

Genome-wide identification of the peptide transporter family in rice and analysis of the PTR expression modulation in two near-isogenic lines with different nitrogen use efficiency

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Abstract

Background: Nitrogen (N) is a major nutrient element for crop yield improvement, but it also causes environmental problems. Therefore, N use efficiency (NUE) enhancement is an important strategy to solve this problem. In plants, the members of the peptide transporter (PTR) gene family are involved in nitrate uptake and transport. In this study, we performed a genome-wide analysis on PTR genes in Nipponbare, R498, and *Oryza glaberrima*, and identified 96, 85, and 78 PTR genes respectively.

Results: Phylogenetic tree showed that the 96 PTR genes could be classified into 8 groups, and the distributions of PTR genes in Asian cultivated rice and African cultivated rice were consistent. The number of PTR gene was higher in peanuts and soybeans, which were 125 and 127, respectively. The 521 PTR genes in rice, maize, sorghum, peanut, soybean and *Arabidopsis* could be classified into 4 groups, and the PTR gene distribution was different between monocots and dicots. In Nipponbare genome, the 25 PTR genes were distributed in 5 segmental duplication regions on chromosome 1, 2, 3, 4, 5, 7, 8, 9, and 10. Rice PTR genes have 0-11 introns and 1-12 exons, and 16 PTR genes have the NPF (NRT1/PTR family) domain. The results of RNA-Seq showed that the number of differentially expressed genes (DEGs) between NIL15 and NIL19 were 928, 1467, and 1586 at three stages, respectively. Under low N conditions, the number of differentially expressed PTR genes increased significantly. The RNA-Seq data was analyzed using WGCNA to predict the potential interaction between genes. We classified the genes with similar expression pattern into one group, and obtained 25 target modules. Among these modules, three modules may be involved in rice nitrogen uptake and utilization, especially the brown module, in which hub genes were annotated as protein kinase that may regulate rice N metabolism.

Conclusions: In this study, we comprehensively analyzed the PTR gene family in rice. Ninety-six PTR genes were identified in Nipponbare genome and twenty-five genes locates on five large segmental duplication regions. The Ka/Ks ratio indicated that many PTR genes had undergone positive selection. The RNA-seq results showed that many PTR genes were involved in rice NUE. Protein kinases may play an important role in regulating NUE in rice. These results will provide a fundamental basis for molecular breeding of NUE in rice.

Background

Rice is one of the most important food crops in the world. As the population continues to grow, the food security issues are becoming more and more prominent^[1]. For rice production, the application of N fertilizer has been the major method to achieve high yield. However, excessive use of N fertilizer not only increases production costs, but also causes serious damage to environment. As the concept of “green super rice” has been proposed^[2], developing the rice varieties with more efficient use of N fertilizers has become the key to solving the contradiction between agricultural production and environmental protection. Recently, researchers have identified several loci that are associated with NUE in rice^[3,4]. In the previous study, we identified a major QTL *qNUE6* for rice NUE on chromosome 6 in rice, and

LOC_Os06g15370 may be the ideal candidate gene^[5]. The annotation for *LOC_Os06g15370* is a peptide transporter, and we named this gene as *OsPTR10*.

Peptide transporter is also known as proton-dependent oligopeptide transporter (POT). The main functions of PTR gene family are: (i) transport dipeptide/tripeptide; (ii) transport nitrate; (iii) transport other substrates. In plants, the main transporters responsible for nitrate uptake and transport are NRT1s and NRT2s. According to their nitrate transport activity, these transporters are also classified into low-affinity and high-affinity transporters. NRT1 family mainly includes low-affinity nitrate transporters and belongs to PTR. Thus, NRT1/PTR is also named as NPF. Tsay et al.^[6] first identified the PTR gene *CHL 1* (*AtNPF6.3* or *AtNRT1.1*) in *Arabidopsis*, which not only has the function of absorbing and transporting nitrate from the roots^[7], but also acts as a nitrate receptor and participates in nitrate response^[8]. Up to now, researchers have identified the functions of 26 NPF genes in *Arabidopsis*^[9].

In rice, it has also been shown that many PTR genes are functionally related to N uptake and utilization^[9]. *OsNRT1* (*OsNRT1.1a*) is the first PTR gene identified in rice^[10] and is located at the same locus as *OsNPF8.9*^[11]. *OsNRT1.1b* encodes a PTR protein with 6 transmembrane domains, and its overexpression can increase rice N uptake under high or low N conditions, while *OsNRT1.1a* only works under high N conditions^[12]. *OsNRT1.1A* localizes on the tonoplast and overexpression of it can up-regulate the genes related to nitrate and ammonium transport^[13]. Hu et al.^[14] showed that *LOC_Os10g40600* encoded a nitrate transporter NRT1.1B. Further studies found that *NRT1.1B* altered the rhizosphere microenvironment by regulating rice root microbial population, thereby affecting NUE between rice subspecies^[15]. *OsNPF2.2* (*OsPTR2*) can unload nitrate from the rice xylem and participate in nitrate transport from root to stem, thus affecting the growth and development of vascular system, or even the whole plant^[16]. Overexpression of *OsPTR6* increases the expression of ammonium transporter gene and the activity of glutamine synthetase; these effects can promote rice growth, but also reduces NUE under high ammonium conditions^[17]. Fang et al.^[18] showed that altering the expression of *OsPTR9* affected NUE, plant growth and production of rice. *OsNPF2.4* plays a role in NO₃-absorption, long-distance transport and redistribution; also, altering its expression indirectly affects the reuse of potassium in roots and stems. Hu et al.^[19] concluded that *OsNPF7.2* played a role in the intracellular distribution of nitrate in roots, thereby affecting rice growth under high nitrate condition. *SP1* encodes a putative PTR transporter that determines rice panicle length^[20]. In summary, these rice PTR genes play an important role in NUE. In rice genome, it's possible that there are still other PTR genes involved in N metabolism pathway.

Here, we use the latest Nipponbare genome to identify PTR genes, and compare them with the PTR genes in R498, *Oryza glaberrima*, maize, sorghum, peanut, soybean and *Arabidopsis*. The main aims of this study are: (i) determine the number of PTR genes in rice; (ii) understand the evolutionary relationship of PTR genes in rice and other 5 plants; (iii) analyze the regulatory network of PTR genes in rice NUE.

Results

Identification of PTR gene in cultivated rice

Among the 27 *Oryza* species, Asian cultivated rice (*Oryza sativa* L.) and African cultivated rice (*Oryza glaberrima* Steud.) are two species that have been domesticated and utilized by humans [21]. Using the conserved region sequence of PTR gene family, 96 PTR genes were identified in Nipponbare (*Oryza sativa* L. ssp. *japonica*) (Table S2), 85 were identified in R498 (*Oryza sativa* L. ssp. *indica*) (Table S3), and 78 were identified in *Oryza glaberrima* (Table S4).

Phylogenetic analysis of PTR genes

Multi-sequence alignment and phylogenetic analysis were performed on the protein sequences of 96 rice PTR genes using MEGA. Based on the evolutionary relationship, the 96 PTR genes were classified into 5 groups, and group I to V contained 20, 15, 26, 4 and 31 genes, respectively. The group V contained the most genes, which could be further classified into four subgroups, Va (3), Vb (4), Vc (8), and Vd (16) (Fig. 1).

To understand the evolutionary relationship of PTR genes between different *Oryza* species or subspecies, phylogenetic analysis was carried out using the PTR genes from Nipponbare, R498 and *Oryza glaberrima*. These PTR genes could be classified into 8 groups: group I contained 73 genes, which was the most, whereas group V had only 11 genes (Fig. 2a). The distribution of PTR genes in the 8 groups were similar among the three *Oryza* species (Table S5). Nipponbare genome was used for the further analysis shown below.

Leguminous plants can form a symbiotic relationship with N-fixing bacteria, which can convert N_2 in the air into NH_3 . Thus, we analyzed the leguminous plants peanuts and soybeans. We also analyzed these species, including the dicotyledonous plant *Arabidopsis*, and the important monocotyledonous plants maize, sorghum and rice. The plant genome sizes of these six species were 2.5 Gb [22], 1.1 Gb [23], 125 Mb [24], 2.3 Gb [25], 730 Mb [26] and 466 Mb [27], respectively. Although the maize genome is large, it only contained 66 PTR genes, whereas the legume genome contained many more PTR genes: 125 for peanuts and 117 for soybeans. This might be related to the need of N transport after N fixation in legumes.

Phylogenetic tree was performed on the protein sequences of 543 PTR genes from the six species (Table S6), and these genes could be classified into 4 groups, I, II, III and IV (Fig. 2b). The gene distribution of rice, maize and sorghum were consistent, and group I contained the most monocots PTR genes. Similarly, the gene distribution of peanut, soybean and *Arabidopsis* were consistent, and group II had the most dicots PTR genes.

The chromosome location and segmental duplication of rice PTR genes

The 96 PTR genes were unevenly distributed on 12 chromosomes in rice. Chromosome 1 had the most PTR genes and chromosome 9 contained the least. The details were shown in Fig. 3. There were 59 PTR genes that formed 17 gene clusters, accounting for 60.4% of the total PTR genes. Except chromosome 7,

8, and 9, all other chromosomes contained PTR gene clusters, and the cluster in 37.79–37.83 Mb region of chromosome 1 was the biggest, containing 9 PTR genes.

The segmental duplication events were analyzed using the 96 PTR genes. The results showed that there were 5 large genomic duplication regions and contained 25 PTR genes: (i) 37.76 Mb –38.75 Mb on chromosome 1 corresponding to 20.31 Mb –21.21 Mb on chromosome 5; (ii) 27.74 Mb –28.95 Mb on chromosome 2 corresponding to 29.11 Mb –30.42 Mb on chromosome 4; (iii) 0.15 Mb –0.21 Mb on chromosome 3 corresponding to 21.76 Mb –21.90 Mb on chromosome 10; (iv) 33.56 Mb –33.92 Mb on chromosome 3 corresponding to 5.31 Mb –6.14 Mb on chromosome 7; (v) 19.49 Mb –19.744 Mb on chromosome 8 corresponding to 12.13 Mb –12.45 Mb on chromosome 9 (Table 1).

Whole genome duplications were detected using the synonymous mutation rate K_s . The rice genome experienced three genome duplication events (Fig. 4). The whole genome duplication event shared by gramineous plants occurred at ~96 million years ago [28]. After that, another two independent genomic duplications events occurred in rice [29,30]. The K_a/K_s ratio reflects the extent to which all nucleotide sequences of a gene are positively selected during differentiation. If K_a/K_s is greater than 1, the gene is positive selection; if K_a/K_s is equal to 1, the gene is neutral selection; if K_a/K_s is less than 1, the gene is purify selection. The K_a/K_s ratios of paralogous gene pairs were 0.08–1.83, and 693 gene pairs were greater than 1. This result indicated that many PTR genes underwent positive selection (Table S7, Fig. 5).

Gene structure and motif composition of rice PTR genes

The information related to the evolution of the gene family can be obtained from gene structure analysis. Thus, we performed a gene structural analysis on the 96 PTR genes (Fig. 6a). In terms of the intron-exon composition, the PTR genes had 0–11 introns and 1–12 exons, and 10 genes contained only 1 exon. Usually, the structurally similar genes have closer evolutionary relationship.

Previous studies have shown that there are three highly conserved domains in most PTR genes [31]. In this study, there were five types of conserved domains in rice PTR genes, which were MFS, RNase_H_like, zf-RV, RT_like superfamily, and PBD. MFS can be further classified into MFS superfamily, MFS_NPF, MFS_NPF1_2, MFS_spinster_like, MFS_NPF5, MFS_NPF7, and MFS_NPF4. By performing conserved gene analysis on PTR genes, we found 77 genes had MFS superfamily domain and 16 genes contained NPF domain (Fig. 6b).

The expression profiles of PTR genes in near isogenic lines

Gene expression profile is often related to its function. Previous studies have shown that the proteins encoded by PTR genes can transport nitrate, playing an important role in plant growth and development. Therefore, we used RNA-Seq to analyze the expression profiles of 96 PTR genes in the near-isogenic lines NIL15 and NIL19 under HN and LN conditions in rice.

The qualities of transcriptome sequencing and sequence alignment results are shown in Table S8. The cDNA library of 18 samples had high sequencing quality and good genome coverage, which was suitable for further analysis. Based on the gene expression levels in different samples, we performed correlation analysis on commonly expressed genes and differentially expressed genes. The average correlation coefficient between biological replicates of the same sample was $r = 0.9357$ (Fig. S1), indicative of good reproducibility and reliable experimental results.

We used the qRT-PCR method to validate 6 PTR genes identified by RNA-Seq. The results showed that the qRT-PCR expression patterns of 6 DEGs were consistent with the RNA-Seq analysis (Fig. S2), suggesting that the RNA-Seq results were reliable for following analysis.

Previous studies have shown that the differences between NIL15 and NIL19 are on chromosomes 6, 8, 9, and 10^[5]. These regions contain 6 PTR genes, which were *LOC_Os06g13200*, *LOC_Os06g13210*, *LOC_Os06g15370*, *LOC_Os06g21900*, *LOC_Os06g38294*, and *LOC_Os10g22560*. The transcriptome sequencing results showed that the number of DEGs between the two lines was 928, 1,467 and 1,586 at three stages (Fig. S3), respectively. After the replacement with LN nutrient solution, the number of DEGs significantly increased first, and then stabilized. We performed functional annotation on DEGs using the GO database (<http://geneontology.org/>), and the classification results were shown in Fig. 7. In the biological process category, metabolic process related genes were the most; in the cellular component category, there were more genes related to the membrane and membrane part; in the molecular function category, transporter activity related genes showed up.

In living organisms, different genes coordinate to perform biological functions, and the same action across different genes forms pathway. We classified and annotated the DEGs using the KEGG database (<https://www.kegg.jp/>), and the classification results were shown in Fig. 8. The main involved pathways were: metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems and human diseases, in which the metabolism pathway contained the most genes. Also, N metabolism-related genes were included in energy metabolism group.

The expression profiles of 96 PTR genes showed in Fig. 9. There were only two differentially expressed PTR genes between the two rice lines at 0 d, which were *LOC_Os06g13210* and *LOC_Os10g02100*. However, at 3 d and 6 d, the number of differentially expressed PTR genes increased to 12 and 7 (Table S9), in which the *LOC_Os04g50950(OsPTR6)*^[17], *LOC_Os06g49250 (OsPTR9)*^[18] and *LOC_Os11g12740 (SP1)*^[20] have been reported to participate in N uptake and transport in rice.

The gene regulation network of NUE in rice

The expression profile data was analyzed using weighted gene co-expression network analysis (WGCNA) to predict the potential interaction between genes. The correlation coefficient of the expression levels between genes was calculated and taken n-degree power, so that the numerical distribution of the correlation coefficient gradually conformed to scale-free distribution. 58,176 genes were screened: (i) at

least 50% of samples are required to have expression; (ii) remove the gene with the smallest variance change of 25%. Finally, 20,282 genes are left for further analysis.

Then, we cluster the samples and find that 0 d, 3 d and 9 d can be separated well (Fig. S4a) Data quality can ensure the reliability of subsequent analysis. Then, soft threshold was selected to construct gene coexpression network (Fig. S4b,c). 20,282 genes were used for WGCNA analysis, and randomly selected 400 genes from gene set and draw expression cluster heatmap of genes (Fig. S5). We classified the genes with similar expression pattern into one group, and identified 25 modules. We analyze the characteristics of the genes in the module and found modules with biological significance (Fig. S6). Subsequently, GO and KEGG databases were used for functional enrichment analysis, and the blue, brown and turquoise modules were related to N metabolism. The blue module contained six NUE-related genes, which were *LOC_Os01g36720* [β^2], *LOC_Os03g62200* [β^3], *LOC_Os04g40410* [β^4], *LOC_Os04g43070* [β^5], *LOC_Os05g39240* [β^6] and *LOC_Os06g49250* [β^8]. The brown module contained *LOC_Os01g61510* [β^3], *LOC_Os01g65000* [β^3], *LOC_Os02g47090* [β^7] and *LOC_Os04g50950* [β^7]. The turquoise module contained *LOC_Os02g02190* [β^8], *LOC_Os02g40710* [β^9], *LOC_Os02g40730* [β^0], *LOC_Os03g13274* [β^0], *LOC_Os03g48180* [β^1], *LOC_Os10g40600* [$\beta^{15,42}$], *LOC_Os11g12740* [β^0] and *LOC_Os12g44100* [β^6].

We constructed a WGCNA for these three modular genes. There were 59 DEGs in the brown module, and *LOC_Os03g29410*, *LOC_Os02g14480*, *LOC_Os04g24220*, *LOC_Os11g39370*, *LOC_Os09g30120* were hub genes in the regulatory network (Table S10, Fig. 10a). The functions of the most connected genes *LOC_Os03g29410*, *LOC_Os04g24220*, *LOC_Os02g14480*, and *LOC_Os11g39370* are related to protein kinase. Based on Go annotation, the functions of these genes were related to activity (GO: 0016301), stress (GO:0006950), biotic stimulus (GO: 0009607), signal transduction (GO: 0007165), metabolic process (GO: 0008152). There were 129 DEGs in the turquoise module, *LOC_Os06g11990*, *LOC_Os10g22590*, *LOC_Os08g44360*, *LOC_Os07g45060*, *LOC_Os07g06680*, *LOC_Os05g08370*, *LOC_Os04g59330* and *LOC_Os02g51710* were hub gene (Table S11, Fig. 10b). Due to the small number of DEGs in the blue module, we were not able to identify the hub genes.

Discussion

Ninety-six PTR genes were identified in Nipponbare genome, which differed from the 84 PTR genes identified by Zhao et al. [31]. The possible reasons are: (i) with the development of sequencing technology, the Nipponbare genome database has been continuously edited and improved [43,44]; (ii) the methods used in the two studies are different. Zhao et al. used BLASTP and BLASTN to search for PTR genes based on the conserved amino acid sequence of PTR domain. Our study used HMMER3 to search for PTR genes based on the conserved DNA sequence of PTR gene family, followed by manual verification.

The 96 PTR genes in rice can be classified into 5 groups, and the number of each group was not same. In addition, *LOC_Os03g13250* and *LOC_Os03g13274* (*OsNRT1*) [β^{10}], *LOC_Os03g01290* and *LOC_Os10g40600* (*OsNRT1.1B*) [β^{14}], *LOC_Os07g21960* and *LOC_Os12g44100* (*OsPTR2*) [β^{16}],

LOC_Os04g50940 and *LOC_Os04g50950* (*OsPTR6*)^[17], *LOC_Os04g36040* and *LOC_Os11g12740* (*SP1*)^[20] were more closely related. Therefore, these 5 genes may participate in N uptake and transport in rice.

Among the Asian cultivated rice, *japonica* rice had more PTR genes than *indica* rice; also, the Asian cultivated rice had more PTR genes than African cultivated rice. Although Nipponbare, R498, and *Oryza glaberrima* contained different numbers of PTR genes, their distributions in the 8 groups were evolutionarily consistent. When comparing rice with maize and sorghum, rice had the smallest genome but contained the most PTR genes. Leguminous plants have rhizobium on the roots, which can convert inorganic N in the air into organic N. We found that peanuts and soybeans had more PTR genes, which may be related to the need of N transport after N fixation. In the phylogenetic relationship, the distribution of PTR genes in monocots and dicots were significantly different, which may be because the two types of plants produced their own unique PTR genes during evolution^[45].

Gene family can be formed by whole genome duplication or polyploidization, tandem duplication, and segmental duplication. In this study, we found that many PTR genes formed by tandem duplication of PTR genes were often closely arranged on the same chromosome. Previous studies have also shown that the genes in the same gene cluster have similar sequences and functions^[46]. By using the PTR gene to analyze segmental duplication events, we found that there were five duplicated blocks in rice genome, which is consistent with the previous results^[29]. Among the 96 PTR genes in rice, 25 were contained within the segmental duplication. Moreover, rice underwent three whole genome duplication events: the first whole genome duplication event shared by all gramineous plants, and the other two chromosomal fragments doubling events occurred independently on rice genome. This result is consistent with previous studies^[29,30,47]. In addition, the PTR genes in the duplication region were clustered into the same group, with closer phylogenetic relationship. There were many PTR gene pairs with Ka/Ks values greater than 1, indicating that non-synonymous mutations lead to functional changes in the PTR gene, making it more suitable for the environmental change^[48].

Gene structure is related its function. Previous studies have shown that there are three conserved motifs in the protein sequences of rice PTR genes^[31]; and all of them are contained in MFS family, which has 12 transmembrane domains^[49]. In this study, we found most PTR genes contained MFS family motifs, and the known rice NUE genes of *OsNRT1*^[0], *OsNRT1.1A*^[3], *OsNRT1.1b*^[2], *OsPTR2*^[6], *OsPTR6*^[7], *OsPTR9*^[8], *OsNPF7.2*^[20] and *SP1*^[21] all contains MFS family domain. Moreover, 16 out of 96 PTR genes have NPF domain. In plants, NPF proteins transport a variety of substrates: nitrates, peptides, amino acids and many more^[11]. Due to the long intron of *LOC_Os11g18044*, the sequence length (10.7 kb) was greater than other PTR genes in rice; moreover, it contained a longer MFS family domain, suggesting that the function of this gene might be more complex.

Transcriptome analysis of the N15 and NIL19 revealed that most of the DEGs are associated with metabolism and transport activation, which is consistent with plant N metabolism pathways. There were only 2 differentially expressed PTR genes under the HN condition, but 12 genes were found under the LN

condition. These results indicated that the PTR genes may play an important role in the N metabolism of rice, and these genes may have interactions. Previous studies have shown that *LOC_Os06g49250* (*OsPTR9*)^[18], *LOC_Os04g50950* (*OsPTR6*)^[17], *LOC_Os11g12740* (*SP1*)^[20] affect the NUE in rice, and these genes were all the differentially expressed PTR genes.

In the three modules that may be associated with rice NUE, each module had a different number of genes involved in N metabolism, but they all contained genes related to the uptake and utilization of ammonium and nitrate, indicating the synergistic expression of ammonium and nitrate genes affects the uptake and utilization of N in rice. This result suggested that N uptake, transport, assimilation and signal transduction involved complex gene regulatory networks in rice^[9].

In the brown module, the hub genes' functional annotations are protein kinase. Protein kinase functions as a protein to regulate gene expression, metabolism, ion channels and other signal transduction pathways. Previous studies have shown that some protein kinases are related to N metabolism. AtCIPK8, a calcineurin B-like (CBL)-interacting protein kinase (*CIPK*) gene, was found to be involved in early nitrate signaling in *Arabidopsis*^[50]. CIPK and mitogen-activated kinase kinase kinase (MEKK) are putative new regulatory proteins involved in the early nitrate signalling^[51]. PbrSLAH3 interacts with calcium-dependent protein kinase 32 (PbrCPK32) and may participate in transporting of nitrate nutrition in pear root^[52]. OsSAPK8 is a counterpart of AtOST1 for the activation of OsSLAC1, which is a nitrate-selective anion channel in rice^[53]. Ca²⁺-sensor protein kinases (CPKs) are master regulators that orchestrate primary nitrate responses^[54]. Furthermore, brassinosteroid (BR) signal kinase BSK3 regulates root elongation under limited N conditions^[55]. *LOC_Os11g39370* is a BRASSINOSTROID INNOVATIVE 1-associated receiver kinase 1 precursor gene. Therefore, the brown module may be related to the regulation of N uptake and utilization in rice. The candidate gene *OsPTR10* in the previous study is included in the brown module, and its function may be related to N metabolism.

Conclusions

In this study, we comprehensively analyzed the PTR gene family in rice. Ninety-six PTR genes were characterized and classified into 5 main groups in the Nippobare genome. The distribution of PTR genes between monocots and dicots was different, and legumes had a greater number of PTR genes. The rice genome experienced three genomic duplication events, and twenty-five rice PTR genes locate on 5 large segmental duplication regions. The Ka/Ks ratio indicated that many PTR genes had undergone positive selection. The structure and motif analysis showed that five types of conserved domains in rice PTR genes. The differentially expressed PTR genes increased significantly under LN conditions. Using WGCNA, there were three modular genes associated with NUE, especially the yellow module. These results provide information for a better understanding of the biological function for the PTR gene and will contribute to the genetic improvement of NUE in rice.

Methods

Genome sequence retrieval and PTR gene identification

The six species genome sequence is downloaded from the following database: Nipponbare (MSU_7.0, <http://rice.plantbiology.msu.edu/index.shtml>), R498^[56] (CANU, <http://www.mbkbase.org/R498/>), *Oryza glaberrima*^[57] (*Oryza_glaberrima_V1*, http://peanutgr.fafu.edu.cn/Genome_Browse.php), *Arabidopsis*^[24] (TAIR10, http://plants.ensembl.org/Arabidopsis_thaliana/Info/Index), *Arachis hypogaea*^[2] (PGR, http://peanutgr.fafu.edu.cn/Genome_Browse.php), *Zea mays*^[5] (B73_RefGen_v4, http://plants.ensembl.org/Zea_mays/Info/Index), *Glycine max*^[3] (*Glycine_max_v2.1*, http://plants.ensembl.org/Glycine_max/Info/Index), and *Sorghum bicolor*^[6] (*Sorghum_bicolor_NCBIv3*, http://plants.ensembl.org/Sorghum_bicolor/Info/Index). The PTR gene family HMM (hidden markov model) file was downloaded from the Pfam (<http://pfam.xfam.org/>). We did a genome-wide search on PTR genes using HMMER3, with e-value cut off = 0.001 and alignment sequence greater than 198 (50% of 395). The protein sequences were extracted and put into the three major databases: CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), SMART (<http://smart.embl.de/>), and PFAM (<http://pfam.xfam.org/>). Then, the resulting homologous family genes were manually verified for domain conservation. If conserved domain was detected in one of the three databases, it was considered as reliable family gene. Finally, the conserved domains and protein sequence were extracted.

Phylogenetic tree construction

ClustalW was used to perform multi-sequence alignment on the identified PTR genes. The NJ tree was constructed using MEGA7, with the bootstrap repeat number of 1000 and other parameters as default. The phylogenetic tree was colored by iTOL (<https://itol.embl.de/>).

Chromosomal distribution and gene duplication

All protein vs all protein alignment was performed using BLAST (Basic Local Alignment Search Tool), and the protein pair with E-value < 0.00001, identity > 90%, and minimum coverage of matching region on query sequence to subject sequence > 75% were extracted as homologous protein. The homologous protein file and the protein coordinate file were input into MCScanX (<http://chibba.pgml.uga.edu/mcscan2/>) for collinear region identification. Finally, the collinear results were presented using Circos (<http://circos.ca/>).

Analysis of gene structure and conserved motif

The PTR gene structure, mainly including UTR (untranslated region), introns and exons, was analyzed through GSDS (<http://gsds.cbi.pku.edu.cn/>). The motif sequence prediction was performed via MEME (<http://meme-suite.org/tools/meme>) and the motif map was presented using TBtools.

Plant materials and hydroponics

This study used the two near-isogenic lines: NIL15 (NIL-13B4, low NUE) and NIL19 (GH998, high NUE). The information about the two lines can be found in previous studies [5]. The evenly germinated seeds were selected and cultured in 96-well cultivation instrument with 1.4 mM NH₄NO₃ (high nitrogen, HN) nutrient solution. The nutrient solution was prepared according to the method in Yoshida et al. [58]. The nutrient solution was changed every 3 days, and the pH was adjusted to ~5.5 using MERS-NaOH. The seeds were cultured in a 28 °C light incubator (13 h light /11 h dark) until the 3-leaf stage. Then, at 3-leaf stage, the nutrient solution was replaced with 0.14 mM NH₄NO₃ (low nitrogen, LN) solution, and samples were taken at 0 d, 3 d, and 6 d, with 3 biologically replicates for each sample.

RNA extraction and sequencing

The RNA of each sample was extracted using Trizol method. The purity and integrity of each RNA sample were examined by agarose gel electrophoresis. The purity of DNA was checked by Nanodrop (Thermo Fisher Scientific, USA) (OD 260/280 ≈ 2.0). RNA concentration was quantified by Qubit (Thermo Fisher Scientific, USA) accurately, and the minimum concentration was 50 ng/ul.

mRNA was isolated from total RNA using the Oligo (dT) coated magnetic beads. Then, the mRNA was randomly fragmented into ~300 bp fragments by fragmentation buffer. With reverse transcriptase and a six-base random primer (random hexamers), the mRNA was subsequently reverse-transcribed into single-strand cDNA, which then formed into a stable double-stranded structure via two-strand synthesis. The constructed library was sequenced using Illumina NovaSeq 6000.

Sequencing quality control and alignment

After quality-control filtering, the clean reads were compared with the reference genome (<http://rice.plantbiology.msu.edu/index.shtml>) to obtain the mapped reads for subsequent analysis. Sequence alignment was performed using TopHat2 [59]. The mapping rate was usually higher than 70% when the reference genome was completely annotated and the experiment was free of contamination.

Quantitative RT-PCR

The expression level of 6 genes were measured by qRT-PCR. The primers were designed based on target gene sequence (Table S1), and *Actin3* was used as a reference [60].

Differentially expressed gene analysis

According to the results of feature counts alignment to reference genome and the annotation file, the gene read counts for each sample were obtained, followed by FPKM (fragments per kilobase million) conversion to obtain standardized gene expression levels. DESeq2 [61] was used to perform statistical analysis on raw counts. The default thresholds were: p-adjust < 0.05 and /log₂FC/ > = 1, which yielded the differential genes between two groups or two samples, whose differential expression folds greater than 2 and *P* value less than 0.05 after multiple tests correction.

Weighted gene co-expression network analysis

After background correction and normalization of gene expression data, the nonstandard genes and less altered genes were filtered, so that the gene correlation intensity fell into scale-free distribution. After data pre-processing, the genes were classified: the genes with similar expression patterns were classified into one type, which was called a module. Then, the relationships among inter-module genes or intra-module genes were analyzed. Key modules were obtained by correlating with phenotypic data, and the module's hub gene was obtained by visualization network analysis.

Declarations

Abbreviations

N: Nitrogen; PTR: Peptide transporter; NUE: Nitrogen use efficiency; NPF: NRT1/PTR family; DEG: Differentially expressed genes; NIL: Near-isogenic line; HMM: Hidden markov model; NJ: Neighbor-joining method; UTR: untranslated region; qRT-PCR: Quantitative RT-PCR; FPKM: Fragments per kilobase million; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; WGCNA: Weighted gene co-expression network analysis

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data analysed during this study are included in this article and its Additional files. Data supporting the findings of this work are available within the paper and its Supplementary Information files. The RNA-seq data that support the findings of this study have been deposited in National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with the accession code PRJNA573824 [<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA573824>].

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XHY and DTL conceived this study. BXN, ZQZ, YYW and QLT performed data analysis. WYZ (Weiyong Zeng), JG, WYZ (Weiyong Zhou) and HFL performed the experiments, XZX and YZ wrote this manuscript. XHY and GFD revised this manuscript. All authors read and approve the paper.

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Table

Due to technical limitations, Table 1 is only available for download from the Supplementary Files section.

Supplementary Files Legend

Additional file 1: Table S1. Primer sequences used for qRT-PCR.

Additional file 2: Table S2. Identification of 96 PTR genes in Nipponbare.

Additional file 3: Table S3. Identification of 85 PTR genes in R498.

Additional file 4: Table S4. Identification of 78 PTR genes in *Oryza glaberrima*.

Additional file 5: Table S5. The distribution of PTR gene in Nipponbare, R498 and *Oryza glaberrima*.

Additional file 6: Table S6. The distribution of PTR genes in six species.

Additional file 7: Table S7. Ka/Ks ratio of PTR gene pairs in rice.

Additional file 8: Table S8. Sequencing data and mapping to Nipponbare genome.

Additional file 9: Table S9. The differentially expressed PTR genes at three stages.

Additional file 10: Table S10. The differentially expressed genes in the brown module.

Additional file 11: Table S11. The differentially expressed genes in the turquoise module.

Additional file 12: Figure S1. The correlation coefficient of 18 samples based on the gene expression levels. The colored box shows correlation coefficient.

Additional file 13: Figure S2. DEGs were validated by qRT-PCR using the same sample as that in RNA-Seq. X-axis represents the stage of 3 treatments, the purple column represents qRT-PCR results in NIL19, the green column represents qRT-PCR results in NIL15, the blue column represents RNA-Seq results in NIL19, and the yellow column represents RNA-Seq results in NIL15. Y-axis represents the relative level of gene expression, RNA-Seq uses $2^{-\Delta\Delta Ct}$ value, and RNA-Seq uses FPKM value. Error bars indicate standard deviations of three biological repetitions.

Additional file 14: Figure S3. The DEGs were screened by DESeq2. A1 vs A2 represents 928 DEGs between NIL15 and NIL19 at 0 d. B1 vs B2 represents 1,467 DEGs between NIL15 and NIL19 at 3 d. C1 vs C2 represents 1,586 DEGs between NIL15 and NIL19 at 0 d.

Additional file 15: Figure S4. Sample cluster dendrogram and soft-thresholding (β) values estimation. *a* Sample cluster dendrogram and clinical trait heatmap of 18 samples based on their expression profile. *b* Analysis of scale-free fit index of each β value from 1 to 20. *c* Analysis of mean connectivity of each β value from 1 to 20. $\beta = 10$ was chosen for subsequent analyses as it has the biggest mean connectivity when the scale-free fit index is up to 0.895.

Additional file 16: Figure S5. The gene network of 400 randomly selected genes using a heat-map plot. The gene dendrogram and module assignment are also shown along the top. The colors, ranging from yellow to red, indicate low to high interconnectedness.

Additional file 17: Figure S6. Identification of modules associated with NUE in two near-isogenic lines. The colors, ranging from blue through yellow to red, indicate low to high correlations.

Figures

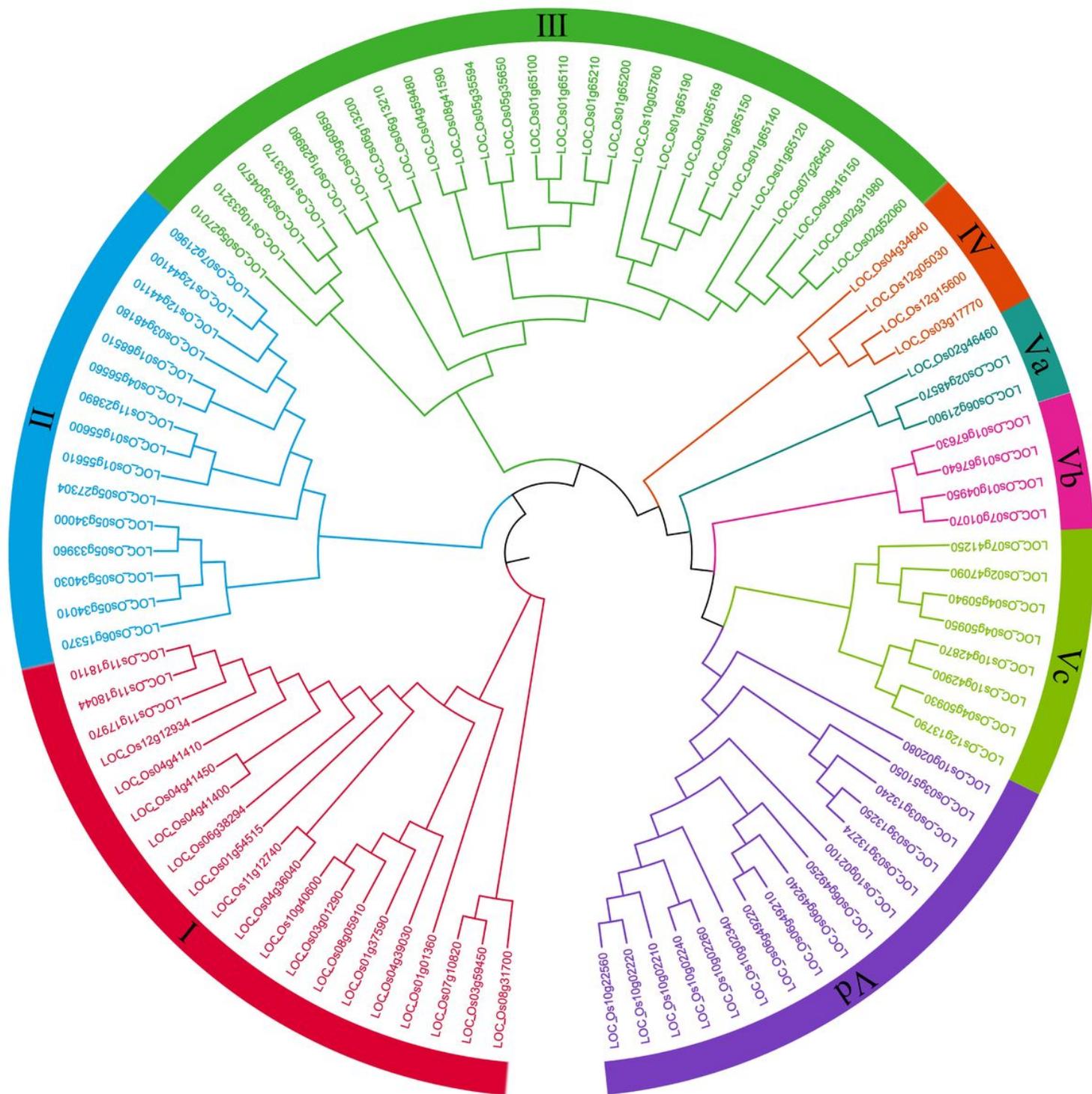


Figure 1

Phylogenetic tree and distribution of PTR genes in Nipponbare. The different-colored arcs indicate different groups. Group I classified into 4 subgroups. Group II, III, IV and V contain 20, 15, 26, 4 and 31 genes. Va, Vb, Vc, and Vd contain 3, 4, 8 and 16 genes.

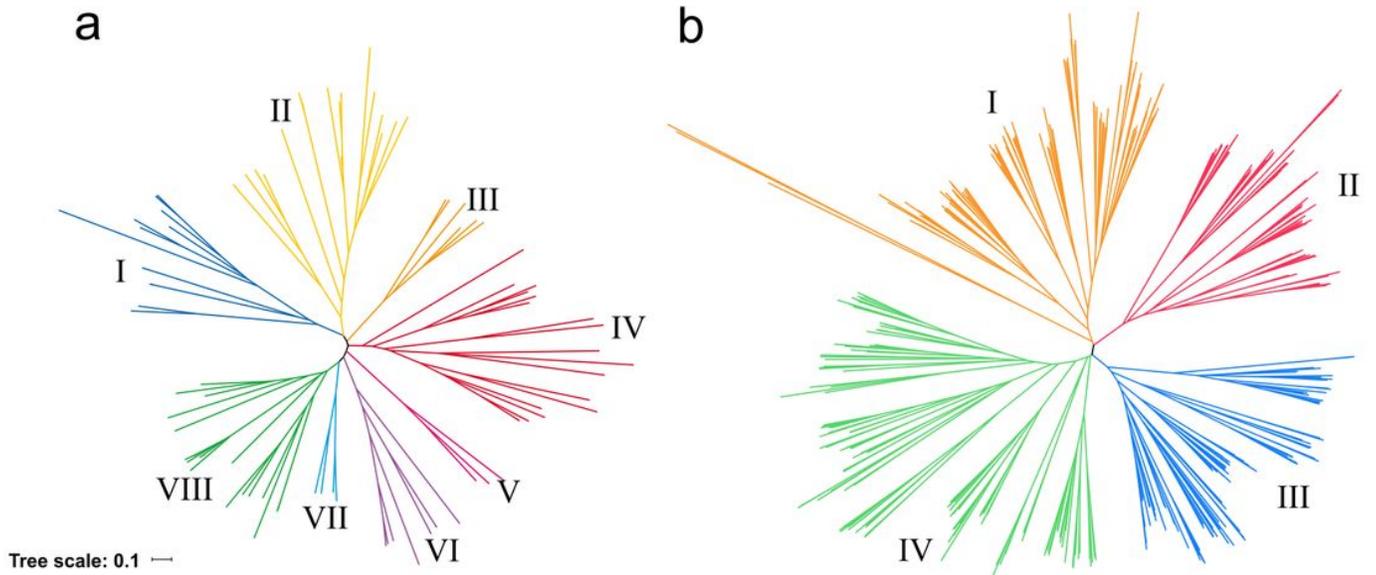


Figure 2

Distribution of PTR genes in different species. a Neighbor-joining tree of 259 PTR genes in Nipponbare, R498 and *Oryza glaberrima*, these genes classified into 8 groups. The different-colored arcs indicate different groups. Group I, II, III, IV, V, VI, VII and VIII contain 29, 73, 17, 46, 15, 23, 11 and 45 genes. b Neighbor-joining tree of 543 PTR genes in rice, maize, sorghum, peanut, soybean and *Arabidopsis*, these genes classified into 4 groups. Group I, II, III and IV contain 140, 87, 135 and 181 genes. Group I contains the most PTR genes of monocots, and group II had the most PTR genes in dicots. The scale bar indicates the simple matching distance.

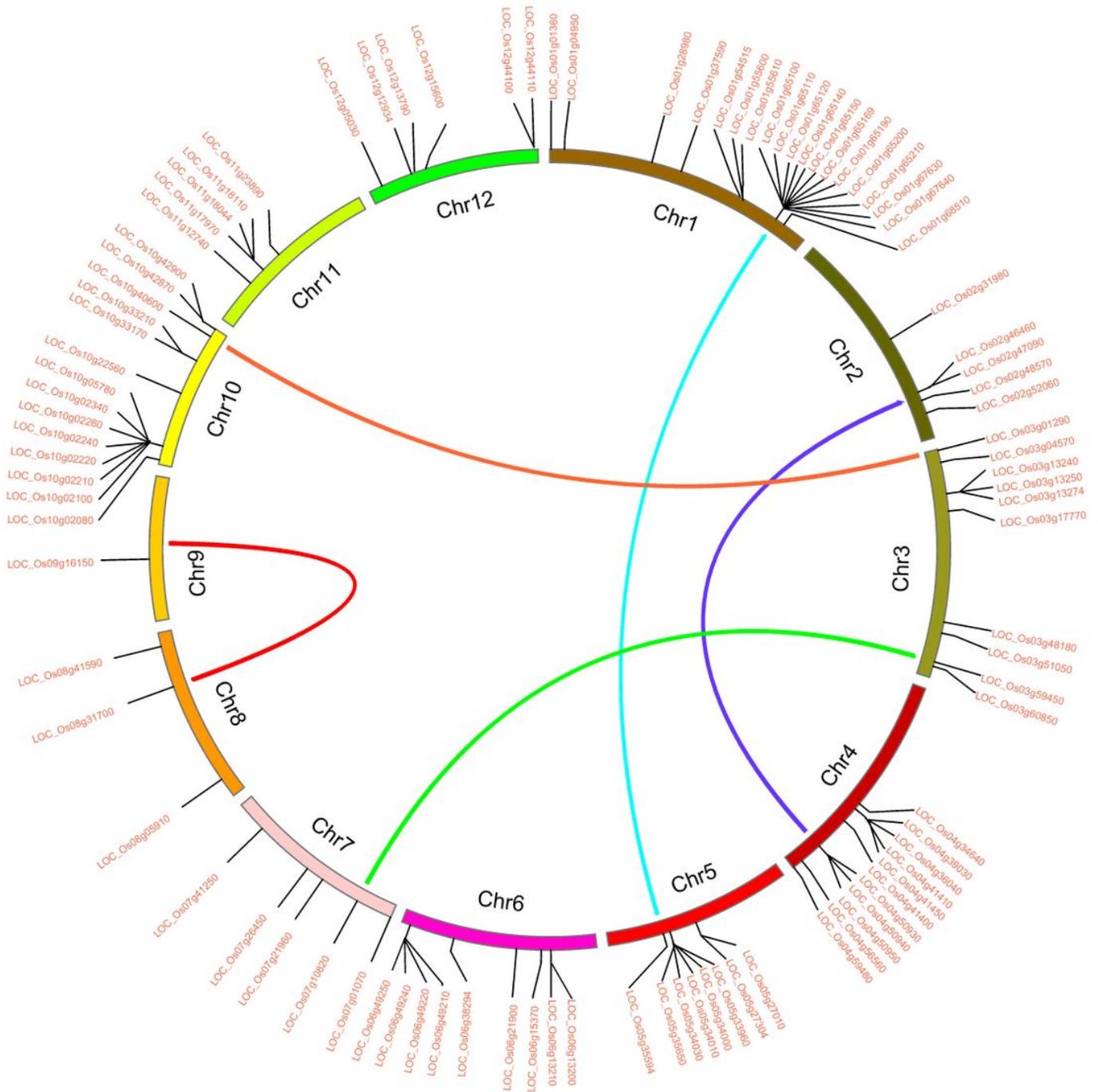


Figure 3

Schematic representations for the chromosomal distribution and segmental duplication events of rice PTR genes. The duplicated blocks were connected with colored lines. The PTR genes tightly arranged together represent gene cluster. The different-colored arcs indicate different chromosomes. The chromosome number is indicated at the bottom of each chromosome.

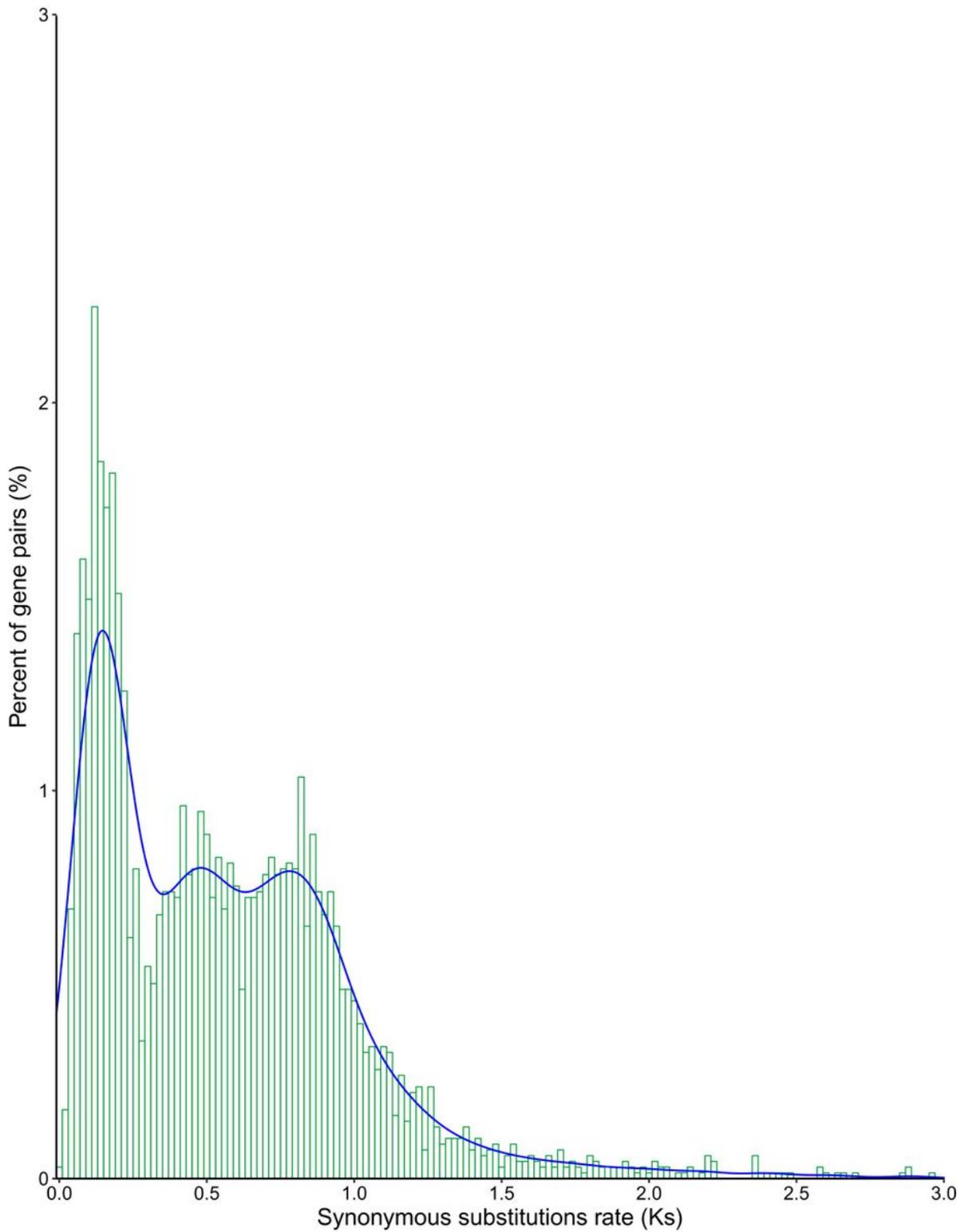


Figure 4

Whole genome duplications of rice using PTR genes. X-axis indicates the synonymous mutation rate K_s . Y-axis indicates PTR paralogous gene pairs. Every peak represents a genome duplication event. The farther away from the origin represents the earlier the genome duplication event occurs.

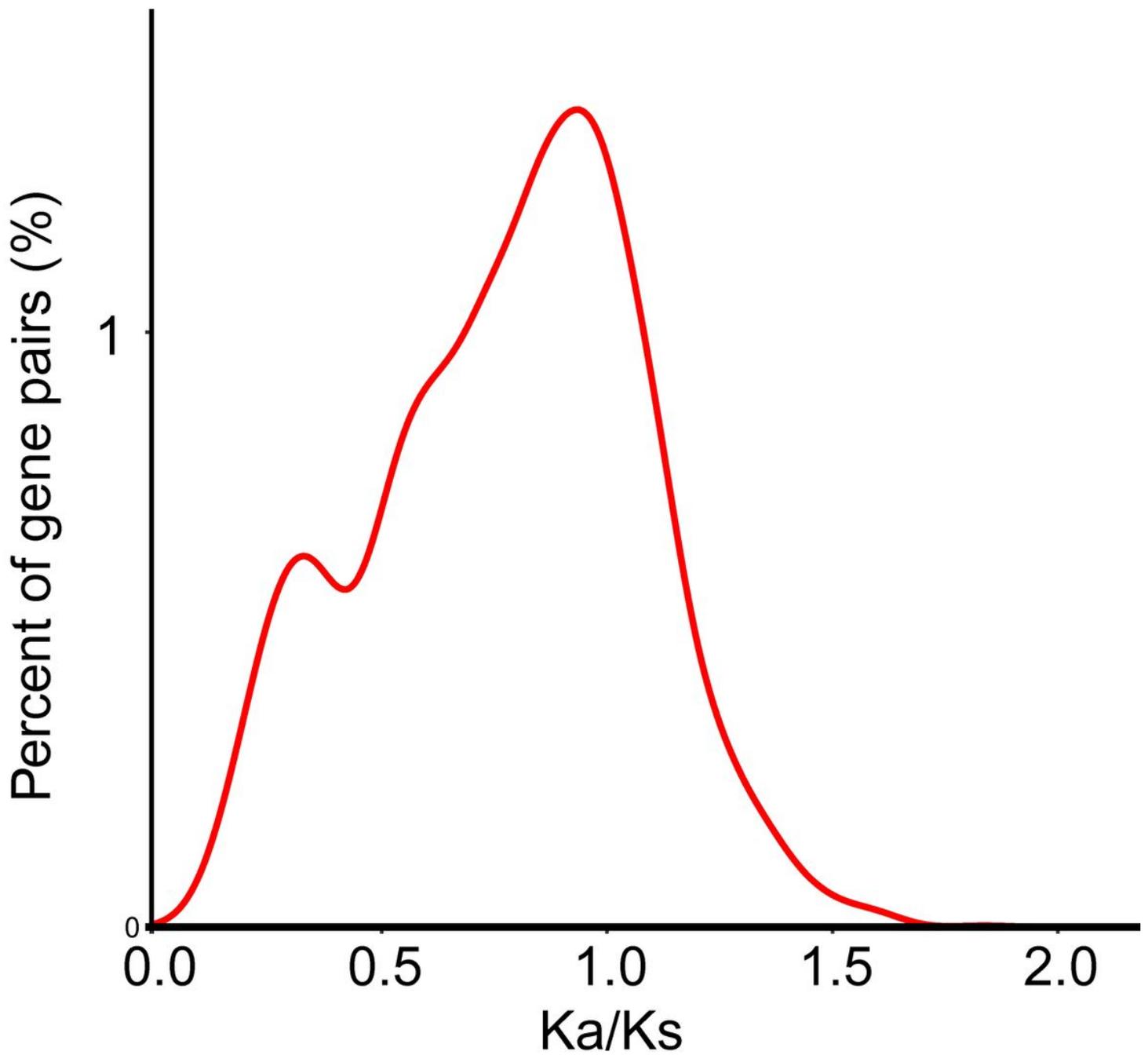


Figure 5

PTR genes underwent positive selection. X-axis indicates Ka/Ks ratio. Y-axis indicates PTR paralogous gene pairs. $Ka/Ks < 1$ represents positive selection; $Ka/Ks = 1$ represent neutral selection; $Ka/Ks > 1$, represents purify selection.

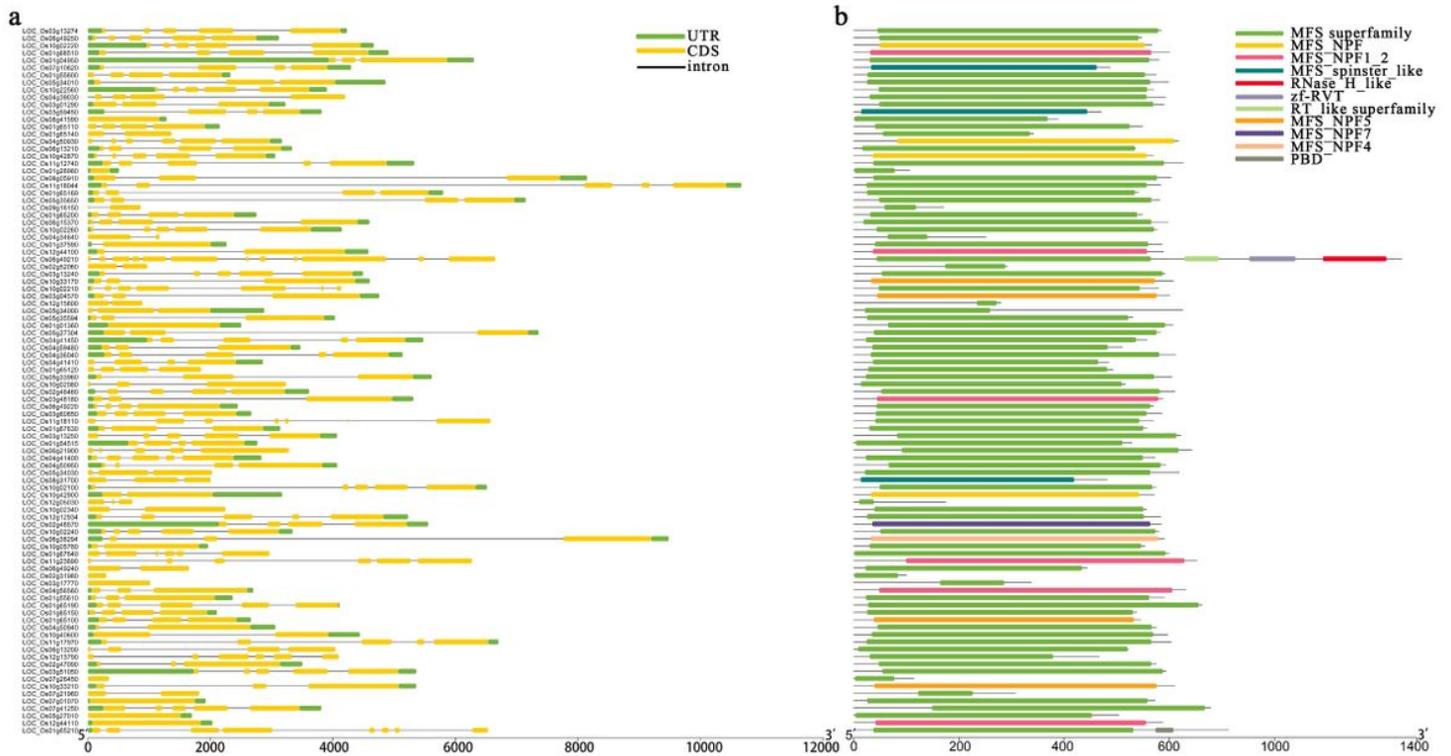


Figure 6

Gene structure and conserved motifs in PTR genes in rice. a Exon-intron structure of pineapple PTR genes. Green boxes indicate untranslated regions; yellow boxes indicate exons; black lines indicate introns. b The motif composition of rice PTR proteins. The eleven type motifs are displayed in different colored boxes. The information for each motif is shown on the right. The length of gene/protein can be estimated using the scale at the bottom.

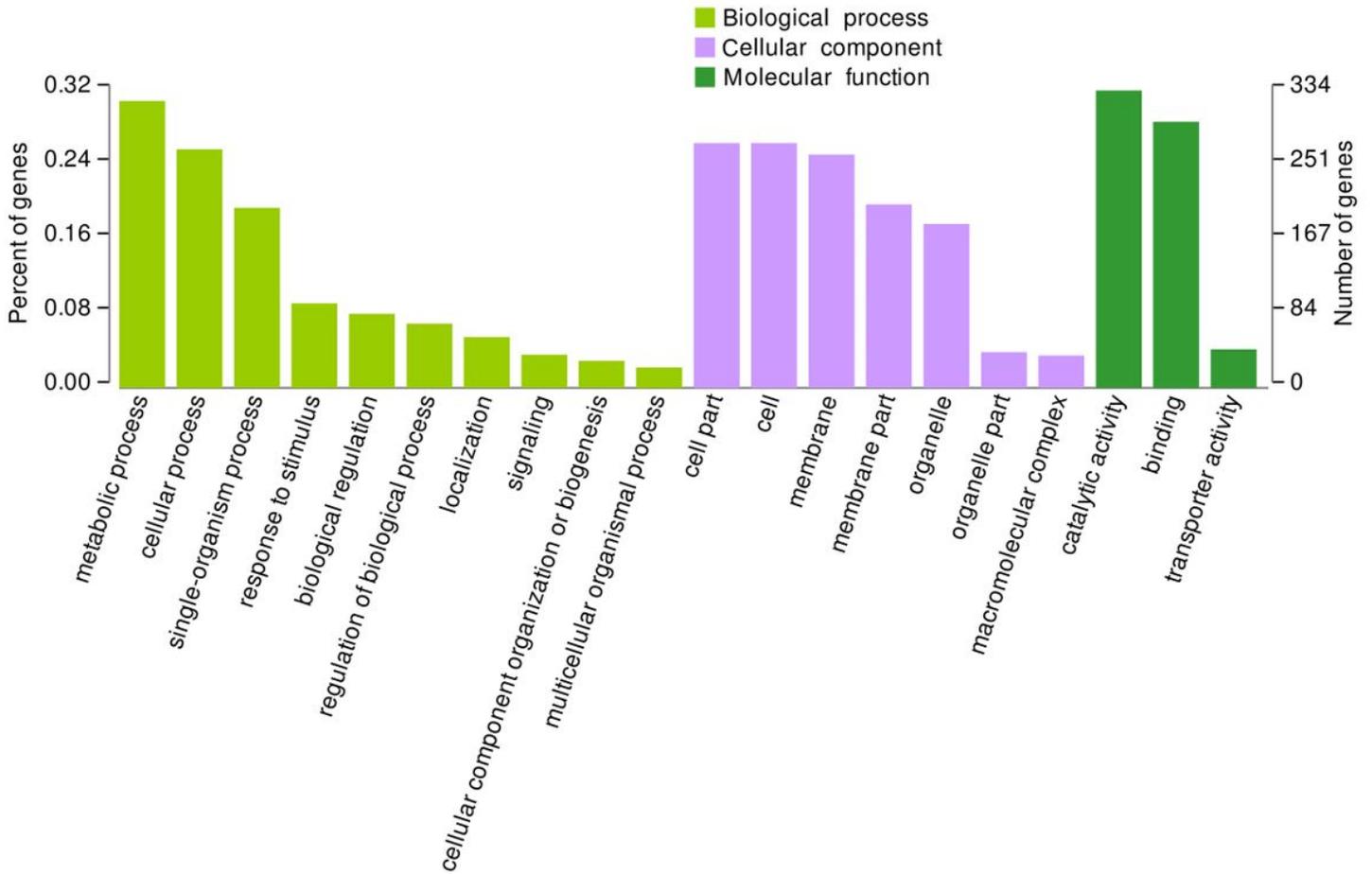


Figure 7

GO functional annotation for DEGs at three stages.

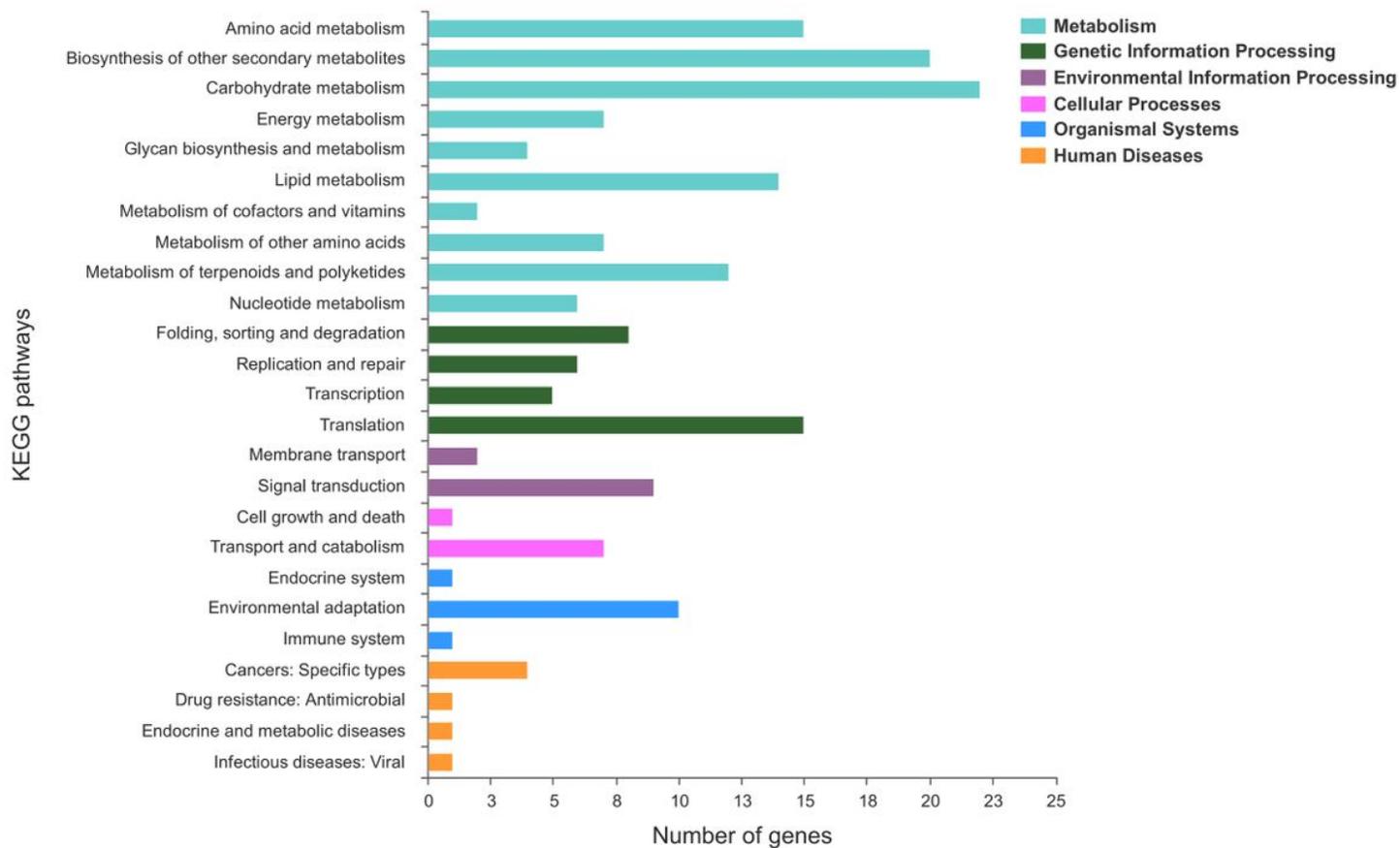


Figure 8

KEGG functional annotation for DEGs at three stages.

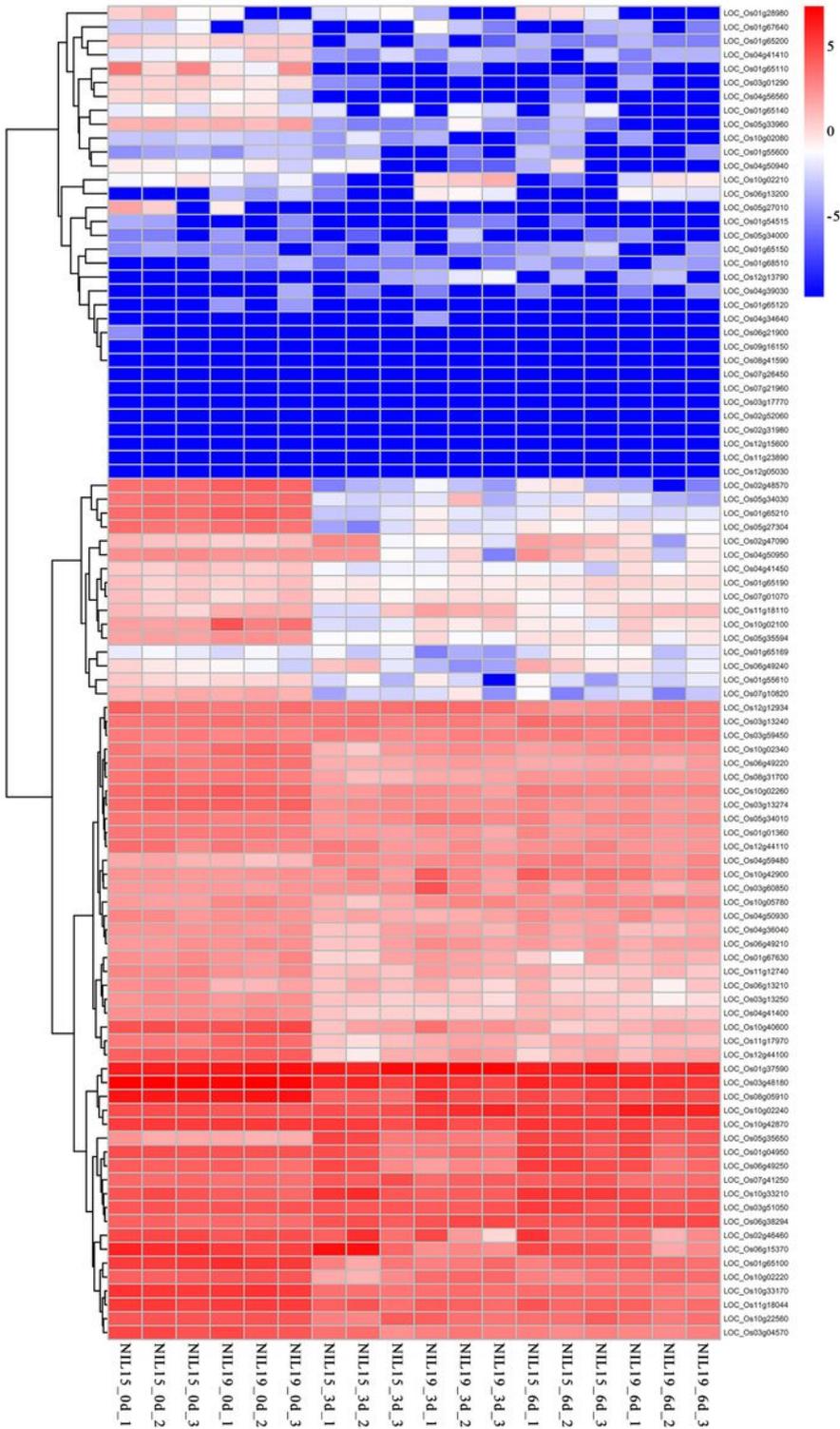


Figure 9

The expression profiles of 96 PTR genes at three stages in two near-isogenic lines. The cluster of PTR gene expression profiles are showed on the left; the gene name is showed on the right.

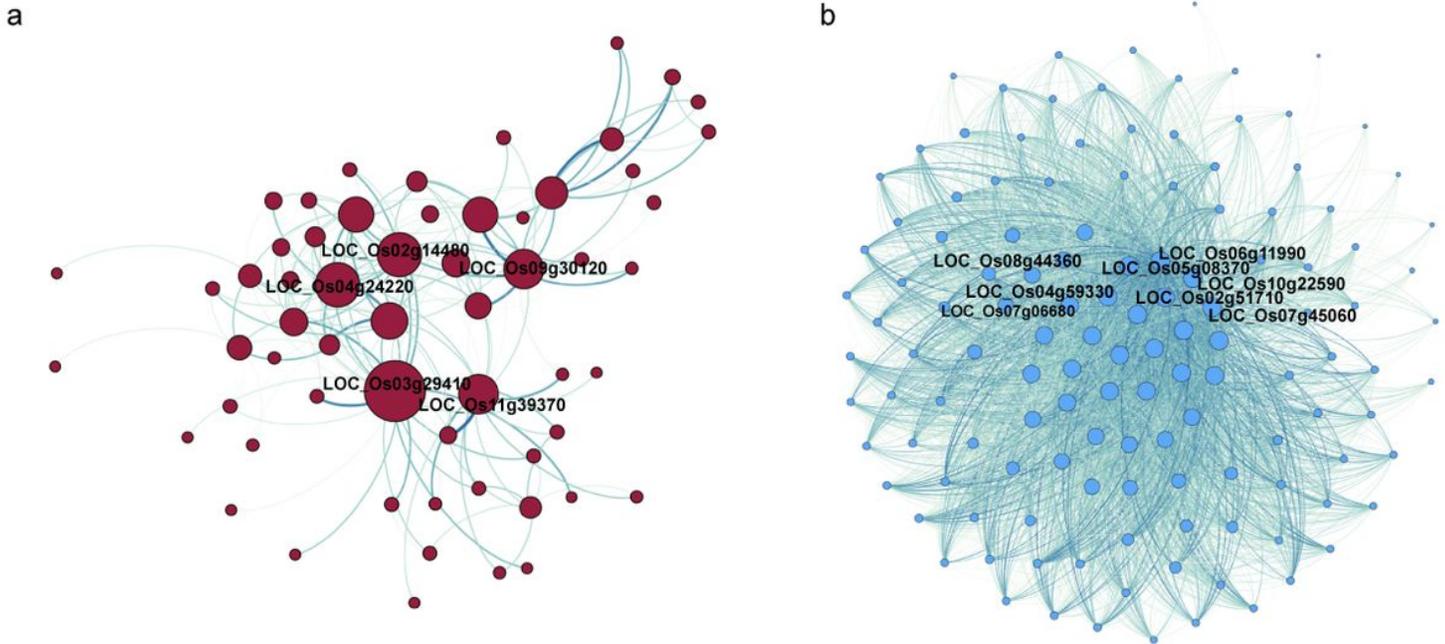


Figure 10

Gene co-expression networks. a 59 DEGs with the highest weight are in the brown module. b 129 differentially expressed PTR genes with the highest weight are in the turquoise module. The top most highly connected genes were signed. The red and blue circles represent DEGs.

Supplementary Files

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