

Article

Identification and Expression Patterns of Four Key Nitrogen Assimilation Enzyme Gene Families in *Malus domestica*

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Abstract

Nitrogen assimilation is vital for apple growth, yield, and quality, with nitrate reductase (NIA), nitrite reductase (NIR), glutamine synthetase (GS), and glutamate synthase (GOGAT) serving as key regulatory enzymes. This study systematically identified these four gene families in apple (*Malus domestica*) through genome-wide analysis and examined their expression patterns under nitrate treatment. In total, 13 genes were identified, 2 *MdNIAs*, 1 *MdNIR*, 7 *MdGSs*, and 3 *MdGOGATs*, with gene lengths ranging from 2577 to 27736 base pairs (bp); *MdGLT1A* had the longest coding sequence (6627 bp). The encoded proteins contained 355–2208 amino acids, with predicted isoelectric points (*pIs*) between 5.55 and 6.63. Subcellular localization analysis predicted distinct compartmentalization: *MdNIA1A* in peroxisomes; *MdGS1* in the cytosol; *MdNIR1*, *MdGS2*, and *MdGLU1* in chloroplasts; and *MdGLT1* in mitochondria/chloroplasts. Functional site prediction revealed multiple phosphorylation and glycosylation sites, with ATP/GTP-binding motifs present only in certain *MdGOGAT* proteins. Protein interaction analysis suggested close associations among these genes and possible interactions with NRT2.1/2.2. Chromosomal mapping showed their distribution across eight chromosomes, while promoter analysis identified diverse cis-acting regulatory elements (e.g., ABRE and G-box). Under nitrate treatment (0–12 h), these genes exhibited distinct expression dynamics: *MdNIA1A* and *B* were rapidly induced (0–6 h) and maintained high expression; *MdNIR1* peaked at 6 h and then declined; *MdGS1.1B* was activated after 6 h; and *MdGS2A*, *MdGLU1*, and *MdGLT1A/B* peaked at 6 h before decreasing. Therefore, these results elucidate the structural and functional divergence of nitrogen assimilation genes in apple and provide a basis for understanding nitrogen utilization mechanisms and developing nitrogen-efficient breeding strategies.

Keywords: *Malus domestica*; Nitrogen assimilation; Nitrate reductase (NIA); Nitrite reductase (NIR); Glutamine synthetase (GS); Glutamate synthase (GOGAT)



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1. Introduction

Nitrogen is an essential nutrient for apple tree growth and development, and its efficient assimilation and utilization are key determinants of tree vigor, fruit yield, and quality and the ecological sustainability of orchards. However, excessive nitrogen fertilizer application in orchard systems leads to low nitrogen use efficiency, higher production costs, reduced fruit quality, and environmental pollution [1]. A comprehensive analysis of nitrogen assimilation in apples is, therefore, critical, given its complex molecular regulation

involving several tightly coordinated enzymes. The enzymes nitrate reductase (NR), nitrite reductase (NiR), glutamine synthase (GS), and glutamate synthase (GOGAT) form a four-step, rate-limiting reaction sequence central to the plant nitrogen assimilation pathway. In most terrestrial plants, including apple trees, nitrate serves as the primary nitrogen source [2]. NR catalyzes the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), which is then reduced to ammonium (NH_4^+) by NiR in plastids. NH_4^+ is incorporated into glutamine (Gln) and glutamate (Glu) via the GS/GOGAT cycle—core reactions that provide nitrogen skeletons for synthesizing amino acids, nucleic acids, and secondary metabolites [3]. Therefore, elucidating the structure, function, gene regulation, and environmental interactions of key enzymes involved in apple nitrogen assimilation is crucial. Such studies will deepen our understanding of nitrogen metabolism in apples, support the development of precise fertilization strategies, enable the breeding of nitrogen-efficient cultivars, and promote the sustainable advancement of the apple industry.

Cytoplasmic nitrate reductase (NR) is a rate-limiting enzyme in plant nitrogen metabolism, directly regulating this process [4]. NR is a dimeric protein complex, with each identical subunit containing three cofactors: a molybdenum cofactor (MoCo), a heme-binding domain (Heme), and a flavin adenine dinucleotide-binding domain (FAD) [5]. In *Arabidopsis*, two NR isoforms—NIA1 and NIA2—are encoded by the *AtNIA1* and *AtNIA2* genes, respectively [6,7]. Among them, NIA2 plays a critical role in nitrogen assimilation and stress tolerance [8]. The *nia1/nia2* double mutant exhibits severe growth retardation and nitrate accumulation, highlighting the enzyme's essential function [9]. In rice, three genes encode NR, with *OsNIA1* contributing predominantly to overall enzyme activity [10]. Functional analysis of *OsNR2* has shown that it enhances effective tiller number, grain yield, and nitrogen fertilizer use efficiency in japonica rice [11]. In apples, the *MdNIA2* gene improves salt and drought tolerance in *Arabidopsis* and callus cultures by modulating nitrogen use efficiency and nitric oxide (NO) metabolism.

The plastid nitrite reductase (NiR) converts NO_2^- produced by NR into NH_4^+ and functions within plastids. NIR1 is a key regulator of nitrogen assimilation, NO homeostasis, and plant growth stress responses [12]. The rice *OsNiR* promoter contains a nitrate-responsive cis-element (NRE). Overexpression of *OsNiR* increases tiller number and overall yield, thereby enhancing nitrogen use efficiency under both high- and low-nitrogen conditions [13]. Research on NiR in fruit trees remains limited, with *PtNiR* identified only once—in Citrus under drought stress [14].

Glutamine synthetase (GS) catalyzes the assimilation of NH_4^+ to produce glutamine. In plants, two functional GS types exist: the prokaryotic GSIIb (GLN2) and the eukaryotic GSIIe (GLN1/GS). Angiosperms generally possess two nuclear gene classes encoding glutamine synthetase II enzymes (GSIIes): cytoplasmic glutamine synthetase (GS1) and plastidic glutamine synthetase (GS2) [15,16]. GS2 is predominantly localized in photosynthetic tissues such as mesophyll cells [1,17,18], whereas GS1 is ubiquitously present across various plant organs and tissues [3]. Simultaneous overexpression of *OsGS1;1* and *OsGS2* in rice improves nitrogen use efficiency (NUE) [19]. In maize, two cytoplasmic glutamine synthetases, *GS1-3* and *GS1-4*, have been identified. Knockout studies show that these enzymes have distinct, non-redundant roles in determining grain yield: *GS1-3* primarily affects grain number, while *GS1-4* influences grain size [20]. In the apple rootstock, genes associated with nitrogen metabolism, such as *MdGS1*, are downregulated [21].

Glutamate synthase (GOGAT) catalyzes the synthesis of glutamic acid from glutamine and α -ketoglutarate and exists in two forms: ferredoxin-dependent (Fd-GOGAT) and NADH-dependent (NADH-GOGAT) [22]. NADH-GOGAT is predominantly found in non-photosynthetic tissues [23,24]. In *Arabidopsis*, two genes, *Glu1* and *Glu2*, encode Fd-GOGAT, while in rice, *OsFd-GOGAT* mutants exhibit chlorosis under natural conditions [25].

Conversely, overexpressing *NADH-GOGAT* in rice enhances grain weight and improves nitrogen utilization [26]. In apple, studies mainly examine how trace elements regulate GOGAT activity during nitrogen metabolism. Under aluminum stress, GOGAT activity in roots and leaves is significantly inhibited. However, magnesium supplementation restores this activity, promoting glutamate synthesis and improving nitrogen metabolism [27]. Similarly, calcium treatment mitigates temperature-induced stress on nitrogen metabolism in apple roots by regulating GOGAT activity [28].

Despite extensive research on nitrogen assimilation in model plants such as *Arabidopsis* and rice, these processes remain poorly understood in perennial fruit trees like apple. Apple is an economically important fruit crop worldwide with significant nutritional and commercial value. Identifying and characterizing its nitrogen-assimilation-related genes are crucial for elucidating nitrogen utilization efficiency mechanisms and developing strategies for improving apple production and sustainability. This study aimed to systematically identify and characterize the four key nitrogen assimilation enzyme gene families (*NIA*, *NIR*, *GS*, and *GOGAT*) in apple (*Malus domestica*) through genome-wide analysis. We explored their structural features, subcellular localization, protein interaction networks, promoter cis-acting elements, and expression profiles under nitrate treatment. These findings provide a foundation for understanding nitrogen utilization in apple and for guiding the development of nitrogen-efficient breeding strategies.

2. Materials and Methods

2.1. Identification of Apple Nitrogen Key Enzyme Genes

The genome of the apple (*Malus domestica*) used in this study is the ‘Golden Delicious’ apple (GDDH13) reference genome (GDDH13 v1.1, <https://iris.angers.inra.fr/gddh13/>, accessed on 26 January 2025) [29]. Two query strategies (blastp and hmmsearch) are used to identify members in this article. The query sequence used for protein homologous alignment is the protein sequence of the pattern species *Arabidopsis thaliana* (L.) Heynh. The sequence is downloaded from the TAIR database (<https://www.arabidopsis.org/>, accessed on 26 January 2025). After screening, the searched apple members were submitted to the SMART website (<http://smart.embl-heidelberg.de/>, accessed on 26 January 2025) for conservative domain confirmation, and we finally obtained the candidate gene [30].

2.2. Characteristic Analysis of Protein

To calculate the molecular weight and theoretical isoelectric point (*pI*), bioinformatic analysis was carried out with the ExPASy online tool (<https://web.expasy.org/protparam/>, accessed on 31 January 2025).

2.3. Subcellular Localization and Signal Peptide Prediction of Protein

The online program WoLFPSORT: Protein Subcellular Localization Prediction (<https://wolfpsort.hgc.jp/>, accessed on 31 January 2025) was used to predict protein subcellular localization, and the online website SignalP (<https://services.healthtech.dtu.dk/services/SignalP-4.1/>, accessed on 31 January 2025) was used to identify lipoprotein signal peptides [31].

2.4. Active Site Prediction of Protein

The PROSITE Server (<http://www.expasy.org/prosite/>, accessed on 14 March 2025) was used to find protein functional sites (glycosylation sites, acylation sites, phosphorylation sites, etc.) [32]. The NetPhos 3.1 Server (<https://services.healthtech.dtu.dk/services/NetPhos-3.1/>, accessed on 14 March 2025) was used to predict and analyze the number of phosphorylation sites of three amino acids (serine, threonine, and tyrosine) [33].

2.5. Protein Interaction Network

Analysis of the interaction network was conducted through the STRING functional protein association network (<https://string-db.org>, accessed on 21 March 2025) and the species source selection model species *Arabidopsis*.

2.6. Gene Structure Analysis of Apple Nitrogen Key Enzyme Genes

Using the Gene Structure View package of TBtools (v1.098691), the CDS, UTR, and intron configurations of apple nitrogen key enzyme genes were examined with an apple genome annotation file (*Malus domestica*.v1.0.consensus.gff) [34].

2.7. Chromosome Localizations of Apple Nitrogen Key Enzyme Genes

The location information of apple nitrogen key enzyme genes was extracted from the gene ID in the apple genome annotation file (*Malus domestica*.v1.0.consensus.gff), and submitted to the online software MG2C (http://mg2c.iask.in/mg2c_v2.0/, accessed on 31 January 2025) for chromosome mapping.

2.8. Promoter Cis-Acting Element Prediction of Apple Nitrogen Key Enzyme Genes

Fragments about 2000 bp upstream of the translation start site of the apple nitrogen key enzyme genes from the apple genomic sequence (GDDH13_1-1_formatted.fasta) used as the promoter regions, and the gene promoter sequence identified above, were submitted to Plant CARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 6 February 2025) for predictive analysis of the promoter cis-acting elements.

2.9. Expression of Key Apple Nitrogen Enzyme Genes Under Different Nitrate Treatments (Including Nitrogen Starvation Treatment)

From the seedlings of '*Malus hupehensis*', 18 seedlings with relatively consistent growth status were selected. The seedlings were first pre-cultured with nitrogen-deficient MS culture medium (ingredients: 1.2 mM KH₂PO₄, 0.1 mM CaCl₂, 1 mM MgSO₄, 0.1 mM Fe salt, trace amount, 1 mM KCl) for one week, and then, 5 mM KNO₃ was added for nitrogen treatment. The entire plant was sampled for 0, 3, 6, and 12 h during the nitrogen treatment.

The total RNA was extracted using the TRIzol Reagent kit (TakaRa, Dalian, China), and the reverse transcription and synthesis of cDNA were performed using the Prime Script RT Reagent Kit with the gDNA Eraser kit. Real-time fluorescence quantitative PCR (qRT-PCR) was carried out according to the method provided by the Ultra SYBR Mixture (High ROX) Reagent (Vazyme Biotech, Nanjing, China). The reaction system was as follows: 2 × UltraSYBR Mixture (High ROX) 10 µL, ddH₂O 7 µL, upstream and downstream primers (10 mM), and cDNA template at 1 µL; the total system was 20 µL. The qRT-PCR reaction process is as follows: pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 65 °C for 10 s; 40 cycles were performed. The fluorescence signal was collected in step 3 of each cycle, and each reaction was repeated 3 times. The design of quantitative primers was verified by the online website Primer3Plus (<https://www.primer3plus.com/>, accessed on 20 May 2025). All primer specificities were verified by Nucleotide BLAST and Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>, accessed on 20 May 2025) of the NCBI. The primers used for the reaction are displayed in Table S1; *Md18S* is the apple intrare gene. Data analysis of relative gene expression amounts was made at $2^{-\Delta\Delta CT}$.

2.10. Statistical Analysis

Each experiment was independently performed in triplicate unless stated otherwise. The significant difference analysis and column chart creation were performed with DPS (DPS software, Sinyosoft, version 15.10, http://www.dpsw.cn/dps_eng/index.html, ac-

cessed on 10 June 2025) and GraphPad Prism 7 Software (GraphPad Software, La Jolla, CA, USA). Data were analyzed by one-way ANOVA with Tukey’s multiple comparison test, presented as means ± SD. Significant differences are indicated by different letters at the $p < 0.05$ level.

3. Results

3.1. Identification and Basic Information of Apple Nitrogen Assimilation Enzyme Genes

After strict screening and confirmation, a total of two *MdNIA* genes, one *MdNIR* gene, seven *MdGS* genes, and three *MdGOGAT* genes were obtained. Statistics showed that the length of each gene sequence is between 2577 and 27736 bp, and the length of the *MdGLT1A* (MD01G1219500) coding sequence of the *MdGOGAT* family reaches 6627 bp. The protein size is between 355 and 2208 aa, and the *pI* value is acidic in 5.55–6.63. See Table 1 for specific information.

Table 1. Information about the NIA, NIR, GS, and GOGAT members found in apple.

Gene Name	Gene ID	Gene Length/bp	CDS Length/bp	Protein Size/aa	Molecular Weight/Da	<i>pI</i> Values (pH)	Best Hits
NIA							
<i>MdNIA1A</i>	MD08G1172400	5403	2709	902	101,608.70	6.24	AT1G77760.1
<i>MdNIA1B</i>	MD15G1357900	5127	2712	903	101,635.65	6.30	AT1G77760.1
NIR							
<i>MdNIR1</i>	MD05G1081500	2907	1758	585	65,321.14	6.63	AT2G15620.1
GS							
<i>MdGS1.1A</i>	MD09G1270600	2577	1071	356	38,971.02	5.78	AT5G37600.1
<i>MdGS1.1B</i>	MD14G1238700	2994	1071	356	39,282.35	5.87	AT5G37600.1
<i>MdGS1.1C</i>	MD17G1268700	3954	1071	356	38,968.06	5.94	AT5G37600.1
<i>MdGS1.4A</i>	MD13G1006600	3314	1068	355	38,892.80	5.55	AT5G16570.1
<i>MdGS1.4B</i>	MD16G1001400	4271	1068	355	38,845.92	6.10	AT5G16570.1
<i>MdGS2A</i>	MD13G1180400	4493	1263	420	46,237.29	6.63	AT5G35630.3
<i>MdGS2B</i>	MD16G1181300	4434	1263	420	46,219.18	6.37	AT5G35630.3
GOGAT							
<i>MdGLU1</i>	MD14G1087500	27,736	4887	1628	176,921.12	6.16	AT5G04140.1
<i>MdGLT1A</i>	MD01G1219500	10,822	6627	2208	242,608.82	6.08	AT5G53460.3
<i>MdGLT1B</i>	MD07G1290500	10,264	6564	2187	240,431.50	6.21	AT5G53460.3

3.2. Active Site Prediction of Apple Nitrogen Assimilation Enzymes

Protein functional sites include various types, such as glycosylation sites, phosphorylation sites, and myristoylation sites. Understanding these active sites can help further study and analyze protein function. In Table 2, we predict the protein active sites of apple nitrogen assimilation enzymes. There are many active sites of various types in *MdNIA*, and there are many protein kinase C phosphorylation sites in part of *MdGOGAT* members. The ATP/GTP-binding site motif A (P ring) is also only present in some *MdGOGAT* members.

3.3. Prediction of Subcellular Localization and Signal Peptide Prediction of Apple Nitrogen Assimilation Enzyme Genes

Subcellular localization prediction can be used to infer the precise location of proteins in cells through calculation models. We found that *MdNIA1A* is mainly located in the peroxisome, *MdNIA1B* is mainly located in the cytoplasm, *MdNIR1* is mainly located in the chloroplast, *MdGS1* members are mainly located in the cytoplasm, *MdGS2* is mainly located in the chloroplast (Table 3), *MdGLU1* is mainly located in the chloroplast, and *MdGLT1A* and *MdGLT1B* are mainly located in the mitochondria and chloroplast. The signal peptide cleavage site of *MdNIR1* may be between amino acids at positions 18 and 19 (Figure 1).

Table 2. Active site prediction of nitrogen assimilation enzymes in apple.

Protein Name	PS00001 N- Glycosylation Sites	PS00004 cAMP and cGMP- Dependent Protein Kinase Phosphorylation Sites	PS00005 Protein Kinase C Phosphorylation Site	PS00006 Casein Kinase II Phosphorylation Site	PS00007 Tyrosine Kinase Phosphorylation Site	PS00008 N- Myristoylation Site	PS00009 Amidation Site	PS00017 ATP/GTP-Binding Positioning Point Motif A (P Ring)
MdNIA1A	9	3	8	16		12	1	
MdNIA1B	10	3	7	17		13	1	
MdNIR1	1		3	6	2	7		
MdGS1.1A	2	1	8	5	2	6		
MdGS1.1B	3	1	7	4	2	7		
MdGS1.1C	2	1	8	5	2	7		
MdGS1.4A	4	1	5	4	1	8		
MdGS1.4B	3	1	7	4	1	6		
MdGS2A	3	1	10	5	2	7		
MdGS2B	3	1	10	5	2	7		
MdGLU1	6	2	21	13	1	28	2	1
MdGLT1A	2	3	37	34	3	40	2	1
MdGLT1B	2	2	34	34	3	41	3	1

Table 3. Subcellular localization and signal peptide of nitrogen assimilation enzymes in apple.

Protein Name	Peroxisome	Mitochondria	Chloroplast	Cellular	Cell Skeleton_Cell Membrane	Cell Membrane	Cell Skeleton	Cell Nucleus	Cytoplasm_ Mitochondria	Chloroplasts_Mitochondria
MdNIA1A	7	4	2							
MdNIA1B	2			7	2	1.5	1.5	1		
MdNIR1			13							
MdGS1.1A				14						
MdGS1.1B				14						
MdGS1.1C				14						
MdGS1.4A				14						
MdGS1.4B				14						
MdGS2A			13							
MdGS2B			12					1		
MdGLU1			14							
MdGLT1A		7.5	6						4.5	
MdGLT1B		4	8.5						2.83333	6.83333

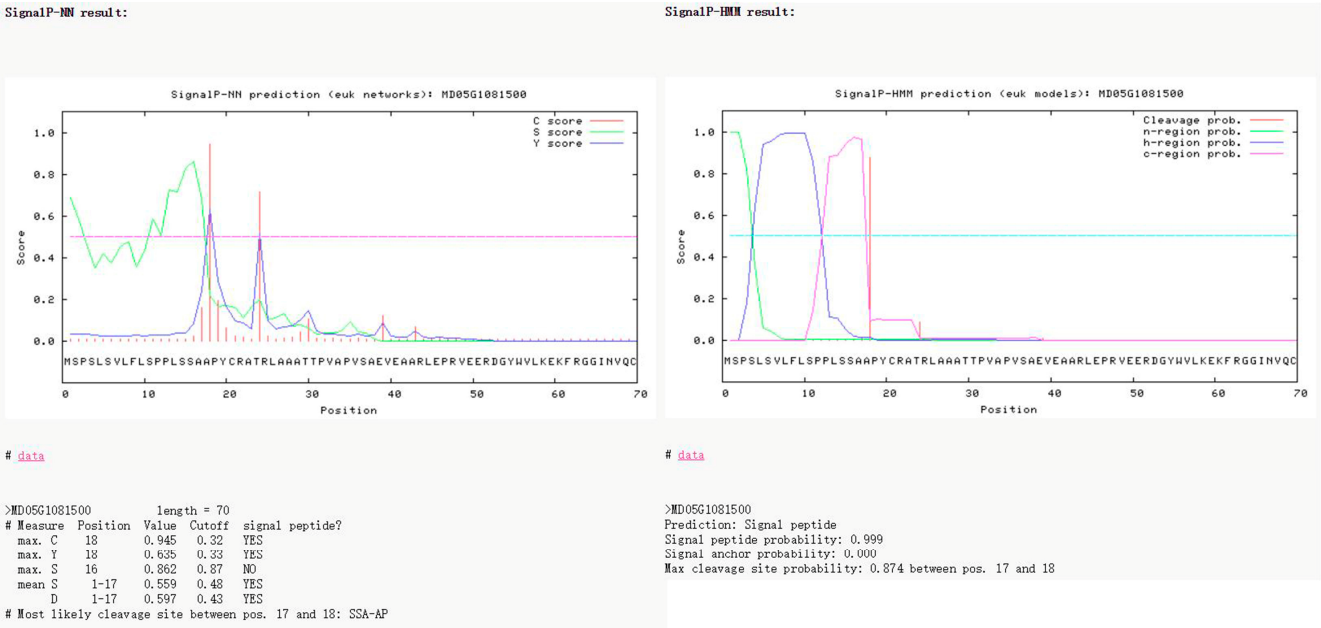


Figure 1. MdNIR1 signal peptide prediction.

3.4. Construction of Interaction Protein Network for Nitrogen Assimilation Enzymes in Apple

In order to predict the potential biological function of apple nitrogen assimilation enzymes, a protein interaction relationship network of key enzymes of nitrogen assimilation in apples was constructed based on the homologous sequences in the model species *Arabidopsis*. In Figure 2, the results show that there is a very close relationship between the proteins of the key enzymes of nitrogen assimilation, and they all have a possible interaction with NRT2.1 and NRT2.2.

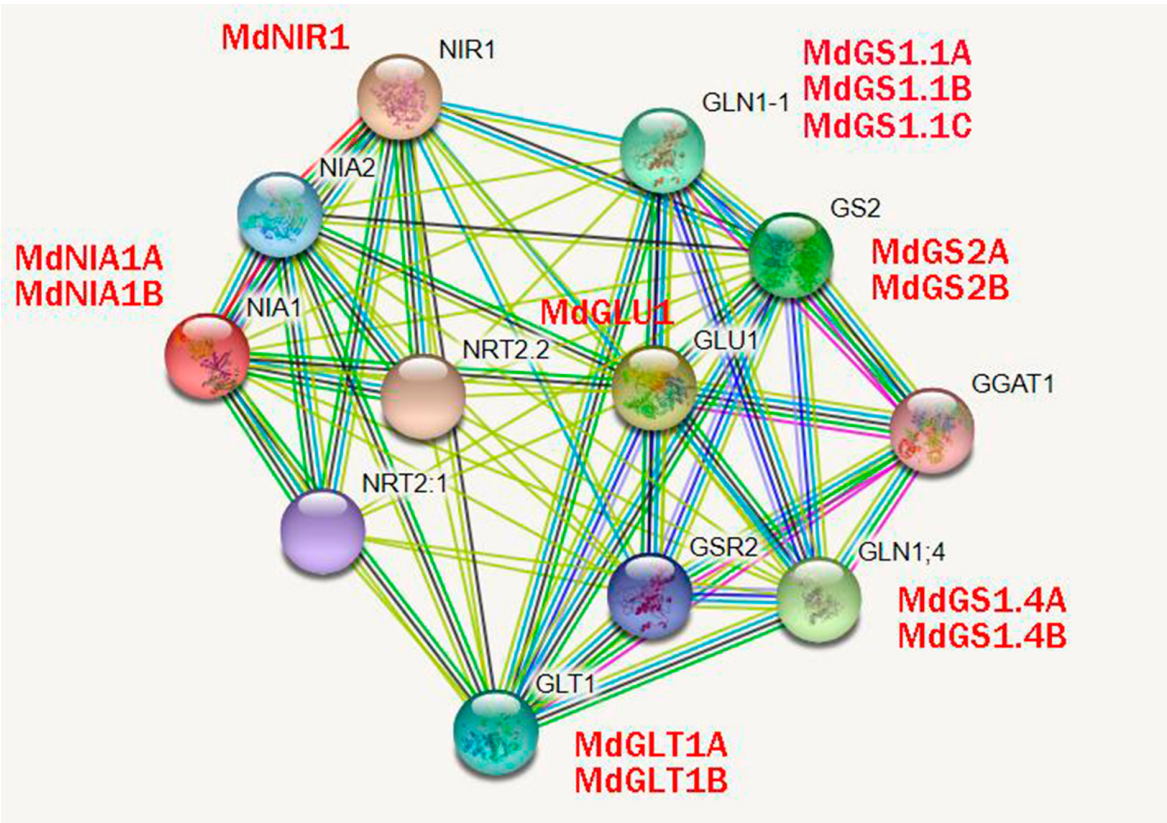


Figure 2. Nitrogen assimilation enzyme protein interaction network in apples and Arabidopsis.

3.5. Chromosome Localization and Gene Structural Analysis of Apple Nitrogen Assimilation Enzyme Genes

Chromosome localization and gene structure analysis were performed on apple nitrogen assimilation enzyme genes using apple genome annotation (*Malus domestica*.v1.0.consensus.gff). Through comparative analysis, it was found that the distribution of each gene was relatively scattered. Among them, MdGLT1.1 is located in chromosome 1, MdNIR1 is located in chromosome 5, MdGLT1.2 is located in chromosome 7, MdNIA1.1 is located in chromosome 8, MdGS1.1A is located in chromosome 9, MdGS1.4A and MdGS2.1 are located in chromosome 13, MdGLU1 and MdGS1.1B are located in chromosome 14, MdNIA1.2 is located in chromosome 15, MdGS14B and MdGS2.2 are located in chromosome 16, and MdGS1.1C is located in chromosome 17 (Figure 3). The introns and exons of each gene were analyzed; the results showed that there were four exons in MdNIA1A, MdNIA1B, and MdNIR1, and the number of exons in the remaining genes was significantly larger (Figure 4).



Figure 3. Chromosomal location of apple nitrogen assimilation enzyme genes.

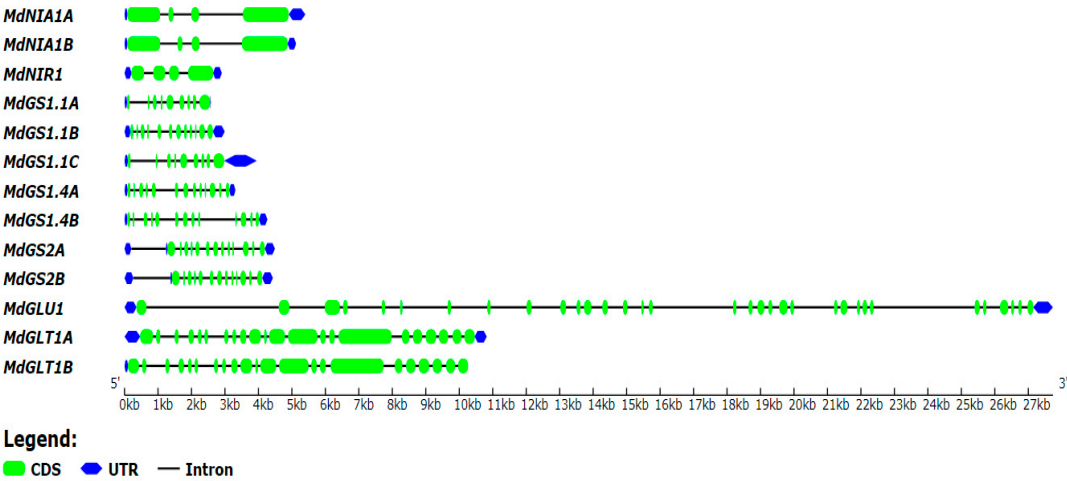


Figure 4. Gene structure (intron/exon) of apple nitrogen assimilation enzyme genes.

3.6. Analysis of Cis-Acting Elements in the Promoter of Apple Nitrogen Assimilation Enzyme Genes

The specific types of cis-acting elements present in the promoter region of a gene play a crucial role in determining the regulatory factors that influence gene expression. To investigate the potential regulatory factors associated with the nitrogen assimilation enzyme genes in apple, we performed a predictive analysis of the cis-acting elements within their promoters. In Table 4, the analysis revealed that *MdNIA1A* contained a maximum of 5 ERF elements, *MdNIA1B* had up to 10 ABRE elements, and *MdNIR1* possessed a maximum of 6 ERF elements. In *MdGS1.1A*, there were up to five ABRE elements, and *MdGS1.1B* contained three ABRE, ARE, and G-box elements each, while *MdGS1.1C* exhibited a maximum of three ARE and CAT-box elements. *MdGS1.4A* had up to three ABRE, three CGTCA-motif, and three TGACG-motif elements, and *MdGS1.4B* had up to five ABRE elements. *MdGS2A* contained a maximum of three ABRE and W-box elements, and *MdGS2B* had up to three G-box elements. *MdGLU1* exhibited a maximum of three CGTCA-motif and TGACG-motif elements. *MdGLT1A* contained up to 5 ABRE, CGTCA-motif, and TGACG-motif elements, and *MdGLT1B* had a maximum of 14 LTR elements. Taken together, the presence of these cis-acting elements suggests that the nitrogen assimilation enzyme genes may be involved in multiple responses, which require further studies to elucidate.

Table 4. Cis-acting elements of apple nitrogen assimilation enzyme gene promoters. Promoter analysis was performed on 2000 bp sequences upstream of the transcription start sites.

Cis-Acting Elements	<i>Md</i> <i>NIA1A</i>	<i>Md</i> <i>NIA1B</i>	<i>MdNI</i> <i>R1</i>	<i>Md</i> <i>GS1.1A</i>	<i>Md</i> <i>GS1.1B</i>	<i>Md</i> <i>GS1.1C</i>	<i>Md</i> <i>GS1.4A</i>	<i>Md</i> <i>GS1.4B</i>	<i>Md</i> <i>GS2A</i>	<i>Md</i> <i>GS2B</i>	<i>MdGL</i> <i>U1</i>	<i>MdGL</i> <i>T1A</i>	<i>MdGL</i> <i>T1B</i>
ABRE	3	10	4	5	3	1	3	5	3	2		5	3
ARE	2	2	1		3	3	1	2	2	1	2	1	3
CAT-box					1	3		1	1				
CGTCA-motif	2	1	3	1	1	1	3	3	1	2	3	5	
ERE	5		6	1				2	1	2	2		1
G-box	2	9	3	1	3		2	3	2	3		3	3
LTR			2	3	1	2			1		1		14
P-box					1	1		1					
TATC-box	1							1					
TC-rich repeats	3	1	1		1				2	2		1	
TCA-element		1				2					1	1	
TGA-element			1			1		1			1	2	1
TGACG-motif	2	1	3	1	1	1	3	3	1	2	3	5	
W-box			1			1		1	3	2	1	1	1

3.7. Expression Analysis of Apple Nitrogen Assimilation Enzyme Genes Under Nitrate Nitrogen Treatment

Following the treatment of apple seedlings with nitrate nitrogen for varying durations (0, 3, 6 and 12h), the expression levels of 13 genes associated with apple nitrogen assimilation enzymes were analyzed, revealing diverse patterns of change (Figure 5). The expression profiles of the *MdNIA1A* and *MdNIA1B* genes were analogous, exhibiting a marked increase in expression from 0 to 6 h, followed by a relatively stable high expression from 6 to 12 h. This suggests that nitrate nitrogen treatment significantly induced transcription in the early phase (0–6h), with expression stabilizing in the later phase. The *MdNIR1* gene expression was slightly upregulated between 0 and 6 h, decreased from 6 to 12 h, and peaked at 6 h, indicating that the gene's expression activation in response to nitrate nitrogen was primarily concentrated around 6 h post-treatment, after which expression was inhibited. The gene expression of *MdGS1.1A*, *MdGS1.1C*, and *MdGS1.4A* demonstrated an overall upward trend, with rapid upregulation from 0 to 6 h and sustained high levels from 6 to 12 h, suggesting that nitrate treatment facilitated their continuous expression,

with approximately 6 h identified as a critical induction point. The expression profile of the *MdGS1.1B* gene remained notably low and stable from 0 to 6 h, followed by a marked increase from 6 to 12 h. This suggests a delayed transcriptional response to nitrate nitrogen, with significant activation occurring after 6 h. Conversely, the *MdGS1.4B* gene exhibited a gradual increase in expression from 0 to 6 h, peaking at 6 h, and subsequently declining sharply by 12 h. This pattern is indicative of a typical short-term high induction followed by rapid attenuation. The expression levels of *MdGS2A*, *MdGLU1*, *MdGLT1A*, and *MdGLT1B* demonstrated an initial increase, peaking around 6 h, before declining, suggesting that these genes are upregulated by nitrate nitrogen between 0 and 6 h, with a subsequent downregulation from 6 to 12 h. This indicates that their response to nitrate nitrogen is most pronounced at the midpoint of the treatment (6 h), with negative regulation occurring thereafter. The expression of the *MdGS2B* gene showed a slight decrease between 0 and 3 h, an upregulation to a peak between 3 and 6 h, and a decline from 6 to 12 h. This gene's response dynamics differ from the others, with the 3 to 6 h window being critical for activation. In summary, there are significant differences in the response patterns of different nitrogen assimilation-related enzyme genes to nitrate nitrogen treatment.

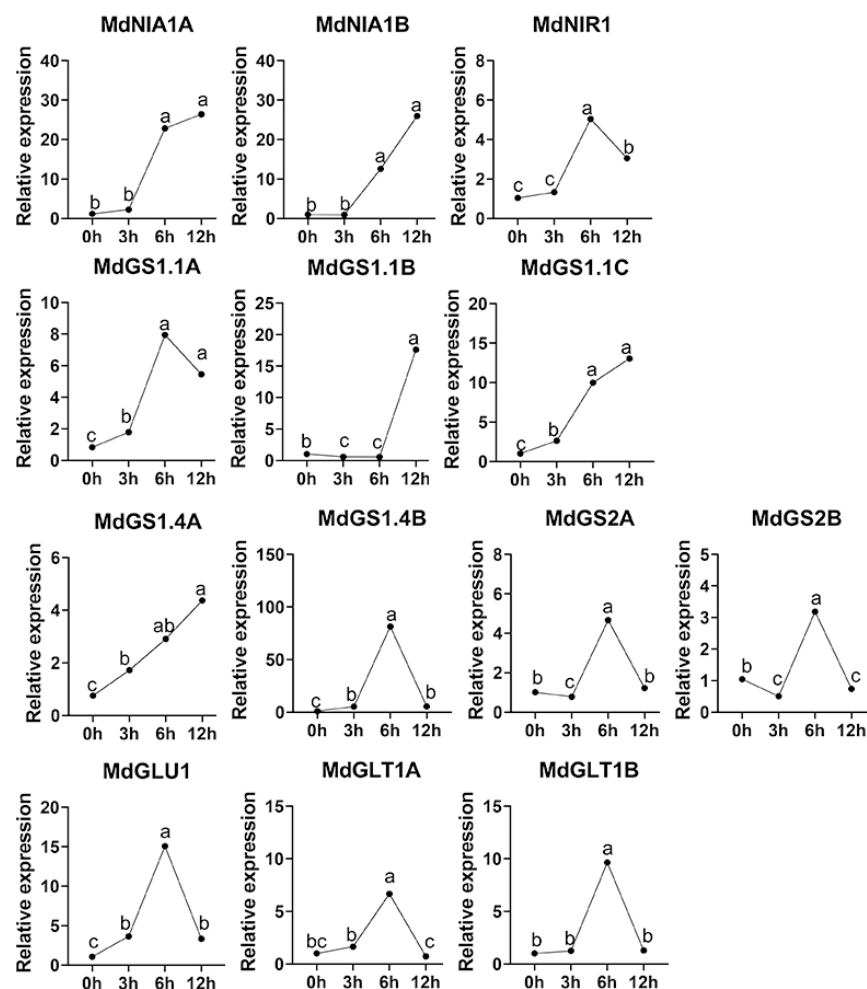


Figure 5. Real-time fluorescence quantitative PCR primers for relative expression of apple nitrogen assimilation enzymes. *Md18S* was used as a control gene. One-way ANOVA with Tukey's multiple comparison test shows significant differences at $p < 0.05$, marked by different letters, $n \geq 3$.

4. Discussion

The present study provides a comprehensive genomic and expression analysis of four key nitrogen assimilation enzyme families (NIA, NIR, GS, and GOGAT) in apple

(*Malus domestica*). By systematically characterizing 13 genes encoding these enzymes, we elucidated their structural diversity, subcellular localization, cis-regulatory elements, and dynamic expression patterns in response to nitrate treatment. This study addresses a significant knowledge gap in nitrogen metabolism in perennial fruit trees and offers new insights into the molecular mechanisms governing nitrogen use efficiency (NUE) in apple.

4.1. Gene Identification and Subcellular Localization Analysis

We identified 13 nitrogen-assimilation-related genes in apple—2 *MdNIAs*, 1 *MdNIR*, 7 *MdGSs*, and 3 *MdGOGATs*—highlighting the evolutionary expansion and functional diversification of these families in woody plants. Gene lengths varied from 2577 to 27,736 bp, and encoded proteins displayed distinct isoelectric points (*pI* 5.55–6.63), suggesting adaptive specialization and potential tissue- or context-specific regulatory roles (Table 1). For instance, the unusually long coding sequence of *MdGLT1A* (6627 bp) may reflect complex post-transcriptional regulation or protein interactions, warranting further functional studies (Table 1). Notably, plant NIR enzymes differ from microbial counterparts; for example, *Bacillus tequilensis* A2 NiR-A2 contains [Fe-S] cluster-binding domains and NADH-binding motifs, with optimal activity at 40 °C and pH 6.0 [35]. In contrast, our study revealed that *MdNIR1* is chloroplast-localized with a *pI* of 6.63 (Tables 1 and 3).

Subcellular localization predictions revealed patterns consistent with enzyme function. *MdNIA1A* localizes to peroxisomes and *MdNIR1* to chloroplasts (Table 3), aligning with their sequential roles in nitrate reduction ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+$). *MdGLT1* exhibits dual localization in mitochondria and chloroplasts, suggesting a role in coordinating energy metabolism and nitrogen recycling (Table 3). Multiple *MdGS* isoforms show compartment-specific localization, *MdGS1* in the cytosol and *MdGS2* in chloroplasts (Table 3), indicating specialized ammonium assimilation pathways and enhancing nitrogen metabolism through compartmentalized carbon–nitrogen interactions.

The 13 genes are distributed across eight chromosomes, identifying potential targets for marker-assisted selection. For example, the co-localization of *MdGS1.4A* and *MdGS2A* on chromosome 13 could facilitate pyramiding favorable alleles to improve NUE (Figure 3). Future studies should assess allelic variation and haplotype diversity among apple cultivars.

4.2. Regulatory Networks and Expression Dynamics

Promoter analysis identified diverse cis-regulatory elements, including ABRE, ERE, and G-box motifs, indicating complex transcriptional regulation by hormones (ABA and ethylene), light, and stress signals. The high frequency of ABRE motifs in *MdNIA1A/B* and *MdGLT1A/B* suggests potential cross-talk between nitrogen assimilation and abiotic stress responses (Figure 4), consistent with findings that ABA modulates nitrate reductase activity in *Arabidopsis* [1]. Light-responsive elements, particularly the G-box in *MdNIR1* and *MdGS2*, suggest coordination between nitrogen metabolism and photosynthesis (Figure 4), as observed in *Arabidopsis*, rice, and wheat [2].

The divergent expression patterns observed under nitrate treatment (0–12 h) indicate a temporally coordinated cascade of nitrogen assimilation. Early induction of *MdNIA1A/B* (0–6 h) promotes rapid nitrate reduction to nitrite, providing substrates for *MdNIR1*, which peaks at 6 h (Figure 5). This sequential activation mirrors the metabolic progression ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_4^+$) and highlights efficient substrate channeling. The delayed induction of *MdGS1.1B* (beyond 6 h) suggests a role in later stages of nitrogen remobilization or nitrogen reallocation under stress (Figure 5). Meanwhile, transient peaks of *MdGS2A*, *MdGLU1*, and *MdGLT1A/B* at 6 h indicate involvement in mid-stage ammonium assimilation, likely supporting rapid amino acid synthesis necessary for growth (Figure 5). These patterns

contrast with rice, where *OsGS1;1* and *OsGS2* are co-induced, indicating distinct regulatory strategies in apple [19].

4.3. Gene Function Comparison and Interaction Networks

Comparative analysis of nitrogen assimilation between apple and model species such as *Arabidopsis* and rice reveals both conserved and divergent features. Notably, apple's single *MdNIR1* aligns with the monocot pattern exemplified by rice *OsNIR* (Table 1), whereas the presence of two *MdNIA* genes reflects the dicot-specific duplication seen in *Arabidopsis* (*NIA1/NIA2*) (Table 1) [8,13]. Additionally, the expansion of the *MdGS* family to seven members and the *MdGOGAT* family to three members exceeds *Arabidopsis* (*GS*: 5; *GOGAT*: 2 *Fd-GOGAT*, 1 *NADH-GOGAT*) (Table 1), suggesting adaptations to the metabolic demands of woody growth and fruit development [36,37]. The functional divergence and dynamic expression of these genes present promising targets for enhancing nitrogen use efficiency (NUE) in breeding programs. Constitutive overexpression of *MdNIA1A/B*, which both maintain high expression under nitrate treatment, could improve early nitrate reduction (Figure 5). In contrast, modulating *MdGS1.1B* or *MdGS2A/B*, which show delayed or transient induction, may optimize ammonium assimilation without imposing metabolic stress (Figure 5).

The predicted protein interaction network indicates links between nitrogen assimilation enzymes and nitrate transporters (*NRT2.1/2.2*), highlighting integrated regulation of nitrogen uptake and assimilation (Figure 2). This aligns with findings in *Arabidopsis*, where *NRT2*-mediated nitrate uptake is closely coordinated with nitrate reductase activity via feedback mechanisms [9]. Experimental validation of these interactions using co-immunoprecipitation or yeast two-hybrid assays could further elucidate the coordination of nitrogen transport and metabolism in apple.

This study identified key genes involved in nitrogen assimilation in apple. Analysis of their structural and functional diversity, along with examination of their expression patterns under nitrate treatment, provides a foundation for improving nitrogen use efficiency in apple cultivation. Future research can leverage these insights to reduce nitrogen fertilizer input without compromising yield or fruit quality, supporting sustainable agricultural practices.

5. Conclusions

This study systematically examined 13 nitrogen assimilation-related genes (2 *MdNIAs*, 1 *MdNIR*, 7 *MdGSs*, and 3 *MdGOGATs*) in apple, revealing structural variation and distinct expression patterns in response to nitrate treatment. Subcellular localization and promoter cis-element analyses further highlight their specialized functions. These genes represent potential targets for breeding nitrogen-efficient apple varieties. Future work should focus on functional validation, mapping regulatory networks, and conducting field trials to evaluate practical applications in sustainable apple production. Such advances could reduce fertilizer dependence while maintaining yield and quality, promoting eco-friendly orchard management.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/nitrogen6040099/s1>: Table S1: Primers used in this study.

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