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# Comparative analysis of NR and NiR gene families reveals nitrite formation mechanisms in two *Toona* species

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## Abstract

**Background** *Toona sinensis* and *Toona ciliata* both belong to the same genus in the Meliaceae family; however, the nitrate and nitrite contents in *T. sinensis* are significantly higher than those in *T. ciliata*. Given the growing emphasis on dietary health, it has become imperative to implement breeding strategies to reduce the excessive nitrite levels in *T. sinensis*. Nitrate reductase (NR) and nitrite reductase (NiR) are crucial enzymes in plant nitrogen metabolism. In this study, we employed a comparative analysis of the NR and NiR gene families in *T. sinensis* and *T. ciliata*. By integrating bioinformatics and expression pattern assessments, we aimed to elucidate the underlying factors contributing to the variance in nitrate and nitrite levels between these two species.

**Results** *T. sinensis* exhibited higher nitrate and nitrite contents than *T. ciliata*. Through comprehensive genome-wide analysis, we identified two *TsNRs* and two *TsNiRs* in *T. sinensis*, compared to one *TcNiR* and two *TcNiRs* in *T. ciliata*, suggesting an expansion of NR members in *T. sinensis* relative to *T. ciliata*. The NR and NiR proteins in *T. sinensis* and *T. ciliata* share high sequence similarity and exhibit close genetic relationships with their counterparts in *Populus trichocarpa* and *Salix purpurea*. The exon–intron structures and conserved motifs of these genes were stringently conserved throughout evolutionary history. All the seven NR and NiR genes in both *T. sinensis* and *T. ciliata* harbor various cis-regulatory elements within their promoter sequences, which are associated with development, stress response, and hormonal regulation. Furthermore, the expression of *TsNiR* and *TcNiR* genes was also found to be tissue-specific. Among them, *TcNR1* and *TsNR2* exhibited the highest expression observed in mature leaves. Especially, the expression level of *TsNR2* in mature leaves was 400-fold higher than in other tissues. In addition, transient overexpression of *TsNiR1* and *TsNiR2* in *T. sinensis* significantly reduced nitrite content.

**Conclusions** The higher nitrite accumulation in *T. sinensis* compared to *T. ciliata* is attributed to its lower nitrite reductase activity, the expansion of the NR gene family, and the elevated expression of *TsNR2* in the leaves.

**Keywords** *Toona sinensis*, *Toona ciliata*, Nitrate reductase gene (NR), Nitrite reductase gene (NiR), Gene expression, Transient expression

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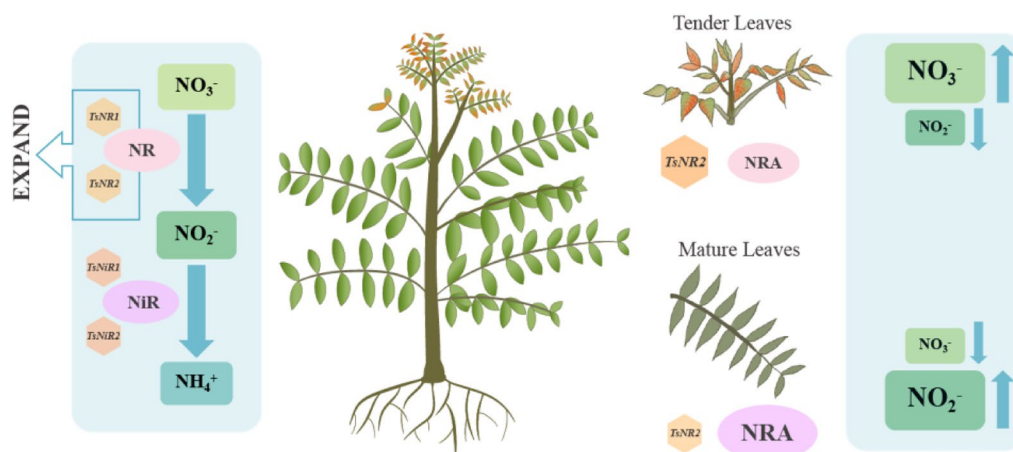
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## Graphic Abstract



## Introduction

*Toona sinensis* (A.Juss.) Roem and *Toona ciliata* Roem are perennial deciduous tree species of the Meliaceae family, commonly referred to as Chinese mahogany due to their high-value timber [1]. *T. sinensis*, commonly called Chinese toon or toon, has been cultivated for over two millennia in tropical to warm temperate regions worldwide [2, 3]. This distinguished tree species is highly esteemed for its fragrant leaves and edible sprouts, valued as a seasonal delicacy and commonly referred to as “tree vegetables” [4]. *T. sinensis* is renowned for its remarkable nutritional and medical values, distinguishing itself among vegetables with its high protein content. In addition, it offers a diverse range of essential nutrients, including fatty acids, amino acids, vitamins, dietary fiber, soluble sugars, and numerous bioactive compounds, such as flavonoids and alkaloids [3, 5, 6]. Beyond its edibility, *T. sinensis* also plays a pivotal role in afforestation efforts, urban and rural greening, timber production, and medicinal applications [7, 8]. *T. ciliata*, a sister species of *T. sinensis*, is highly regarded not only as a valuable timber resource but also for its significant market potential, attributed to the medicinal properties of its roots, stems, and leaves [1, 9, 10]. Therefore, both of these tree species embody a remarkable blend of edible, ecological, and economic value, making them truly invaluable assets.

Both *T. sinensis* and *T. ciliata* exhibit sensitivity to nitrogen, with nitrogen application significantly promoting their growth. Plants primarily assimilate nitrogen from soil in the forms of nitrates and ammonium salts [11]. Nitrates are absorbed by plant roots and subsequently transported through the xylem to the aerial parts of the plants. Nitrate reductase (NR), located in

the cytoplasm, catalyzes the reduction of nitrates to nitrites, thereby initiating nitrogen assimilation and impacting both nitrate utilization efficiency and nitrite accumulation [12, 13]. However, excessive nitrite accumulation in plant cells can be toxic [14]. When humans consume vegetables with excessive nitrite accumulation, most of the nitrite is excreted in the urine, while a small fraction enters the gastrointestinal tract. There, it interacts with endogenous amines, forming reactive nitrides, including potentially carcinogenic N-nitrosamines, which pose substantial health hazards [15, 16]. The efficiency of NR and nitrite reductase (NiR) in primary nitrogen assimilation plays a crucial role in regulating plant growth and development [13, 17]. NR, localized either in the cytoplasm or anchored to the cell membrane, acts as a rate-limiting enzyme during nitrate reduction. It accomplishes the nitrate-to-nitrite conversion by donating two electrons via NADH and NADPH [18]. In eukaryotes, three types of nitrate reductase have been identified, with the NADH-specific NR being prevalent in higher plants [19, 20]. The second type, NAD(P)H-bispecific NR, is rare and has been identified in *Betula pendula*, where it occurs in both homologous and heterogeneous forms. This variant coexists with the NADH-specific NR in soybeans and monocotyledons, such as maize, rice, and barley. Finally, the NADPH-specific NR is uniquely found in fungi and mosses [21]. NiR, the subsequent enzyme in the nitrate assimilation pathway, facilitates the transfer of six electrons from degraded ferredoxin to nitrite, leading to its reduction [22]. NiR, encoded by nuclear genes, is transported to the chloroplast, where a 330-amino-acid signal peptide is excised during

transmembrane passage. NiR is primarily localized in C3 plant leaf chloroplasts, mesophyll cell chloroplasts, and plastids of non-photosynthetic tissues in C4 plants [23, 24]. A diverse array of environmental and internal factors regulates the activity of plant nitrate reductase. Environmental influences encompass nitrogen source, light intensity, O<sub>2</sub>/CO<sub>2</sub> ratio, pH levels, and temperature. In addition, internal regulators such as metabolites and plant hormones also play a significant role in modulating this enzyme's function [24, 25].

The overexpression of NR or NiR genes significantly elevates mRNA levels and enhances nitrogen uptake in plants. Notably, the cDNA of the NiR gene has been successfully cloned across various higher plant species, including *Arabidopsis thaliana* [26], rice (*Oryza sativa* L.) [27], maize (*Zea mays*) [28], tobacco (*Nicotiana tabacum* L.) [29], spinach (*Spinacia oleracea* L.) [30], Chinese cabbage (*Brassica campestris* L. ssp. *Chinensis* (L.) Makino) [31], and cotton (*Gossypium hirsutum*) [32]. The overexpression of the *NtNiR* gene in tobacco can double the NiR activity [29]. Furthermore, overexpression of the spinach *NiR* gene in *Arabidopsis thaliana* effectively modulates the assimilation of nitrite (NO<sub>2</sub><sup>-</sup>), highlighting its essential role in regulating nitrogen homeostasis [26]. The *AtNiR* gene was introduced into tobacco to mitigate nitrite accumulation, resulting in transgenic lines exhibiting a fivefold increase in NiR activity compared to the wild-type plant, along with reduced nitrite content in selected lines. Therefore, the precise regulation of nitrogen assimilation through the NR and NiR metabolic pathways presents a viable strategy to prevent the harmful overaccumulation of nitrite in plants, protecting human health and optimizing nutrient utilization.

During the growth and maturation of vegetables, nitrites tend to accumulate, posing potential risks such as increased gastrointestinal cancer, methemoglobinemia, and other health concerns when consumed in high amounts [33, 34]. Notably, the leaves of *T. sinensis* are known to accumulate high levels of nitrite [35]. In our study of the *Toona* population, we found that *T. ciliata*, a close relative of *T. sinensis*, has accumulated a small amount of nitrite (Figure S1). As a result, these two *Toona* species provide ideal models for investigating the molecular mechanisms underlying differential nitrite accumulation. Considering the potential significance of the NR and NiR gene families, we performed a comparative genome-wide identification of NR and NiR genes between *T. sinensis* and *T. ciliata*. We also analyzed and compared their gene structures, phylogenetic relationships, protein motifs, chromosomal locations, collinearity, and expression patterns. Then *TsNiR1* and *TsNiR2* were cloned and performed transient expression of these genes in *T. sinensis* to verify their effects on

nitrite content. This study provides crucial insights into the mechanisms of nitrite formation and gene regulation.

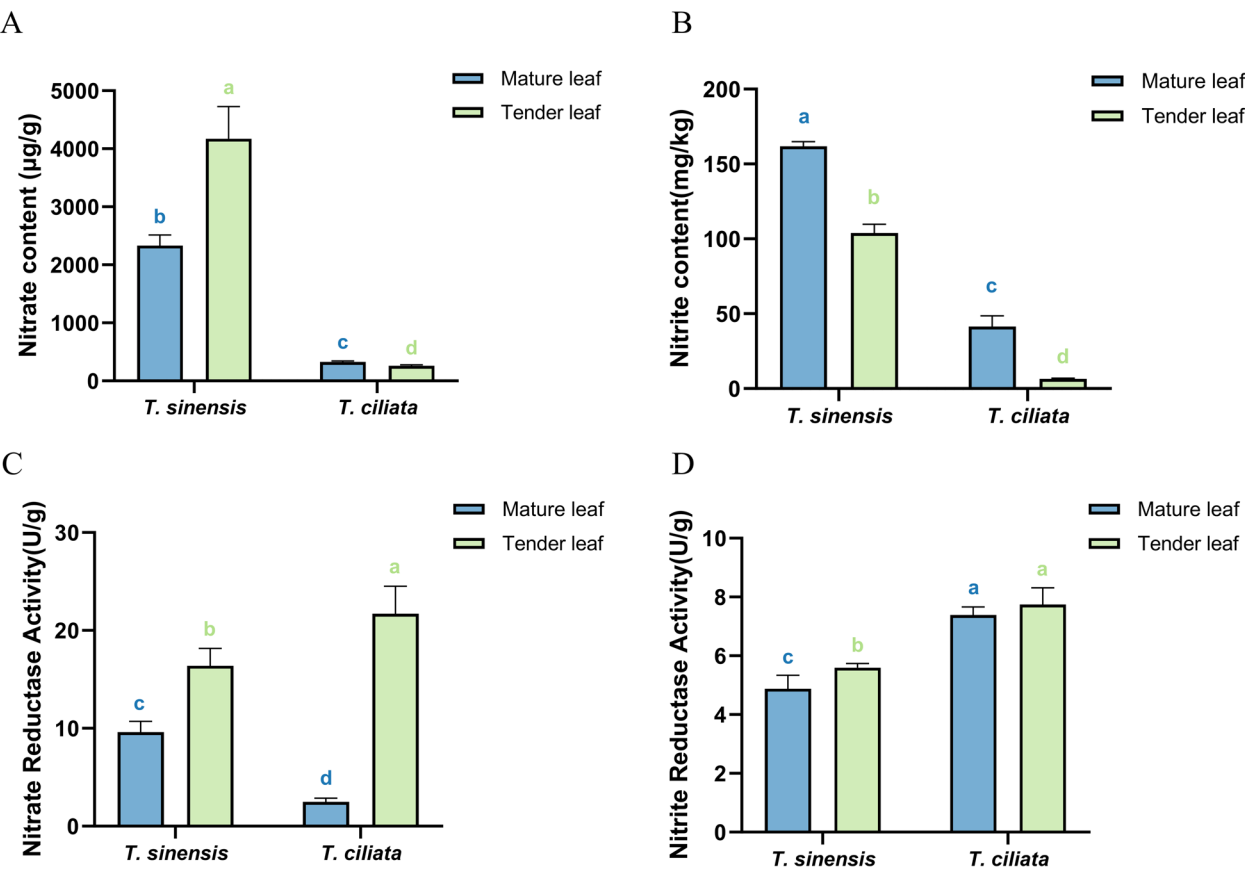
## Results

### *T. sinensis* displays higher nitrate and nitrite contents than *T. ciliata*

The nitrate and nitrite contents in *T. sinensis* were significantly higher than those in *T. ciliata*, with both differences reaching statistical significance (Fig. 1A, B). In *T. sinensis*, nitrate levels were elevated in tender leaves compared to mature leaves (Fig. 1A), whereas nitrite levels were comparatively higher in mature leaves (Fig. 1B). In contrast, both nitrate and nitrite contents in *T. ciliata* were consistently higher in mature leaves than in tender leaves (Fig. 1A, B). In both germplasm sources, except for nitrite reductase activity (NiRA) in *T. ciliata*, nitrate reductase activity (NRA) and NiRA were consistently higher in tender leaves compared to mature leaves (Fig. 1C, D). In addition, the NiRA activity in *T. sinensis* was markedly lower than in *T. ciliata*, possibly leading to the accumulation of nitrite as a substrate.

### NR members expand in *T. sinensis* compared to *T. ciliata*

Based on their phylogenetic relationships, the seven NR and NiR members were identified and named as *TsNR1*, *TsNR2*, *TsNiR1*, *TsNiR2*, *TcNR1*, *TcNiR1*, and *TcNiR2* (Table 1). A physicochemical analysis of the NR proteins revealed that each consisted of 902 amino acids, with molecular weights ranging from 102,051.34 to 102,217.78 Da. The isoelectric points (pIs) of these proteins varied between 6.3 and 6.78. Furthermore, all NR proteins exhibited enhanced stability, as indicated by instability indices below 40. The lipophilicity indices ranged between 77.66 and 79.92, and the overall hydrophilicity index was negative, confirming the hydrophilic nature of all NR proteins. The molecular weight of *TcNR1* was notably lower than those of the other *TsNR* proteins, with *TcNR* also exhibiting stable hydrophilic properties. The physicochemical properties of *TcNiR* proteins from *T. ciliata* closely resemble those of *T. sinensis*. Their molecular weights, ranging from 64,336.81 to 66,597.72 Da, are smaller than those of the NR proteins. The pIs of the NiR proteins range from 5.54 to 6.2, indicating a varied charge environment. The instability indices for both *TsNiR* and *TcNiR2* proteins are below 40, indicating their stability. The aliphatic indices of *TsNiR1* and *TsNiR2* are 88.42 and 88.43, respectively, while the overall hydrophilicity index is negative, confirming the hydrophilic nature of all *TsNiR* and *TcNiR* proteins. This comprehensive characterization highlights the consistent



**Fig. 1** Significant differences in the samples of *T. sinensis* and *T. ciliata*. **A** Nitrate content; **B** nitrite content; **C** nitrate reductase activity; **D** nitrite reductase activity. Data are represented as the mean  $\pm$  SD of three independent replicates, and different letters indicate statistically significant differences after SNK multiple comparisons ( $p < 0.05$ )

**Table 1** Basic characteristics of identified TsNRs and TsNiRs in *T. sinensis* and *T. ciliata*

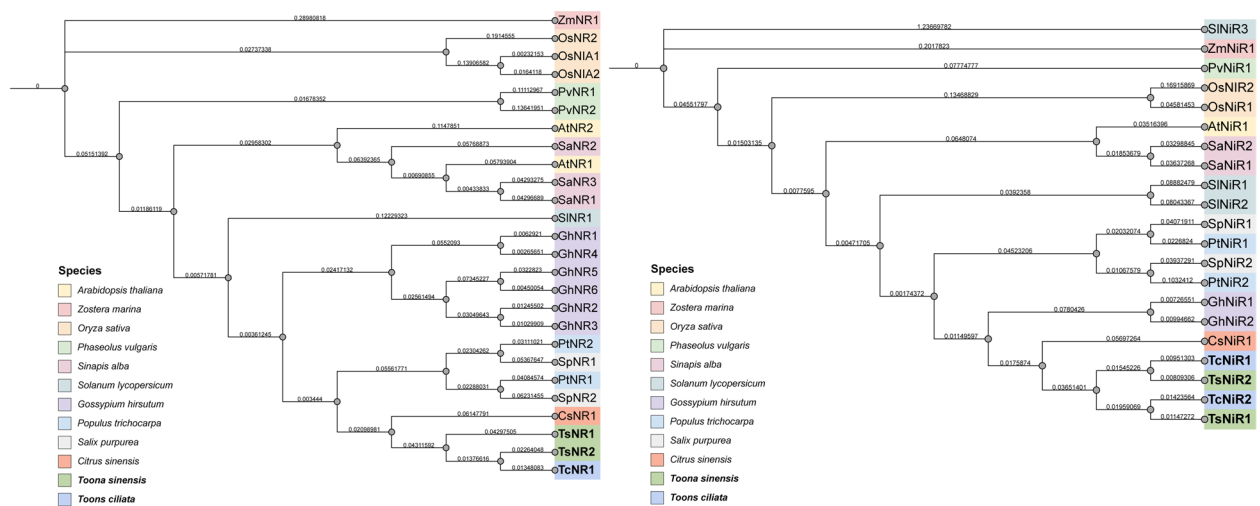
|     | Gene          | Sequence ID   | Number of amino acid | Molecular weight | pI   | Instability index | Aliphatic index | Grand average of hydropathicity |
|-----|---------------|---------------|----------------------|------------------|------|-------------------|-----------------|---------------------------------|
| NR  | <i>TsNR1</i>  | Maker00008733 | 902                  | 102,217.78       | 6.3  | 37.1              | 79.92           | − 0.378                         |
|     | <i>TsNR2</i>  | Maker00000389 | 902                  | 102,051.34       | 6.78 | 38.82             | 77.66           | − 0.422                         |
| NiR | <i>TsNiR1</i> | Maker00010156 | 595                  | 66,597.72        | 6.2  | 39.85             | 88.42           | − 0.267                         |
|     | <i>TsNiR2</i> | Maker00014338 | 575                  | 64,336.81        | 5.54 | 38.67             | 88.43           | − 0.299                         |
| NR  | <i>TcNR1</i>  | Tci09G000250  | 902                  | 101,925.05       | 6.56 | 40.69             | 77.56           | − 0.442                         |
| NiR | <i>TcNiR1</i> | Tci27G008440  | 590                  | 66,004.71        | 5.74 | 41.17             | 86.53           | − 0.31                          |
|     | <i>TcNiR2</i> | Tci28G004970  | 593                  | 66,219.28        | 6.2  | 36.1              | 88.55           | − 0.252                         |

and stable nature of these proteins in both *T. sinensis* and *T. ciliata*.

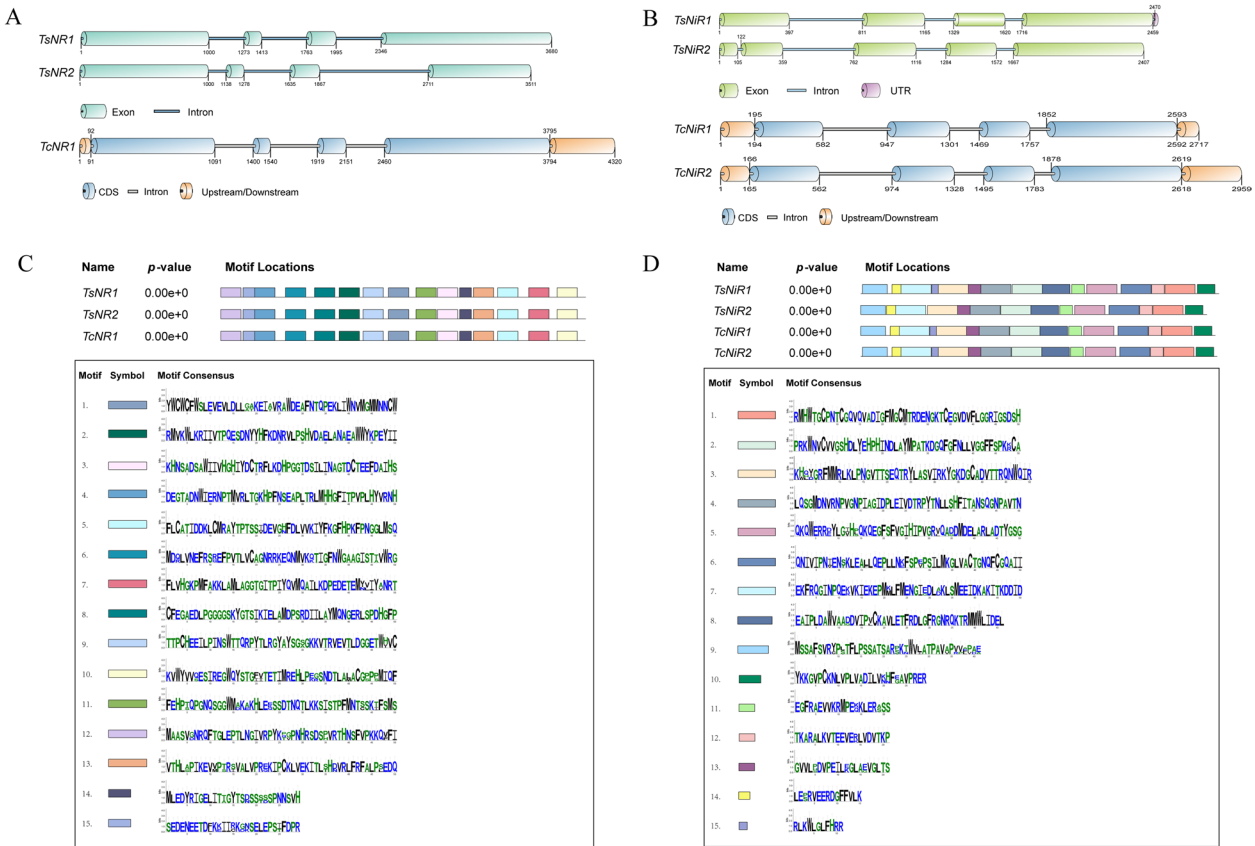
**NR and NiR gene families are evolutionarily conserved in both *T. sinensis* and *T. ciliata***

Phylogenetic analysis of the NR and NiR families, along with those from other species, indicated that proteins

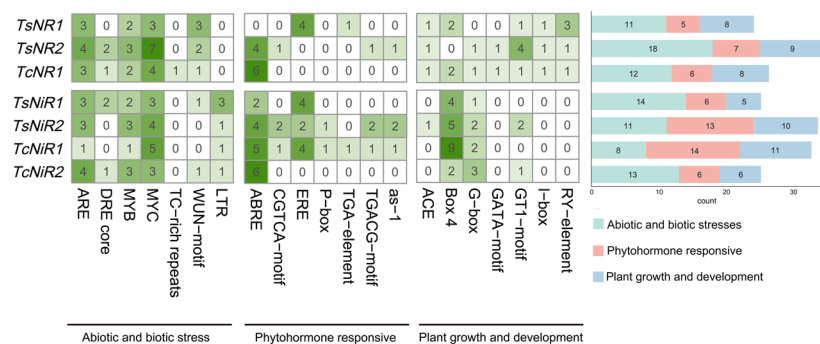
from *T. sinensis* and *T. ciliata* are most closely related, followed by poplar and willow (Fig. 2). The intron/exon composition of the NR and NiR genes in *T. sinensis* was identified using the GSDS tool and visualized with the IBS2.0 tool (Fig. 3A, B). The results revealed that members of the same gene family exhibited similar structures. *TsNR1* (Maker00008733) and *TsNR2* (Maker00000389)



**Fig. 2** Phylogenetic relationships of NR and NiR family proteins in *T. sinensis*, *T. ciliata* and other plant species. **A** Phylogenetic tree of NR family proteins. **B** Phylogenetic tree of NiR family proteins



**Fig. 3** Motifs and exon-intron structure of NR, NiR genes in *T. sinensis* and *T. ciliata*. **A** Exon-intron structure analysis of *TsNR* and *TcNR* genes. **B** Exon-intron structure analysis of *TsNiR* and *TcNiR* genes. **C** Conserved motif analysis of the *TsNRs* and *TcNRs*. **D** Conserved motif analysis of the *TsNiRs* and *TcNiRs*



**Fig. 4** Cis-regulatory elements of *NR*, *NiR* genes in *T. sinensis* and *T. ciliata*

of the *NR* gene family both contain four exons and three introns, with gene lengths exceeding 3500 bp. *TsNiR1* (Maker00010156) and *TsNiR2* (Maker00014338) in *T. sinensis* were phylogenetically clustered, each containing three introns and four exons, with gene lengths less than 2500 bp. This indicates that the *NR* and *NiR* gene families are evolutionarily conserved, while the diversity in exon/intron structures within the *NiR* gene families suggests functional differentiation among their descendant genes. To explore the potential functions of the *NR* and *NiR* family members, we used the MEME tool for conserved motif analysis of their protein sequences (Fig. 3C, D). These results showed that motifs 1–10 were highly conserved in *NR* family members, belonging to the NADPH domain. Motifs 1–10 are also conserved in *NiR* members, specific to the ferredoxin–nitrite reductase domain.

#### Cis-regulatory elements analysis of *NRs* and *NiRs*

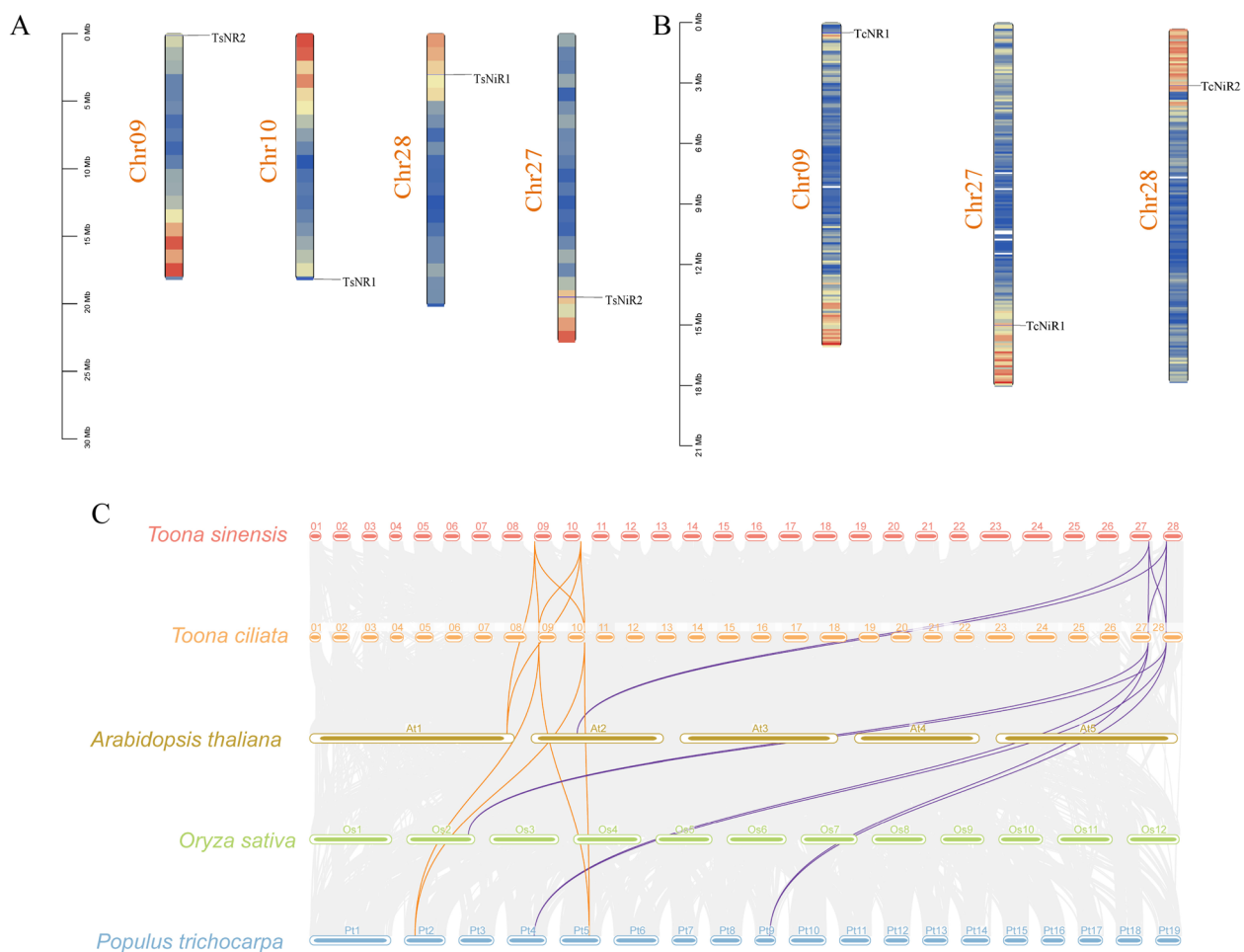
Cis-regulatory elements analysis revealed three categories of cis-acting elements in all promoters, which are associated with plant growth and development, phytohormone responses, and biotic and abiotic stresses [36]. The same cis-acting elements identified in both *TsNR* and *TcNR* promoters include antioxidant response element (ARE), MYB-binding element (MYB), MYC-binding element (MYC), injury response element (WUN-motif), stress response element (STRE), injury response element (WUN-motif), light response elements (ACE, GT1-motif), light response elements (I-box), and seed-specific expression elements (RY-element). There are also many identical cis-elements in *TcNiR* and *TsNiR* promoters, such as ARE, MYB, MYC, low-temperature response elements (LTR), abscisic acid response element (ABRE), and light response elements (Box 4, G-box) (Fig. 4). These results suggest that the expression of *TsNR/NiR* and *TcNR/NiR* genes may be regulated by external environmental factors, such as light, moisture, and stress. Besides environmental stresses, plant hormones such as abscisic acid, jasmonic acid, and others may also regulate the expression of this gene family.

#### Chromosomal distribution and collinearity analysis of *TsNRs* and *TsNiRs*

The two *TsNR* members and two *TsNiR* members were clearly positioned on four distinct chromosomes (Fig. 5A, B). *TsNR1* is located on chromosome 10, while *TsNR2* resides on chromosome 9. *TsNiR1* and *TsNiR2* are located on chromosomes 28 and 27, respectively. Similarly, *TcNR1* is found on chromosome 9, whereas *TcNiR1* and *TcNiR2* are mapped to chromosomes 27 and 28, respectively. To explore into the potential biological roles of *TsNR* and *TsNiR* genes in *T. sinensis*, a collinearity analysis was conducted to identify homologous gene pairs between *T. sinensis* and several other species, including *A. thaliana*, *O. sativa*, *P. trichocarpa*, and *T. ciliata* (Fig. 5C). The results revealed that all four *NR/NiR* genes in *T. sinensis* have homologous genes in *A. thaliana*, *O. sativa*, *P. trichocarpa*, and *T. ciliata*. Among the species examined, *T. sinensis* and *T. ciliata* displayed the strongest genetic homology, suggesting a close evolutionary relationship.

#### The expression patterns of *TsNR/NiRs* and *TcNR/NiRs* in various tissues

To investigate whether *NR* and *NiR* gene expression is associated with nitrite accumulation and metabolism, we performed quantitative real-time PCR (qRT-PCR) analysis on different tissues, including apical apex, tender leaves, mature leaves, petioles, tender stems (internodes 2–4), old stems (internodes 7–8), xylem and phloem (Fig. 6). The expression levels of all *NR* and *NiR* genes were predominantly higher in mature leaves compared to tender leaves, except for *TsNR1*, which was expressed at levels too low to detect. Notably, *TsNR2* and *TcNR1* exhibited high expression levels in mature leaves, followed by old stems, phloem, and tender leaves (Fig. 6A, D). Remarkably, the relative expression level of *TsNR2* in mature leaves was 400-fold higher than in other tissues, suggesting that *TsNR2* is the primary gene responsible for nitrite accumulation (Fig. 6A). *TcNiR1* and *TsNiR2* demonstrated the most pronounced difference between



**Fig. 5** Distribution of *NR/NiR* genes on the chromosomes of *T. sinensis* and *T. ciliata* and synteny analysis of *NR/NiR* gene with other species. **A** Distribution of *TsNR* and *TsNiR* genes on the chromosomes of *T. sinensis*. **B** Distribution of *TcNR* and *TcNiR* genes on the chromosomes of *T. ciliata*. **C** Synteny analysis of *TsNR* and *TsNiR* genes in the genomes among *T. sinensis*, *T. ciliata*, *A. thaliana*, *O. sativa*, and *P. trichocarpa*

mature and tender leaves (Fig. 6C, E). The expression profiles of *NR* and *NiR* genes demonstrated notable tissue specificity, suggesting their distinct roles in nitrite metabolism and accumulation across various plant tissues.

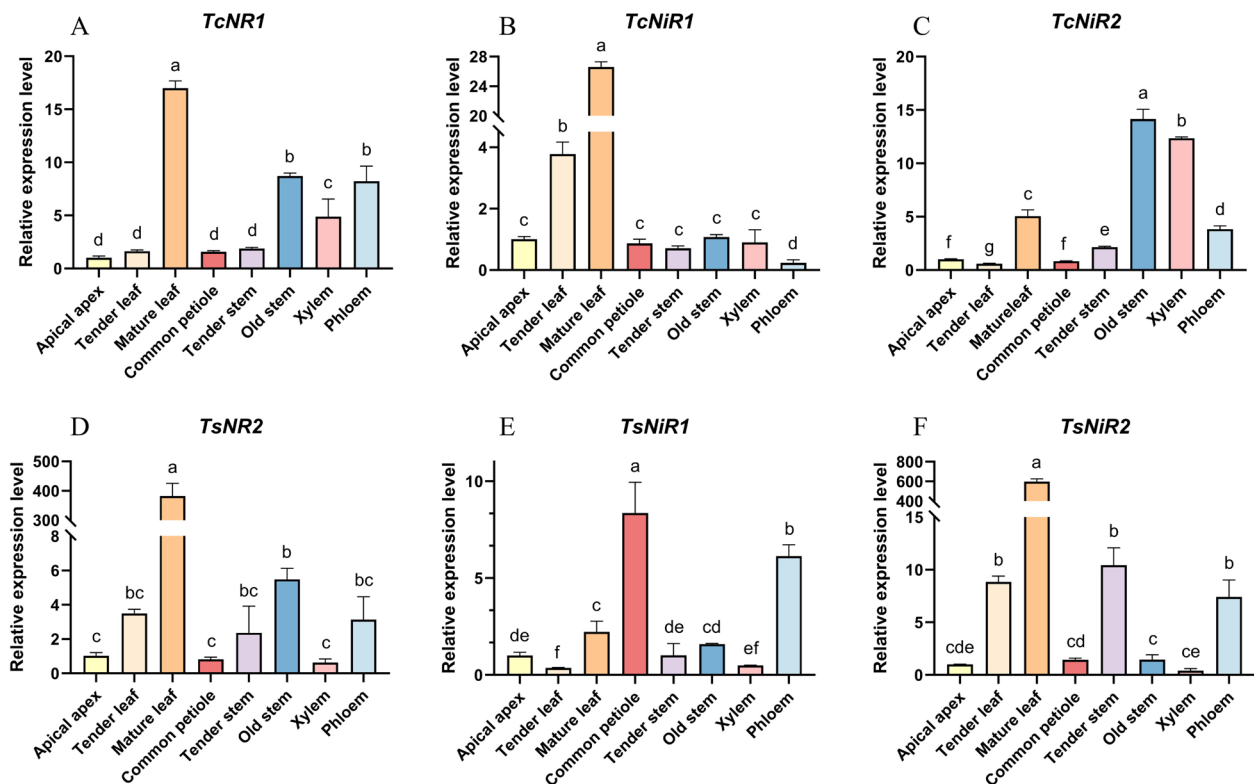
#### *TsNiR1* and *TsNiR2* reduce the nitrite content of *T. sinensis*

To further validate whether *TsNiR1* and *TsNiR2* are related to the nitrite content in *T. sinensis*, recombinant plasmids containing pBI121-*TsNiR1*-GFP, pBI121-*TsNiR2*-GFP, and an empty vector control were introduced into plants using the *Agrobacterium*-mediated transient transformation method. The seedlings were stained using a vacuum method and cultured in the dark for 24 h. The transformed seedlings were then grown under normal conditions for two days (Fig. 7A). After DNA extraction, PCR amplification was performed using 35S-F vector primers, and the reverse primer of the amplified gene. The results showed that the plants

transiently expressing *TsNiR1* and *TsNiR2* contained an approximately 1800 bp band, while the target bands were not detected in the empty vector control (Fig. 7B). The leaves of plants overexpressing *TsNiR1* or *TsNiR2* plants exhibited significantly reduced nitrite levels (Fig. 7C). These results indicate that the overexpression of *TsNiR1* and *TsNiR2* in *T. sinensis* significantly reduces nitrate and nitrite contents.

#### Discussion

*NR* and *NiR* are pivotal enzymes in plant nitrogen metabolism in most higher plants and algae [17]. *NR* catalyzes the reduction of nitrate ( $\text{NO}_3^-$ ) to nitrite ( $\text{NO}_2^-$ ), representing the first step in nitrogen assimilation, and affecting both nitrate utilization efficiency and nitrite accumulation levels. [37, 38]. The rapid reduction of nitrite is crucial as excessive nitrite accumulation in plant cells can be toxic [14, 39]. Through the coordinated

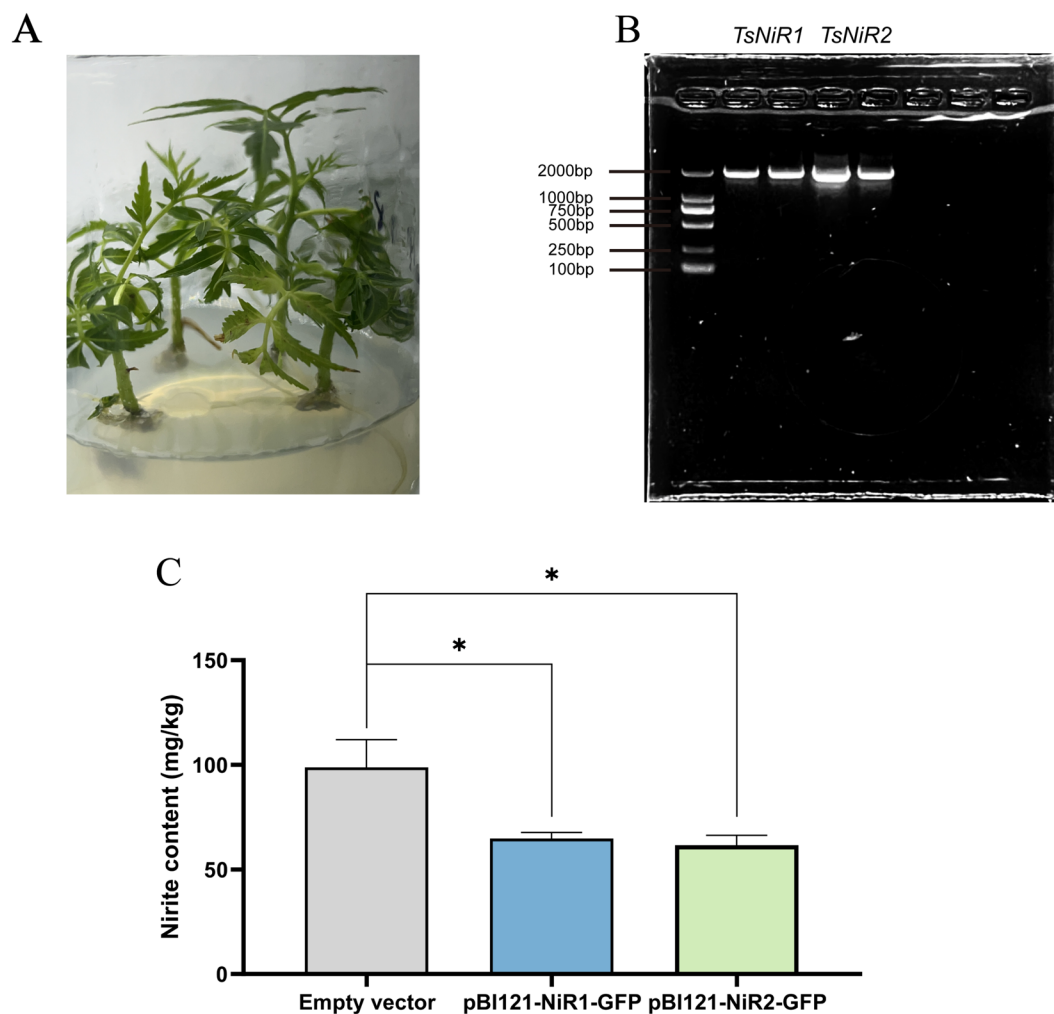


**Fig. 6** Expression profiles of NR and NiR genes. **A** Relative expression level of *TsNR2* in different tissues of *T. sinensis*. **B** Relative expression level of *TsNiR1* in *T. sinensis*. **C** Relative expression level of *TsNiR2* in *T. sinensis*. **D** Relative expression level of *TcNR1* in *T. ciliata*. **E** Relative expression level of *TcNiR1* in *T. ciliata*. **F** Relative expression level of *TcNiR2* in *T. ciliata*. Data are represented as the mean  $\pm$  SD of three independent replicates, and different letters indicate statistically significant differences ( $p < 0.05$ )

action of NR and NiR, plants efficiently convert inorganic nitrate from the environment into organic nitrogen compounds, preventing nitrite accumulation and safeguarding normal physiological metabolic processes [13]. *T. sinensis*, a popular spring seasonal vegetable and one of the rare woody species contains high levels of both nitrate and nitrite in its leaves, raising concerns regarding potential health risks associated with its consumption [7, 40, 41]. In this study, we measured nitrate content, nitrite content, NRA, and NiRA in *T. sinensis* and *T. ciliata*. In *T. sinensis*, the nitrite content in mature leaves was higher than in tender leaves, while the nitrate content was lower in mature leaves than tender leaves (Fig. 1). The NRA and NiRA in mature leaves of *T. sinensis* were lower than those in the tender leaves. The higher nitrate content in tender leaves requires more active NR for efficient metabolism. While this would typically result in higher nitrite levels in tender leaves, the enhanced activity of nitrite reductase in tender leaves ensures rapid conversion, leading to lower nitrite accumulation compared to mature leaves. The nitrite content and NR activity in the tender leaves of *T. sinensis* were also found to be consistently higher than in the stems, indicating that nitrate

reduction primarily occurs in leaves [42]. The findings of this study suggest that both nitrate content and nitrate reductase activity are consistently higher in the tender leaves of *T. sinensis* than in the stems. These results indicate that nitrate reduction in *T. sinensis* likely occurs primarily in the mature leaves.

In this study, two *TsNR* genes and two *TsNiR* genes were identified in the genome of *T. sinensis*. These genes were identical to those found in *P. trichocarpa* (two *PtNRs* and two *PtNiRs*), and *S. purpurea* (two *SpNRs* and two *SpNiRs*). However, there is only one NR in *T. ciliata*, which is one less than in *T. sinensis*. The phylogenetic tree analysis showed that *TsNRs* of *T. sinensis* were closely related to those in *T. ciliata*, *P. trichocarpa*, *S. purpurea*, and *C. sinensis*, especially to *T. ciliata*, which belongs to the same genus *Toona* in the Meliaceae family, with high sequence similarity between *TsNRs* and *TcNRs*. Similarly, the *TsNiRs* and *TcNiRs* branches were more clustered, indicating a close relationship. The exon–intron structure of genes is highly conserved during evolution, serving as a reliable marker of distant phylogenetic relationships [43, 44]. Analyzing the exon–intron structures of gene family members can uncover critical evolutionary



**Fig. 7** Validation and phenotypic characterization of transiently transformed *TsNiR1* and *TsNiR2* in *T. sinensis* plants. **A** *T. sinensis* seedlings with targeted transient gene expression. **B** Validation of transiently transformed *TsNiR1* and *TsNiR2* in *T. sinensis*. **C** Variations in nitrite content in *T. sinensis* after transient overexpression of *TsNiR1* and *TsNiR2*

relationships that may not be evident through basic sequence comparisons alone [45]. Intron gains and losses play crucial roles in gene family evolution. Intron gain can occur through the insertion of new exon fragments within existing introns, while intron loss can happen through the deletion of exon fragments. Intron gain and loss events contribute to gene structural diversification and influence gene regulation and function [46, 47]. In the *TsNiR* gene family, *TsNiR2* contains an additional exon segment compared to *TsNiR1*, demonstrating structural diversification between the two *TsNiR* genes. It has been shown that the first and last exons are typically the longest in a gene, suggesting the necessity of retaining larger exons at both the beginning and end of the coding sequence [43, 45]. This pattern is also observed in the *TsNR* and *TsNiR* genes, where the first or last exon is the longest across all four gene structures, suggesting

that selectively retaining larger first and last exons may be essential for transcription, splicing, or translation. The conserved structural domains present in the two *TsNR* and two *TsNiR* proteins are identical, indicating that members of the *NR* and *NiR* families are highly conserved throughout evolution.

Gene expression profiling across various tissues can elucidate the functions of specific genes in distinct tissues [48]. A radish *NiR* gene, *RsNiR*, exhibited higher expression in leaves and roots, and lower expression in petioles, stems, and lateral roots [49]. In this study, given the significant variance in nitrate and nitrite contents between *T. sinensis* and *T. ciliata*, which are both members of the same genus, we conducted qRT-PCR analyses across various tissues to determine if there were substantial differences in the expression of *NR* and *NiR* genes between these species. Analysis revealed that both *TcNR1* and

*TsNR2* were highly expressed in mature leaves. However, *TsNR2* expression in mature leaves of *T. sinensis* was exponentially greater than in any other tissue, suggesting that this elevated expression may lead to enhanced reduction of nitrate to nitrite, thereby resulting in higher nitrite accumulation in the mature leaves of *T. sinensis* compared to *T. ciliata*. Moreover, *TsNiR1*, functioning as a nitrate reductase, did not exhibit elevated expression in mature leaves relative to other tissues, further contributing to nitrite accumulation in mature leaves of *T. sinensis*. Besides, the *RsNiR* from radish was predominantly expressed in green tissues rather than non-green tissues [49]. However, in *T. sinensis*, the highest expression of *TsNiR* was detected in the female flower, a non-photosynthetic tissue. In tobacco, overexpressing the nitrite reductase gene (*AtNiR*) from *A. thaliana* significantly reduced the nitrite content in transgenic plants [26]. These findings on the expression and regulation of *TsNiR* genes provide valuable insights into the mechanisms underlying nitrite accumulation in *T. sinensis*. These results lay the groundwork for breeding low-nitrite cultivars, which could be an essential step toward improving the nutritional quality of *T. sinensis*.

## Conclusions

In this study, we revealed that *T. sinensis* accumulates higher levels of nitrite and nitrate compared to *T. ciliata*. The higher nitrite accumulation in mature leaves of *T. sinensis* can be attributed to increased NR activity, combined with relatively lower NiR activity in comparison to tender leaves. NR members expanded in *T. sinensis* compared to *T. ciliata*. NR and NiR gene families are evolutionarily conserved in both *T. sinensis* and *T. ciliata*. The expression levels in all mature leaves were significantly higher than those in tender leaves. The higher nitrite accumulation in *T. sinensis* compared to *T. ciliata* is attributed to several factors: its lower nitrite reductase activity, the expansion of NR gene family, and the elevated expression of the *TsNR2* gene in its leaves. In addition, overexpression of *TsNiR1* and *TsNiR2* significantly reduced leaf nitrite content. The findings presented herein provide valuable insights for further exploration of the functional disparities between NR and NiR gene family in *T. sinensis* and *T. ciliata* and for elucidating the regulatory networks governing nitrate and nitrite accumulation and metabolism in *T. sinensis*.

## Methods

### Measurement of indices related to nitrogen metabolism

Leaves from both tender and mature leaves of *T. sinensis* and *T. ciliata* varieties were collected. The nitrate and nitrite contents, along with NRA and nitrite reductase NiRA, were quantified using assay kits, which include

G0409W and G0431W from Suzhou Grace Biotechnology Co., LTD. (Suzhou, China), and BC0085 and BC1545 from Beijing Solarbio Science and Technology Co., LTD. (Beijing, China). Each experiment underwent three biological replicates. The specific prescription methods followed the instructions provided with each kit.

### Identification of NR and NiR genes in *T. sinensis* and *T. ciliata*

Using two *Arabidopsis* NR protein sequences and three rice NR proteins as baits, BLAST (Basic Local Alignment Search Tool) was employed to identify the NR coding genes in *T. sinensis* comprehensively [50]. We use the hidden Markov model (HMM) profile of NR (Pfam: PF00173, PF02665, PF02613) from the Pfam database to search for NR protein sequences in *T. sinensis*, which was retrieved from the genome database for *T. sinensis* var. 'heiyouchun' (<https://db.cngb.org/search/project/CNP0000958/>) [51, 52]. The candidate NR-encoding genes were manually checked for domains using NCBI Conserved Domain tool (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Finally, a total of two *TsNRs* were identified, and designated *TsNR1* to *TsNR2*. In the same way, we identified two *TsNiRs* in *T. sinensis* by BLAST and hmmersearch (Pfam: PF01077). Taking the same approach, one *TcNR* and two *TcNiRs* were identified from the *T. ciliata* genome [53]. To detect the physio-chemical properties of the *TsNR*, *TsNiR*, *TcNR*, and *TcNiR* proteins, all high-confidence sequences were selected to estimate their molecular weights (MWs) and isoelectric points (pIs) and other properties using ExPASy ([https://web.expasy.org/computer\\_pi/](https://web.expasy.org/computer_pi/)) [54].

### Phylogenetic analysis of NR and NiR family members

To explore the evolutionary relationships of NR and NiR proteins among *T. sinensis*, *T. ciliata* and other plant species, phylogenetic analysis of NR and NiR proteins from 12 plants was conducted using MEGA11 with the neighbor-joining (NJ) method [55]. Those protein sequences originated from *A. thaliana* (AtNR, AtNiR), *O. sativa* (OsNR, OsNiR), *Citrus sinensis* (CsNR, CsNiR), *Gossypium hirsutum* (GhNR, GhNiR), *Phaseolus vulgaris* (PvNR, PvNiR), *P. trichocarpa* (PtNR, PtNiR), *S. purpurea* (SpNR, SpNiR), *Sinapis alba* (SaNR, SaNiR), *Zostera marina* (ZmNR, ZmNiR), *Solanum lycopersicum* (SlNR, SlNiR). All the protein sequences used for the phylogenetic tree has been provided in Supplementary Table S1, which were collected from the Phytozome database (<https://phytozome.jgi.doe.gov/>) [56].

### Gene structure, conserved motif, and cis-regulatory elements analysis

The conserved motif and exon–intron organization of the gene structure were analyzed to further understand

the development of the *TsNR/NiR* and *TcNR/NiR* gene family. The exon–intron structures were obtained using the genome annotation information (GFF files), and the online Gene Structure Display Server (<http://gsds.gao-lab.org/>) was utilized for analyzing the gene structure [57]. The MEME website (<http://meme-suite.org/tools/meme>) was employed to predict conserved motifs in the protein sequences of NR and NiR family members [58]. The maximum number of conserved motifs was set to 10, with other conditions set to default. The upstream 2000 bp sequences of each gene were extracted from the genome sequence to analyze the cis-acting elements within the promoter region. The sequences were retrieved using the PlantCARE website (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>), and the potential roles of cis-elements in the promoter regions were subsequently filtered using Excel [59]. Then the results were visualized using TBtools and Adobe Illustrator [60].

#### Plant materials and expression analysis

One variety of *T. sinensis* (CAF1108) and one variety of *T. ciliata* (CAF0606) were collected from the germplasm resource nursery at Xinsha Island, Hangzhou, Zhejiang Province in June 2023, and tender leaves and mature leaves were taken, respectively. The collected samples were immediately frozen in liquid nitrogen. Total RNA was then extracted using the RNAprep Pure Polysaccharide Polyphenol Plant Total RNA Extraction Kit (DP441, Tiangen, Beijing, China). Reverse transcription was performed using the MonScript RTIII Super Mix with dsD-Nase (Two-Step) (Monad Biotechnology, Wuhan, Hubei, China) qRT-PCR was performed using the TB Green® Premix Ex Taq II (Tli RNaseH Plus) (RR420, Takara, Dalian, China). The  $2^{-\Delta\Delta C_t}$  method was utilized to quantify the relative expression levels of the target gene [61]. Various tissues, including the apical apex, tender leaves, old leaves, common petioles, tender stems, old stems, xylem, and phloem, were collected and uniformly processed.

#### Transient overexpression of target gene in *T. sinensis*

The overexpression vectors pBI121–*TsNiR1*–GFP and pBI121–*TsNiR2*–GFP were constructed and introduced into *T. sinensis* seedlings using *Agrobacterium tumefaciens* strain GV3101. The seedlings were first incubated in darkness for 24 h, followed by incubation under normal conditions for an additional 2 days. Subsequently, leaf samples were collected for DNA extraction. PCR and agarose gel electrophoresis were performed to identify the target gene bands, using the 35S forward primer from the vector and the reverse primer specific to the target

gene. The presence of target gene bands was confirmed through PCR based on extracted genome DNA. Nitrite content was determined using the G0431W kit (Grace Biotechnology, Suzhou, China).

#### Abbreviations

|                              |                                                  |
|------------------------------|--------------------------------------------------|
| NR                           | Nitrate reductase                                |
| NiR                          | Nitrite reductase                                |
| NO <sub>3</sub> <sup>−</sup> | Nitrate ion                                      |
| NO <sub>2</sub> <sup>−</sup> | Nitrite ion                                      |
| NRA                          | Nitrate reductase activity                       |
| NiRA                         | Nitrite reductase activity                       |
| MWs                          | Molecular weights                                |
| pIs                          | Isoelectric points                               |
| qRT-PCR                      | Quantitative real-time polymerase chain reaction |

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40538-025-00728-8>.

Supplementary material 1. Nitrate and nitrite contents across various populations of *T. sinensis* and *T. ciliata*.

Supplementary material 2. NR and NiR genes from different species

Supplementary material 3. Primer sequences for qRT-PCR of *TsNR/NiRs* and *TcNR/NiRs*.

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#### Author contributions

Y.W. was responsible for data processing, chart preparation, experimentation, and drafting of the original manuscript. J.H.D. and Y.R.F. contributed to conducting supplementary experiments and data curation. H.F.Y. supervised the writing review and editing. X.J.H. and J.L. contributed to conceptualization, writing review, editing, and funding acquisition. All authors will be notified at each stage of manuscript processing, including submission, revisions, and reminders. The authors have read and approved the final manuscript.

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#### Availability of data and materials

No datasets were generated or analysed during the current study.

#### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare no competing interests.

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