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Genome-wide identification of Phosphate Transporter gene family in *Trichoderma harzianum* and expression in response to biotic and abiotic stress

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ABSTRACT

Phosphorus is an essential nutrient for organismal growth and development, and its absorption and transport primarily depend on the phosphate transporter (PHT) family. Although the functions of PHTs have been extensively studied in plants and algae, the researches on PHTs in microorganisms, particularly in *Trichoderma* species, remain limited. In this study, we identified 22 PHT family members in *Trichoderma harzianum* T-aloe through a genome-wide analysis. Phylogenetic analysis classified these members into three major clusters: Cluster I (8 members), Cluster II (12 members), and Cluster III (2 members). Furthermore significant differences among cluster members in gene structure, protein topology, subcellular localization, conserved motifs, and promoter cis-elements were revealed. Notably, the pronounced induction of all TrPHT genes under phosphate deficiency, coupled with their suppression under salt stress, suggests a finely-tuned regulatory mechanism that coordinates phosphate homeostasis with stress adaptation. In addition, *TrPHT1/2/4/15/22* were induced in response to *Fusarium graminearum* stress. The *TrPHT1/4/22* mutants exhibited significantly decreased total phosphorus content, accompanied by compromised antifungal efficacy against *F. graminearum* and reduced salt stress tolerance. Together, our results provide foundational insights into the PHT family in *T. harzianum* and demonstrate that TrPHTs are critical for maintaining phosphorus homeostasis and mediating responses to both biotic and abiotic stresses.

Keywords: Trichoderma; phosphate transporters; biotic stress; abiotic stress; Fusarium graminearum

1. Introduction

Phosphorus (P) is one of the most important macronutrients for living organisms, and participates in many biochemical and energy metabolic processes, including photosynthesis, signal transduction, and respiration (Ham et al., 2018; Wang et al., 2018). P is also an essential building blocks for nucleic acids, phospholipids and ATP (Lorenzo-Orts et al., 2020). Land plants primarily access P in the form of inorganic phosphate (Pi). However, Pi is mostly immobile in soil and tends to be fixed by metal ions, resulting in very low concentrations of plant-available Pi (Yibo Zhang et al., 2022). However, cytosolic Pi concentration can reach micromolar level (60-80 μM), even up to the millimolar level (1-10 mM), which causes a significant Pi concentration gradient between soil and plant cell. Correspondingly, plant has evolved some specialized phosphate transporter proteins (PHTs) to transfer Pi from soil into the cells (Z. Wang et al., 2021). Based on protein sequence, subcellular localization, and function, PHTs are divided into five subfamilies, named as PHT1, PHT2, PHT3, PHT4 and PHT5, respectively, which play a crucial role in maintaining phosphate homeostasis between cytoplasm and other subcellular compartments (Li et al., 2020; Wei et al., 2022; Xu et al., 2019).

PHT1 was the first gene that reported in plant, and complemented the *pho84* mutant phenotype in *Arabidopsis thaliana* (Muchhal et al., 1996). Since then, comprehensive and systematic researches on PHT1 have been conducted in other species, including but not limited to *Arabidopsis thaliana* (Ayadi et al., 2015; Mudge et al., 2002), rice (Chang et al., 2019; Jia et al., 2017), maize (Liu et al., 2016; Nagy et al., 2006), and wheat (Grün et al., 2018; Zhang et al., 2019). PHT1 is located on the plasma membrane of root cells, and PHT1-type proteins are defined as the major contributors to the Pi uptake system (Ceasar et al., 2017). PHT2 proteins and several PHT4 members are located on thylakoid membranes or envelope membrane of chloroplast/plastid, and they are responsible for ATP synthesis or phosphate homeostasis and starch accumulation in chloroplast (Guo et al., 2008; Irigoyen et al., 2011; Karlsson et al., 2015; Liu et al., 2019). PHT3, a mitochondrial phosphate transporter, is not only essential for the conversion of ADP to ATP, but also plays a crucial role in balancing phosphate homeostasis in the cytoplasm and mitigating heavy metal stress or other stress (Tao et al., 2024; Zhu et al., 2012). PHT5 proteins, also named as vacuolar phosphate transporters (VPTs), transfer cytosolic Pi into the vacuolar lumen to regulate phosphate homeostasis in plant cell (Jia et al., 2021; Xu et al., 2019).

Establishing symbiotic relationship with fungi is another important strategy for plant to cope with phosphorus deficiency. Plants transfer shoot-sourced carbohydrates into the rhizosphere in the form of lipids or sugar to promote rhizosphere microbial growth, and in turn receive water and mineral nutrients from fungi, especially nitrogen and phosphorus (Jiang et al., 2017; Romero-Munar et al., 2024; Zhao et al., 2023). Among the symbiotic system, arbuscular mycorrhizal (AM) fungi is predominant and more than 70% of land plant can form a symbiosis with AM fungi (Shi et al., 2023). Host plants primarily rely on PHT1 to absorb Pi that originated from AM fungi. *OsPHT1;11* and *OsPHT1;13* were specifically induced by AM fungi, and participated in the development of arbuscular mycorrhizal symbiosis in rice (Güimil et al., 2005; Yang et al., 2012). In addition, OsPHR2 (phosphate starvation response in *Oryza sativa*), core transcription factor in phosphate signaling, can partially activate the mycorrhizal-related gene and arbuscular mycorrhizal symbiosis maybe belong to a part of PHR-mediated phosphate starvation response (Shi et al., 2021; Das et al., 2022).

Phosphate is primarily taken up by fungi through phosphate transporters located on the extensive fungi extraradical mycelium (Zhao et al., 2023). RiPT7, a plasma membrane-located phosphate transporter in *Rhizophagus irregularis*, facilitates bidirectional phosphate transport in a Pi-dependent manner (Xie et al., 2022). *Trichoderma* spp. is widely used as a biocontrol agent that can effectively reduce the incidence of soilborne diseases. Besides, *Trichoderma* spp. can release some secondary metabolites or IAA to promote plant growth (Zin and Badaluddin, 2020). *T. longibrachiatum* HL167, a salt-tolerant strain, could enhance the K^+/Na^+ ratio of cowpea, thereby alleviating salt stress-induced phytotoxicity (Liu et al., 2023). *Trichoderma* metabolites can simultaneously upregulate genes related to both salicylic acid and jasmonic acid defense pathways in plants and enhance the activity of defense enzymes such as phenylalanine ammonia-lyase (PAL) and peroxidase (POD), thereby coordinating the plant’s growth–defense balance and effectively mitigating pathogen damage (Guzmán-Guzmán et al., 2023). This ability to enhance host stress resistance through chemical interaction is an important ecological adaptation strategy for Trichoderma to successfully colonize the rhizosphere and assist plants in coping with biotic stress. Previously researches have indicated that *Trichoderma* spp. can increase plant Pi content and promote the transfer of Pi to host plant (Cai et al., 2015; De Jaeger et al., 2011). However, compared with the in-depth understanding of its secondary metabolism and induced resistance, the members and function of PHT1 gene family in *Trichoderma* spp. have not yet been explored, and the knowledge of their functions in biotic and abiotic stress is still limited.

To address the above concerns, PHT gene family was firstly identified based on *Trichoderma* spp. genomic information in this study. 22 *TrPHTs* genes were identified in *T. harzianum*. by comprehensive genome-wide analysis herein. We analyzed their subcellular localization, gene structure, and response to pathogens and abiotic stress. These findings on the TrPHT family provide a crucial resource and pave the way for further investigation into phosphorus metabolism and stress adaptation in *Trichoderma* spp.

2. Materials and Methods

2.1 *Trichoderma* spp. strain and growth conditions

The *T. harzianum* T-aloe (GenBank Accession No. KC753766) isolated from aloe was provided by Zhoukou Normal University. The pathogenic strain of *Fusarium graminearum* PH-1 was provided by Dr. Qi Luo, Zhoukou Normal University. *T. harzianum* T-aloe and *Fusarium graminearum* were propagated on PDA medium and incubated at 28℃ for 7 days.

To investigate the response of *TrPHT* to phosphorus stress, the *T. harzianum* discs (5 mm diameter) were placed on phosphorus-supplemented and phosphorus-deprived media. The culture medium recipe is as follows: 5 mM potassium nitrate (KNO_3), 2 mM calcium nitrate [$Ca(NO_3)_2$], 2 mM magnesium sulfate ($MgSO_4$), 70 μM boric acid (H_3BO_3), 14 μM manganese chloride ($MnCl_2$), 1 μM zinc sulfate ($ZnSO_4$), 0.2 μM sodium molybdate (Na_2MoO_4), 0.01 μM cobalt chloride ($CoCl_2$), 0.5 μM copper sulfate ($CuSO_4$), 0.01 μM ferrous sulfate ($FeSO_4$), 20 g/L glucose, and 15 g/L agar, sterilized by autoclaving at 121°C for 20 minutes. For the phosphorus treatment, 2 mM sodium dihydrogen phosphate (NaH_2PO_4) was added to the culture medium prior to sterilization. After 5 days, fresh mycelial was harvested, frozen in liquid nitrogen, and stored at -80℃ for subsequent expression analysis.

To investigate the response of *TrPHT* to salt stress, the *T. harzianum* discs (5 mm diameter) were placed on PDA medium supplemented with either salt-free or 200 mM NaCl. After 3 days, fresh mycelial was harvested, frozen in liquid nitrogen, and stored at -80℃ for subsequent expression analysis.

For the pathogen treatment, the *T. harzianum* discs (5 mm) and the *F. graminearum* discs (5 mm) were placed on opposite sides of the PDA medium, and the *Trichoderma* mycelium at the confrontation zone between *T. harzianum* and *F. graminearum* interface was collected for further analysis. After 3 days, fresh mycelial was harvested, frozen in liquid nitrogen, and stored at -80℃ for subsequent expression analysis.

2.2 Identification of the *TrPHT* gene family in *T. harzianum*

To obtain the candidate *TrPHT* genes in *T. harzianum*, the PHO84 and PHO89 protein sequences were downloaded from UniProt (https://www.uniprot.org/), and used as query sequence to search for all possible TrPHTs by TBtools (Chen et al., 2020). The obtained sequences were submitted to NCBI CDD database (https://www.ncbi.nlm.nih.gov/) and InterProScan (https://www.ebi.ac.uk/interpro/) database to identify the conserved structure domains. The molecular weight and *pI* were calculated by ExPASy (https://web.expasy.org/compute_pi/). WoLF PSORT was employed to predict subcellular localization (https://wolfpsort.hgc.jp/). Topology structure was performed on TMHMM and visualized with TMPRED.

2.3 Phylogenetic analysis and multiple sequence alignment

To provide an evolutionary context for the preliminary classification of the identified TrPHT gene family, a phylogenetic tree was constructed using the 22 TrPHT sequences along with reference sequences from taxa with well-annotated PHTs. These included the canonical transporters from *Saccharomyces cerevisiae* (PHO84, PHO89), arbuscular mycorrhizal fungi (*Glomus intraradices*, *Glomus versiforme*), and *Chlamydomonas reinhardtii*. This selection was based on the availability of functionally characterized sequences in public databases. Neighbor-joining method was used. Multiple sequences were alignment using MEGA X and visualized with Jalview.

2.4 Gene structure and motif analysis

The putative TrPHT protein sequences were submitted to MEME online to search for the conserved motifs with the following parameters: site distribution was set as zero or one, the number of motif was set as 10, and the other options were set as default parameters. The conserved domain and the number of introns and exons of genes were visualized by TBtools according to the GFF3 file (Chen et al., 2020).

2.5 Cis-acting elements analysis of the promoter in *TrPHT* genes

The promoter region, 2000 bp sequence upstream of the *TrPHT* genes, was used to predict cis-acting elements by PlantCare online with some manual adjustments. Motif distribution was visualized by TBtools (Chen et al., 2020).

2.6 Generation of TrPHT1/4/22 mutants

The generation of TrPHT1/4/22 mutants was performed by a similar method as previous studies (Chen et al., 2025; Wang et al., 2016; Wen et al., 2023). In brief, to generate the *TrPHT1/4/22* mutants, a knockout construct was assembled by ligating the HYG cassette between the genomically amplified upstream and downstream sequences of the target gene (TrPHT1/4/22-up::HYG::*TrPHT1/4/22*-down). This construct was transformed into *T. harzianum* protoplasts using homologous recombination. Putative transformants were screened and verified by PCR.

2.7 RNA extraction and quantitative real-time PCR analysis

Total RNA was isolated using an RNA extraction kit (Promega) following the manufacturer’s instructions. The cDNA was synthesized using a Moloney Murine Leukemia Virus Reverse Transcriptase cDNA Synthesis Kit (Promega) according to the manufacturer’s instructions. RT-qPCR was performed as described (Li et al., 2020). The primers used for RT-qPCR are listed in Table S1.

2.8 Determination of total phosphorus content in mycelium

Trichoderma harzianum mycelium was harvested after 3 days of cultivation on PDA plates, followed by drying in an oven. The dried samples were digested with 5 mL of concentrated sulfuric acid (H_2SO_4) at 300 °C. Total phosphorus content was then determined using the molybdenum-blue colorimetric method as previously described (Xu et al., 2019). Briefly, the digested solution was appropriately diluted, mixed with reaction buffer containing ammonium molybdate, antimony potassium tartrate and ascorbic acid, and incubated for 30 min. Absorbance was measured at 700 nm with a spectrophotometer, and the phosphorus concentration was calculated from a standard curve of KH_2PO_4. Results were normalized to the dry weight of the mycelium.

2.9 Statistical analyses

Statistical analyses were performed using SPSS 27.0. Data are presented as mean ± standard deviation (SD) from at least three independent biological replicates. Significant differences between means were determined by Student’s t-test, with $P < 0.05$ considered statistically significant. Figures were created using GraphPad Prism 9.

2.10 Accession number

Sequence data of this article can be found in the UniProt or GeneBank database. Accesion number for PHTs are as follows: PHO84 (P25297), PHO89 (P38361), (P38361), GvPT (Q00908), GiPT (XP_025183371). CrPTs can be found in (L. Wang et al., 2021).

3. Results

3.1 Identification of PHT Gene Family in *T. harzianum* T-aloe

To investigate *PHT* genes in *T. harzianum* T-aloe, PHT protein sequences of *Saccharomyces cerevisiae*, PHO84 (UniProt Accession No. P25297) and PHO89 (UniProt Accession No. P38361), were employed as BLAST query sequences, and genomic databases of T-aloe strain were filtered according to E value < 1E-5. Based on previous studies, fungal PHTs have some classical domain, such as phosphate-H^+ symporter (PHS), glycerol-3-phosphate inorganic phosphate (GlpT) transporter and major facilitator superfamily (MFS). Therefore, we followed these criteria to identify putative PHTs. At present, a total of 22 putative PHTs were found in T-aloe strain (Table S2 and S3). Physicochemical properties of 22 putative PHTs, including gene symbol, gene length, open reading frame (ORF) length, protein length, molecular weight, isoelectric point (*pI*), predicted transmembrane domain (TM) number, predicted long hydrophilic loop (loop after) and N/C terminal location, are listed in Table 1. Amino acid length, relative molecular weight and *pI* ranged from 370 (TrPHT17) to 738 (TrPHT2) aa, 40.42 (TrPHT17) to 80.52 (TrPHT2) kDa, 5.31 (TrPHT10) to 9.03 (TrPHT2) respectively. Subcellular localization prediction results showed that all TrPHTs located in the plasma membrane. Transmembrane and topology prediction indicated that twenty TrPHTs have 10 – 12 transmembrane domains, and fifteen TrPHTs have amino and carboxyl terminals (Supplemental Figure 1). NCBI and InterproScan analysis showed that all proteins contain a classical phosphate transporter domain (Table S4), and most proteins contain a large hydrophilic loop between the fifth and sixth TMDs. Together, these data suggest that 22 putative PHTs may play an important role in phosphate transport

Table 1. Key features of all 22 putative PHTs in *T. harzianum*

Gene symbol	strand	Gene length (bp)	ORF length (bp)	pretion length (aa)	*Mol* wt (kDa)	pI	predicted localization	TM number	Hydrophilic loop after	N	C
TrPHT1	+	1919	1722	573	61.83	7.13	PM	11	6	Ext	Cyt
TrPHT2	-	2577	2217	738	80.52	9.03	PM	12	6	Ext	Ext
TrPHT3	-	2464	1887	628	69.73	8.62	PM	11	5	Ext	Cyt
TrPHT4	-	2059	1986	661	74.30	6.23	PM	9	4	Cyt	Ext
TrPHT5	+	1699	1422	473	50.64	7.51	PM	10	-	Cyt	Cyt
TrPHT6	+	1642	1452	483	52.80	6.19	PM	12	-	Cyt	Cyt
TrPHT7	+	1673	1488	495	53.66	6.66	PM	10	2	Cyt	Cyt
TrPHT8	-	1903	1716	571	62.18	6.07	PM	12	6	Cyt	Cyt
TrPHT9	-	1756	1632	543	59.85	6.26	PM	11	5	Cyt	Ext
TrPHT10	+	1906	1674	557	61.78	5.31	PM	12	6	Cyt	Cyt
TrPHT11	+	1919	1704	567	62.41	7.67	PM	12	6	Cyt	Cyt
TrPHT12	+	1836	1725	574	64.18	8.71	PM	12	6	Cyt	Cyt
TrPHT13	+	1762	1632	543	60.10	7.05	PM	11	6	Cyt	Ext
TrPHT14	+	1813	1542	513	56.65	8.10	PM	12	6	Cyt	Cyt
TrPHT15	+	1734	1563	520	56.17	6.01	PM	10	5	Cyt	Cyt

TrPHT16	-	1707	1584	527	56.61	5.91	PM	11	-	Cyt	Cyt
TrPHT17	+	1489	1113	370	40.42	7.08	PM	10	2	Cyt	Cyt
TrPHT18	-	1728	1662	553	60.30	6.61	PM	12	6	Cyt	Cyt
TrPHT19	-	1670	1614	537	58.90	6.35	PM	12	6	Cyt	Cyt
TrPHT20	+	1981	1653	550	60.19	6.90	PM	12	6	Cyt	Cyt
TrPHT21	-	1883	1551	516	57.22	9.02	PM	10	5	Cyt	Cyt
TrPHT22	-	2022	1833	610	65.03	9.01	PM	9	6	Cyt	Ext

Note: +: The mRNA sequence is positive strand; -: The mRNA sequence is negative strand; PM: plasma membrane; Cyt: N/C terminal locates in cytoplasmic; Ext: N/C terminal locates in extracellular.

3.2 Phylogenetic analysis and multiple sequences alignment of TrPHTs

To better explore phylogenetic relationships among *PHT* genes in different species, a phylogenetic tree was constructed by neighbor-joining method using PHT protein sequences from *T. harzianum* T-aloe, *Chlamydomonas reinhardtii*, *Saccharomyces cerevisiae*, *Glomus intraradices* and *Glomus versiforme* in MEGA X. The 22 putative TrPHTs was divided into three monophyletic groups, named as cluster I to III (Figure 1). Cluster I consists of TrPHT1/2/3/4/5/6/7/16, and they share high similarity with PHO84 and CrPTAs, which belong to phosphate-H^+ symporter. This indicated cluster I may transport phosphate coupled with protons. Consistently, TrPHT1/2/3 have a phosphate-H^+ symporter domain (Table S4). TrPHT8-14 and TrPHT17-21 belong to cluster II, and all members have MFS domain or sugar transporter domain. TrPHT15/22 fall into cluster III, and all members share high similarity with PHO89 and CrPTBs, which belong to the phosphate-Na^+ symporter.

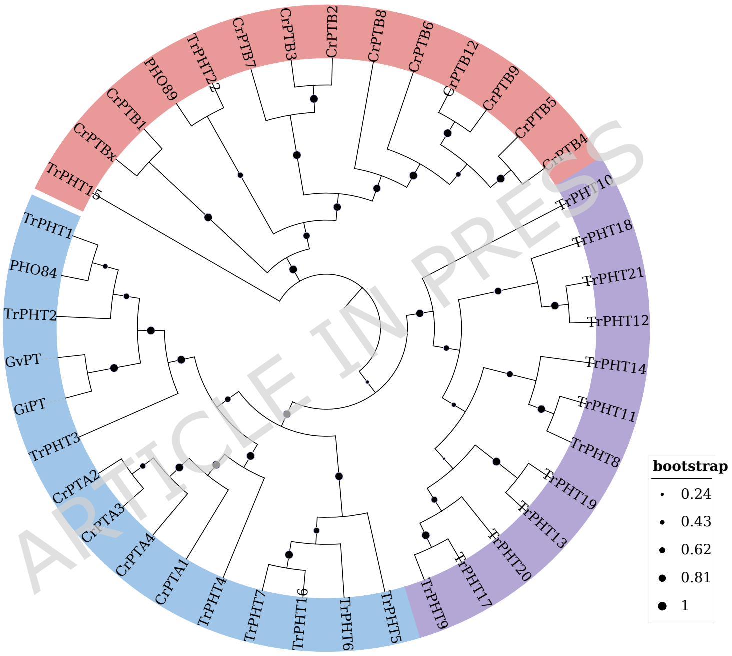

Figure 1. Phylogenetic analyses of phosphate transporter genes in *T. harzianum* (Tr), *Chlamydomonas reinhardtii* (Cr), *Saccharomyces cerevisiae* (PHO84, PHO89), *Glomus intraradices* (Gi), and *Glomus versiforme* (Gv). All PHT proteins were divided into three clusters represented by different colors. Blue, purple and red color represent cluster I, II, and III, respectively. The phylogenetic tree was constructed by the neighbor-joining method using MEGA-X software with 1000 bootstrap replicates.

Previously studies reported that phosphate transporter has a significant motif (GGDYPLSATIxSE), and this region is also conserved in fungal phosphate transporter (GGDYPLSxxIxSE) (Karandashov and Bucher, 2005). To further analysis the phylogenetic relationships among PHT, an alignment of the conserved region was performed using MUSCLE. However, we found cluster III has a low sequence similarity with cluster I and cluster II (Supplemental Figure 2), therefore, cluster III is removed from the downstream investigation. Results showed that GGDYPLSxxIxSE motif is relatively conserved in T-aloe strain (Figure 2). The Gly residue in the motif region is highly conserved and followed by other conserved residues including Asp/Glu (third position in the motif). In addition, the last residue Glu (last position in the motif) is also conserved. These conserved residues may play a vital role in transporting phosphate into cytosol.

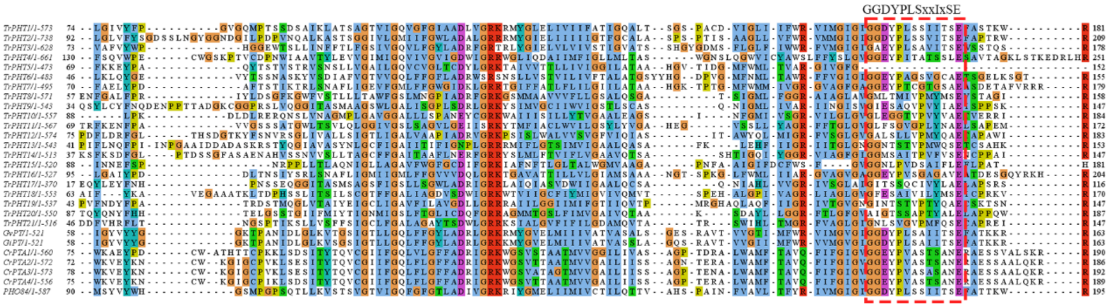

Figure 2. Multiple sequence alignment of signature motif in cluster I and cluster II. The alignment was constructed by MUSCLE and visualized by Jalview. No color means that the similarly between amino acid residues of TrPHT members at this site is less than 35%. The conserved motif sequence was indicated inside the red box.

3.3 Motif and gene structure analysis of *TrPHTs*

To further demonstrate the characteristic of TrPHTs proteins, an online MEME analysis was performed to identify additional motifs. Phylogenetic tree showed that all TrPHTs could be classified into three groups as described in the former section (Figure 3a). Ten conserved motifs were predicted except TrPHT22 (Figure 3b and Table S5). Previous reported fungal PHT conserved motif was matched with motif 5 precisely. In addition, motif 1 and 2 was also conserved in TrPHTs (except TrPHT22). Cluster I and cluster II contained motif 4, 7 and 8 (except motif 4 in TrPHT16, motif 7 not in TrPHT16). While some motifs were common to TrPHTs, the others were unique to some clusters. For example, motif 9 was only present in cluster I. Motif 3 and 10 were unique to cluster II (except motif 3 in TrPHT4). Notably, the distribution of motifs relative to the PHT-specific loop region differed between clusters: it resided between Motif 8 and Motif 9 in Cluster I but between Motif 6 and Motif 3 in Cluster II (see Figure 3b).

To further understand the structure of *TrPHT* genes, the exon-intron arrangements and protein domain were analyzed to obtain insights into structural diversity. The number of introns ranged from 1 to 5, and most of *TrPHT* genes (14/22) had 2-3 introns (Figure 3c). TrPHTs from the different cluster had its own distinct domain.

Figure 3. Phylogenetic relationship, architecture of the conserved protein motifs and gene structure in *TrPHTs*. a The phylogenetic tree was constructed based on the full-length sequences of TrPHT proteins. b The conserved motifs of TrPHT proteins. The motifs are displaced in different colored boxes. The sequence information for each motif is provided in Additional file1: Table S5. c Exon-Intron structure of *TrPHTs*. Green boxes represent coding sequence (CDS), and other colored boxes represent the canonical PHTs domains.

3.4 Cis-acting elements analysis of *TrPHTs*

Transcription factors interact with cis-acting elements to regulate gene expression precisely. To identify the transcriptional regulation of *TrPHTs* gene, the cis-acting elements in the promoter regions were analyzed. The PlantCare database was utilized for cis-acting element prediction. This choice was based on the functional conservation of many stress- and hormone-responsive regulatory elements across eukaryotes, and the aim to conduct a comprehensive scan for potential regulatory motifs within the TrPHT promoters. Some manually criterions were carried to screen functional cis-acting elements, specifically removing the CAAT-box and TATA-box, and deleting elements without names and without annotations. Finally a total of 677 putative cis-acting elements were obtained, including 291 environmental stress responsive cis-acting elements, 305 phytohormone responsive cis-acting elements, 40 transcription factor responsive cis-acting elements, and 41 development responsive cis-acting elements (Figure 4 and Table S6). Light responsive cis-acting elements was the most abundant in environmental related elements (Figure 4b), G-box, I-box and spl were the top three elements (120, 17 and 17 respectively). 148 and 98 elements were involved in response to abscisic acid (ABA) and methyl jasmonate (MeJA) respectively (Figure 4c). The P1BS (GNATATNC) is core cis-acting element in phosphorus signalling networks, and this element is present in the promoter regions of Pi starvation-induced genes. To identify P1BS element, we searched the GNATATNC sequence in the promoter regions of *TrPHT* genes. Ten P1BS elements were identified in eight of the TrPHTs genes promoter regions (Figure 5).

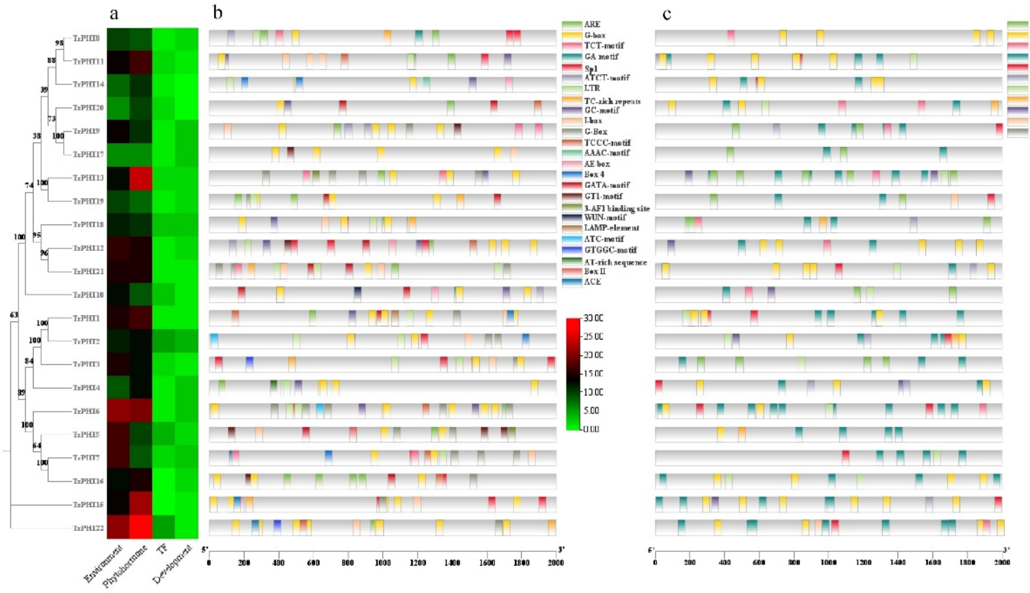

Figure 4. Prediction of cis-acting elements in *TrPHTs*. a Numbers of cis-acting elements detected in the promoter region of each *TrPHT* gene. All cis-acting elements were divided into four types. b Kind, quanitity and position of environmental stress-related elements in *TrPHTs*. c Kind, quanitity and position of phytohormone stress-related elements in *TrPHTs*.

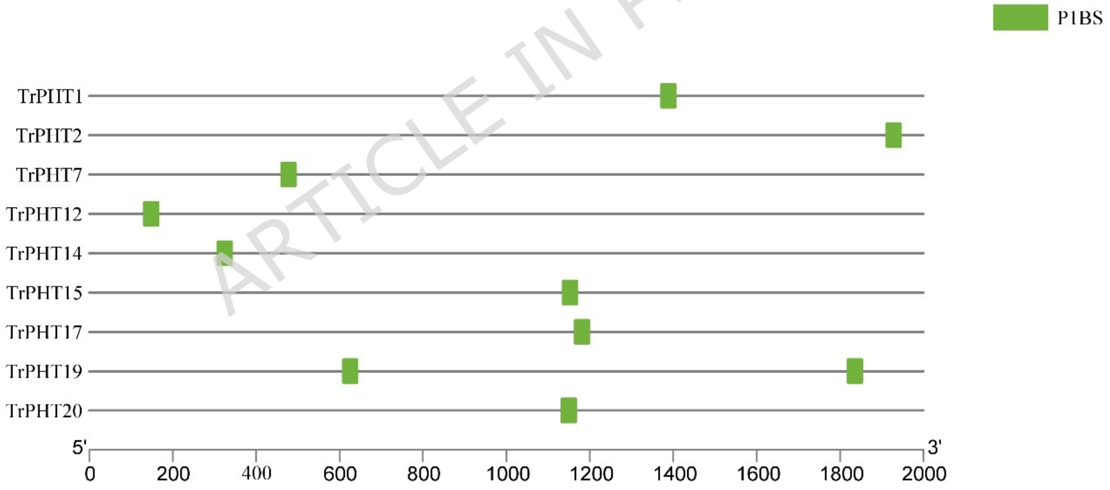

Figure 5. The schematic diagram of P1BS motif in *TrPHTs* promoter.

3.5 Expression of TrPHTs in response to biotic and abiotic stress

Organisms primarily absorb phosphorus through phosphate transporters. To explore the expression profiles of *TrPHT* genes under deprivation starvation, *T. harzianum* T-aloe was grown in phosphorus-supplemented and phosphorus-deprived media, respectively. After 5 days, fresh mycelia were collected for RT-qPCR analysis. The promoter regions of *TrPHT1/2/15* harbor P1BS motifs, and these genes were significantly induced under phosphorus deficiency (Figure 6), indicating that their expression is regulated by the core phosphorus signal regulator PHRs (hypothetically present). However, some *TrPHT* genes lacking the P1BS element in their promoter regions were also significantly induced under phosphorus deficiency (Figure 6), suggesting that their expression is regulated by additional transcription factors. These findings confirm that the *TrPHTs* gene family performs essential functions in the phosphorus uptake of *T. harzianum* in response to phosphate starvation.

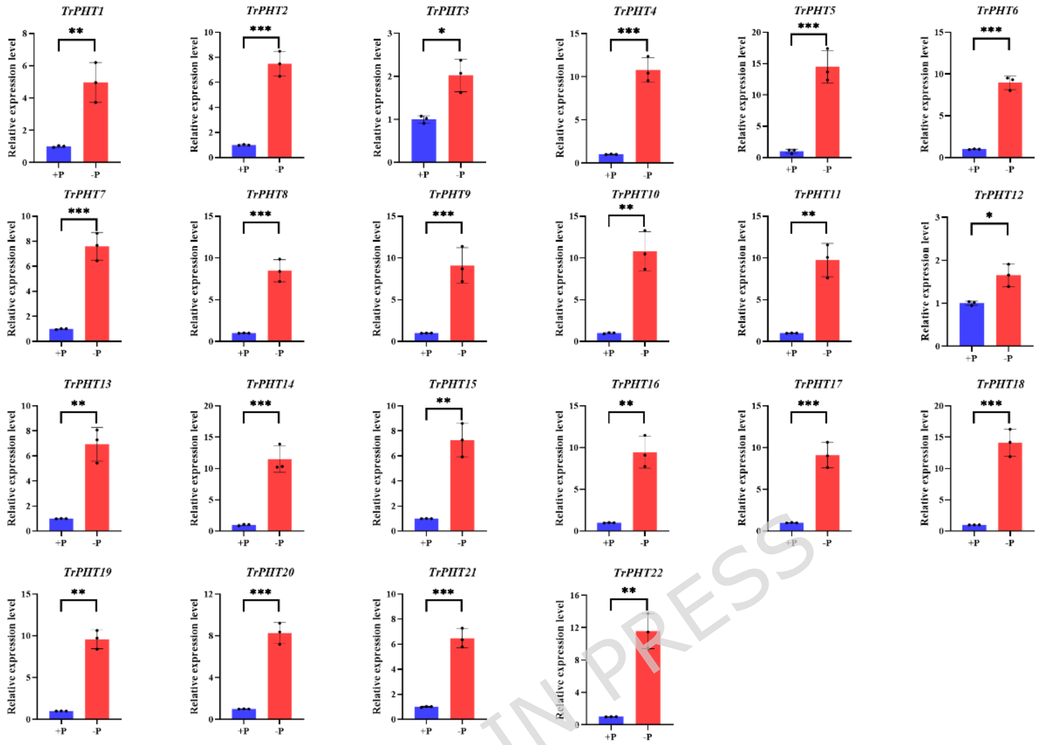

Figure 6. Profile changes of transcript levels of *TrPHT* genes in response to phosphate deprivation. The mean expression values were calculated from three independent biological replicates and are relative to ck-inoculated controls. *, *P* ≤ 0.05 and **, *P* ≤ 0.01 by the Student's t-test.

T. harzianum exhibited significant growth inhibition against *F. graminearum*. To investigate the potential involvement of *TrPHTs* in this antagonistic process, we conducted dual-culture confrontation assays. After 5 days of co-cultivation, fresh mycelia from the confrontation zone between *T. harzianum* and *F. graminearum* were collected for RT-qPCR analysis. Most phosphate transporter genes showed no significant differential expression compared to the control treatment (Figure 7). However, *TrPHT10* expression was significantly downregulated (Figure 7). Intriguingly, *TrPHT1/4/12/15/22* exhibited significant upregulation in the confrontation zone (Figure 7). This dichotomous regulation pattern implies specialized roles for distinct PHT family members during mycoparasitism, with the upregulated cluster potentially facilitating competitive phosphate acquisition at the confrontation interface.

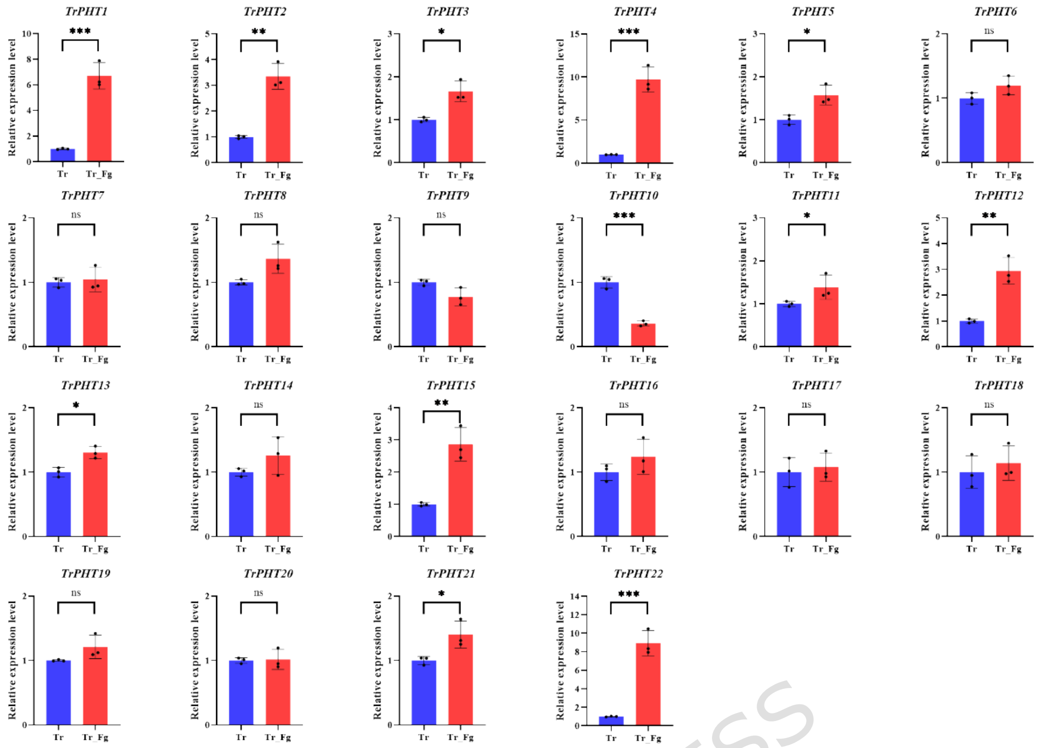

Figure 7. Profile changes of transcript levels of *TrPHT* genes in response to pathogen. The mean expression values were calculated from three independent biological replicates and are relative to ck-inoculated controls. *, $P \leq 0.05$ and **, $P \leq 0.01$ by the Student's t-test.

T. longibrachiatum has been reported to exhibit high salt stress tolerance (Liu et al., 2023). Parallel investigations were conducted to quantify salt stress-induced alterations in phosphate transporter activity. All *TrPHTs* were significant suppressed under salt stress conditions compared to control conditions (Figure 8). This comprehensive suppression suggests a potential trade-off between salt tolerance and phosphate acquisition mechanisms in this fungal species.

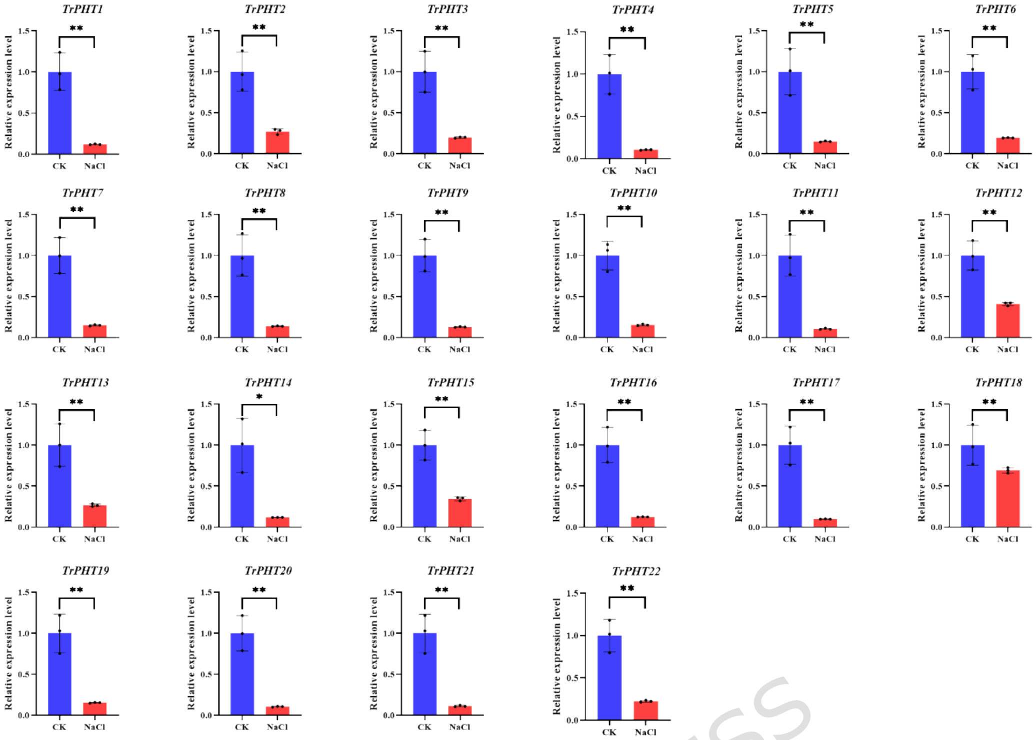

Figure 8. Profile changes of transcript levels of *TrPHT* genes in response to salt stress. The mean expression values were calculated from three independent biological replicates and are relative to ck-inoculated controls. *, $P \leq 0.05$ and **, $P \leq 0.01$ by the Student's t-test.

3.6 *TrPHTs1/4/22* mutants exhibit impaired tolerance to both biotic and abiotic stress

To further investigate the roles of phosphate transporters in *T. harzianum* under biotic and abiotic stress, one phosphate transporter from each category (TrPHT1/4/22) was randomly selected for gene knockout by homologous recombination (Supplemental Figure 3). The *pht1*-24, *pht*4-22, and *pht*22-20 mutants exhibited significant growth impairment, with growth rates reduced by approximately 21%, 23%, and 26%, respectively, compared to the wild-type T-aloe strain (Figure 9a). To further explore the function of these transporters in phosphorus uptake, total phosphorus content was measured, which revealed marked deficiencies in the mutants. The phosphorus levels in *pht1*-24, *pht*4-22, and *pht*22-20 decreased by 34.85%, 17.59%, and 13.02%, respectively, relative to T-aloe, indicating the critical role of TrPHTs in phosphorus uptake and fungal development (Figure 9b). In dual-culture confrontation assays against *F. graminearum*, all mutants displayed significantly weakened antagonistic capacity, with inhibition rates reduced by 20.24%, 54.76%, and 41.67%, respectively (Figure 9c and 9e). Similarly, under salt stress conditions, the mutants showed notable growth defects, with growth rates decreased by 20%, 18%, and 22%, respectively (Figure 9d). Take together, these results demonstrate that phosphate transporters in *T. harzianum* play indispensable roles in mediating both biotic and abiotic stress responses.

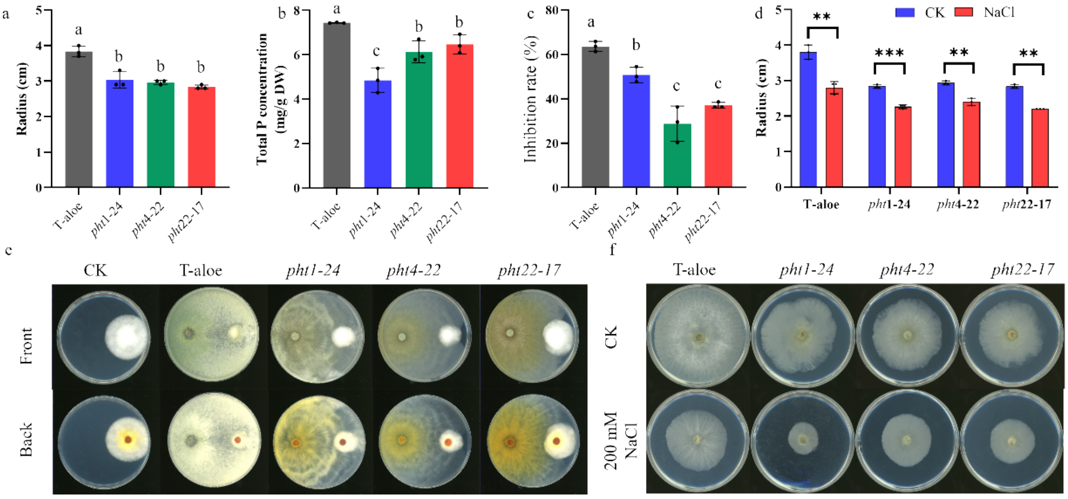

Figure 9. Physiological changes of *TrPHT1/4/22* mutants responding to biotic and abiotic stress. a: The radius of the *TrPHT1/4/22* mutants after 3 days of growth on PDA medium. b: Total phosphorus content of the *TrPHT1/4/22* mutants. c: Inhibition rate of the *TrPHT1/4/22* mutants against *F. graminearum*. d: Colony radius of the *TrPHT1/4/22* mutants under salt stress conditions for 3 days. e: Antagonistic phenotype of the *TrPHT1/4/22* mutants against *F. graminearum*. f: Phenotypic alteration of the *TrPHT1/4/22* mutants under salt stress conditions for 2 days.

4. Discussion

Phosphorus is a fundamental macronutrient. It plays a pivotal role in the growth and metabolic processes of living organisms. As the predominant form of phosphorus assimilated by plants and microorganisms, phosphate is primarily acquired through dedicated phosphate transporter systems (Wang et al., 2018). While extensive studies have elucidated phosphate transport mechanisms in plants, the characterization of fungal phosphate transporters remains relatively understudied (Chien et al., 2018). *Trichoderma*, renowned as effective biocontrol agents in sustainable agriculture, have demonstrated remarkable capacity to enhance phosphorus acquisition in crops, thereby promoting plant growth and increasing yield potential (Cai et al., 2015; De Jaeger et al., 2011). However, current understanding of the molecular mechanisms governing phosphate uptake and translocation in *Trichoderma* remains fragmentary, with particular knowledge gaps existing regarding the specific contributions of phosphate transporters in these processes. Elucidating the functional properties of these transporters in *Trichoderma* could yield significant agricultural implications, potentially offering novel strategies to improve phosphorus utilization efficiency in cropping systems, particularly under phosphate-limiting soil conditions.

In this study, we conducted a systematic genome-wide characterization of the entire PHT1 gene family in *Trichoderma* species to identify candidate genes involved in phosphorus homeostasis. A total of 22 *TrPHT* genes were identified in the *Trichoderma* genome (Table 1). Phylogenetic analysis revealed that the 22

TrPHT genes can be divided into three distinct clusters. Similar to terrestrial plants, members of Cluster 1 contain the PHS domain and are capable to promote phosphate uptake through the H^+/Pi symporter (Lorenzo-Orts et al., 2020). In addition, the genome of *Trichoderma* contains a Na^+/Pi symporter system that may promote phosphate absorption. Previous studies have shown that under alkaline conditions, the phosphate transport capacity of the Na^+/Pi symporter is significantly increased (Martinez and Persson, 1998). PTB, which contains a Na^+/Pi symporter, is only found in green algae and bryophytes, and has been lost in terrestrial plant species(Jia et al., 2021; Wang et al., 2020). The number of putative PHT transporters identified in *Trichoderma* (22) is notably higher than in model plants like *Arabidopsis thaliana* (9 PHT1 members). This expansion may be linked to *Trichoderma's* saprophytic lifestyle and exceptional adaptability to diverse, often nutrient-poor, soil environments. A larger transporter repertoire could enhance phosphate acquisition efficiency from complex organic and inorganic sources. The results indicate that the recruitment of different Pi transporters in different species is closely linked to changes in their environmental conditions.

While all identified TrPHTs were predicted to localize to the plasma membrane, this contrasts with the diversification observed in plants, where PHT families (PHT1-PHT5) are targeted to various organelles such as the chloroplast (PHT2), mitochondrion (PHT3), Golgi, and tonoplast (PHT4/PHT5) to orchestrate intracellular phosphate partitioning (Jia et al., 2021). In *Trichoderma*, the primary interface for environmental phosphate scavenging is likely the plasma membrane. However, the presence of organellar phosphate transporters in fungi cannot be ruled out. For instance, in the alga *Chlamydomonas reinhardtii*, CrPTC1 is a tonoplast-localized transporter crucial for vacuolar phosphate storage (L. Wang et al., 2021). The exclusive plasma membrane prediction for TrPHTs suggests that other, structurally distinct transporter families might be responsible for organellar phosphate flux in *Trichoderma*. Future studies employing fluorescent protein tagging or organellar proteomics are needed to validate these predictions and identify the full complement of phosphate transporters.

The phylogenetic classification of TrPHTs in this study was conducted within the context of currently available, well-annotated reference sequences. While sequences from phylogenetically closer filamentous ascomycetes were not included due to their lack of systematic annotation in public databases, our primary aim was to establish a functional clustering framework. This framework is robustly supported by the conserved domain architecture of all TrPHT members (Table S4), each containing signature phosphate transporter domains (e.g., Pho4-like or MFS domains) as confirmed by InterPro analysis. Thus, the combination of phylogenetic placement and conserved domain validation provides strong evidence for the functional identity of the clusters we define. It should be noted that the phylogenetic analysis in this study, while robust, was conducted primarily with sequences from evolutionarily distant reference species due to the current lack of comprehensively annotated PHT homologs from closely related filamentous ascomycetes in public databases. Future studies incorporating such data, as genomes of related fungi become more fully annotated, will provide even finer resolution of the evolutionary relationships within the fungal PHT family.

All of the TrPHT proteins contain a highly conserved phosphate transporter domain, which belongs to one of the following families: PHS, GlpT, or MFS. Notably, these transporters share the core signature sequence "GGDYPLSATIxSE", which aligns perfectly with motif 5 and shows remarkable conservation across diverse species (Karandashov and Bucher, 2005; Zhang et al., 2019). In addition, our motif analysis revealed two additional conserved sequences (motif 1 and motif 2) in TrPHT proteins that exhibit significant sequence similarity to those reported in Chlamydomonas (Wang et al., 2020), wheat (Zhang et al., 2019), and rice (Liu et al., 2011). This remarkable evolutionary conservation of these motifs across kingdoms suggests they may serve as valuable molecular signatures for future identification and characterization of phosphate transporters. However, consistent with previous study (Wang et al., 2020), our analysis failed to detect any conserved motifs in the Na^+/Pi symporter TrPHT22. This complete absence of conserved sequence features strongly suggests this transporter has undergone significant functional specialization within the PHT family, potentially adapting to unique physiological roles or environmental niches distinct from other family members.

The identification of diverse cis-acting elements in *TrPHT* promoters reveals an intricate transcriptional regulatory system that likely enables *Trichoderma* to adapt to fluctuating phosphorus availability. The predominance of light-responsive elements suggests an intriguing connection between photosynthesis and phosphate homeostasis. This may reflect an evolutionary adaptation to coordinate energy metabolism with nutrient acquisition. OsPHT2 functions as a chloroplast-localized phosphate transporter whose expression is induced by both phosphate deficiency and light. Mutations in this transporter not only impair rice photosynthesis and carbohydrate accumulation but also modulate flavonoid biosynthesis, consequently affecting UV-B tolerance and ultimately grain yield (Liu et al., 2019). The OsPHO1;2 transporter mediates Pi translocation to leaves, enhancing photosynthetic efficiency through increased leaf Pi content, which collectively contributes to yield improvement (Ma et al., 2024). UV-PHR cross-talk forms a bidirectional regulatory module: UV represses PHR activity to downregulate phosphate acquisition, while PHR in turn transcriptionally activates UV-protective genes to mitigate photodamage (Ren et al., 2025). Notably, the abundance of phytohormone-responsive motifs, particularly to ABA and MeJA, implies potential cross-kingdom signaling mechanisms that may mediate *Trichoderma*-plant interactions during symbiotic phosphorus exchange. The ABA signaling component ABI5, a transcription factor induced under phosphate deficiency, directly activates the expression of phosphate transporters PHT1;1 to enhance Pi uptake (Yu Zhang et al., 2022). While the presence of P1BS elements in some transporters confirms conservation of the core phosphorus signaling pathway, their absence in other phosphorus-responsive *TrPHTs* points to the existence of additional transcription factors may be involved in their regulation in fungi.

In agricultural ecosystems, crops are frequently faced multiple stresses, which significantly affect crops growth and yield, even their survival (Ren et al., 2025). Adequate phosphorus supply could activate plant immunity and enhance rhizosphere microbiome function related to disease suppression, thereby significantly inhibiting tomato bacterial wilt caused by *Ralstonia solanacearum* (Cao et al., 2024). In this study, we found that the antagonistic interaction specifically activated a subset of phosphate transporters (TrPHT1/2/4/15/22), indicating that phosphorus acquisition is essential for *Trichoderma*'s biocontrol efficacy. In addition, *TrPHT1/2/15*, contain P1BS cis-elements in promoter regions, exhibited dual induction under both phosphate deficiency and pathogen invasion. These findings suggest that PHR transcription factors may orchestrate a coordinated response linking phosphate acquisition and biotic stress responses in *Trichoderma*. The observation that all *TrPHT* genes were upregulated under phosphate deficiency suggests widespread functional redundancy within the family. This coordinated response may be a robust strategy to ensure survival during sudden phosphate scarcity. Alternatively, this generalized induction could result from the prolonged (several days) acclimation period used in our experiment, potentially triggering a broad starvation response that activates the entire phosphate regulon. Salt stress is another major abiotic stress that severely impacts crop productivity. Phosphorus supply could alleviate the levels of oxidative stress caused by salt stress (Muhammad et al., 2024). Our data reveal a complex, dual role for *TrPHTs* in salt stress adaptation. While transcript levels of most *TrPHT* genes were suppressed under salt stress, the increased salt sensitivity of *TrPHT1/4/22* mutants indicates that these transporters are nonetheless required for tolerance. This apparent paradox suggests that basal-level PHT activity, rather than induced expression, is critical during salt stress. The mechanism is likely multifaceted and indirect. Efficient phosphate uptake and homeostasis are prerequisites for maintaining energy metabolism (ATP), phospholipid membranes, and nucleic acid integrity—all processes challenged under ionic stress.

To the best of our knowledge, this is the first study to systematically investigate TrPHTs in *T. harzianum*, concurrently profiling their responses to both biotic (pathogen interaction) and abiotic (salt/phosphate stress) challenges. Our work highlights the TrPHT family as a key regulators that links phosphate homeostasis with stress adaptation in *T. harzianum*. The coordinated regulation of these transporters in response to diverse environmental signals underscores a sophisticated mechanism behind Trichoderma' ecological versatility and its role in improving phosphorus nutrition for plants. These insights not only advance the fundamental knowledge of nutrient sensing in fungi but also present strategic targets for developing Trichoderma-based solutions to enhance crop resilience and soil health in sustainable agricultural systems.

5. Conclusion

Through genome-wide analysis, we deciphered the TrPHTs family in *T. harzianum*, comprising 22 members phylogenetically classified into three motif-defined clusters. Our results elucidate a transcriptional paradigm wherein TrPHTs expression is enhanced by phosphate deficiency but inhibited by salt stress, positioning these transporters at the interface of nutrient sensing and environmental signaling. The pathogen-specific induction of a subset of TrPHTs further links phosphorus status to biotic stress pathways. Together, these results establish an initial framework for understanding how the TrPHT family contributes to phosphate homeostasis and stress adaptation in *Trichoderma*, offering a basis for further exploration of its potential in sustainable agriculture.

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Author’s Contributions

Yibo Zhang: Formal analysis, Data curation, Investigation, Writing – original draft, Writing – review and editing. **Chaofeng Dong:** Formal analysis, Methodology, Visualization. **Zhen Liu:** Formal analysis, Validation. **Xiaodong Yang:** Formal analysis, Methodology, Data curation. **Dongxu Lu:** Formal analysis, Investigation. **Li Wang:** Formal analysis, Methodology, Investigation. **Huifang Zhang:** Formal analysis, Validation; **Qingdi Yan:** Formal analysis, Visualization. **Junchang Li:** Formal analysis, Software. **Cunjie Li:** Data curation, Visualization. **Fuli Zhang:** Supervision, Conceptualization, Writing – review and editing ,Funding acquisition.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data Availability Statement

The datasets generated and/or analyzed during the current study are included in this published article and its Supplementary Information files. The genome sequences of Trichoderma harzianum T-aloe used for gene identification are available from the corresponding author upon reasonable request. All TrPHT protein sequences identified in this study are provided in Supplementary Table S2.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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