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Genomic survey of NPF and NRT2 transporter gene families in five inbred maize lines and their responses to pathogens infection

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ABSTRACT

Besides manipulating nitrate uptake and allocation, nitrate transporters (NRTs) are also known to play crucial roles in pathogen defense and stress response. By blasting with the model NRT genes of poplar and Arabidopsis, a total of 408 gene members were identified from 5 maize inbred lines in which the number of NRTs ranged from 72 to 88. Phylogenetic analysis showed that the NRT genes of maize were classified into NRT1/PTR (NPF), NRT2 and NRT3 subfamilies, respectively. Marked divergence of the duplication patterns of NRT genes were identified, which may be a new basis for classification and identification of maize varieties. In terms of biotic stress, *NRT2.5A* showed an enhanced expression during the pathogen infection of *Colletotrichum graminicola*, while *NRT1c4C* was down-regulated, suggesting that maize NRT transporters may have both positive and negative roles in the disease resistance response. This work will promote the further studies of NRT gene families in maize, as well as be beneficial for further understanding of their potential roles in plant-pathogen interactions.

1. Introduction

Nitrogen (N) is an important factor for shaping plant-pathogen interactions. Various plant pathogens have evolved diverse strategies for acquiring N sources from their host [1]. N acquisition is essential for pathogen growth and colonization during infection of plants [2]. A biotrophic pathogen *Erysiphe necator* was found to manipulate host N distribution in grapevine and Arabidopsis, suggesting that increased N transport may promote its infection [3]. On the other hand, N availability can affect plant resistance to pathogens by the regulation of plant immunity [4]. N content in plant tissues can be modified by pathogen infection, resulting in the enhancement of plant defense responses [5]. However, the relationship between nitrate transport and pathogen resistance is still poorly understood in plant-pathogen interactions.

Among several different forms of N, nitrate (NO_3^-) is the most abundant source of N available to plants. Plants have evolved the high- and low-affinity nitrate transport system to efficiently absorb NO_3^- from soil and then transport around the whole plant. Over the last two or three decades, four families of transporters functioning in NO_3^- transport have been identified in plants including nitrate transporter 1 (NRT1)/

peptide transporter (PTR) family (NPF), NRT2 family, chloride channel (CLC) family, and slowly activating anion channel (SLAC) family, which have been well reviewed [6,7]. Their functions have also been described elsewhere [7,8]. It should be noted that many NRT2 family members are unable to transport NO_3^- alone without the help of the partner protein, nitrate assimilation related protein (NAR2), which is also named NRT3 [7].

Further investigations provided new insights into the functions of NRTs beyond NO_3^- transport in planta, such as regulation of plant-microbe interaction, as well as pathogen response. OsNRT1.1B, a rice nitrate transporter and sensor, can regulate the root microbiota, thus altering rhizosphere microenvironment and improving N use efficiency of *indica* varieties [9,10]. The Arabidopsis peptide transporter AtNPF5.2 is induced by biotic and abiotic stresses and participates in plant defense against virulent bacterial pathogens [11]. On the other hand, negative function of NRTs in the plant immune system has also been reported. The Arabidopsis mutant *lin1*, in which expression of the *NRT2.1* gene was blocked, displayed enhanced resistance against the necrotrophy fungus *Plectosphaerella cucumerina*, with no associated fitness costs [12]. The *AtNRT2.5* T-DNA mutant lines were more resistant to *Pseudomonas*

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syringae pv. *tomato* pv *tomato* DC3000 inoculation than wild-type plants, suggesting a direct role for *NRT2.5* in plant defense [13].

Maize (*Zea mays* L.) is the most important food and feed crop worldwide, and the uptake and transport of NO_3^- in maize have been widely studied [14–16]. For example, a recent study revealed that *ZmNRT1.5/NPF7.9*, a low-affinity bidirectional nitrate transporter, is responsible for delivering NO_3^- from maternal tissues to the developing endosperm of seed [17]. However, to our knowledge, a comprehensive genome-wide analysis of the NRT family in maize is still lacking. One study mentioned that there were 97 members of the NRTs/NPFs family being identified in maize [18]. Although a basic phylogenetic analysis of NPF and NRT2 gene families in maize has been reported, which conducted using an old genome version of B73 line [19]. However, several important questions about gene structure, motifs identification, chromosomal location, tissue-specific expression patterns and stress responses remain largely unclear in maize.

At present, the maize genome assembled to chromosomal level of 5 inbred lines B73, Mo17, PH207, small-kernel (SK) line and *Zea mays* ssp. *Mexicana* (Mex) has been available, which provide new resources for comprehensive analysis of nitrate transporter family in maize and investigations of their functions in plant-pathogen interactions at a genome-wide scale. In this paper, we focus on maize NPF and NRT2 transporter gene families, which have been studied intensively in model plants such as Arabidopsis and rice. A comprehensive analysis of maize NPF and NRT2 transporter gene families in 5 inbred lines was performed at the whole genome level. The object of present investigations was to (1) study the structure, location and duplication of NPF and NRT2 transporter members in 5 maize inbred lines, (2) study their transcription profiles under biotic stress conditions.

2. Materials and methods

2.1. Identification NRT members in maize

The complete genome data of B73 (v 4.0), Mo17 (v CAU-1.0), PH207 (v 1.0), the small-kernel inbred line (SK, v 1.0) and *Zea mays* ssp. *Mexicana* (Mex, v 1.0) were downloaded from Maize Genetics and Genomics Database (<https://www.maizegdb.org/>) [20]. Model protein sequences of Arabidopsis [21] and poplar [22] (Table S1) were obtained from previous studies. Blast [23] and HMMER [24] programs were used to identify candidate NRT members in maize with the *E*-value cutoff of 10^{-5} . The final NRT members which contain the NPF or NRT domains were confirmed using NCBI-CDD (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) [25].

2.2. Multiple sequence alignment, conserved motifs identification and phylogenetic analysis

Multiple sequence alignments of confirmed NRT amino acid sequences were performed by using ClustalW [26] with the default parameters. The phylogenetic trees of the five maize genomes were constructed by MEGA [27] (version 7.0.21) with neighbor-joining (NJ) methods and 1000 bootstrap replications. NRT members of maize were named according to their phylogenetic relationship with PtNRTs. The phylogenetic trees was represented using the online tool ITOL [28] (<https://itol.embl.de/>). The conserved motifs of NRT members were determined by MEME [29] program (<http://meme-suite.org/tools/meme>) with *E*-value <0.05. Sequences that were too short or do not contain domains were deleted.

2.3. Gene location and duplication analysis

The location information of NRT members were obtained from the genome annotation files. Mapchart was then used to show the position of genes on each chromosome. Before collinearity was analyzed, self-comparison of each maize genome was performed by BLAST. The

segmental duplication landscape and tandem duplication were calculated by MCScanX [30]. Only the duplicate segments contained NRT members were retained by using in-house Perl scripts. CIRCOS [31] was used to construct the gene location and syntonic map. Non synonymous (KA) and synonymous (KS) values were calculated with TBtools [32] for each duplicated gene pair. The distribution diagram based on Ks values was plotted using R package ggplot2 [33].

2.4. Expression analysis

The expression of NRT members in different tissues was analyzed by using published maize data (PRJNA137659) under normal N condition [34]. Three GEO datasets (GSE107562, GSE152249 and GSE135613) were used to study the expression pattern of NRT genes under low N stress [35–37]. In addition, the transcriptome data for gray leaf spot (GLS) disease (GSE137198) [38] and bacterial wilt disease (GSE133302) were downloaded and reanalyzed. The expression data of NRT members were extracted from the total expression data by in-house Perl script. The fragments per kilobase million (FPKM) of the NRT genes were converted into dimensionless data, and bubble plots were drawn with TBtools. R packages limma [39] and GEOquery [40] were used to screen the differentially expression genes. Principal component analysis (PCA) and principal coordinates analysis (PCoA) were performed based on the expression data using R packages ggplot2, vegan [41] and ggbplot [42]. Enhanced Volcano package was employed to draw the volcano plot. The MA plot was completed by ggplot2 and ggrepel.

2.5. Plant materials and transcriptome sequencing

The experiment was conducted in the greenhouse of Plant Protection Institute, Chinese Academy of Agricultural Sciences in 2019. The transcriptome sequencing was performed on a typical maize variety B73. For biological stress treatment, the maize leaves were inoculated with water and *Colletotrichum graminicola*. The first sampling of maize leaves was performed at 0 h after treatment of water. Subsequent samples of maize leaves were taken at 24, 40, 60 and 96 h after inoculation with *C. graminicola*. Three biological replicates of leaf samples were collected at each time point. Total RNA was extracted from leaf samples using Trizol method. Then, the RNA was analyzed by agrose gel electrophoresis. RNA-seq libraries was finally constructed with the Illumina standard mRNASeq Prep Kit (TruSeq RNA and DNA Sample Preparation Kits). Transcriptome sequencing was performed on Illumina Hi-seq 2000 Sequencer platform. The transcriptome data is available in Zenodo (<https://www.zenodo.org/record/6559214#.Y7VARcit-jh>). Fragments per kilobase of exon model per million mapped reads (FPKM) of maize genes were calculated. The multi-mapping reads were directly filtered out during progress of quantification. The heatmap was created by R package pheatmap [43] based on the transformed FPKM with Z-score standardization. The *C. graminicola* treated transcriptome sequencing raw data used in this study has been uploaded to the NCBI (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE210899>).

2.6. Quantitative real-time PCR (qRT-PCR) analysis

The experiment described above including the RNA extraction was repeated in the same greenhouse in 2021. A maximum of 1 μg total RNA was used for synthesizing cDNA with HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). qRT-PCR was performed in a 20 μl volume, which included 5 μl diluted cDNA template, 0.5 μl each specific primers, 10 μl RealStar Green Fast Mixture with ROXII (GenStar, Beijing, China) and 4 μl ddH₂O. The PCR cycling conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s; *ZmActin* was used as stable reference genes. Gene specific primer pairs for qRT-PCR (listed in Table S2) were designed by NCBI Primer BLAST. Three biological replicates were performed to ensure the accuracy of results. The relative expression of the target genes was determined using

the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were conducted using GraphPad [44] software. The nitrate contents of the leaves with three biological replicates under the treatment of *C. graminicola* at four time points were determined using the Plant Nitrate Nitrogen Activity Assay Kit (Solarbio, Beijing, China).

3. Results

3.1. Identification and phylogenetic analysis of NPF and NRT2 genes in the genome of 5 maize inbred lines

NRTs genes from *Arabidopsis thaliana* and poplar were extracted as references to search in B73, Mo17, PH207, SK and Mex genome using BLASTP program with *E*-value of $1e^{-5}$. Thereafter, NCBI-CDD search was used to search the NRT domains in the candidate members. The proteins lacking MFS or PTR domains were removed. The characteristics of NRT genes were listed in Table 1, a total of 349 NPF, 33 NRT2 and 26 NRT3 genes were identified in the genomes of five varieties (Table S3). The smallest NRTs family was found in Mex genome with 72 members, which was 11 members less than that in B73 genome. The average numbers of introns in each subfamily varied from 0.67 (NRT2) to 3.12 (NRT1). Significant length divergence of the amino acid sequences in the three subfamilies was also confirmed. The average length of proteins in NRT1/PTR subfamily was 556 bp much longer than that in NRT2 (434 bp) and NRT3 (316 bp).

To determine the evolutionary relationship of NRT genes among the five genomes, phylogenetic trees were constructed by MEGA using the NJ method. NRT members were named based on the sequence similarities with poplar NRT genes. As shown in Fig. 1a, all the identified NRT members in B73 were classified into three groups, NRT1/PTR, NRT2 and NRT3. The majority of the NRT members belong to NRT1/PTR subfamily. NRT3 subfamily had the least members. Similar results were also found among the other four maize genomes (Fig. S1). Moreover, a comprehensive phylogenetic tree was constructed based on all NRTs members of the 5 maize species. As shown in Fig. 1b, similar to the results of B73, NRT2 and NRT3 subfamilies showed a closer evolutionary relationship. NRT1 subfamily can be subdivided into four branches: NRT1a, PTR, NRT1c and NRT1d, and their new name following the nomenclature used by L eran et al. [45] was listed in Table S4.

3.2. Gene structure and motif analysis of NPF and NRT2 genes

In order to obtain more information of NRT genes, the motif and exon/intron structures were analyzed (Fig. 2). The number of exons of 3 examined members varied from 1 (like B73NRT3.2A) to 22 (MexNRT1a1A). Most of the NRT1/PTR family had 4 exons in which the first three were short and the last one was relatively long. Among the 5 genomes, the exon/intron structure of NRT2.4 showed a high degree of consistency with only one exon. The structure pattern of NRT3 subfamily was more similar to that of NRT1/PTR subfamily. In addition, in-house Perl scripts were used to count the number of introns. As shown in Table 1, NRT1 subfamily members contained the most introns (average

3.12), which were almost twice or even five times larger than that of NRT3 and NRT2, respectively. Motif analysis showed that the NRT1/PTR subfamily contained all motifs (motif 1 to motif 10). As shown in Fig. 2, the NRT2 and NRT3 subfamily contained significantly fewer motifs. Considering the important transfer function of NRT genes, the transmembrane domain of these genes was predicted by TMHMM using default parameters (Table S5). The quantitative characters among the three subfamilies were distinct. There are about 10 transmembrane domains in NRT1/PTR subfamily, but individual genes differ greatly. However, NRT3 subfamily contained few transmembrane domains in which NRT3.1 gene consistently contained 3 transmembrane domains. No transmembrane domain of NRT3.2 genes was found in 5 maize inbred lines.

3.3. Chromosomal localization of NPF and NRT2 genes in the genome of 5 maize inbred lines

The position of the identified NRT members were mapped to chromosomes by Mapchart software (Fig. 3). Obviously, chromosome 1 contained the most abundant NRT genes among the 5 maize inbred lines, ranging from 15 (B73) to 22 (Mo17 and SK). On the contrary, the fewest NRT genes (only 2 or 3) were located on chromosome 7, which has moderate chromosomes size. It is worth mentioning that some NRT genes were located in the same regions on each chromosome, for example, the NRT1.1 gene distributed on the distal region of each chromosome 1, and the NRT3.2 gene distributed near the central region of chromosome 7. The similar chromosome localization pattern in 5 maize inbred lines indicate well conservation of NRT family members among the lengthy evolutionary and biological engineering process.

3.4. Divergent duplication pattern of NRT gene in 5 maize genomes

To explore the expansion pattern, we analyzed the segmental duplication of NRT genes. Among the NRT involved segmental duplications, most of the segments were duplicated once, except for a few segments were duplicated twice or more. Moreover, divergent patterns of NRT gene duplication were found in 5 maize genomes. In B73, SK and Mex inbred lines (Fig. 4a, c and e), the sparse and minor duplications of NRT members were identified. However, Mo17 and PH207 contained more NRT genes which involved in large-scale duplications (Fig. 4b and d). For instance, the large duplication (red line) between chromosomes 4 and 5 consistently contained the NRT1.3 and NRT1.5 genes. Similar large-scale duplications also appeared between chr3 and chr8 (green in Mo17, orange in PH207), chr2 and chr10 (dark green).

To investigate the evolutionary constraints of maize NRT genes, the Ka/Ks values of duplicated NRT gene pairs were calculated (Table S6). The Ka/Ks values of Mo17, PH207, SK and Mex were all >1. However, the Ka/Ks values of B73 showed a reverse change trend, ranging from 0.1 to 0.7. We also calculated the pairwise synonymous distance (Ks) among the paralogs of collinear blocks in 5 genomes. The peak Ks frequency of Mex and SK inbred line showed similar pattern (0.025 and 0.022, respectively), indicating a close genetic relationship (Fig. 4f). These results were consistent with previous studies on small-kernel inbred line with tropical background of local varieties. According to estimates, about 10% of maize genome regions were introgressed from Mex. However, little is known about the more detailed genetic relationship between Mex and SK. Further, the Ks peaks of Mo17 and PH207 were 0.2553 and 0.2586, respectively. Segmental and tandem duplications are the most commonly evaluated mechanisms for gene family expansion. We investigated the proportion of tandem and segmental duplications among NRT genes in maize. As shown in Table 1, a total of 52 (61.90%) and 55 (67.90%) NRT genes of Mo17 and PH207 belonged to segmental duplications. Inversely, the tandem duplications of in B73, SK and Mex inbred lines accounting for 43.37%, 53.41% and 50.00%, respectively.

Table 1
Statistics information of the identified NRT members.

Maize inbred lines	NRT1/PTR	NRT2	NRT3	Total	Tandem	Segmental
B73	68	7	8	83	36	15
Mo17	70	10	4	84	27	52
PH207	72	7	2	81	25	55
SK	75	6	7	88	47	26
Mex	64	3	5	72	36	22
Number of intron (Ave)	3.12	0.67	1.54	–	–	–
Length of protein (Ave)	556	434	316	–	–	–

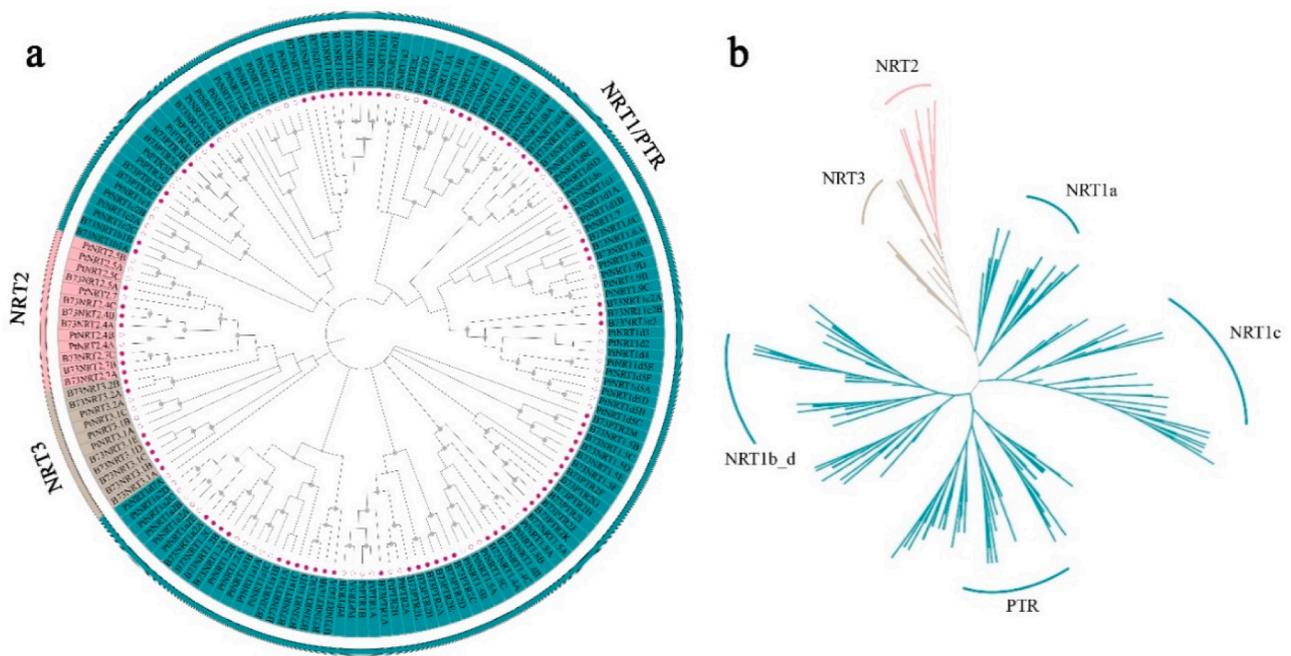


Fig. 1. Phylogenetic analysis of NRT members in the genome of B73 (a) and 5 maize inbred lines (b). NRTs of B73 and poplar were marked with solid circle and hollow circle, respectively. Phylogenetic trees were built using MEGA7 with Neighbor-Joining method and bootstrap of 1000 replications.

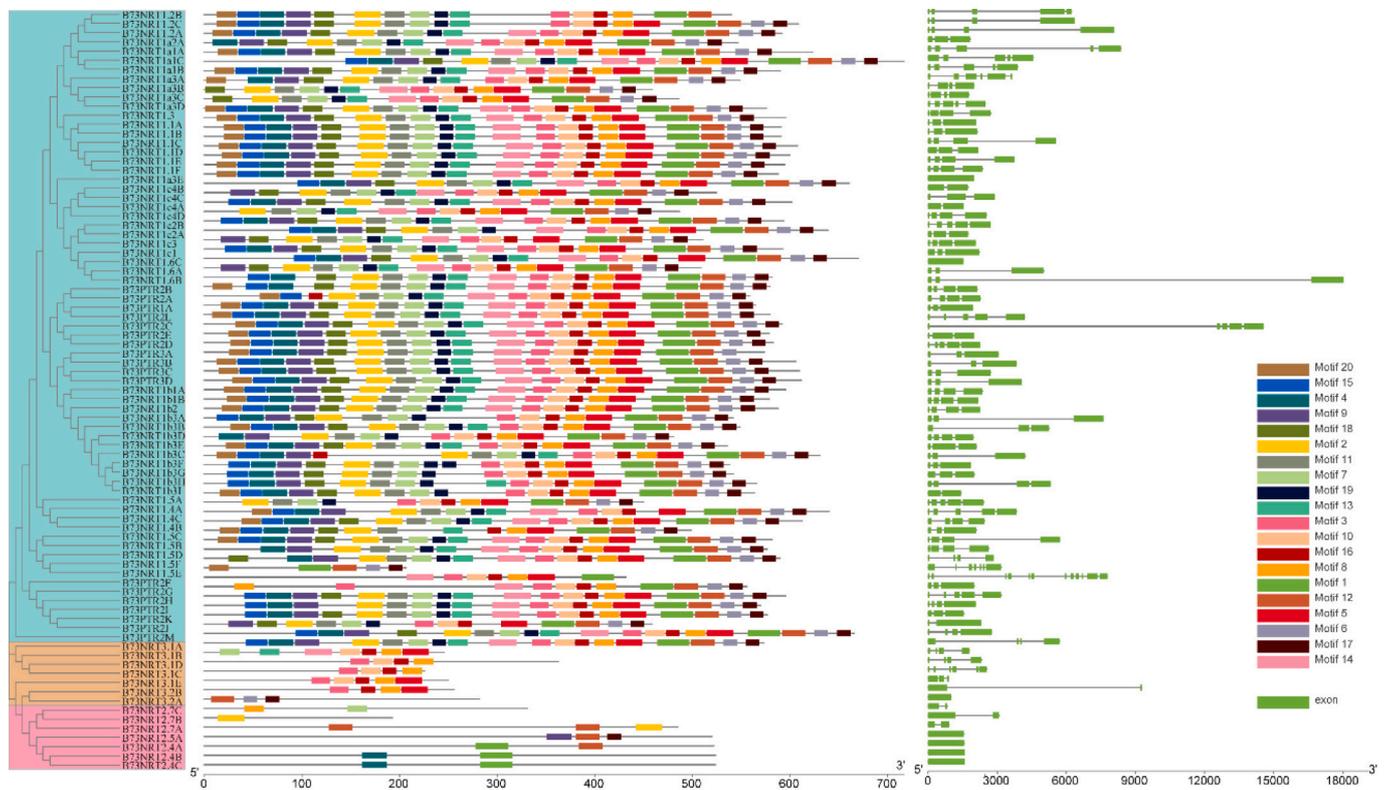


Fig. 2. Phylogenetic relationship, motif and gene structure analysis of B73 NRT members. Coloured bars in the left part of the figure refer to different motifs. Green bars in the right part of the figure indicate coding sequences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. NRT genes showed tissue-specific expression pattern

To characterize the expression profile of maize NPF and NRT2 genes, we assessed the RNA-seq data in four tissues obtained from the published GSE27004. Based on the normalized expression data, principal

component analysis (PCA, Fig. 5a) and principal coordinates analysis (PCoA, Fig. 5b) was completed with Bray-Curtis matrix. PCA and PCoA ($R = 0.87$, p value = 0.001) totally explained 62.7% and 73.39% of the global variations, respectively. Interestingly, genes in the four tissues distinctly separated from each other, indicating a high tissue-specific

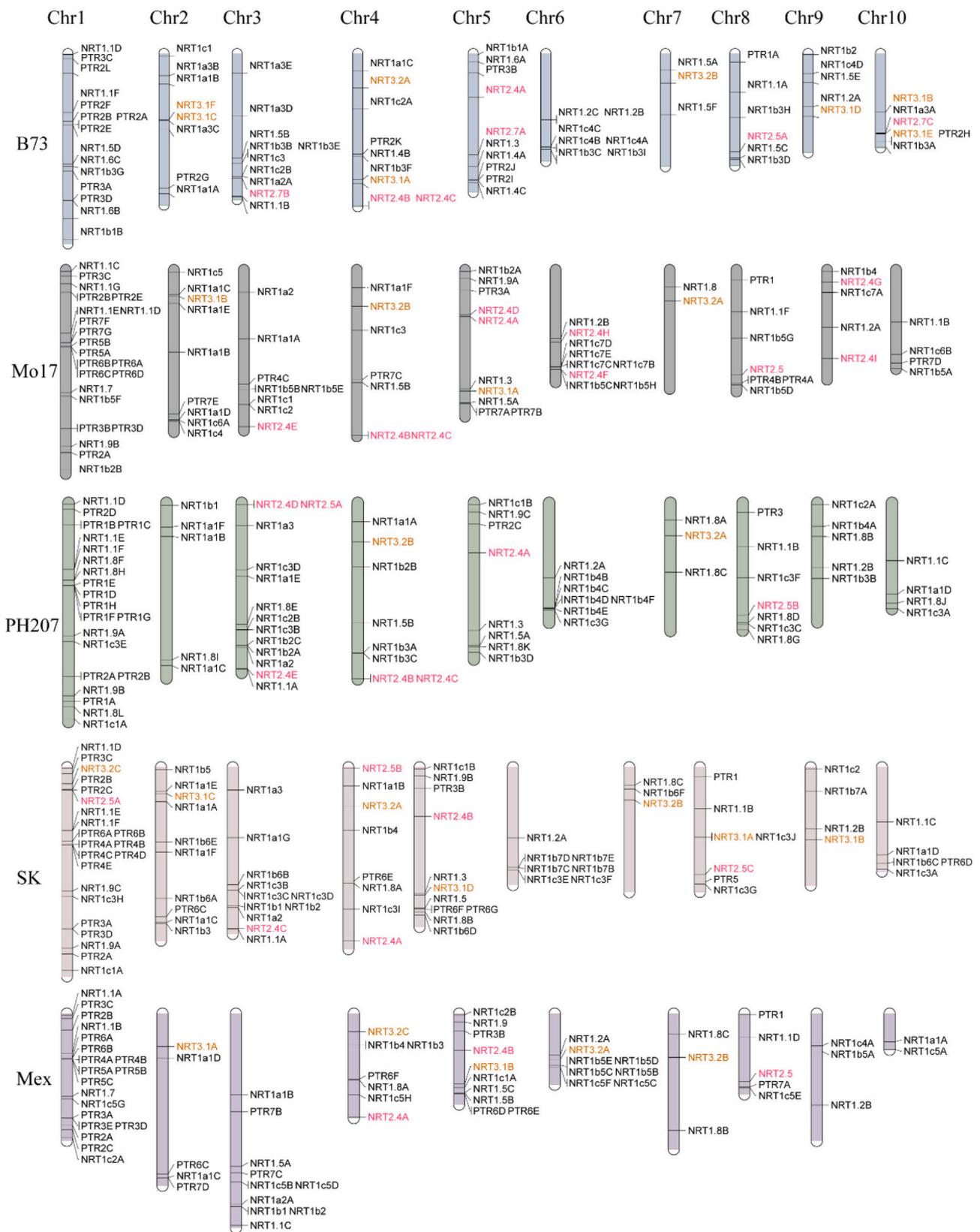


Fig. 3. Chromosomal locations for NRT genes in 5 maize genomes. Each colour corresponds to a maize specie. The length of the bar refers to the size of chromosome. MapChart was used to generate the figure. NRT1 and PTR members were highlighted in green, NRT2 in red and NRT3 in brown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

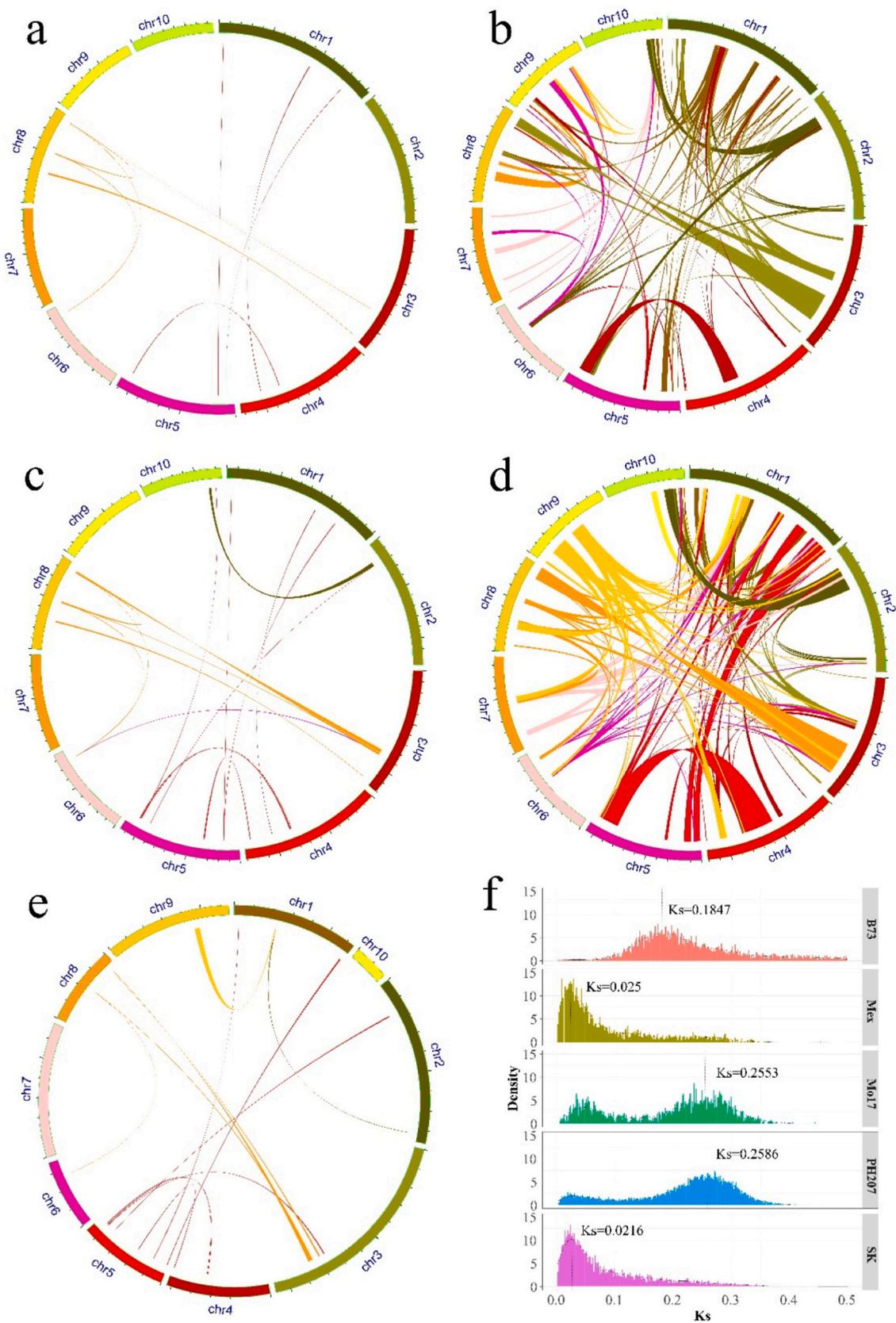


Fig. 4. The synteny analysis of NRT gene family in B73 (a), Mo17 (b), SK (c), Ph207 (d) and Mex (e). Chromosomes are shown in different colours. Only the collinear blocks contain the NRT members were linked and shown in this figure. Ks peaks of the 5 maize genomes (f). Peak curve of the five maize genomes was shown in different colours. X-axis refers to ks value, Y-axis represents numbers of the ks values.

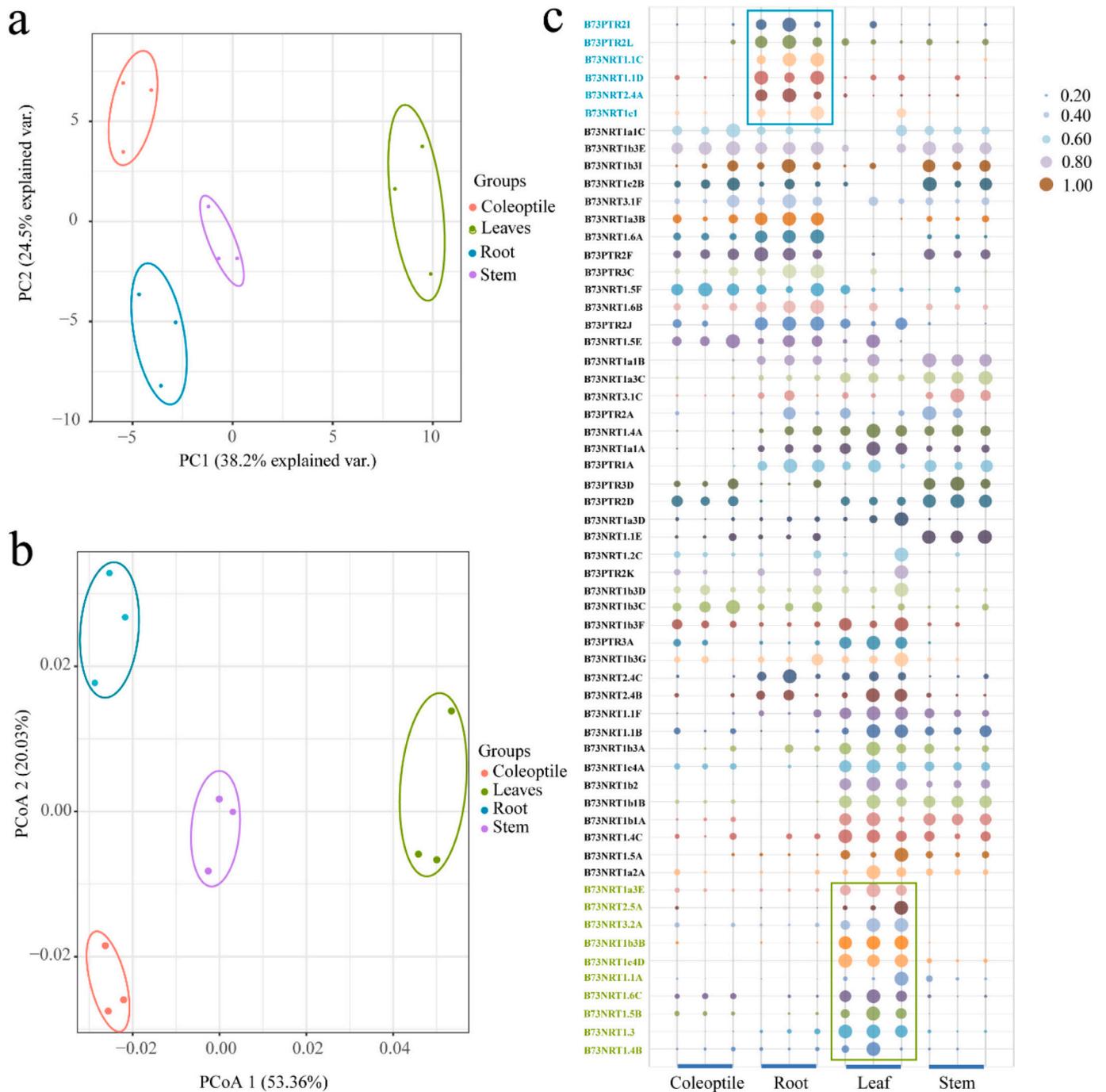


Fig. 5. The tissue-specific expression pattern of NRT genes in B73 based on GSE27004. (a) The principal component analysis (PCA) with Bray-Curtis matrix based on the expression data of NRT genes. (b) The principal co-ordinates analysis (PCoA) with Bray-Curtis matrix based on the expression data. (c) Bubble diagram based on the expression data of different tissues of B73 with dimensionless method.

expression pattern. In the PC1 direction, the order of tissue cluster was root, coleoptile, stem and leaf which might uncover the functional changes of NRT members. The expression depicted by bubble diagram with dimensionless method also showed the tissue-specific pattern of NRT genes (Fig. 5c). Pairwise comparison of expression levels in different tissues was used to verify whether the difference was significant. Some genes showed high expression only in root tissues, such as *NRT1.1c*, *NRT1.1D*, *NRT2.4A*, *PTR2I* and *PTR2L*. Moreover, the genes specifically expressed in leaves were mainly involved in NRT1 subfamily, such as *NRT1.3*, *NRT1.4B*, *NRT1.5B*, *NRT1.6C*, *NRT1c4D*, *NRT1.1A*, *NRT1b3B* and *NRT1a3E*. And several NRT2 and NRT3 members also

showed the leaf specific pattern, such as *NRT2.5A* and *NRT3.2A*. There was no gene showing stem-specific or coleoptile-specific expression pattern. Some NRT members showed a quite widely expression pattern in different tissues. For instance, *NRT1d3E*, *PTR2F*, *PTR2D*, *PTR1A*, *PTR2A* and *PTR2J* universally expressed in two or three tissues.

To evaluate the response of maize NRT genes to low N stress, the transcript profile of NRT genes were specially extracted from three previous studies. In two different maize inbred lines (Lo5 and T250), several NRT genes were up-regulated under low N stress. Only the expression of *NRT2.5A* in the root was induced in both inbred lines under the low N stress (Fig. S2A and Fig. S2B). As for leaf tissue, *NRT1c3*

were suppressed and no high-affinity N transporter were found to be induced under low N stress (Fig. S2C and S2D).

3.6. Maize NPF and NRT2 expression pattern in response to pathogens

To investigate the influence of pathogenic bacteria on NRT members, we analyzed the transcriptome data of B73 responses to *Pantoea stewartii* vascular infection. As shown in Fig. S3, 6 NRT genes, including *NRT2.7B*, *NRT1c4C*, *NRT1a3E*, *NRT1b3E*, *PTR2H* and *NRT1c1*, were up-regulated under the infection of *P. stewartii*. Four NRT genes such as *PTR3D*, *NRT2.5A*, *NRT1a3D* and *NRT1b2* showed the down-regulated expression when responding to *P. stewartii*. Among the 10 genes, the expressions of 4 NRT genes in NRT1/PTR subfamily were significantly different ($|\log_2(\text{FC})| \geq 1$, p -value < 0.05). In addition, the influence of gray leaf spot (GLS) disease on NRT genes was analyzed by high-throughput sequencing. Under GLS infection, the expressions of *PTR1A*, *NRT1.1C*, *NRT1c3*, *NRT1a3E*, *PTR1A*, *NRT1.1C*, *NRT1.2A* and *NRT2.5A* were significantly different (Table S7). Combining the both diseases, genes in NRT1/PTR subfamily have pathogen-inducible expression profile to pathogenic bacterium and fungus.

To further confirm whether the expression of NRT genes were influenced by pathogen infection, maize leaves were sampled at 0 h, 24 h, 40 h, 60 h and 96 h post inoculation with wild-type strain *Colletotrichum graminicola*, the causing agent of maize anthracnose disease. The results of RNA-seq indicated that some NRT genes were significantly induced or repressed by the *C. graminicola* infection (Fig. 6a). A total of 6201, 3423, 4699 and 8303 genes were found differently expressed at 24 h, 40 h, 60 h and 96 h after the inoculation of pathogen, respectively ($|\log_2(\text{FC})| > 1$, p -value < 0.05). Among them, 19, 10, 16 and 19 NRT members showed the significant differential expression at the four time

points. To be specific, *NRT1.6C*, *NRT1c4C*, *NRT2.5A*, *PTR3B*, *NRT3.2A* and *PTR3C* significantly regulated by *C. graminicola* infection at all the four time points (Fig. 6b, Fig. S4). Among the five genes, *NRT1.6C*, *NRT3.2A* and *NRT1c4C* were continuously repressed in the early infection. On the contrary, the expression of *PTR3B* and *PTR3C* were induced at all the four time points. Interestingly, the expression of *NRT2.5A* and *NRT1c4C* was significantly regulated by *P. stewartii* and *C. graminicola*, suggesting the two genes may play important roles in the early infection of plant pathogens.

To better understand the co-expression pattern and the regulatory network of NPF and NRT2 genes, weighted correlation network analysis was constructed based on the RNA-seq data. The expression of each subfamily member in four time points compared with the control group was diagramed a pie chart divided into four parts (Fig. 7b). Interestingly, NRTs specifically expressed in leaves, like *NRT1c4C*, *NRT1b3B* and *NRT1.6C* were clustered together when facing the pathogen stress. To validate expressions of the maize NRT genes, four B73NRTs with significantly differential expression in leaves based on RNA-seq analysis were selected to investigate their expression levels under *C. graminicola* treatment by qRT-PCR (Fig. 7a). Results showed the expression trend of these four genes by qRT-PCR were highly consistent with the that of RNA-seq analysis. *NRT1c4C* was significantly down-regulated by *C. graminicola* infection over the 96-h period. The overall expression trend of *NRT1b3B* and *NRT1.6C* was decreased. On the contrary, the expression of *NRT2.5A* was up-regulated at 40 h. Marked down-regulation of nitrate N contents were observed after spraying the spore suspension of *C. graminicola*. The overall findings highlight the hidden role of NRT1 subfamily in the protecting plants from pathogens by inhibiting the transport and absorption of N.

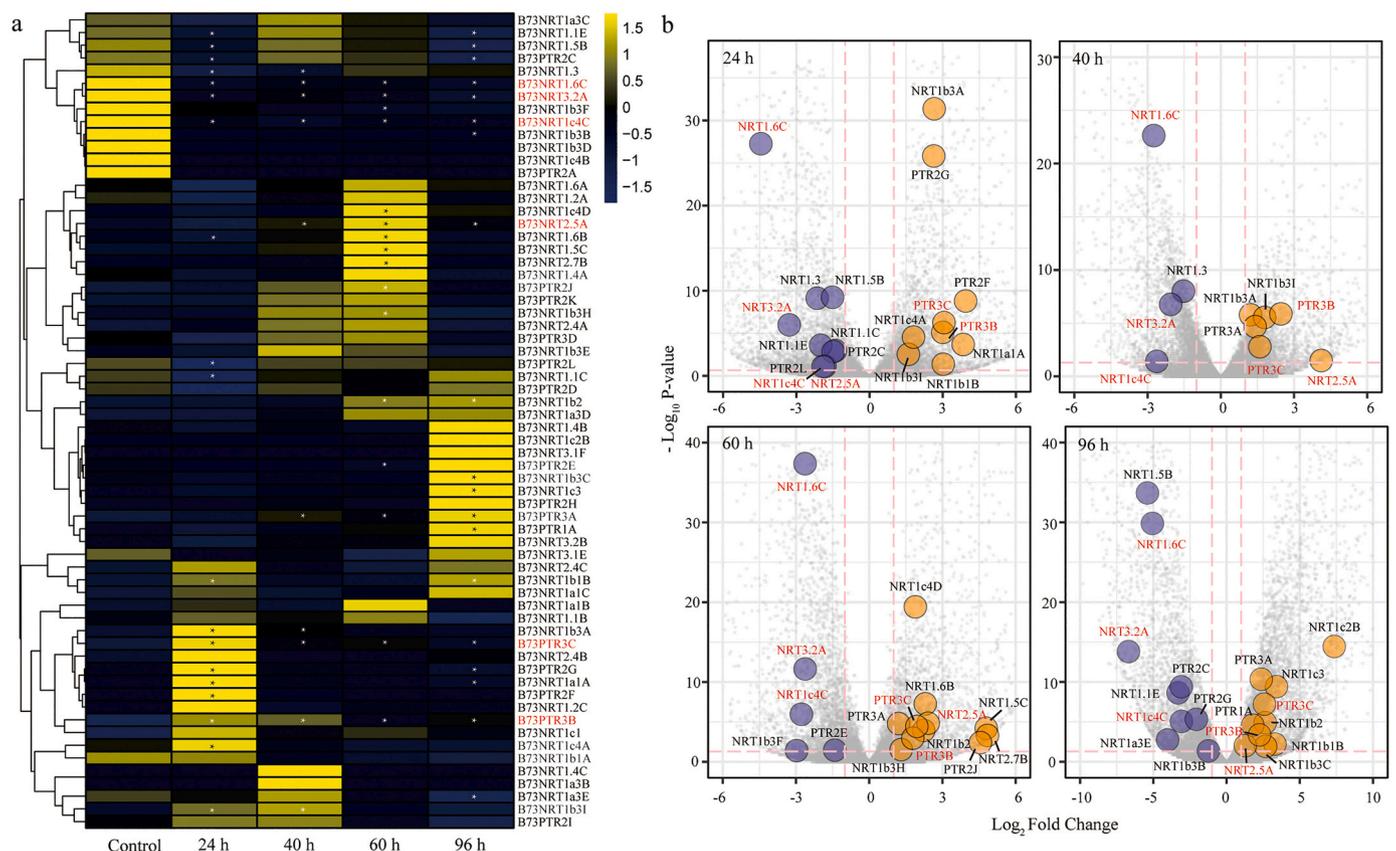


Fig. 6. Expression of NRT genes in B73 with the treatments of water and *Colletotrichum graminicola*. (a) The genes marked red were differentially expressed at 24, 40, 60 and 96 h post inoculation of *C. graminicola*. (b) Volcano plot of all the maize genes under *C. graminicola* at 24, 40, 60 and 96 h post inoculation. Up-regulated NRT members were marked yellow and down-regulated NRT members were marked blue ($|\log_2(\text{FC})| \geq 1$, p -value < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

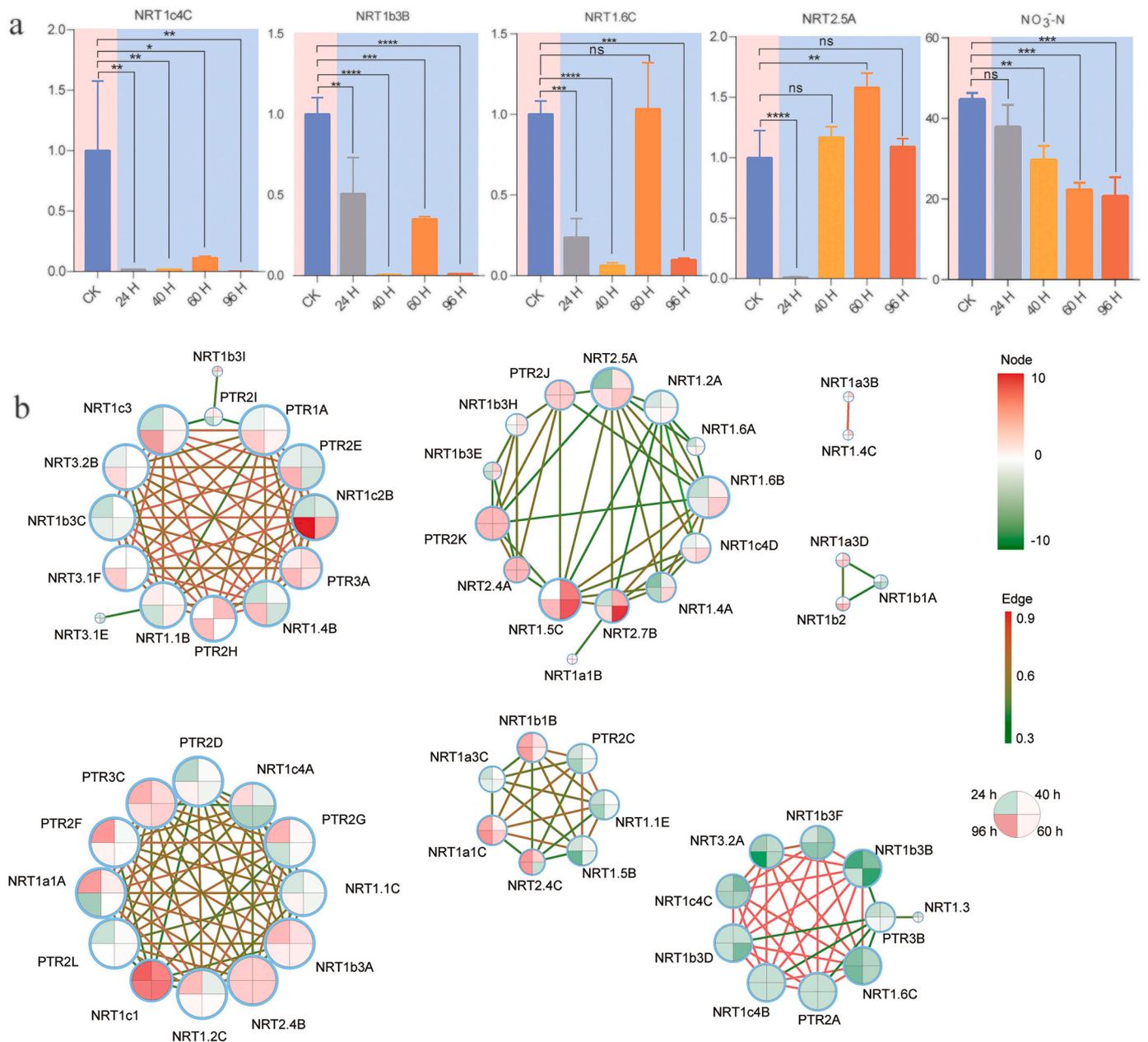


Fig. 7. The expression of 4 selected NRT genes in response to *C. graminicola* by qRT-PCR, nitrate contents (a) and weighted correlation network analysis (b) of all the NRT genes. One-way ANOVA test with the multiple comparisons was used for the *C. graminicola* treatment at different time points. Nodes in the network graph were replaced by pie charts divided into four parts which represented the log FC values of samples at 24 h (upper-left part), 40 h (upper-right part), 60 h (lower-right part) and 96 h (lower-left part).

4. Discussion

4.1. Identification and phylogenetic analysis of NPF and NRT2 genes in 5 maize inbred lines

Nitrate is not only the main source of inorganic N in plants, but also acts as a signal molecule to activate the expression of a series of genes, triggering the nitrate response and promoting the efficient use of N [46]. As nitrate receptors, nitrate transporters can sense nitrate signal and trigger the expression of downstream response genes [40]. Previous studies of an individual NRT gene have been reported in Arabidopsis, poplar and pineapple [47]. However, the NPF and NRT2 gene families in maize have been largely uncharacterized. In this study, a comprehensive identification of NPF and NRT2 genes were performed in 5 inbred lines B73, Mo17, PH207, SK and Mex.

High consistency in the number of subfamily genes was found in 5 maize inbreds. NRT1/PTR family contained the most gene members, accounting for 86% of the total. Only several members were found in NRT2 and NRT3 subfamilies. The distribution pattern of subfamily members was consistent with the previous researches in Arabidopsis, poplar and pineapple. Significant structural diversities were found among the 3 NRT subfamilies. NRT1/PTR subfamily contained the most introns, with an average of 3 introns per gene, twice as many as NRT3 and five times as many as NRT2. According to previous reports, the rate of intron loss after fragmented replication was faster than that of intron gain, indicating that the NRT1/PTR of maize might be older in the course of evolution [48]. Few paired segmental and tandem duplication NRT genes contained the same number of introns indicated that functional divisions might occur after replication events.

4.2. Tissues-specific expression pattern of NPF and NRT2 genes related with their functions

The uptake and transport of nitrate depend on the cooperation of different NRT genes. NRT1.1 (primitive name CHL1) was firstly identified in Arabidopsis, and was found to have nitrate and pH dependent regulatory effects. In this study, *NRT1.1C* and *NRT1.1D* were found to highly expressed in root tissues. It was consistent with previous research that revealed the widely expression of *AtNRT1.1* in roots and shoots [49]. However, the other four NRT1.1 genes (*NRT1.1A*, *NRT1.1B*, *NRT1.1E*, and *NRT1.1F*) were high expressed in leaf tissues. This is understandable in view of the multiple functions of *NRT1.1* genes which participate in both low and high-affinity nitrate transport, as well as function as a nitrate sensor to activate the expression of nitrate related genes in plants [50]. It has been reported that *NRT1.1* was highly expressed in guard cells, which was involved in promoting the stomatal opening [51]. In contrast to roots, no gene was specifically expressed in stem tissues. However, NRT1/PTR subfamily (*NRT1a3E*, *NRT1b3D*, *PTR2K* and *PTR3D*) were found in stem. This may be due to several NRT1 genes located in xylem for transporting nitrate from root to the aboveground parts [52]. Furthermore, recent integration of NRT1/PTR genes highlighted its new function, mainly involving in the transport of phytohormone, glucosinolates and short chain polypeptide [21]. For maize, other natural substrates of NRT1/PTR need to be further studied.

Our results revealed that the expression levels of NRT2 (*NRT2.5A*, *NRT2.4B*) and NRT3 (*NRT3.2A*) genes were higher in leaf than those in other 3 tissues. A strong leaf-specific expression of NRT2 gene (*AtNRT2.7*) was also discovered in Arabidopsis and poplar [22]. Interestingly, it seemed that *NRT2.5* and *NRT3.2* showed a functional relevance, which had been repeatedly proved in poplar, dicots and grass species [22]. Though it is known that NRT3 is not a nitrate transporter, the mechanism about how NRT3 regulates NRT2 is still unclear.

4.3. Duplications involving in NPF and NRT2 genes diverge largely among the 5 maize species

Besides polyploidization, segmental duplication is also an important impetus for the evolution which occurs frequently in diploid plant genomes [53]. Redundant genes originated from duplication were considered to be the driving force of evolution [54]. The identified NRT gene sizes of B73, Mo17, PH207 and SK were consistent between 81 and 88, suggesting that they may have experienced the same duplication event. The ancestor species Mex contains 72 NRT genes slightly less than the other four modern species, which may mean that new gene replication events were artificially caused in the later breeding work [55]. Divergent pattern of NRT gene duplications were found among the 5 maize genomes. B73, SK and Mex contained sparse and small duplications, while Mo17 and PH207 contained more large duplicate fragments. The replicated NRT subfamily genes Mo17 and PH207 were well preserved. However, the duplicate segments in B73, SK and Mex seem to be subjected to stronger natural selection and resulted in more gene loss or species-specific evolution in maize genomes. Moreover, most of the Ka/Ks values of NRT genes were >1, which suggested that NRT genes had experienced relatively rapid evolution and positive selection [56].

By comparing the numbers of the NRT members in five maize inbred lines, the variation of the NRT numbers was mainly from the NRT2 subfamily in Mex, which was two or three times less than other inbred lines. The NRT2 members play critical roles in plant physiological processes. Current studies have demonstrated that the functional differences of NRT2 members were based upon their tissue distribution. For example, it has been reported that *NRT2.1* and *NRT2.2* were expressed alternately in the root cortex and epidermis to transport nitrate, while *NRT2.3* was predominantly in young leaves [57,58]. *NRT2.4* was mainly expressed in the epidermis of lateral roots and phloem near young leaves [59]. *NRT2.5* was expressed in both young leaves and roots and regulated by nitrate supply [59]. In this study, *NRT2.5A* showed up-

regulated expression under both low N and biological stress, implying its functional role in plant resistance against abiotic and biotic stress. More in-depth analyses are still needed to determine whether long-term evolution or artificial intervention that leads to the expansion of NRT2 membership.

In addition to segmental duplication, tandem duplication is also an important factor in genome expansion [60]. Our results showed that the ratio patterns between tandem and segmental duplications were different in the 5 maize genomes. Mo17 and PH207 contained more segmental duplications, while the other three maize inbred lines had the opposite results. More than half of the NRT genes in Mo17 and PH207 were associated with segmental duplication, indicating that segmental duplication played an important role in NRT gene expansion. Compared with the other 2 maize inbred lines, more NRT genes of B73, SK and Mex were associated with tandem duplications, indicating that the three might be large-scale of chromosomal anomalies, unequal crossing over and transposon insertions in these 3 genomes [61]. Previous researches have proposed some models for functional divergence of redundant genes, such as sub-functionalization, non-functionalization and neo-functionalization [62]. The divergent expression patterns of NRT genes in different subfamilies indicate that the functional segregation has occurred among these genes.

4.4. NPF and NRT2 genes in maize responses to pathogens infection

The important roles of nutrients in regulating plant diseases have been investigated for decades. N is one of the most important nutrients for impacting on susceptibility/resistance to crop diseases [63]. Comprehensive analysis of the transcriptome data of gray leaf spot disease and bacterial wilt disease confirmed the down-regulated expression of NRT1/PTR subfamily members following pathogen attacks. This finding was in accordance with previous reports, which demonstrated that nitrogen uptake and transport would be reduced when disease occurred [64]. Bellegarde et al. have demonstrated that N deficiency can lead to excessive production of reactive oxygen species (ROS) [65]. The normal ROS level is closely related to the regulation of plant development and defense in plant. We speculated that the *NRT2.5A* might maintain high levels of N to balance the level of ROS during plant disease. In addition, the susceptibility of plants to pathogens is influenced by N level. Some defense genes have been found to overexpress upon infection under high N level. Therefore, *NRT2.5A* may be directly or indirectly related to the expression of some resistance genes.

5. Conclusions

In this study, a total of 408 NRT members were identified in 5 maize genomes and classified into NRT1/PTR, NRT2 and NRT3 subfamilies. Systematically analysis of the conserved motif composition, gene structure and chromosomal locations revealed the similar features of NRTs among the 5 maize genomes. Duplication analysis demonstrated the marked divergence of the NRT genes duplication patterns between B73, SK, Mex and Mo17, Ph207. Furthermore, the tissue-specific expression pattern of the NRT genes and their response to pathogens were identified based on the transcript data. The expression level of *NRT2.5A* and *NRT1c4C* significantly up- and down-regulated during the pathogen infection, respectively, suggesting that they may play important roles in host-pathogen interaction through both positive and negative regulatory mechanisms. Collectively, these findings provided important information for further investigation of the divergent function of ZmNRTs and their regulatory roles in plant-pathogen interactions.

CRedit authorship contribution statement

Xinyao Xia: Conceptualization, Methodology, Software, Writing – original draft. **Qiuhe Wei:** Data curation. **Chunxia Xiao:** Visualization.

Yiping Ye: Formal analysis. **Zhiqiang Li:** Software, Validation. **Cécile Marivingt-Mounir:** Methodology. **Jean-François Chollet:** Investigation. **Wende Liu:** Funding acquisition, Project administration. **Hanxiang Wu:** Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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