PtrAMT1;2	Potri.019G023600
PtrAMT1;3	Potri.008G173800	PtrAMT1;4	Potri.002G255100
PtrAMT1;5	Potri.002G255000	PtrAMT1;6	Potri.009G045200
PtrAMT2;1	Potri.009G045200	PtrAMT2;2	Potri.016G121400
PtrAMT3;1	Potri.001G305400	PtrAMT3;2	Potri.019G000800
PtrAMT4;1	Potri.002G047000	PtrAMT4;2	Potri.018G033500
PtrAMT4;4	Potri.013G040400	AtAMT1;1	At4g13510
AtAMT1;2	At1g64780	AtAMT1;3	At3g24300
AtAMT1;4	At4g28700	AtAMT1;5	At3g24290
AtAMT2;1	At2g38290	LeAMT1;1	P58905
LeAMT1;2	O04161	LeAMT1;3	Q9FVN0
LjAMT1;1	Q9FSH3	LjAMT1;2	Q7Y1B9
LjAMT1;3	Q70KK9	LjAMT2;1	Q93X02
OsAMT1;1	Os04g43070	OsAMT1;2	Os02g40710
OsAMT1;3	Os02g40730	OsAMT2;1	Os05g39240
OsAMT2;2	Os01g61550	OsAMT2;3	Os01g61510
OsAMT3;1	Os01g65000	OsAMT3;2	Os02g34580
OsAMT3;3	Os03g62200	OsAMT4;1	Os12g01420
TaAMT1;1	AAS19466	TaAMT1;2	AAS19467
TaAMT1;3	AAR27052	TaAMT2;1	AAR87397