Genome-Wide Identification and Expression Analysis of AMT and NRT Gene Family in Pecan (Carya illinoinensis) Seedlings Revealed a Preference for NH$_4^+$-N

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Abstract: Nitrogen (N) is a major limiting factor for plant growth and crop production. The use of N fertilizer in forestry production is increasing each year, but the loss is substantial. Mastering the regulatory mechanisms of N uptake and transport is a key way to improve plant nitrogen use efficiency (NUE). However, this has rarely been studied in pecans. In this study, 10 AMT and 69 NRT gene family members were identified and systematically analyzed from the whole pecan genome using a bioinformatics approach, and the expression patterns of AMT and NRT genes and the uptake characteristics of NH$_4^+$ and NO$_3^-$ in pecan were analyzed by aeroponic cultivation at varying NH$_4^+$/NO$_3^-$ ratios (0/0, 0/100, 25/75, 50/50, 75/25, 100/0 as CK, T1, T2, T3, T4, and T5). The results showed that gene duplication was the main reason for the amplification of the AMT and NRT gene families in pecan, both of which experienced purifying selection. Based on qRT-PCR results, CiAMTs were primarily expressed in roots, and CiNRTs were majorly expressed in leaves, which were consistent with the distribution of pecan NH$_4^+$ and NO$_3^-$ concentrations in the organs. The expression levels of CiAMTs and CiNRTs were mainly significantly upregulated under N deficiency and T4 treatment. Meanwhile, T4 treatment significantly increased the NH$_4^+$, NO$_3^-$, and NO$_2^-$ concentrations as well as the Vmax and Km values of NH$_4^+$ and NO$_3^-$ in pecans, and Vmax/Km indicated that pecan seedlings preferred to absorb NH$_4^+$. In summary, considering the single N source of T5, we suggested that the NH$_4^+$/NO$_3^-$ ratio of 75:25 was more beneficial to improve the NUE of pecan, thus increasing pecan yield, which provides a theoretical basis for promoting the scale development of pecan and provides a basis for further identification of the functions of AMT and NRT genes in the N uptake and transport process of pecan.

Keywords: NH$_4^+$; NO$_3^-$; AMT; NRT; pecan

1. Introduction

Ammonium nitrogen (NH$_4^+$) and nitrate nitrogen (NO$_3^-$) are the two main forms of nitrogen (N) that plants can absorb and use. The absorption, transport, and assimilation of NH$_4^+$ and NO$_3^-$ in plants have been studied extensively. Physiologically, NH$_4^+$ and NO$_3^-$ uptake and translocation systems were identified mainly by root absorption kinetics, which were classified into two types: high-affinity transport systems (HATs) and low-affinity transport pathways (LATs) [1]. Vmax (maximum ion uptake rate) and Km (Mie’s constant) are the two main parameters in the kinetic equation for root nutrient uptake, which can quantitatively characterize the plant uptake of nutrient ions. In general, a larger Vmax value indicates that the plant has a great uptake potential for a certain ion, and the number of the ion transport carrier protein on the cell membrane determines the size of Vmax. Km indicates the affinity between the ion absorbed by the root system and the uptake site (transport carrier), and the greater the affinity, the smaller the Km value [2]. Previous
AMTs carry proteins that actively transport NH$_4^+$ across biological cell membranes, and they are divided into three main subfamilies: AMT/MEP/Rh (Ammonium transporter/Methylamine permease/Rhesus protein) [6]. The AMT family can usually be divided into two subfamilies, namely AMT1 and AMT2 [7]. Both AMT1 and AMT2 show high affinity for NH$_4^+$, but members of the AMT1 subfamily play a more important role in high-affinity NH$_4^+$ uptake [8]. AMT2 has a more complex gene structure and protein profile than AMT1 [9]. The AMT1 subfamily of Arabidopsis thaliana has five members, while AMT2 has low homology with the five AMT1 subfamily members and belongs to the MEP subfamily [10]. Expression of AtAMT1;1 in roots is significantly correlated with N uptake, whereas expression of AtAMT1;2 in roots is insensitive to changes in N concentration [11]. AtAMT1;4 mediate the intake of NH$_4^+$ at the pollen plasma membrane [12]. NH$_4^+$ is regulated into the cytoplasm through AtAMT1;1 and AtAMT2;1, which are primarily expressed in leaves [13]. In addition, AtAMT2;2 may play a role in the transport of NH$_4^+$ from the roots to the ground [10]. A study by Couturier et al. on AMT proteins in poplar (Populus L.) found that the expression of PtrAMT1;2 was influenced by intracellular N concentration. Most PtrAMT1s were preferentially expressed in roots, while most PtrAMT2s were majorly expressed in stems, and PtrAMT3;1 was expressed only in senescing leaves [14].

The absorption systems, corresponding genes, and regulatory mechanisms of NO$_3^-$ by plants are different from those of NH$_4^+$. The uptake of NO$_3^-$ by plants is the process of pumping protons out of the cell through the H$^+$/ATPase in the plasma membrane, creating pH and electrical ($\Delta$Ψ) gradients across the plasma membrane that allows the NO$_3^-$ transporter to take up NO$_3^-$ into the cell [15]. Four families of transporters are known to contribute to nitrate uptake and transport in plants: the nitrate transporter protein family [4,5].

N absorption and utilization pattern of plants and the molecular regulation mechanism, important economic tree species introduced for many years in China, pecan still has the economic value of the kernels [30]. However, as an important economic tree species, indigenous to the United States and Mexico [29]. Pecans have a good development prospect in China because of the high content of various nutrients and the important economic value of the kernels [30]. However, as an important economic tree species introduced for many years in China, pecan still has the problem of insufficient yield, and low nitrogen use efficiency (NUE) is an important factor leading to the underproduction of pecan. The key way to improve NUE is to master the N absorption and utilization pattern of plants and the molecular regulation mechanism,
but there is no systematic and in-depth research on pecan in this subject. In this study, we determined the absorption characteristics of NH$_4^+$ and NO$_3^-$ in different N forms. We identified 10 AMT and 69 NRT gene family members in pecan and classified them into different evolutionary subfamilies. Then, we analyzed their gene structure, replication events and expression patterns to explore their functions in varying N forms, and we provide a theoretical basis for improving the NUE of pecan.

2. Results

2.1. Identification and Sequence Analysis of AMT and NRT Gene Family Members in Pecan

A total of 11 AMT candidates were identified that contained AMT or AMT-like repeats, and 95 NRT candidates were identified that contained NRT or NRT-like repeats. After validation of AMT and NRT structural domains by Pfam and NCBI CD-search, 10 were identified as AMT gene family members, and 69 were identified as NRT gene family members. The AMT and NRT genes were renamed according to the Arabidopsis gene names as well as the NCBI blastp results for subsequent analysis. Table S3 provided details of AMTs and NRTs.

Sequence analysis of pecan AMT and NRT gene family members revealed that the number of exons in AMTs ranged from one to five, and in NRTs from two to nine. The CDS length of AMTs ranged from 471 to 1542 bp, and in NRTs from 1053 to 2826 bp. Most AMTs (9/10) and NRTs (56/69) were stable proteins with a low protein instability index (instability index < 40). GRAVY analysis showed that the hydration of AMT and NRT proteins in pecan was greater than 0, indicating that these proteins are hydrophobic. Subcellular localization predictions showed that most AMTs (9/10) localized to the cell membrane and most NRTs (64/69) localized to the vesicles.

2.2. Phylogenetic Analysis of AMTs and NRTs in Different Species

To decipher the evolutionary relationships and functional associations of pecan AMTs and NRTs, phylogenetic trees were constructed using pecan AMT and NRT proteins and other plants, respectively (Figures 1 and 2). According to the phylogenetic trees, all AMT proteins were divided into two distinct evolutionary branches: AMT1 and AMT2 with strong support (Bootstrap = 100%). The AMT2 was further divided into three clusters: AMT2a, AMT2b, and AMT2c. AMT1 was the largest branch and included 34 AMTs (four CiAMTs), AMT2a included two CiAMTs, AMT2b included three CiAMTs, and AMT2c had no pecan AMT gene family members.

All NRT proteins were divided into three main clades: NRT1/PTR, NRT2, and NRT3 subfamily. The NRT1 formed four subclasses, named NRT1a, NRT1b, NRT1c, and NRT1d, and included 62 CiNRTs and 52 AtNRTs. NRT2 included five CiNRTs and seven AtNRTs, while NRT3 included two CiNRTs and two AtNRTs. The evolutionary tree had 48 sister pairs, the majority of which were paralogous proteins, 38 pairs in total (21 pairs in pecan and 17 pairs in Arabidopsis), and 10 pairs of orthologous proteins. Only the NRT3 subfamily had no orthologous proteins, while all other clades contained orthologous and paralogous proteins.
characteristic motifs associated with members of the AMT gene family. Only 2–3 motifs in NRT2 were identical to the NRT1 subfamily, while NRT3 had no identical motifs to the NRT1 subfamily. We analyzed the structural domains of pecan AMT and NRT proteins using Pfam search and found that only one conserved Ammonium_trasp domain existed in all pecan AMT proteins, and they were all located at similar positions, while three conserved structural domains existed in pecan NRT proteins (Figures 3C and 4C, Table S4).

The NRT1 subfamily had two conserved structural domains: PTR2 and MSF_1; NRT2 had only the MSF_1 structural domain; NRT3 contained only the NAR2 structural domain. In addition, we analyzed the exons/introns of the pecan AMT and NRT genes to study the structural diversity. The results showed that the AMT1s contained no intron, and the AMT2s contained 2–4 introns (Figure 3D); the NRT1s had 2–9 introns, the NRT2s and NRT3s contained 1–2 introns (Figure 4D). In conclusion, the phylogenetic correlation between gene structure and prediction strongly supports a close evolutionary relationship between paired genes within the same subfamily.

Figure 1. Phylogenetic analysis of AMT gene family in pecan (Carya illinoinensis), poplar (Populus trichocarpa), apple (Malus domestica), peach (Amygdalus persica L.), tomato (Lycopersicon esculentum Miller), rice (Oryza sativa L.) and Arabidopsis (Arabidopsis italiana). AMT proteins from seven species were divided into two subfamilies (AMT1 and AMT2). The AMT2 was further divided into three clusters (AMT2a, AMT2b, and AMT2c).

2.3. Phylogenetic Tree, Conserved Motif, Conserved Domain, and Gene Structural Analyses of CiAMTs and CiNRTs

To better understand the evolutionary relationships and functional associations of pecan AMT and NRT genes, we constructed unrooted phylogenetic trees with pecan AMT and NRT proteins, respectively (Figures 3A and 4A). According to MEME analysis, we found 10 motifs in most of the pecan AMT and NRT proteins (Figures 3B and 4B, Table S5). Motifs 1 to 7 were found in all AMT subfamilies, suggesting that these motifs may be characteristic motifs associated with members of the AMT gene family. Only 2–3 motifs in NRT2 were identical to the NRT1 subfamily, while NRT3 had no identical motifs to the NRT1 subfamily. We analyzed the structural domains of pecan AMT and NRT proteins using Pfam search and found that only one conserved Ammonium_trasp domain existed in all pecan AMT proteins, and they were all located at similar positions, while three conserved structural domains existed in pecan NRT proteins (Figures 3C and 4C, Table S4).
The NRT1 subfamily had two conserved structural domains: PTR2 and MSF_1; NRT2 had only the MSF_1 structural domain; NRT3 contained only the NAR2 structural domain.

Figure 2. Phylogenetic analysis of NRT gene family in pecan (Carya illinoinensis) and Arabidopsis (Arabidopsis thaliana). NRT proteins from two species were divided into three subfamilies (NRT1, NRT2 and NRT3). The NRT1 was further divided into four clusters (NRT1a, NRT1b, NRT1c, and NRT1d).
In addition, we analyzed the exons/introns of the pecan AMT and NRT genes to study the structural diversity. The results showed that the AMT1s contained no intron, and the AMT2s contained 2–4 introns (Figure 3D); the NRT1s had 2–9 introns, the NRT2s and NRT3s contained 1–2 introns (Figure 4D). In conclusion, the phylogenetic correlation between gene structure and prediction strongly supports a close evolutionary relationship between paired genes within the same subfamily.

2.4. Synteny Analysis of CiAMTs and CiNRTs

To determine the replication events of pecan AMT and NRT genes, we performed a synteny analysis of the pecan genome. The results showed that there were five duplicated gene pairs among the ten members of the pecan AMT gene family, two of which originated from tandem duplication and three from segmental duplication (Figure S1). There are 103 duplicated gene pairs among 69 members of the pecan NRT gene family, of which 9 originated from tandem duplication and 94 from segmental duplication. This suggested that fragment replication events played an important role in the expansion of the AMT and NRT gene families in pecan.

To examine the selection type of duplicate gene pairs in the pecan AMT and NRT gene families, the Ka/Ks ratios were analyzed for duplication events (Table S6). Ka/Ks < 1 means the gene is subjected to purifying selection, Ka/Ks > 1 means the gene underwent positive selection, and Ka/Ks = 1 means neutral evolution. The Ka/Ks values of all duplicate gene pairs were less than 1, indicating that the amplification of the pecan AMT and NRT genes was mainly influenced by purifying selection.
Figure 4. Phylogenetic analysis, conserved motif, conserved domain and gene structural of the NRT genes in pecan. Phylogenetic analysis of the NRT genes in pecan (A). The conserved motifs of the NRT genes in pecan (B). The conserved domain of the NRT genes in pecan (C). The gene structure of the NRT genes in pecan (D).
2.5. Effect of N Forms on Quantitative qRT-PCR Analysis of AMT and NRT Gene Expression Levels in Pecan

The results of qRT-PCR analysis of CiAMTs showed that the relative expression levels of CiAMTs were significantly affected by different N forms (Figure 5). The relative expression levels of almost all CiAMTs were higher in roots than in leaves, indicating that CiAMTs mainly worked in roots. In the roots, CiAMT1.1 was significantly upregulated under T3 and T4 ($p < 0.05$), CiAMT1.3a was significantly upregulated only under T5 ($p < 0.05$), and CiAMT1.3b, CiAMT1.4 and CiAMT3.1a were all significantly upregulated only under T4 ($p < 0.05$). CiAMT2.1 was significantly upregulated under T1, T3, and T4 ($p < 0.05$), CiAMT2.2 was significantly upregulated under T4 and T5 ($p < 0.05$), and CiAMT3.1b was significantly upregulated under T1, T2, and T3 ($p < 0.05$). The relative expression of CiAMT3.3 was significantly upregulated under all N form treatments, with the most significant in T4 and T5 ($p < 0.05$). In leaves, all of them showed significant downregulated except CiAMT2.1, CiAMT2.2, CiAMT3.1b, and CiAMT3.3, which were significantly upregulated under individual treatments ($p < 0.05$).

Figure 5. Relative expression of pecan CiAMT genes under varying NH$_4^+$:NO$_3^-$ ratios. The expression levels of CiAMT in pecan leaves and roots after varying NH$_4^+$:NO$_3^-$ ratio treatments were quantified by qRT-PCR, with Actin as the reference gene. Different capital letters indicate significant differences in leaves ($p < 0.05$), and different lowercase letters indicate significant differences in roots ($p < 0.05$).
Based on the results of previous studies [16], some members of the Arabidopsis and rice NRT gene families have been shown to be associated with NO$_3^-$ uptake and transport. According to the phylogenetic tree, we selected 16 pecan CiPawNRTs corresponding to them and further analyzed them using qRT-PCR (Figure 6). The qRT-PCR results showed that CiNRTs showed different expression patterns in different N forms as well as in different pecan organs. Except for CiNPFP2.13, CiNPFP4.6, CiNPFP5.5, CiNPFP6.3a, CiNPFP6.4, CiNRT2.5, and CiNRT2.7, which showed relative higher expression in leaves than in roots under most N form treatments, most of the other CiNRTs showed higher expression in roots than in leaves. In leaves, CiNPFP2.4 and CiNPFP2.13 under T1, CiNPFP5.5 under T1 and T4, CiNPFP7.2 under T3, CiNRT2.7 under T1, and CiNPFP2.7b, CiNPFP4.6, CiNPFP5.9a, CiNPFP5.10b under T5 showed significantly upregulated expression ($p < 0.05$). In roots, CiNPFP1.2a was significantly upregulated under T3 and T4 ($p < 0.05$), and CiNPFP2.7b was significantly upregulated under T2 and T4 ($p < 0.05$). CiNPFP2.4 and CiNPFP2.11b were significantly upregulated only with T3 ($p < 0.05$), while CiNPFP5.10b was significantly upregulated only with T4 ($p < 0.05$). CiNPFP2.13, CiNPFP6.4, and CiNRT2.7 were significantly downregulated under each N form treatment ($p < 0.05$), CiNPFP4.6 was significantly downregulated under T1, T2, and T5 ($p < 0.05$), while CiNPFP7.3a was significantly downregulated under T1, T2, T3, and T5 ($p < 0.05$). CiNPFP5.5 was significantly upregulated under T2 as well as significantly downregulated under T3 ($p < 0.05$), CiNPFP6.2 was significantly upregulated under T2 as well as significantly downregulated under T1, T3, and T5 ($p < 0.05$), and CiNPFP6.3a was significantly upregulated under T4 as well as significantly downregulated under T1, T2, and T3 ($p < 0.05$). CiNPFP7.2 was significantly upregulated under T3 and T4 and downregulated under T5 ($p < 0.05$). CiNRT2.5 was significantly upregulated under T2 and T3 and downregulated under the other treatments ($p < 0.05$).

2.6. Effect of N Forms on NH$_4^+$, NO$_3^-$ and NO$_2^-$ Concentration in Pecan

To further investigate the response mechanisms of pecan AMT and NRT genes to N forms, we measured the concentrations of NH$_4^+$, NO$_3^-$, and NO$_2^-$ in pecan under different N forms (Figure 7). The results showed that there was no significant difference in the NH$_4^+$ concentrations of pecan in all organs under different N forms. Except for the T5 treatment, NH$_4^+$ concentrations under all other treatments showed greater in roots than in leaves and stem ($p < 0.05$) and no significant difference between leaves and stems. The variability of NO$_3^-$ concentrations in each organ of pecan varied among treatments, and T5 showed significantly greater than CK and T3 in leaves ($p < 0.05$), and no significant differences were found between T1, T2, T4, and other treatments. In the stems, no significant differences were found between treatments. In the roots, it showed that T4 was significantly greater than CK ($p < 0.05$), and T4 was not significantly different from the other treatments. Except for T3 and T5 treatment, the NO$_3^-$ concentrations of pecan under all other treatments showed greater leaves than roots ($p < 0.05$) and no significant difference between stems, leaves, and roots. There was no significant difference in NO$_2^-$ concentrations in all organs of pecan under different treatments, while the variability of NO$_2^-$ concentrations in different organs under each treatment varied. CK, T1, and T5 showed no significant difference between stems and roots, and both of them were significantly greater than leaves ($p < 0.05$), T2 and T3 showed no significant difference between leaves and roots, and both of them were significantly greater than stems ($p < 0.05$), while T4 showed no significant variation in different organs.
Figure 6. Relative expression of selected pecan CiNRT genes under varying NH$_4^+$:NO$_3^-$ ratios. The expression levels of selected CiNRT in pecan leaves and roots after varying NH$_4^+$:NO$_3^-$ ratio treatments were quantified by qRT-PCR, with Actin as the reference gene. Different capital letters indicate significant differences in leaves (p < 0.05), and different lowercase letters indicate significant differences in roots (p < 0.05).

At the mean level, pecan NH$_4^+$ concentrations were significantly greater in the T4 than in the other treatments (p < 0.05), with no significant differences between the other treatments. The pecan NO$_3^-$ concentrations revealed no significant difference between T1, T4, and T5, and both of them were significantly greater than CK, T2, and T3 (p < 0.05). There was no significant difference between T2 and T1, T3 and T4, T2 was significantly greater than CK (p < 0.05), and no significant difference between CK and T3. The variability of pecan NO$_2^-$ and NH$_4^+$ concentrations was consistent, suggesting that T4 was significantly greater than the other treatments (p < 0.05), with no significant differences between the other treatments.
Figure 7. Differences of NH$_4^+$, NO$_3^-$, and NO$_2^-$ concentrations of pecan under varying NH$_4^+$:NO$_3^-$ ratios. Differences of NH$_4^+$ concentrations in pecan leaves, stems, and roots under varying NH$_4^+$:NO$_3^-$ ratios (A). The mean values of NH$_4^+$ concentrations in pecan leaves, stems, and roots under varying NH$_4^+$:NO$_3^-$ ratios (B). Differences of NO$_3^-$ concentrations in pecan leaves, stems, and roots under varying NH$_4^+$:NO$_3^-$ ratios (C). The mean values of NO$_3^-$ concentrations in pecan leaves, stems, and roots under varying NH$_4^+$:NO$_3^-$ ratios (D). Differences of NO$_2^-$ concentrations in pecan leaves, stems, and roots under varying NH$_4^+$:NO$_3^-$ ratios (E). The mean values of NO$_2^-$ concentrations in pecan leaves, stems, and roots under varying NH$_4^+$:NO$_3^-$ ratios (F). Upper capital letters indicate significant differences between organs (p < 0.05), and lowercase letters indicate significant differences between varying NH$_4^+$:NO$_3^-$ ratios (p < 0.05).

2.7. Effect of N Forms on the Uptake Kinetics of NH$_4^+$ and NO$_3^-$ in Pecan

We also determined the kinetic properties of pecan NH$_4^+$ and NO$_3^-$ uptake under different N forms (Figure 8). The results showed that the uptake rates of NH$_4^+$ and NO$_3^-$...
under T2, T3, and T4 were still in a significant upward trend at the ion concentration of 2000 μmol·L⁻¹; the uptake rates of NH₄⁺ and NO₃⁻ under CK, T1, and T5 leveled off at the medium ion concentration of 1000 μmol·L⁻¹.

The root uptake rates were processed data according to the Hofstee transformation equation to obtain the maximum uptake rate (Vₘₐₓ) and the Mee’s constant (Kₘ) of pecan for different N forms with significant coefficients of determination R² (Table 1). Different N forms showed significant effects on Vₘₐₓ and Kₘ of NH₄⁺ and NO₃⁻ (p < 0.05), both showing T₄ > T₃ > T₂ > CK > T₁ > T₅. This indicates that the involvement of a certain proportion of NH₄⁺ accelerated the uptake of NH₄⁺ and NO₃⁻ by pecans, but the affinity decreased, while NH₄⁺ above a certain proportion decreased the uptake rate and increased the affinity. Except for the T₁ treatment, the Vₘₐₓ/Kₘ of NH₄⁺ was greater than that of NO₃⁻ under all treatments, indicating that the rate of NH₄⁺ uptake by pecan was greater than that of NO₃⁻.

Table 1. N uptake kinetics of pecan roots under varying NH₄⁺:NO₃⁻ ratios. Lowercase letters indicate significant differences between varying NH₄⁺:NO₃⁻ ratios (p < 0.05).

| Treatment | NH₄⁺-N | NO₃⁻-N |
|-----------|--------|--------|
|           | Vₘₐₓ/ (μmol·g⁻¹·h⁻¹) | Kₘ/ (mmol·L⁻¹) | Vₘₐₓ/Kₘ | Vₘₐₓ/ (μmol·g⁻¹·h⁻¹) | Kₘ/ (mmol·L⁻¹) | Vₘₐₓ/Kₘ |
| CK        | 3.00 ± 0.21 d | 3.40 ± 0.07 d | 0.89 | 0.975 | 3.30 ± 0.01 c | 4.18 ± 0.47 c | 0.81 | 0.996 |
| T1        | 2.11 ± 0.00 d | 1.85 ± 0.18 e | 1.17 | 0.977 | 1.90 ± 0.02 d | 1.62 ± 0.14 d | 1.19 | 0.994 |
| T2        | 6.96 ± 0.42 c | 7.89 ± 0.21 c | 0.88 | 0.966 | 7.33 ± 0.52 b | 8.65 ± 0.18 b | 0.85 | 0.979 |
| T3        | 9.52 ± 0.93 b | 10.80 ± 0.43 b | 0.88 | 0.911 | 9.05 ± 0.78 a | 10.43 ± 0.36 a | 0.86 | 0.954 |
| T4        | 11.13 ± 0.93 a | 12.01 ± 0.39 a | 0.92 | 0.942 | 10.10 ± 0.55 a | 11.10 ± 0.35 a | 0.91 | 0.957 |
| T5        | 1.68 ± 0.02 d | 1.72 ± 0.10 e | 0.98 | 0.978 | 1.60 ± 0.01 d | 1.68 ± 0.12 d | 0.97 | 0.995 |

3. Discussion

3.1. Functional Differentiation of AMT and NRT Gene Family in Pecan Genome

The biological functions of AMT and NRT gene families in pecan are poorly understood. Therefore, we report for an earlier time identification of 10 AMT and 69 NRT gene family members from pecan, confirming that direct orthologs of AMT and NRT proteins should be highly conserved evolutionarily throughout the plant.

Studies on Arabidopsis AMT proteins have shown that AtAMTs had a prominent role in NH₄⁺ assimilation at the cell membranes [10,11,31], and studies of flowering Chinese
cabbage (*Brassica campestris*) also indicated that BeAMT2 was located on the plasma membrane [32], which was consistent with the predicted results of subcellular localization in this study (Table S3), and this is the optimal cellular structure for maintaining stable $\text{NH}_4^+$ concentrations in plants. AtNPF5.11, AtNPF5.12, and AtNPF5.16 were identified to function in the process of NO$_3^-$ from the vesicle to the cytoplasm, thereby regulating NO$_3^-$ distribution between roots and shoots [33]. In contrast, the predicted subcellular localization of the pecan NRT genes in this study showed that most of the NRT1s were localized to the vacuoles (Table S3), suggesting that pecan NRT1s may mostly act in the transport of NO$_3^-$ between the vacuoles and the cytoplasm. There were also some NRT1s localized on the cell membrane, while all NRT2s were all localized to the cell membrane, suggesting that this fraction of NRT proteins may mediate assimilation and efflux of NO$_3^-$ in plants as well as inter-subcellular transport, which was consistent with the findings of cassava (*Manihot esculenta*) [34]. According to the predicted results of subcellular localization, the subcellular localization of all NRT3s differed significantly from NRT1 and NRT2 in pecan, with CiNRT3.1 localized to the nucleus and CiNRT3.2 localized on the cell membrane, cell wall, chloroplast, and vacuoles (Table S3), suggesting that the pecan NRT3s had more complex structures and functions.

3.2. Effect of N Forms on the Absorption Characteristics of $\text{NH}_4^+$ and NO$_3^-$ in Pecan

$\text{NH}_4^+$ and NO$_3^-$ are the two main forms of N absorbed and utilized by plants, and previous studies have shown that a nutrient mixture of $\text{NH}_4^+$ and NO$_3^-$ can improve crop yield and quality compared to a single source of N [35]. Therefore, understanding the best $\text{NH}_4^+:\text{NO}_3^-$ ratios provides the possibility to improve the N utilization efficiency of plants. We investigated the effect of varying $\text{NH}_4^+:\text{NO}_3^-$ ratios on the absorption and transport of N by measuring the concentrations of different N forms in the pecan organs (Figure 7).

This study showed that the concentrations of different N forms in pecan were tissue-specific. The $\text{NH}_4^+$ concentrations of pecan were significantly higher in roots than in leaves and stems, while NO$_3^-$ and NO$_2^-$ concentrations were significantly higher in leaves than in stems and roots, which was consistent with the finding that $\text{NH}_4^+$ was majorly assimilated in roots and NO$_3^-$ was mostly translocated to leaves for storage or assimilation [36]. T4 significantly increased the total $\text{NH}_4^+$ concentrations of pecan, primarily in the roots, suggesting that T4 was more favorable to promote $\text{NH}_4^+$ absorbed by pecan roots, but had no effect on $\text{NH}_4^+$ transport between organs. All $\text{NH}_4^+:\text{NO}_3^-$ ratio treatments increased the total NO$_3^-$ concentrations, primarily in the leaves, indicating that the feeding of $\text{NH}_4^+$ and NO$_3^-$ mainly promoted the translocation of pecan NO$_3^-$ from roots to leaves, and possibly the acclimation of NO$_3^-$ in the leaves. Compared to T1, T3 reduced the total pecan NO$_3^-$ concentrations, which was in agreement with the results of studies in pepper (*Capsicum annuum*) [37]. T4 significantly increased the total NO$_2^-$ concentrations of pecan, indicating that T4 promoted the conversion of NO$_3^-$ to NO$_2^-$. 

Previous conclusions on the effects of the simultaneous presence of $\text{NH}_4^+$ and NO$_3^-$ on each other’s uptake were varied, with some suggesting that they have a facilitative or inhibitory effect [38,39], and that plant absorption of $\text{NH}_4^+$ and NO$_3^-$ is limited by the maximum uptake threshold [40]. In this study, the uptake rates of $\text{NH}_4^+$ and NO$_3^-$ gradually saturated with increasing substrate concentrations under a single N source, while they remained on an increasing trend under mixed N source treatments, with the T4 treatment being the most obvious, indicating that $\text{NH}_4^+$ and NO$_3^-$ promoted each other’s intake in this study, as evidenced by the magnitude of Vmax values of pecan. However, single N source treatment increased the affinity of $\text{NH}_4^+$ and NO$_3^-$, and the combined application of $\text{NH}_4^+$ and NO$_3^-$ decreased their affinity instead, which was generally consistent with the results of Kamminga-Van Wijk and Prins [41]. Vmax/Km is also commonly used to indicate plant preference for $\text{NH}_4^+$ and NO$_3^-$ uptake [42], suggesting that pecans may be more biased toward $\text{NH}_4^+$ absorption.
3.3. Effect of N Forms on the Expression of CiAMTs and CiNRTs

The ingestion and transport of NH$_4^+$ and NO$_3^-$ in plants is majorly mediated by AMT and NRT gene family members. Therefore, we investigated the expression modes of these genes in varying organs and N forms to further understand the effect of N forms on N uptake and transport in pecan.

In the present study, the relative expression levels of almost all CiAMTs were higher in roots than in leaves, indicating that the addition of NH$_4^+$ and NO$_3^-$ majorly stimulated the expression of CiAMTs in roots. Studies have shown that AtAMT1.1, AtAMT1.2, AtAMT1.3, and AtAMT1.5 are the principal transporter proteins that take up high-affinity NH$_4^+$ into Arabidopsis roots, with AtAMT1.1 and AtAMT1.3 responsible for approximately two-thirds of the high-affinity NH$_4^+$ uptake capacity in the root; the CiAMTs may have the same function [31,43]. The results of this study showed that all CiAMT1s were upregulated in roots of T4, except for CiAMT1.3a, which was upregulated in roots under T5, suggesting that T4 may have improved the absorption of high-affinity NH$_4^+$ by the pecan root. In this study, CiAMT1s were mostly upregulated in leaves under N lack, but numerous studies have shown that expression levels of AMT1s were upregulated in roots under N limited conditions [14,43,44]. This may be due to the variation caused by the longer duration of N deficiency in this study, or it is possible that the expression of CiAMT1s in leaves was not only affected by N lack, but may also be involved in some other regulatory mechanisms [45]. Previous studies have shown that peach (Prunus persica) PpeAMT3.4 was primarily expressed in roots [46], and the expression pattern of CiAMT2s was similar to that of CiAMTs, being expressed mostly in roots and almost significantly upregulated at all timepoints under T4.

Studies on Arabidopsis suggested that AtNPF6.3/AtNRT1.1 was not only an amphiphilic NO$_3^-$ transport protein but might also act as a NO$_3^-$ sensor under low NO$_3^-$ conditions [47]. The expression level of CiNPF6.3a was significantly upregulated in roots under T4, suggesting that it may carry both translocation and signaling functions at this time. AtNPF4.6/AtNRT1.2 was not only a low-affinity NO$_3^-$ transporter protein that plays a role in NO$_3^-$ influx [21], but also an abscisic acid (ABA) transporter protein that positively regulated the ABA response [48]. CiNPF4.6 expression was significantly upregulated in roots under T3 and in leaves under T5, perhaps because CiNPF4.6 worked primarily on NO$_3^-$ influx under T3, while it worked mostly on ABA regulation under T5. MtNPF6.8/MtNRT1.3 of Medicago truncatula was an amphiphilic NO$_3^-$ transport protein and was upregulated by the absence of NO$_3^-$ [49]. This was the same as the results of our study, where CiNPF6.4/CiNRT1.3 expression was significantly upregulated in roots under CK and in leaves under T5. AtNPF7.3/AtNRT1.5 mediated root-to-stem transport, and the same conclusion was found for ZnNPF7.3/ZnNRT1.5 in the Zygophyllum xanthoxylum, which also contributed to the uptake of NO$_3^-$ [50]. The expression levels of CiNPF7.3a/CiNRT1.3a were significantly upregulated in roots, leaves under CK, and roots under T4, indicating that both N scarcity and T4 may promote NO$_3^-$ transport from roots to stems. Moreover, AtNPF7.2/AtNRT1.8 was phylogenetically similar to AtNPF7.3 [51], while CiNPF7.2/CiNRT1.8 was also significantly upregulated in roots under T4 and may function similarly to CiNPF7.3. NPF2.13/NRT1.7 and NPF1.2/NRT1.11 were proven to be involved in the transfer and redistribution of NO$_3^-$ from xylem or the NO$_3^-$ containing tissues to the phloem [23], CiNPF2.13 was largely induced by N limitation and T3, while CiNPF1.2a was mainly induced by N limitation, and T4. NPF2.11/NRT1.10 was identified to be involved in thioglucoside transport [52], and CiNPF2.11 expression was significantly upregulated under CK, suggesting that CiNPF2.11 may resist N deficiency stress by regulating the concentrations of thioglucosides. AtNRT2.5 was a plasma-membrane localized high-affinity NO$_3^-$ transporter protein that mediated NO$_3^-$ acquisition, and reactivation under N deficient conditions [27], and CiNRT2.5 in this study exhibited the same expression pattern. The transcript levels of NRT2.7 in Fraxinus mandshurica were both upregulated in leaves due to N limitation [53], whereas CiNRT2.7 in this study was significantly expressed in roots under CK and in leaves under T1, which may be caused by species differences.
4. Materials and Methods

4.1. Plant Materials and Experimental Design

In the study, aeroponic cultivation trials were carried out in the greenhouse of the campus of Nanjing Forestry University from 18 April to 9 June 2021 and from 4 May to 21 June 2022. The plant materials and experimental design refer to Chen et al. [29]. In the case of the same N supply, the five ammonia-to-nitrate ratios (NH$_4^+$:NO$_3^-$) were 100:0, 75:25, 50:50, 25:75, and 0:100, corresponding to T1, T2, T3, T4, and T5, respectively. The nutrient solution without N was used as the control (CK). Each treatment was repeated 3 times, each with 6 seedlings. Regulation of the NH$_4^+$:NO$_3^-$ ratios for each treatment was achieved with specific source compounds (Table S1). Samples were taken after 45 days of treatment for further determination.

4.2. Identification of AMT and NRT Genes in Pecan

To identify the pecan AMT and NRT genes, we obtained all of the protein sequences of pecan from the Phytozome v13 database (https://phytozome-next.jgi.doe.gov/info/CillinoensisPawnee_v1_1 (accessed on 1 November 2021)). The hidden Markov model (HMM) profiles of the AMT domain (PF00909) and NRT domain (PF07690, PF00854, PF16974), downloaded from the Pfam database (http://pfam.xfam.org/ (accessed on 1 February 2022)) [54], were used to identify the pecan AMT and NRT proteins by using HMM search through HMMER3.0 program (www.hmmer.org (accessed on 1 February 2022)) with default parameters [55]. As multiple AMT and NRT proteins were corresponding to a specific gene in several cases, only one protein sequence corresponding to each gene was retained for further detailed analysis. The Pfam database was used to confirm the presence of these conserved domains of the screened genes. The biophysical properties such as amino acid length (AA), molecular weights (MWs), theoretical isoelectric points (pIs), and grand average of hydration (GRAVY) of pecan AMT and NRT proteins were estimated by ExPASy ProtParam server (http://web.expasy.org/protparam (accessed on 1 February 2022)) [56]. Transmembrane helices (TMHs) were determined using the TMHMM tools (http://www.cbs.dtu.dk/services/TMHMM-2.0/ (accessed on 1 February 2022)), prediction of subcellular localization information for pecan AMT and NRT proteins using the Cell-PLoc 2.0 software (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/ (accessed on 1 February 2022)) [57].

4.3. Phylogenetic Analysis

The Arabidopsis AMT and NRT protein sequences were downloaded from the TAIR database (https://www.arabidopsis.org/ (accessed on 1 November 2021)) [58]. The poplar, apple, pear, tomato, and rice AMT protein sequences were downloaded from the Phytozome database. Phylogenetic trees were constructed by MEGA 7.0 with the following settings: the neighbor-joining (NJ) method, 1000 bootstrap replicates, the Jones–Taylor–Thornton (JTT) model, and pairwise deletion [59]. The evolutionary tree was visualized using the online tool Evolview (http://evolgenius.info/ (accessed on 1 March 2022)) [60]. Because CiAMT3.1c is a partial sequence, it was not included in the phylogenetic tree.

4.4. Analysis of Gene Structure, Conservative Motifs, and Domains

The pecan genome sequence files and the General Feature Format (GFF) annotation files were downloaded from the Phytozome database. We used the online MEME tool (http://MEME-suite.org/ (accessed on 1 April 2022)) for topic prediction, keeping the maximum number of topics at 10 [61]. TBtools visualizes AMT and NRT gene structures, conservative motifs, and domains [62].

4.5. Synteny Analysis

For detecting syntenic blocks, the whole genome sequence file and the GFF annotation file of pecan were used to identify all duplication events in the pecan genome using
MCScanX software [63]. Then, the AMT and NRT gene families were analyzed for synteny and visualized using TBtools.

4.6. Estimation of the Ka/Ks Values

Multiple sequence alignment of full-length coding sequences (CDS) in the AMT and NRT gene families in pecan was performed using MEGA 7 software and further used to calculate nonsynonymous (Ka) and synonymous (Ks), and the Ka/Ks ratios. Ks values were commonly used to determine the time since gene duplication, and the selection pressure of duplication event was determined by the Ka/Ks ratio.

4.7. Cis-Regulatory Elements Analysis

To investigate potential cis-regulatory elements in the promoters of the pecan AMT and NRT genes, a 1000 bp region upstream of the AMT and NRT genes was retrieved from the pecan genome sequences. Then, the cis-regulatory elements were predicted using PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 1 April 2022)) [64] and screened and visualized by TBtools software (Figures S2 and S3).

4.8. Protein–Protein Interaction Network Prediction

Protein–protein interaction networks of AMT and NRT gene families were analyzed by String (https://string-db.org/ (accessed on 1 April 2022)) [65].

4.9. RNA Collection and qRT-PCR Expression Analysis

According to the manufacturer’s protocol, the total RNA was extracted from the leaves and roots of pecan using a Universal Plant Total RNA Extraction Kit (Bioteke, Beijing, China) and stored at −80°C until further use. The purity and integrity of the isolated total RNA were analyzed by agarose gel electrophoresis and Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). First-strand cDNA was synthesized using a cDNA Synthesis Kit (HiScript® RIII RT SuperMix for qPCR +gDNA wiper, Vazyme, Nanjing, China). The qRT-PCR was performed on a 7500 Real-Time PCR system (Applied Biosystems™, Foster City, CA, USA) using a Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The specific primers were synthesized by Tsingke Biotechnology Ltd. (Nanjing, China), and the details of the primers were provided in Supplementary Table S2. The PCR parameters applied here were as follows: 95°C for 30 s, followed by 40 cycles of 5 s at 95 and 30 s at 60°C. The Actin gene was used as an internal reference gene [66], and the relative expression levels of pecan AMT and NRT genes were determined using the 2^−ΔΔCt method [67]. Values represent mean calculated from three biological replicates and three technological repeats.

4.10. Measurement of NH₄⁺, NO₃⁻ and NO₂⁻ Concentration in Pecan

The NH₄⁺ concentrations of pecan roots, stems and leaves were determined according to the Berthelot reaction [68]. NO₃⁻ concentrations were determined according to the method suggested by Patterson et al. [69]. The NO₂⁻ concentrations were determined by the method of Ogawa et al. [70].

4.11. Kinetic Characterization of NH₄⁺ and NO₃⁻ Uptake in Pecan

The kinetic characteristics of NH₄⁺ and NO₃⁻ uptake in pecan seedlings were determined by the conventional depletion method, the ion concentrations in the solutions to be measured were determined after preparing different concentrations of NH₄⁺ and NO₃⁻ for 24 h incubation of the plants, the net rate of ion uptake per unit fresh root per unit time was calculated, and the kinetic parameters of uptake were mathematically derived. The concentration of NH₄⁺ and NO₃⁻ was determined by referring to 2.10.
4.12. Data Analysis

Before analysis of variance (ANOVA), data were checked for normality and homogeneity of variances. One-way ANOVA was performed to test the effects of different N forms on NH$_4^+$, NO$_3^-$, NO$_2^-$ concentrations, absorption kinetic characteristics and relative gene expression of pecan seedlings. Differences were considered significant at $p < 0.05$.

The kinetic parameters of NH$_4^+$ and NO$_3^-$ uptake were calculated using the Hofstee transformation of the Michaelis–Menten kinetic equation: $V = C \times \frac{V_{\text{max}}}{(K_m + C)}$, where $C$ represents the ion concentration, $V$ indicates the net ion uptake rate, $V_{\text{max}}$ indicates the maximum uptake rate, and the $K_m$ value represents the root uptake site for ion affinity. Non-linear regression fitting and graphing were performed using SPSS to obtain the $V_{\text{max}}$ and $K_m$. The $\alpha$ value represents the competitive ability of the plant root system for nutrient uptake, $\alpha = \frac{V_{\text{max}}}{K_m}$.

All statistical analyses were performed with SPSS 23.0 software (Version 23.0, Chicago, IL, USA). All charts were drawn with Excel (Version 2019, Redmond, WA, USA) and SigmaPlot (Version 14.0, Barcelona, Spain).

5. Conclusions

We identified 10 AMT and 69 NRT genes in the pecan genome, and the analysis showed that the biophysical properties, gene structure, and expression levels of CiAMTs and CiNRTs were strongly associated with pecan NH$_4^+$ and NO$_3^-$ uptake. Combining the effects of different N form treatments on the expression levels of CiAMTs and CiNRTs, the N concentrations of pecan, and the uptake rate of NH$_4^+$ and NO$_3^-$, we concluded that pecan preferred NH$_4^+$ and that the NH$_4^+$/NO$_3^-$ ratio of 75:25 was more favorable to improve the N uptake capacity of pecan seedlings. This study provides a basis for further identification of the functions of AMT and NRT genes in N uptake and transport of pecan, and it provides a theoretical basis for the application of an optimal proportion of N fertilizer to improve NUE, thereby increasing pecan yield and promoting pecan industrialization.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232113314/s1.

Author Contributions: F.P. conceived and designed the study. M.C. collected experimental data, analyzed, and wrote the manuscript. M.C. and J.L. participated in the collection of samples. M.C. and J.X. performed the experiments. P.T. and K.Z. provided help in data analysis and in improving the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a grant from the National Key R&D Program of China (2021YFD1000403) and the Central Financial Forestry Science and Technology Extension Demonstration Funds Program (Su (2022) TG04).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nacry, P.; Bouguyon, E.; Gojon, A. Nitrogen Acquisition by Roots: Physiological and Developmental Mechanisms Ensuring Plant Adaptation to a Fluctuating Resource. *Plant Soil* 2013, 370, 1–29. [CrossRef]
2. Van Tichelen, K.K.; Colpaert, J.V. Kinetics of Phosphate Absorption by Mycorrhizal and Non-Mycorrhizal Scots Pine Seedlings. *Physiol. Plant.* 2000, 110, 96–103. [CrossRef]
3. Wang, L.; Macko, S.A. Constrained Preferences in Nitrogen Uptake across Plant Species and Environments: Plant Nitrogen Preference. *Plant Cell Environ.* 2011, 34, 525–534. [CrossRef] [PubMed]
4. Gazzarrini, S.; Lejay, L.; Gojon, A.; Ninneemann, O.; Frommer, W.B.; von Wirén, N. Three Functional Transporters for Constitutive, Diurnally Regulated, and Starvation-Induced Uptake of Ammonium into Arabidopsis Roots. *Plant Cell* 1999, 11, 937–948. [CrossRef]
5. Bai, H.; Euring, D.; Volmer, K.; Janz, D.; Polle, A. The Nitrate Transporter (NRT) Gene Family in Poplar. *PLoS ONE* 2013, 8, e72126. [CrossRef] [PubMed]
6. Ariz, I.; Boeckstaens, M.; Gouveia, C.; Martins, A.P.; Sanz-Luque, E.; Fernández, E.; Soveral, G.; von Wirén, N.; Marin, A.M.; Aparicio-Tejo, P.M.; et al. Nitrogen Isotope Signature Evidences Ammonium Deprotonation as a Common Transport Mechanism for the AMT-Mep-Rh Protein Superfamily. *Sci. Adv.* 2018, 4, eaar3599. [CrossRef]
7. Huang, L.; Li, J.; Zhang, B.; Hao, Y.; Ma, F. Genome-Wide Identification and Expression Analysis of AMT Gene Family in Apple (Malus domestica Borkh.). *Horticulturae* **2022**, *8*, 457. [CrossRef]
8. Song, S.; He, Z.; Huang, X.; Zhong, L.; Liu, H.; Sun, G.; Chen, R. Cloning and Characterization of the Ammonium Transporter Genes *BaAMT1;1* and *BaAMT1;3* from Chinese Kale. *Hortic. Environ. Biotechnol.* **2017**, *58*, 178–186. [CrossRef]
9. Castro-Rodríguez, V.; Assaf-Casals, I.; Pérez-Tienda, J.; Fan, X.; Avila, C.; Miller, A.; Cánovas, F.M. Deciphering the Molecular Basis of Ammonium Uptake and Transport in Maritime Pine. *Plant Cell Environ.* **2016**, *39*, 1669–1682. [CrossRef]
10. Giehl, R.F.H.; Laginha, A.M.; Duan, F.; Rentsch, D.; Yuan, L.; von Wirén, N. A Critical Role of AMT2;1 in Root-To-Shoot Translocation of Ammonium in *Arabidopsis*. *Mol. Plant* **2017**, *10*, 1449–1460. [CrossRef]
11. Shelden, M.C.; Dong, B.; de Bruxelles, G.L.; Trevaskis, B.; Whelan, J.; Ryan, P.R.; Howitt, S.M.; Udvardi, M.K. Arabidopsis Nitrate Transporters, AtAMT1;1 and AtAMT1;2, Have Different Biochemical Properties and Functional Roles. *Plant Soil* **2001**, *231*, 151–160. [CrossRef]
12. Yuan, L.; Graff, L.; Loqué, D.; Kojima, S.; Tsuchiya, Y.N.; Takahashi, H.; von Wirén, N. AtAMT1;4, a Pollen-Specific High-Affinity Ammonium Transporter of the Plasma Membrane in *Arabidopsis*. *Plant Cell Physiol.* **2009**, *50*, 13–25. [CrossRef] [PubMed]
13. Ludewig, U.; Neuhäuser, B.; Dynowski, M. Molecular Mechanisms of Ammonium Transport and Accumulation in Plants. *FEBS Lett.* **2007**, *581*, 2301–2308. [CrossRef]
14. Couturier, J.; Montanini, B.; Martin, F.; Brun, A.; Blaudez, D.; Chalot, M. The Expanded Family of Ammonium Transporters, AtAMT1;1 and AtAMT1;2, Have Different Biochemical Properties and Functional Roles. *Plant Cell Physiol.* **2012**, *53*, 85–122. [CrossRef] [PubMed]
15. Liu, K.H.; Huang, C.Y.; Tsay, Y.F. CHL1 Is a Dual-Affinity Nitrate Transporter of *Arabidopsis* Involved in Multiple Phases of Nitrate Uptake. *Plant Cell* **1999**, *11*, 865–874. [CrossRef]
16. Tsay, Y.-F.; Chiu, C.-C.; Tsai, C.-B.; Ho, C.-H.; Hsu, P.-K. Nitrate Transporters and Peptide Transporters. *FEBS Lett.* **2007**, *581*, 2290–2300. [CrossRef]
17. Huang, N.-C.; Liu, K.-H.; Lo, H.-J.; Tsay, Y.-F. Cloning and Functional Characterization of an *Arabidopsis* Nitrate Transporter Gene That Encodes a Constitutive Component of Low-Affinity Uptake. *Plant Cell* **1999**, *11*, 1381–1392. [CrossRef] [PubMed]
18. Segonzac, C.; Boyer, J.-C.; Ipotesi, E.; Szponarski, W.; Tillard, P.; Touraine, B.; Sommerer, N.; Rossignol, M.; Gibrat, R. Nitrate Efflux at the Root Plasma Membrane: Identification of an *Arabidopsis* Excretion Transporter. *Plant Cell* **2007**, *19*, 3760–3777. [CrossRef] [PubMed]
19. Iqbal, A.; Qiang, D.; Alamzeb, M.; Xiangru, W.; Huiping, G.; Hengheng, Z.; Nianchang, P.; Xiling, Z.; Meizhen, S. Untangling the Molecular Mechanisms and Functions of Nitrate to Improve Nitrogen Use Efficiency. *J. Sci. Food Agric.* **2021**, *100*, 904–914. [CrossRef]
20. Krapp, A.; David, L.C.; Chardin, C.; Girin, T.; Marmagne, A.; Leprince, A.-S.; Chaillou, S.; Meyer, C.; Daniel-Vedele, F. Nitrate Transport and Signalling in *Arabidopsis*. *J. Exp. Bot.* **2014**, *65*, 789–798. [CrossRef]
21. Ma, H.; Zhao, J.; Feng, S.; Qiao, K.; Gong, S.; Wang, J.; Zhou, A. Heterologous Expression of Nitrate Assimilation Related-Protein DsNAR2.1/NRT3.1 Affects Uptake of Nitrate and Ammonium in Nitrogen-Starved *Arabidopsis*. *Int. J. Mol. Sci.* **2021**, *22*, 4027. [CrossRef] [PubMed]
22. Li, W.; Wang, Y.; Okamoto, M.; Crawford, N.M.; Siddiqi, M.Y.; Glass, A.D.M. Dissection of the AtNRT2.1:AtNRT2.2 Inducible High-Affinity Nitrate Transporter Gene Cluster. *Plant Physiol.* **2007**, *143*, 425–433. [CrossRef]
23. Leznheva, L.; Kiba, T.; Feria-Bourrelier, A.-B.; Lafouge, F.; Boutet-Mercey, S.; Zoufane, P.; Sakakibara, H.; Daniel-Vedele, F.; Krapp, A. The *Arabidopsis* Nitrate Transporter NRT2.5 Plays a Role in Nitrate Acquisition and Remobilization in Nitrogen-Starved Plants. *Plant J.* **2014**, *80*, 230–241. [CrossRef]
24. Kotur, Z.; Mackenzie, N.; Ramesh, S.; Tyerman, S.D.; Kaiser, B.N.; Glass, A.D.M. Nitrate Transport Capacity of the Arabidopsis Thaliana NRT2 Family Members and Their Interactions with AtNAR2.1. *New Phytol.* **2012**, *194*, 724–731. [CrossRef]
25. Chen, M.; Zhu, K.; Tan, P.; Liu, J.; Xie, J.; Yao, X.; Chu, G.; Peng, F. Ammonia–Nitrate Mixture Dominated by NH4+–N Promoted Growth, Photosynthesis and Nutrient Accumulation in Pecan (*Carya illinoinensis*). *Forests* **2021**, *12*, 1808. [CrossRef]
26. Zhu, K.; Fan, P.; Liu, H.; Tan, P.; Ma, W.; Mo, Z.; Zhao, J.; Chu, G.; Peng, F. Insight into the CBL and CIPK Gene Families in Pecan (*Carya illinoinensis*): Identification, Evolution and Expression Patterns in Drought Response. *BMC Plant Biol.* **2022**, *22*, 221. [CrossRef]
27. Yuan, L.; Loqué, D.; Kojima, S.; Rauch, S.; Ishiyama, K.; Inoue, E.; Takahashi, H.; von Wirén, N. The Organization of High-Affinity Ammonium Uptake in *Arabidopsis* Roots Depends on the Spatial Arrangement and Biochemical Properties of AMT1-Type Transporters. *Plant Cell* **2007**, *19*, 2636–2652. [CrossRef] [PubMed]
32. Zhu, Y.; Hao, Y.; Liu, H.; Sun, G.; Chen, R.; Song, S. Identification and Characterization of Two Ammonium Transporter Genes in Flowering Chinese Cabbage (Brassica campestris). Plant Biotechnol. 2018, 35, 59–70. [CrossRef] [PubMed]

33. He, Y.-N.; Peng, J.-S.; Cai, Y.; Liu, D.-F.; Guan, Y.; Yi, H.-Y.; Gong, J.-M. Tonoplast-Localized Nitrate Uptake Transporters Involved in Vacular Nitrate Efflux and Reallocation in Arabidopsis. Sci. Rep. 2017, 7, 6417. [CrossRef] [PubMed]

34. You, S.; Wang, Y.; Zhang, T.; Zhu, Y.; Ren, N.; Jiang, X.; Zhou, Y. Genome-Wide Identification of Nitrate Transporter 2 (NRT2) Gene Family and Functional Analysis of MeNRT2.2 in Cassava (Manihot esculenta Crantz). Gene 2022, 809, 146038. [CrossRef]

35. Zhu, Y.; Qi, B.; Hao, Y.; Liu, H.; Sun, G.; Chen, R.; Song, S. Appropriate NH₄⁺/NO₃⁻ Ratio Triggers Plant Growth and Nutrient Uptake of Flowering Chinese Cabbage by Optimizing the FH Value of Nutrient Solution. Front. Plant Sci. 2021, 12, 724–739. [CrossRef]

36. Oh, K.; Kato, T.; Xu, H.L. Transport of Nitrogen Assimilation in Xylem Vessels of Green Tea Plants Fed with NH₄⁺-N and NO₃⁻-N. Plosphere 2008, 18, 222–226. [CrossRef]

37. Zhang, J.; Lv, J.; Dawuda, M.M.; Xie, J.; Yu, J.; Li, J.; Zhang, X.; Tang, C.; Wang, C.; Gan, Y. Appropriate Ammonium-Nitrate Ratio Improves Nutrient Accumulation and Fruit Quality in Pepper (Capsicum annuum L.). Agronomy 2019, 9, 683. [CrossRef]

38. Ruan, L.; Wei, K.; Wang, L.; Cheng, H.; Zhang, F.; Wu, L.; Bai, P.; Zhang, C. Characteristics of NH₄⁺ and NO₃⁻ Fluxes in Tea (Camellia Sinensis) Roots Measured by Scanning Ion-Selective Electrode Technique. Sci. Rep. 2016, 6, 38370. [CrossRef]

39. Wu, S.; Hua, J.; Lu, Y.; Zhang, R.; Yin, Y. Characteristics of NH₄⁺ and NO₃⁻ Fluxes in Taxodium Roots under Different Nitrogen Treatments. Plants 2022, 11, 894. [CrossRef]

40. Tang, Y.; Gao, F.; Yu, Q.; Guo, S.; Li, F. The Uptake Kinetics of NH₄⁺ and NO³⁻ by Lettuce Seedlings under Hypobaric and Hypoxic Conditions. Sci. Hortic. 2015, 197, 236–243. [CrossRef]

41. Kamminga-Van Wijk, C.; Prins, H.B.A. The Kinetics of NH₄⁺ for Nitrate and Phosphate Uptake by Douglas Fir from Single N-Solutions and from Solutions Containing Both NH₄⁺ and NO₃⁻. Plant Soil 1993, 151, 91. [CrossRef]

42. Nishikawa, T.; Tarutani, K.; Yamamoto, T. Nitrate and Phosphate Uptake Kinetics of the Harmful Diatom Coscinodiscus Wailesii, a Causative organism in the Bleaching of Aquacultured Porphyra thalli. Harmful Algae 2010, 9, 563–567. [CrossRef]

43. Loqué, D.; Yuan, L.; Kojima, S.; Gojon, A.; Wirth, J.; Gazzarrini, S.; Ishiyama, K.; Takahashi, H.; Von Wiren, N. Additive Contribution of AMT1;1 and AMT1;3 to High-Affinity Ammonium Uptake across the Plasma Membrane of Nitrogen-Deficient Arabidopsis Roots. Plant J. 2006, 48, 522–534. [CrossRef]

44. Kumar, A.; Silim, S.N.; Okamoto, M.; Siddiqi, M.Y.; Glass, A.D.M. Differential Expression of Members of the AMT1 Gene Family Encoding Putative High-Affinity NH₄⁺ Transporters in Roots of Oryza sativa Subspecies Indica. Plant Cell Environ. 2003, 26, 907–914. [CrossRef]

45. Camañes, G.; Cerezo, M.; Primo-Millo, E.; Gojon, A.; García-Agustín, P. Ammonium Transport and CitAMT1 Expression Are Regulated by N in Citrus Plants. Planta 2008, 229, 331. [CrossRef]

46. You, S.; Wang, Y.; Li, Y.; Li, Y.; Tan, P.; Wu, Z.; Shi, W.; Song, Z. Cloning and Functional Determination of Ammonium Transporter PpeAMT3;4 in Peach. BioMed Res. Int. 2020, 2020, e2147367. [CrossRef] [PubMed]

47. Wei, W.; Hu, B.; Li, A.; Chu, C. NRT1.1 in Plants: Functions beyond Nitrate Transport [Cover Story]. J. Exp. Bot. 2020, 71, 4373–4379. [CrossRef]

48. Zhang, L.; Yu, Z.; Xu, Y.; Ren, Y.; Zhang, S.; Yang, G.; Huang, J.; Yan, K.; Zheng, C.; et al. Regulation of the Stability and ABA Import Activity of NRT1.2/NPF4.6 by CEPR2-Mediated Phosphorylation in Arabidopsis. Mol. Plant 2021, 14, 633–646. [CrossRef]

49. Morère-Le Paven, M.-C.; Viau, L.; Hamon, A.; Vandecasteele, C.; Pellizzaro, A.; Bourdin, C.; Laffont, C.; Lepetit, M.; Frugier, F.; et al. Characterization of a Dual-Affinity Nitrate Transporter MtNRT1.3 in the Model Legume Medicago Truncatula. J. Exp. Bot. 2011, 62, 5595–5605. [CrossRef]

50. Yuan, J.-Z.; Cui, Y.-N.; Li, X.-T.; Wang, F.-Z.; He, Z.-H.; Li, X.-Y.; Bao, A.-K.; Wang, S.-M.; Ma, Q. ZmNPF7.3/NRT1.5 from the Xerophyte Zygophyllum Xanthoxylum Modulates Salt and Drought Tolerance by Regulating NO₃⁻, Na⁺ and K⁺ Transport. Environ. Exp. Bot. 2020, 177, 104123. [CrossRef]

51. Li, J.-Y.; Fu, Y.-L.; Pike, S.M.; Bao, J.; Tian, W.; Zhang, Y.; Chen, C.-Z.; Zhang, Y.; Li, H.-M.; Huang, J.; et al. The Arabidopsis Nitrate Transporter NRT1.8 Functions in Nitrate Removal from the Xylem Sap and Mediates Cadmium Tolerance. Plant Cell 2010, 22, 1633–1646. [CrossRef]

52. Nour-Eldin, H.H.; Andersen, T.G.; Burow, M.; Madsen, S.R.; Jørgensen, M.E.; Olsen, C.E.; Dreyer, I.; Hedrich, R.; Geiger, D.; Halkier, B.A. NRT/PTR Transporters Are Essential for Translocation of Glucosinolate Defence Compounds to Seeds. Nature 2012, 488, 531–534. [CrossRef] [PubMed]

53. Zhao, X.; Zhang, X.; Liu, Z.; Lv, Y.; Song, T.; Cui, J.; Chen, T.; Li, J.; Zeng, F.; Zhan, Y. Comparing the Effects of N and P Deficiency on Physiology and Growth for Fast- and Slow-Growing Provenances of Fraxinus Mandshurica. Forests 2021, 12, 1760. [CrossRef]

54. El-Gebali, S.; Mistry, J.; Bateman, A.; Eddy, S.R.; Luciani, A.; Potter, S.C.; Qureshi, M.; Richardson, L.J.; Salazar, G.A.; Smart, A.; et al. The Pfam Protein Families Database in 2019. Nucleic Acids Res. 2019, 47, D427–D432. [CrossRef] [PubMed]

55. Finn, R.D.; Clements, J.; Eddy, S.R. HMMPR Web Server: Interactive Sequence Similarity Searching. Nucleic Acids Res. 2011, 39, W29–W37. [CrossRef]

56. Artimo, P.; Jonnalagedda, M.; Arnold, K.; Baratin, D.; Csardi, G.; de Castro, E.; Duvaud, S.; Flegel, V.; Fortier, A.; Gasteiger, E.; et al. ExPaSy: SIB Bioinformatics Resource Portal. Nucleic Acids Res. 2012, 40, W597–W603. [CrossRef]
57. Chou, K.-C.; Shen, H.-B. Cell-PLoc 2.0: An Improved Package of Web-Servers for Predicting Subcellular Localization of Proteins in Various Organisms. Nat. Sci. 2010, 2, 1090. [CrossRef]
58. Rhee, S.Y.; Beavis, W.; Berardini, T.Z.; Chen, G.; Dixon, D.; Doyle, A.; Garcia-Hernandez, M.; Huala, E.; Lander, G.; Montoya, M.; et al. The Arabidopsis Information Resource (TAIR): A Model Organism Database Providing a Centralized, Curated Gateway to Arabidopsis Biology, Research Materials and Community. Nucleic Acids Res. 2003, 31, 224–228. [CrossRef]
59. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 2016, 33, 1870–1874. [CrossRef]
60. Subramanian, B.; Gao, S.; Lercher, M.J.; Hu, S.; Chen, W.-H. Evolview v3: A Webserver for Visualization, Annotation, and Management of Phylogenetic Trees. Nucleic Acids Res. 2019, 47, W270–W275. [CrossRef]
61. Bailey, T.L.; Boden, M.; Buske, F.A.; Frith, M.; Grant, C.E.; Clementi, L.; Ren, J.; Li, W.W.; Noble, W.S. MEME Suite: Tools for Motif Discovery and Searching. Nucleic Acids Res. 2009, 37, W202–W208. [CrossRef] [PubMed]
62. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. Mol. Plant 2020, 13, 1194–1202. [CrossRef] [PubMed]
63. Wang, Y.; Li, J.; Paterson, A.H. MCScanX-Transposed: Detecting Transposed Gene Duplications Based on Multiple Colinearity Scans. Bioinformatics 2013, 29, 1458–1460. [CrossRef] [PubMed]
64. Lescot, M.; Déhais, P.; Thys, G.; Marchal, K.; Moreau, Y.; Van de Peer, Y.; Rouzé, P.; Rombauts, S. PlantCARE, a Database of Plant Cis-Acting Regulatory Elements and a Portal to Tools for in Silico Analysis of Promoter Sequences. Nucleic Acids Res. 2002, 30, 325–327. [CrossRef] [PubMed]
65. Szklarczyk, D.; Morris, J.H.; Cook, H.; Kuhn, M.; Wyder, S.; Simonovic, M.; Santos, A.; Doncheva, N.T.; Roth, A.; Bork, P.; et al. The STRING Database in 2017: Quality-Controlled Protein–Protein Association Networks, Made Broadly Accessible. Nucleic Acids Res. 2017, 45, D362–D368. [CrossRef]
66. Zhu, K.; Fan, P.; Mo, Z.; Tan, P.; Feng, G.; Li, F.; Peng, F. Identification, Expression and Co-Expression Analysis of R2R3-MYB Family Genes Involved in Graft Union Formation in Pecan (Carya illinoinensis). Forests 2020, 11, 917. [CrossRef]
67. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−∆∆CT Method. Methods 2001, 25, 402–408. [CrossRef]
68. Bräutigam, A.; Gagneul, D.; Weber, A.P.M. High-Throughput Colorimetric Method for the Parallel Assay of Glyoxyllic Acid and Ammonium in a Single Extract. Anal. Biochem. 2007, 362, 151–153. [CrossRef]
69. Patterson, K.; Cakmak, T.; Cooper, A.; Lager, I.; Rasmusson, A.G.; Escobar, M.A. Distinct Signalling Pathways and Transcriptome Response Signatures Differentiate Ammonium- and Nitrate-Supplied Plants. Plant Cell Environ. 2010, 33, 1486–1501. [CrossRef]
70. Ogawa, T.; Fukuoka, H.; Yano, H.; Ohkawa, Y. Relationships between Nitrite Reductase Activity and Genotype-Dependent Callus Growth in Rice Cell Cultures. Plant Cell Rep. 1999, 18, 576–581. [CrossRef]