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Characterization of the PtPHT family in *Poncirus trifoliata* and identification of a regulatory module, PtRap2.12–PtPHT1;2, under drought stress

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Abstract

Phosphate transporters (PHTs) are $\text{H}_2\text{PO}_4^-/\text{H}^+$ transporters that facilitate inorganic phosphate uptake from the soil and its distribution within plants. In citrus, seasonal summer drought constrains yield and limits phosphate uptake. However, the roles of *PHT* genes in drought response remain poorly understood. In this study, we identified 20 *PtPHTs* in the *Poncirus trifoliata* genome and classified them into five subgroups (I to V) based on phylogenetic relationships. Promoter analysis revealed abundant *cis*-acting elements associated with abiotic stress and phytohormone responses, while synteny analysis indicated that gene duplication contributed to *PtPHT* family expansion. Expression profile showed that several genes, including *PtPHT1;1*, *PtPHT1;2*, *PtPHT1;3*, *PtPHT3;3*, *PtPHT4;3*, *PtPHT5;1*, and *PtPHT5;2* were significantly upregulated by drought, with *PtPHT1;2* exhibiting the strongest response (about 16.4 folds). Silencing *PtPHT1;2* markedly reduced phosphorus (P) accumulation in transgenic citrus calli, confirming its functional role in inorganic phosphate uptake. Yeast one-hybrid (Y1H) screening identified Related to AP2.12 (PtRap2.12), an APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factor, as an upstream regulator of *PtPHT1;2*. This interaction was validated by Y1H and dual-luciferase assays, demonstrating that PtRap2.12 directly binds to and activates the *PtPHT1;2* promoter. Functional analysis further showed that overexpression of *PtRap2.12* enhances drought tolerance of transgenic plants, as indicated by improved growth, reduced membrane damage, elevated antioxidant enzyme activities, and increased P accumulation. Moreover, transgenic plants displayed upregulated expression of *NtPHT1;1* in shoots and *NtPHT1;4* in roots of tobacco (*Nicotiana tabacum*) under drought conditions. In summary, this study provides a comprehensive analysis of *PtPHT* genes in *P. trifoliata* and highlights the role of the PtRap2.12–*PtPHT1;2* module in regulating both phosphate uptake and drought tolerance, offering a theoretical basis for breeding citrus rootstocks with enhanced resistance to drought and phosphate deficiency.

Keywords Phosphate transporters (PHTs), Drought stress, Transcription factor, *Poncirus trifoliata*

Introduction

Phosphorus (P) is an essential macronutrient for plant growth and development and is involved in vital metabolic processes such as respiration, photosynthesis, and

cell division. Adequate P supply is critical for crop productivity, influencing plant growth, yield, and fruit quality (Gahoonia and Nielsen 2004; Wu et al. 2021). Plants primarily acquire P from the soil in the form of inorganic phosphate (Pi) through root uptake systems (Chiou and Lin 2011). The absorption, translocation, and intracellular distribution of Pi are mediated by phosphate transporters (PHTs), which are therefore indispensable for

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maintaining P homeostasis (López-Arredondo et al. 2014; Wang et al. 2017; Madison et al. 2023).

The first *PHT* gene, *PHO84*, was identified in yeast (Bun-Ya et al. 1991), and since then, numerous *PHT* genes have been characterized in diverse plant species, including *Arabidopsis* (Muchhal et al. 1996), rice (Paszowski et al. 2002), and soybean (Tamura et al. 2012). Based on sequence homology and subcellular localization, PHTs are primarily categorized into five subfamilies: PHT1, PHT2, PHT3, PHT4, and PHT5. Members of the PHT1 subfamily are primarily responsible for Pi uptake from the soil and its subsequent translocation within plants (Shin et al. 2004; Chien et al. 2022). The PHT2 subfamily, represented by *AtPHT2;1* in *Arabidopsis thaliana*, functions as a plastid-localized Pi cotransporter (Daram et al. 1999; Versaw and Harrison 2002). PHT3 and PHT4 subfamilies are localized to mitochondria and Golgi apparatus, respectively, where they regulate intracellular Pi transport (Versaw and Garcia 2017). In contrast, PHT5 members are localized to the vacuolar membrane and mediate Pi sequestration into vacuoles, thereby contributing to cellular Pi homeostasis (Hassler et al. 2012; Wang et al. 2015a; Luan et al. 2018). Collectively, these subfamilies coordinate Pi uptake, allocation, and intracellular storage, ensuring effective Pi utilization and homeostasis in plants.

Drought is a major environmental constraint in citrus-producing regions and severely limits citrus growth and productivity (Dahro et al. 2023). Seasonal drought, particularly during the citrus fruit expansion stage, adversely affects both the quality and yield of citrus, leading to increased acidity, reduced nutrient uptake by roots, and decreased mineral nutrient content in leaves (Nagatoshi et al. 2023; Ma et al. 2024). Drought stress diminishes the solubility of mineral elements in soil, induces osmotic stress and ultimately impairs nutrient acquisition and assimilation (Buljovic and Engels 2001; Rennenberg et al. 2006). Additionally, drought has been shown to inhibit Pi uptake and reduce P concentrations in tomato and citrus plants (Subramanian et al. 2006; Ma and Fan 2007). To cope with Pi deficiency induced by drought, plants activate a range of adaptive responses, including the upregulation of *Pi-starvation-induced* (*PSI*) genes, such as *PHTs*. (Devaiah et al. 2007; Kavka and Polle 2017). Increasing evidence suggests that the Pi starvation response (PSR) is closely linked with drought signaling pathways, indicating potential crosstalk between these two stress responses. However, the regulatory mechanisms underlying PHT-mediated Pi uptake and transport under drought stress in citrus remain largely unexplored.

Trifoliate orange (*Poncirus trifoliata* (L.) Raf) is widely used as a rootstock in citrus production due to its strong tolerance to abiotic stresses, particularly drought,

compared with other commonly used rootstocks, such as *Citrus sinensis* × *P. trifoliata*, *Citrus paradisi* × *P. trifoliata*, *Citrus junos* and *Citrus reticulata* (Guo et al. 2002; Hayat et al. 2021). As the root system determines water and nutrient uptake, rootstock performance directly influences scion growth, fruit yield and quality, and stress adaptability (Albacete et al. 2015; Morales Alfaro et al. 2023). Despite the importance of PHTs in nutrient acquisition and stress adaptation, their roles in *P. trifoliata*, especially under drought conditions, remain poorly understood.

In this study, a genome-wide analysis of the *PHT* gene family was conducted in *P. trifoliata*. The phylogenetic relationships, gene structures, conserved domains, and *cis*-acting elements in the promoters and syntenic analyses of the *PtPHT* genes were examined. Additionally, the expression patterns of *PtPHT* genes under drought stress were assessed. To elucidate the regulatory mechanisms governing *PtPHT* expression, yeast one-hybrid (Y1H) screening and dual-luciferase (LUC) assays were conducted to identify upstream transcription factors. These findings provide new insights into the molecular basis of Pi uptake and drought adaptation in citrus and offer potential targets for improving rootstock performance under water-limited conditions.

Materials and methods

Plant material and treatment

Uniform one-year-old *P. trifoliata* seedlings were cultivated in a greenhouse using a substrate mixture of grass charcoal, sawdust, and fine river sand (1:2:1 v/v). Before the plants were subjected to the drought treatment, the plants were uniformly managed in the greenhouse. Substrate moisture content was monitored using a TDR300 soil moisture probe (Spectrum Technologies, Aurora, IL, USA).

The experiment included two treatments: a well-watered control (CK) and a drought stress group. In the CK group, plants were irrigated thoroughly every 2 d to maintain substrate relative water content (RWC) at 70% and 80%. For the drought treatment, irrigation was withheld, and lateral root samples were collected at four stages corresponding to substrate RWC ranges of 55–65%, 25–35%, 17–21%, and 11–15%. At each sampling point, six seedlings were harvested per treatment and divided into three biological replicates. Root samples were surface-sterilized with 75% ethanol, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction. Calli derived from ‘Bingtang’ sweet orange (*Citrus sinensis*) were used for genetic transformation. Tobacco (*Nicotiana benthamiana*) plants for subcellular localization and genetic transformation were maintained in growth chambers under a 16 h light/8 h dark cycle, with a light intensity of 5000 lux at 25 °C.

Identification and characterization of PHT members in *P. trifoliata*

The *P. trifoliata* genome assembly (v1.0) was obtained from the Citrus Pangenome to the Breeding Database (<http://citrus.hzau.edu.cn/>). The protein sequences of 20 *Arabidopsis* PHT family members were retrieved from the TAIR database (<https://www.arabidopsis.org/index.jsp>), and their structural features were annotated using UniProt (<https://www.uniprot.org/>). *Arabidopsis* PHT protein sequences served as queries for bidirectional BLASTP analysis ($E\text{-value} \leq 1 \times 10^{-5}$) against the *P. trifoliata* genome using TBtools. Subsequent secondary screening and identification of *P. trifoliata* PHT family protein sequences were conducted using the BLAST website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and the Conserve Domain Database (CDD; <https://www.ncbi.nlm.nih.gov/Structure/cdd/>). After removing redundant sequences, the confirmed *P. trifoliata* PHT proteins were subjected to physicochemical characterization using ExPASy ProtParam (<https://web.expasy.org/protparam>), including the prediction of molecular weights and isoelectric points.

Structural and evolutionary analysis of *PtPHT* members

Conserved motifs of *PtPHT* family members in *P. trifoliata* were identified using the MEME suite (<https://meme-suite.org/meme/doc/meme.html>) with default parameters. Conserved domains were further analyzed using CDD. TBtools facilitated the visualization of motif distribution, conserved domains, and gene structure. Collinearity analysis of the *P. trifoliata* genome was conducted using MCScanX to identify gene duplication events, including tandem and segmental duplications. Synteny relationships of *PtPHT* genes were further analyzed between *P. trifoliata*, *Arabidopsis thaliana*, and *Citrus sinensis*. Paralogous and orthologous gene pairs among these species were identified, and syntenic relationships were visualized using TBtools.

The protein sequences of PHT family members in *C. sinensis* were retrieved. Multiple sequence alignment of PHT family proteins from *A. thaliana*, *P. trifoliata* and *C. sinensis* was performed using MEGA7. Based on the aligned sequences, a phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap replicates under the LG + G substitution model.

Analysis of promoter *cis*-acting elements and transcription of *PtPHT* genes in *P. trifoliata*

The 2-kb upstream promoter sequences of *PtPHT* genes were extracted from the *P. trifoliata* genome and analyzed for *cis*-acting regulatory elements using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/>

[http/](http://)). The distribution of identified elements was visualized using TBtools.

Drought-related *PtPHT* genes were identified by transcriptome analysis under four different drought conditions. Uniform one-year-old *P. trifoliata* seedlings were subjected to controlled drought treatment, and substrate moisture was monitored using TDR300. After 45 d of growth under well-watered conditions ($RWC \approx 70\%$), lateral roots were collected as control samples. Subsequent samples were collected under drought conditions, with RWC s ranging from 54 to 57%, 29% to 33%, and 21% to 24%, respectively. For each treatment, three independent biological replicates of root samples were collected. Total RNA extraction, library construction, and RNA sequencing were performed by BioMarker Technologies (Beijing, China). Gene expression levels were quantified as fragments per kilobase of transcript per million mapped reads (FPKM) using HTSeq0.6.1. Differentially expressed genes were identified using edgeR (v3.38.4) with thresholds of $|\log_2 FC| \geq 1$ and false discovery rate ≤ 0.05 . Heatmaps of drought-responsive *PtPHT* gene expression were generated using the R package pheatmap (v1.0.12).

RNA extraction, cDNA synthesis, and reverse transcription quantitative PCR (RT-qPCR) validation

Total RNA was extracted from *P. trifoliata*, 'Bingtang' sweet orange calli, and tobacco using the SteadPure Plant RNA Kit (Accurate Biology, China) following the manufacturer's protocols. RNA quality was assessed by spectrophotometric ratios ($A_{260}/A_{280} > 1.8$; Implen P330) and agarose gel electrophoresis (2.0%).

First-strand cDNA was synthesized from 1 μ g of total RNA using the Evo M-MLV RT Kit (Accurate Biology, China) and subsequently diluted five-fold for use as a template in quantitative PCR. Based on the transcriptome data, seven drought-induced *PtPHT* genes were selected for validation. Gene-specific primers were designed using Beacon Designer 8 and synthesized by Tsingke (Beijing, China) (Table S1). Data analysis employed the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) with *PtActin* or *NtActin* as the endogenous control. RT-qPCR validation was performed using a Bio-Rad CFX96 with a Talent RT-qPCR PreMix kit (Tiangen, China). The amplification program consisted of an initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 15 s, and 72 °C for 15 s. A melting curve analysis was conducted from 65 °C to 95 °C with a continuous fluorescence measurement at a rate of 0.5 °C per second. No-template reactions and triplicate technical replicates were performed on the RT-qPCR plate.

Silencing of *PtPHT1;2* by virus-induced gene silencing (VIGS) in calli

A 427 bp fragment of *PtPHT1;2*, encompassing the 3'-end CDS region, was cloned and inserted into the pTRV2 vector (tobacco rattle virus 2, driven by the *CaMV35S* promoter). This recombinant plasmid was designated TRV-*PtPHT1;2*. The empty pTRV2 vector without an insert, named TRV, served as the VIGS control. Both constructs were introduced individually into the GV3101 strain of *Agrobacterium tumefaciens* (Tsingke, Beijing). The transfection of calli was performed following previously described methods (Ma et al. 2024). After infection, the calli were maintained in a growth chamber under controlled conditions. Samples of control and TRV-*PtPHT1;2* calli were collected at 4 d post-infection for RNA extraction and gene expression analysis. Total P content was measured at 14 d post-infection.

Measurement of total P content in plants

The P content in both the calli and tobacco samples was measured using inductively coupled plasma-mass spectrometry (ICP-MS, PerkinElmer, USA) by determining the specific mass-to-charge ratio ($m/z=31$). Prior to analysis, samples were dried to constant weight, and P content was calculated based on dry weight (DW) as described previously (Dai et al. 2022).

Y1H, dual LUC and transcription activation assays

Y1H assays were performed using the Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech, USA) following the manufacturer's instructions. Root samples of *P. trifoliata* collected at four different drought stages were pooled for cDNA library construction using the CloneMiner II cDNA Library Construction Kit (Invitrogen, USA). A 2-kb promoter fragment of *PtPHT1;2* was inserted into the pABAI vector to generate the bait construct, which was then transformed into *Saccharomyces cerevisiae* Y1HGold.

Library screening was conducted using the Matchmaker Gold System (Clontech, USA) under dual selection: SD/-Leu basal medium and 100 ng ml⁻¹ Aureobasidin A (AbA) suppression. Positive clones were selected and inoculated into SD/-Leu/AbA liquid medium. After overnight incubation with shaking, yeast plasmids were extracted using a small-scale plasmid extraction kit (Coolaber, Beijing), followed by PCR amplification with universal primers. PCR products were verified by agarose gel electrophoresis and subsequently sequenced. Candidate genes were annotated using the NCBI database. For one-to-one interaction validation, the *PtRap2.12*-pGADT7 construct was co-transformed with the bait strain and plated on SD/-Leu medium

containing AbA. Primer sequences used for Y1H construction are detailed in Table S1.

The 2-kb *PtPHT1;2* promoter was cloned upstream of the firefly luciferase coding sequence in pGreen0800II-LUC to construct the pro*PtPHT1;2::LUC* reporter construct. For co-transfection, *35S::PtRap2.12* was used as the effector. Both constructs were introduced into the GV3101 strain with a pSoup helper plasmid (Tsingke, Beijing). For transient expression, leaves from 5- to 6-week-old tobacco plants were co-infiltrated with reporter and effector strains at a 1:5 (v/v) ratio. LUC activity was measured 72 h post-infiltration using the Dual Luciferase Reporter Assay System (Promega, USA), with Renilla luciferase (REN) serving as an internal control. The fluorescence signals were captured using a NEWTON 7.0 imaging system (Vilber Lourmat, France). LUC activity was calculated as the LUC/REN ratio, with eight biological replicates per sample. The primers used for constructing LUC vectors are detailed in Table S1.

For transcriptional activation assays, the full-length *PtRap2.12* coding sequence, as well as truncated fragments corresponding to the N-terminal (75 amino acids) and C-terminal (90 amino acids) regions, were cloned into the pGBKT7 vector. The recombinant constructs, along with empty pGBKT7 vector control, were transformed into *Saccharomyces cerevisiae* AH109 via the lithium acetate method. The yeast transformants were selected on SD/-Trp medium and subsequently transferred to SD/-Trp/-His/-Ade medium supplemented with X-gal (40 µg mL⁻¹) to assess transcriptional activation activity. Primer sequences used for pGBKT7 construct generation are detailed in Table S1.

Subcellular localization and functional identification of *PtRap2.12* in tobacco

For subcellular localization, the *35S::PtRap2.12::GFP* construct was co-expressed with *HY5::mCherry* (nuclear marker) in tobacco leaves, while *35S::GFP* served as a control. After 48 h of incubation in the dark at 25 °C, subcellular localization patterns were captured using a Zeiss Axio Imager 2 fluorescence microscope (Zeiss, Germany).

For functional analysis, the full-length coding sequence of *PtRap2.12* (Pt7g011690) was inserted into the pBI121-GFP vector under the control of the *CaMV35S* promoter to generate the *35S::PtRap2.12* overexpression construct. The construct was introduced into the GV3101 strain for subsequent tobacco transformation, following an established method (Zhang et al. 2009) with minor modifications.

To evaluate the role of *PtRap2.12* in drought response, transgenic and wild-type (WT) tobacco plants were subjected to drought stress induced by 10% (w/v) PEG-6000

for 10 d. The electrolyte leakage of tobacco shoots was measured as described previously (Wu et al. 2016). Malondialdehyde (MDA) content and the activities of superoxide dismutase (SOD) and peroxidase (POD) were quantified using commercial kits (Solarbio, China) according to the manufacturer's instructions. Total P content in shoots and roots was also measured.

In addition, the expression levels of two tobacco homologs of *PtPHT1;2* (*NtPHT1;1* and *NtPHT1;4*) in roots and leaves under normal and PEG-6000-induced drought conditions were analyzed by RT-qPCR. Each treatment included three independent biological replicates.

Statistical analysis

The data were processed using Microsoft Office Excel 2007. Statistical analysis was performed using SPSS Statistics 19.0 software. Differences between groups were evaluated using one-way analysis of variance (ANOVA) or Student's *t*-test, as appropriate. Figures were generated using TBtools and GraphPad Prism 6.03. Differences were considered statistically significant at $P < 0.05$.

Results

Genome-wide identification of PtPHTs in *P. trifoliata*

A total of 20 PtPHT proteins were identified in *P. trifoliata* through verification with universal protein

resource protein analysis, bidirectional BLAST and CDD conserved domain analysis. These 20 PtPHTs were named according to the nomenclature established for the *Arabidopsis* PHT family. Physicochemical property analysis (Table 1) revealed that the predicted PtPHT protein varied in length from 277 to 757 amino acids, with molecular weights ranging from 30.32 and 83.78 kDa and theoretical isoelectric points between 6.04 to 9.71. The deduced PtPHT proteins were predicted to localize to various subcellular locations: PtPHT1 and PtPHT4 proteins were primarily localized to the cell membrane, PtPHT2 proteins to chloroplast, PtPHT3 proteins to mitochondrion, and PtPHT5 proteins to vacuoles.

Phylogenetic relationships and classification of PHTs

To investigate the evolutionary relationships of PHT proteins, a phylogenetic tree was constructed using 56 PHT sequences from *A. thaliana*, *C. sinensis* and *P. trifoliata*. The analysis revealed that all PHTs clustered into five subfamilies: PHT1, PHT2, PHT3, PHT4, and PHT5 (Fig. 1). Among these, the PHT1 subfamily was the largest in *P. trifoliata*, comprising seven members and accounting for 35% of the total PtPHT proteins. In contrast, the PHT2 subfamily was the smallest, with only one member identified in each of the three

Table 1 Characteristics of *Poncirus trifoliata* phosphate transporter (PtPHT) proteins

No	Gene Name	Gene ID	Amino acid length (aa)	Chromosome position	Molecular weight (Mw; kDa)	Isoelectric point (pI)	GRAVY	Subcellular location prediction
1	<i>PtPHT1;1</i>	Pt9g007710.1	538	Chr9:5639850–5643603	58.75	9.09	0.328	Cell membrane
2	<i>PtPHT1;2</i>	Pt3g030800.1	541	Chr3:36333323–36337025	59.22	8.95	0.276	Cell membrane
3	<i>PtPHT1;3</i>	Pt9g007720.1	516	Chr9:5653481–5656782	56.46	8.96	0.411	Cell membrane
4	<i>PtPHT1;4</i>	Pt9g018680.1	535	Chr9:24887835–24889439	59.45	8.31	0.292	Cell membrane
5	<i>PtPHT1;5</i>	Pt4g020070.1	549	Chr4:19798371–19800017	59.72	8.88	0.365	Cell membrane
6	<i>PtPHT1;6</i>	PtUn001980.1	277	ChrUn:2440984–2442222	30.32	6.04	0.278	Cell membrane
7	<i>PtPHT1;7</i>	Pt5g000400.1	546	Chr5:289002–292594	60.33	7.94	0.257	Cell membrane
8	<i>PtPHT2;1</i>	Pt4g006610.1	575	Chr4:3184035–3187384	60.15	9.26	0.571	Chloroplast
9	<i>PtPHT3;1</i>	Pt8g000200.1	351	Chr8:130586–134856	38.74	9.21	0.012	Mitochondrion
10	<i>PtPHT3;2</i>	Pt7g020410.1	319	Chr7:21380295–21382901	34.70	9.36	0.222	Mitochondrion
11	<i>PtPHT3;3</i>	Pt4g014410.3	372	Chr4:11849405–11852924	39.72	9.29	0.108	Mitochondrion
12	<i>PtPHT3;4</i>	Pt2g008850.1	369	Chr2:8367885–8371001	39.06	9.32	0.259	Mitochondrion
13	<i>PtPHT4;1</i>	Pt8g011670.1	513	Chr8:9345867–9350819	56.19	8.92	0.406	Cell membrane
14	<i>PtPHT4;2</i>	Pt8g011480.1	757	Chr8:9250090–9257166	83.78	9.60	−0.117	Cell membrane
15	<i>PtPHT4;3</i>	Pt6g013690.1	505	Chr6:9679199–9683698	53.91	9.71	0.475	Cell membrane
16	<i>PtPHT4;4</i>	Pt3g038390.1	616	Chr3:41687506–41693568	68.42	9.08	0.161	Cell membrane
17	<i>PtPHT4;5</i>	Pt1g022950.1	536	Chr1:24522464–24529476	57.88	7.56	0.33	Cell membrane
18	<i>PtPHT4;6</i>	Pt8g000170.1	439	Chr8:109388–121329	48.01	9.59	0.500	Cell membrane
19	<i>PtPHT5;1</i>	Pt3g006940.1	698	Chr3:7458721–7458959	78.29	5.91	0.209	Vacuole
20	<i>PtPHT5;2</i>	Pt3g039240.1	705	Chr3:42168778–42176661	78.76	6.48	0.184	Vacuole

species. Overall, PtPHT proteins grouped closely with their homologs from *A. thaliana* and *C. sinensis*, indicating a high degree of evolutionary conservation.

Motif and gene structure analysis of PtPHTs

Conserved motif analysis revealed that PtPHT proteins within the same subfamily shared highly similar motif compositions and arrangements (Fig. 2a; Table S2). Among them, the PtPHT1 subfamily exhibited the highest degree of conservation, characterized by a greater number of motifs and a consistent motif order across members. Gene structure analysis showed clear differences among subfamilies (Fig. 2b). *PtPHT1* genes contained fewer exons but exhibited relatively longer exon lengths compared with other *PtPHT* genes. Most *PtPHT* genes contain both 5' and 3' untranslated regions. Domain analysis further supported subfamily classification. All *PtPHT1* genes contained the 2A0109 phosphate transport domain, whereas the *PtPHT2* genes harbored the PHO4 domain. *PtPHT3* genes contained mitochondrial transport domains, *PtPHT4* genes possessed Major

Facilitator Superfamily (MFS) transporter domains, and *PtPHT5* genes contained both SYG1/PHO81/XPR1 (SPX)-MFS and MFS domains. The diversity of conserved domains among PtPHT proteins suggests functional specialization in phosphate transport and subcellular distribution. Overall, the consistency between motif composition, gene structure, and conserved domains strongly supports the phylogenetic classification and indicates functional conservation within subfamilies.

Chromosome distribution and synteny analysis of *PtPHT* genes

PtPHT genes were unevenly distributed across nine chromosomes of the *P. trifoliata* genome. Several duplicated gene pairs were identified, including *PtPHT1;1/1;2*, *PtPHT3;1/3;2*, *PtPHT3;3/3;4*, *PtPHT4;2/4;3*, and *PtPHT5;1/5;2* were identified as segmentally duplicated genes (Fig. 3a). These results indicated that the segmental duplication has played a major role in the expansion of the PtPHT gene family. To further investigate the evolutionary relationships of *PtPHT* genes, comparative

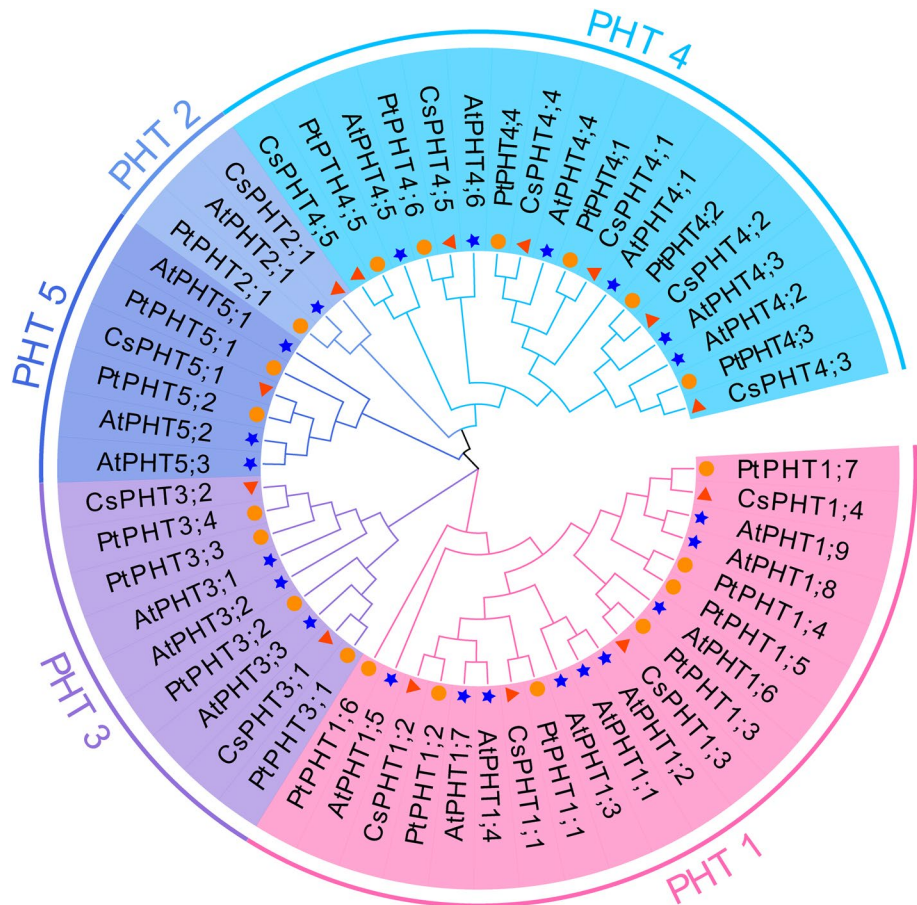


Fig. 1 Phylogenetic analysis of phosphate transporter (PHT) members from *Arabidopsis thaliana*, *Citrus sinensis* and *Poncirus trifoliata*. Blue stars, red triangles, and orange dots represent PHT proteins from *A. thaliana*, *C. sinensis* and *P. trifoliata*, respectively

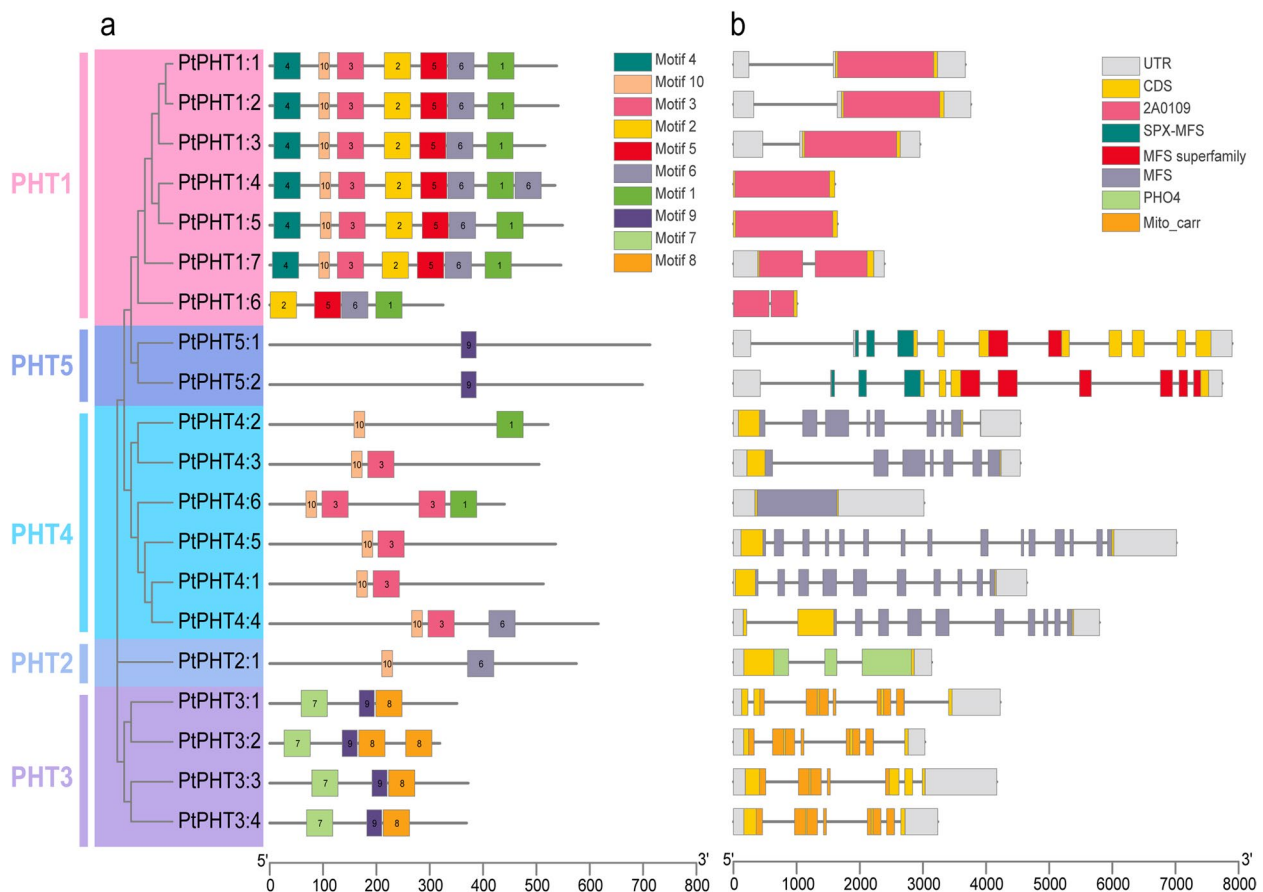


Fig. 2 Motif, conserved domain and gene structure analysis of *Poncirus trifoliata* phosphate transporters (*PtPHTs*). **a** Motif analysis of *PtPHT* members; each motif is marked with a number in a colored box. **b** Conserved domain and gene structure of *PtPHT* members. UTR, untranslated region; CDS, coding Sequence; 2A0109, a phosphate:proton symporter domain; MFS, Major Facilitator Superfamily; SPX-MFS, Syg1/Pho81/Xpr1-MFS; Mito_carr, mitochondrial carrier protein domain

synteny analyses were performed between *P. trifoliata*, *A. thaliana*, and *C. sinensis*. A total of 18 homologous gene pairs were identified between *P. trifoliata* and *A. thaliana*, with chromosome 3 of *P. trifoliata* containing the highest number of homologous gene pairs (six pairs) (Fig. 3b). In addition, 23 homologous gene pairs were found between *P. trifoliata* and *C. sinensis*. These results indicate a high degree of conservation of *PHT* genes across species and suggest that gene duplication and divergence have contributed to the evolution and functional diversification of the *PtPHT* family.

Analysis of *cis*-acting elements in *PtPHT* promoters

Cis-acting elements in the *PtPHT* promoter regions were categorized into two groups. As shown in Fig. 4, the first group comprised hormone-responsive elements, such as TGA-elements, auxin-responsive region (AuxRR)-core, gibberellin-responsive element (GARE)/P-box/TATC-box, TGACG-motif/CGTCA-motif, abscisic acid-responsive elements (ABREs), ethylene-responsive

elements (EREs), and salicylic acid-responsive (TCA) elements, which mediated responses to various phytohormones, including auxin, gibberellin, methyl jasmonate, abscisic acid, ethylene and salicylic acid. The second group consisted of abiotic stress-responsive elements, including TC-rich repeats/W-boxes, wound-responsive (WUN) motifs, MYB binding sites (MBSs), low-temperature-responsive elements (LTRs) and anaerobic-inductive elements (AREs). These elements were associated with environmental stresses such as defense responses, wounding, drought, low temperature and anaerobic conditions.

Analysis of the *PtPHT* gene promoter regions revealed the presence of different types and numbers of *cis*-acting elements, which suggests that *PtPHT* genes may be regulated by various phytohormones and abiotic stress factors. For instance, *PtPHT1;1*, *PtPHT4;3*, *PtPHT4;5*, and *PtPHT5;1* contained six or more ABREs. Similarly, *PtPHT1;1*, *PtPHT1;3*, *PtPHT2;1*, *PtPHT3;1*, *PtPHT3;3*, *PtPHT3;4*, *PtPHT4;3*, *PtPHT4;4*, and *PtPHT5;1*

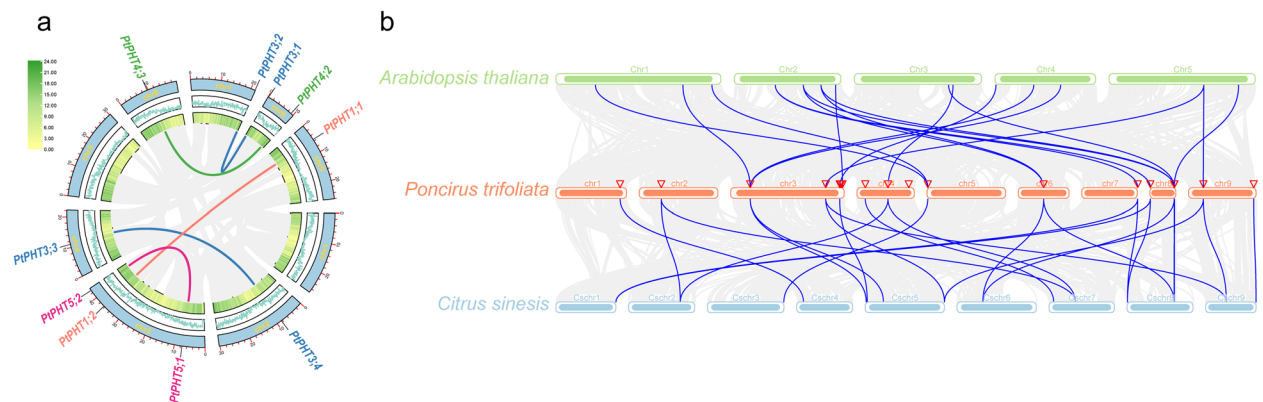


Fig. 3 Synteny analysis of phosphate transporter (PHT) genes in *Arabidopsis thaliana*, *Citrus sinensis* and *Poncirus trifoliata*. **a** Chromosome distribution and segmental duplication of *PtPHT* genes in *P. trifoliata*. Colored lines indicate duplicated *PtPHT* gene pairs. The color scale bar denotes gene density profile. **b** Comparative synteny analysis of *PHT* genes among *A. thaliana*, *C. sinensis* and *P. trifoliata*. The duplications of *PtPHT* genes in *P. trifoliata* are displayed with colored lines. Blue lines indicate syntenic *PtPHT* gene pairs, while gray lines represent other collinear genes pairs

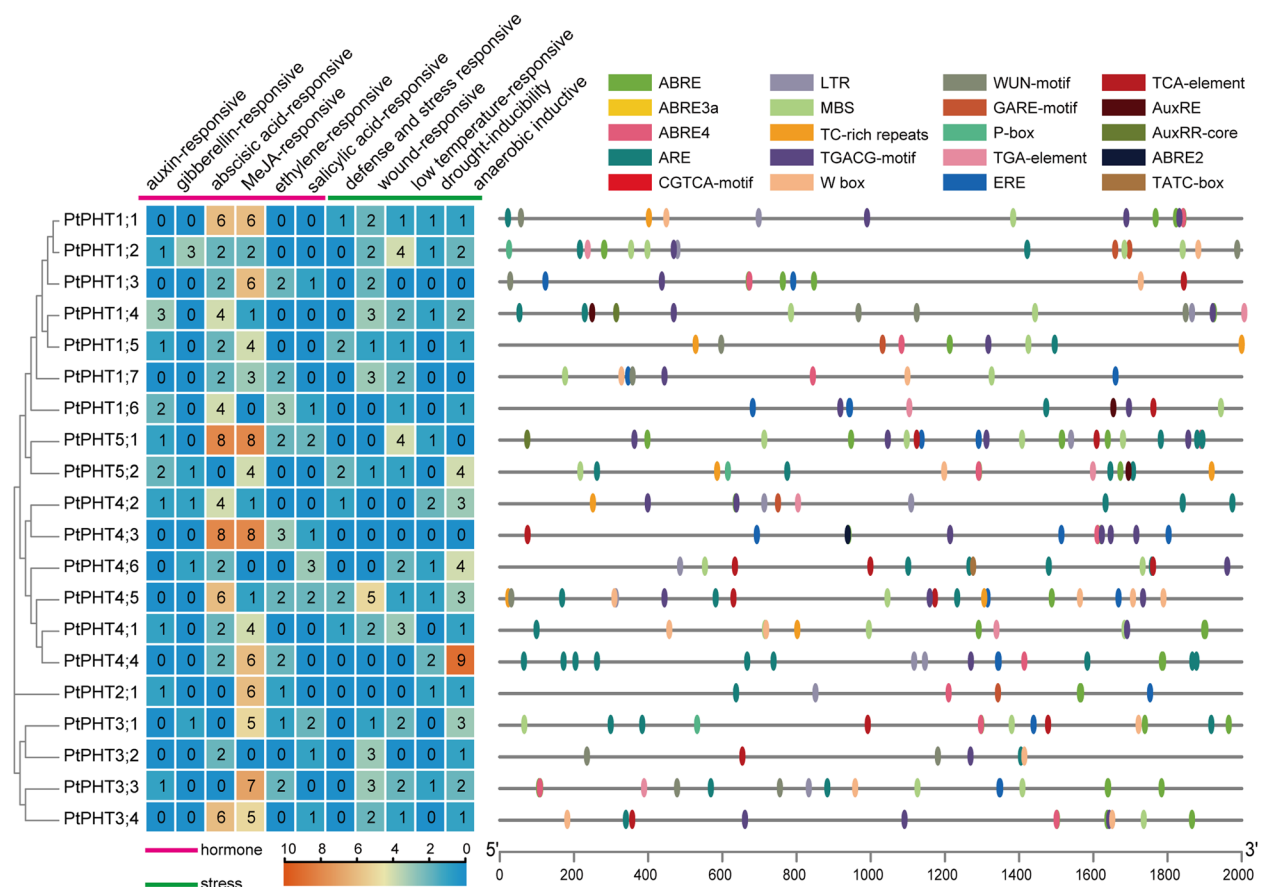


Fig. 4 Putative *cis*-acting elements and transcription factor-binding sites in the promoters of *Poncirus trifoliata* phosphate transporter (*PtPHT*) genes. The grid colors and numbers indicate the quantity of different *cis*-acting elements in the *PtPHT* genes, with a gradient from blue (low) to orange (high). Colored boxes represent the types of *cis*-acting elements and their locations in each *PtPHT* gene. ABRE, abscisic acid-responsive element; ARE, anaerobic-inducible elements; LTR, low-temperature responsive element; MBS, MYB binding site; WUN-motif, wound-responsive motif; GARE-motif, gibberellin-responsive element; ERE, ethylene-responsive element; TCA-element, salicylic acid-responsive element; AuxRE, auxin-responsive element; AuxRR, auxin-responsive region

contained five or more methyl jasmonate-responsive elements. Additionally, several abiotic stress-responsive elements, such as WUN elements, LTRs and AREs were broadly detected in the promoter regions of *PtPHT* genes. These findings suggest that the *PtPHT* gene family is potentially involved in diverse phytohormones and stress responses.

Transcriptional profiles of *PtPHT* genes in response to drought and RT-qPCR validation

Transcriptome analysis revealed that *PtPHT* genes exhibited distinct expression patterns in response to drought stress, which could be broadly classified into two groups: upregulated and downregulated genes (Fig. 5a). Eight *PtPHT* genes were upregulated, whereas eight were downregulated under drought conditions. In *P. trifoliata* roots, the expression levels of *PtPHT1;2*, *PtPHT1;3*, *PtPHT3;3*, *PtPHT4;3*, and *PtPHT4;5* increased under drought stress. Notably, *PtPHT1;2* and *PtPHT3;3* displayed a late-response pattern, with expression levels continuing to rise as drought severity increased. Several genes, including *PtPHT1;1*, *PtPHT3;4*, *PtPHT4;2*, *PtPHT4;5*, *PtPHT4;6*, *PtPHT5;1*, and *PtPHT5;2* were expressed under both well-watered and drought-stress

conditions. In contrast, *PtPHT2;1*, *PtPHT3;1*, *PtPHT4;1*, and *PtPHT4;4* were downregulated, suggesting that it was suppressed by drought or functioned in other plant organs. RT-qPCR analysis of selected genes confirmed the transcriptome results (Fig. 5b). The expression of *PtPHT1;2* increased progressively with drought stress but declined sharply under severe dehydration (RWC 11–15%). A similar trend was observed for *PtPHT1;1*, *PtPHT1;3*, *PtPHT3;3*, and *PtPHT4;3*, which were strongly induced during early drought (RWC 55–65%) and subsequently decreased as stress intensified. In contrast, *PtPHT5;1* and *PtPHT5;2* showed sustained upregulation across all drought stages. The findings suggest that different subfamilies exhibit distinct stress responses to drought stress. Among them, *PtPHT1;2* displayed the most significant response, with a 16.4-fold upregulation, highlighting its potential role in drought adaptation in *P. trifoliata*.

Silencing *PtPHT1;2* reduced the total P content in calli

Given the strong induction of *PtPHT1;2* under drought conditions, we hypothesized that *PtPHT1;2* regulates P accumulation in citrus. To test this, *PtPHT1;2* was silenced in ‘Bingtang’ sweet orange calli using VIGS

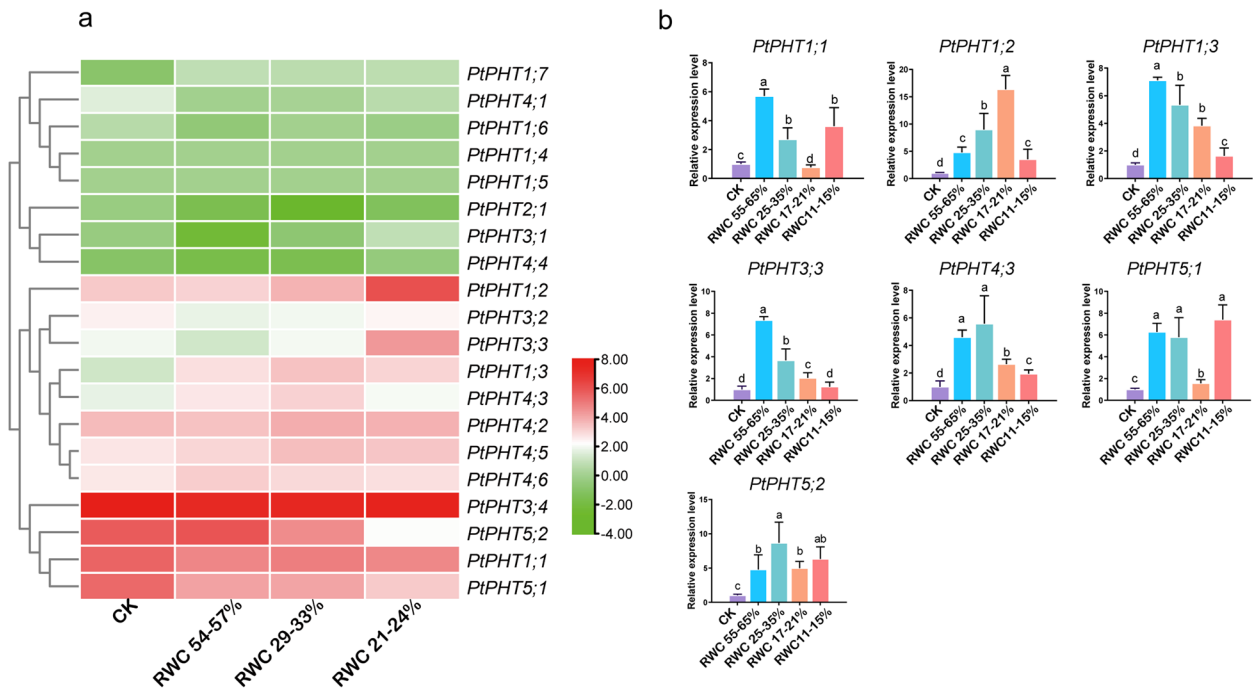


Fig. 5 Expression profiles of *Poncirus trifoliata* phosphate transporter (*PtPHT*) genes under drought stress. **a** FPKM values of *PtPHT* genes in *P. trifoliata* roots under different types of drought stress. CK, a well-watered control treatment; RWC, relative water content. **b** RT-qPCR validation of seven candidate *PtPHT* genes in *P. trifoliata* roots in response to various levels of drought. Colored boxes represent the $\log_2$ -transformed values. Each value represents the mean $\pm$ SD of three biological replicates. Different letters indicate statistically significant differences ($P < 0.05$)

(Fig. 6a). RT-qPCR revealed that the TRV-*PtPHT1;2* construct was successfully transiently expressed in the calli (Fig. 6b). A significant decrease in P was observed in the TRV-*PtPHT1;2-1* and TRV-*PtPHT1;2-2* calli compared with that in the TRV control calli (Fig. 6c). These results indicated that *PtPHT1;2* serves as a vital Pi transporter and participates in Pi uptake in citrus.

PtRap2.12 positively regulates *PtPHT1;2* expression under drought stress

Given the pronounced induction of *PtPHT1;2* under drought stress and its role in phosphate uptake and transport, its upstream regulatory mechanism was further investigated. *Cis*-acting element analysis of the *PtPHT1;2* promoter revealed the presence of multiple hormone- and stress-responsive elements, including a dehydration-responsive element (DRE), suggesting potential regulation by drought-associated transcription factors. To identify candidate regulators, a Y1H library screening was performed using the *PtPHT1;2* promoter as bait. A total of 22 candidate proteins were identified as potential interactors (Table S3). Among these, *PtRap2.12*, an APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factor, was selected for further validation. Y1H assays confirmed that *PtRap2.12* directly binds to the *PtPHT1;2* promoter. Yeast cells co-transformed with pro*PtPHT1;2*-BD and *PtRap2.12*-AD grew normally on SD/-Leu medium supplemented with 100 ng mL⁻¹ AbA, similar to the positive control, whereas no growth was observed for the negative control (Fig. 7a).

To further assess the regulatory effect of *PtRap2.12*, a dual-luciferase (LUC) assay was performed in *N. benthamiana* leaves. Co-expression of the effector (35S::*PtRap2.12*) with the reporter (pro*PtPHT1;2*::LUC)

resulted in a significant increase in LUC activity compared with the control (Fig. 7b), consistent with enhanced fluorescence signals.

Together, these results indicate that *PtRap2.12* directly binds to the *PtPHT1;2* promoter and positively regulates its transcription, suggesting a role for *PtRap2.12* in mediating phosphate transport in response to drought stress.

Identification and functional analysis of *PtRap2.12*

The expression of *PtRap2.12* was strongly induced by drought stress, with a pronounced increase observed at RWC levels of 17–21%, followed by a decline under more severe stress (Fig. 8a), suggesting a role in early drought signal transduction. Subcellular localization revealed that the fluorescence signal from the 35S::*PtRap2.12*::GFP fusion protein was observed exclusively in the nucleus (Fig. 8b), confirming that *PtRap2.12* was a nucleus-localized protein. To further characterize its function, transcriptional activation activity was assessed in yeast. Deletion constructs lacking either the N- or C-terminal regions were generated and expressed in AH109 yeast cells. Yeast cells harboring the N-terminal deletion construct grew normally on selective medium (SD/-Trp/-Ade/-His/X-α-gal), whereas those with the C-terminal deletion did not, indicating that the transcriptional activation domain resides in the C-terminus of *PtRap2.12* (Fig. 8c).

To investigate its biological function, *PtRap2.12* was overexpressed in tobacco, generating three independent transgenic lines (OE#1, OE#2, and OE#3) (Fig. 9a, b). After 10 d of treatment with 10% (w/v) PEG-6000, significant differences were observed between the wild-type (WT) and *PtRap2.12* transgenic tobacco lines.

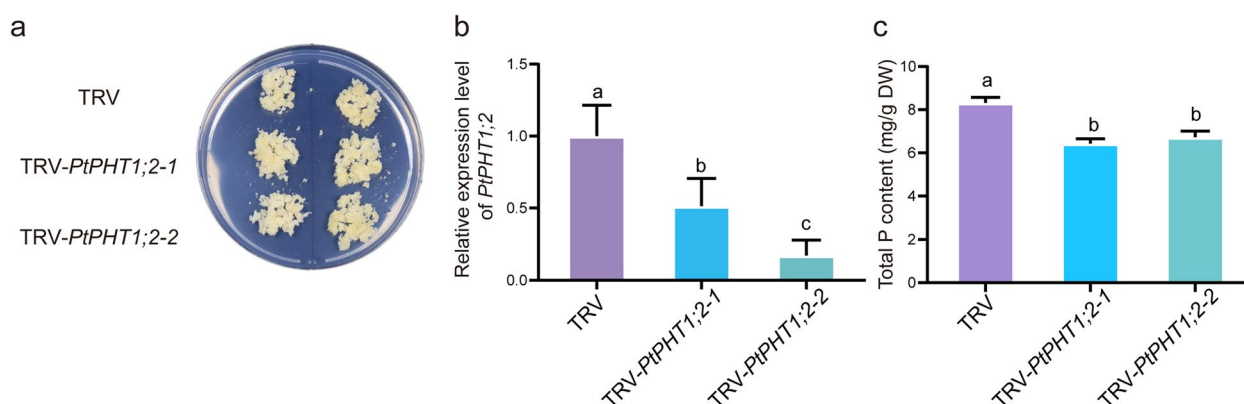


Fig. 6 Total Phosphorus (P) content in the *Poncirus trifoliata* phosphate transporter 1;2 (*PtPHT1;2*)-silenced calli. **a** Phenotype of control and *PtPHT1;2*-silenced (VIGS) citrus calli. **b** Relative expression level of *PtPHT1;2* in control (TRV) and *PtPHT1;2*-silenced (TRV-*PtPHT1;2*) lines. **c** Total P content in the calli of the control and *PtPHT1;2*-silenced lines. TRV represents the empty pTRV2 vector used as the VIGS control. Different letters indicate statistically significant differences ($P < 0.05$)

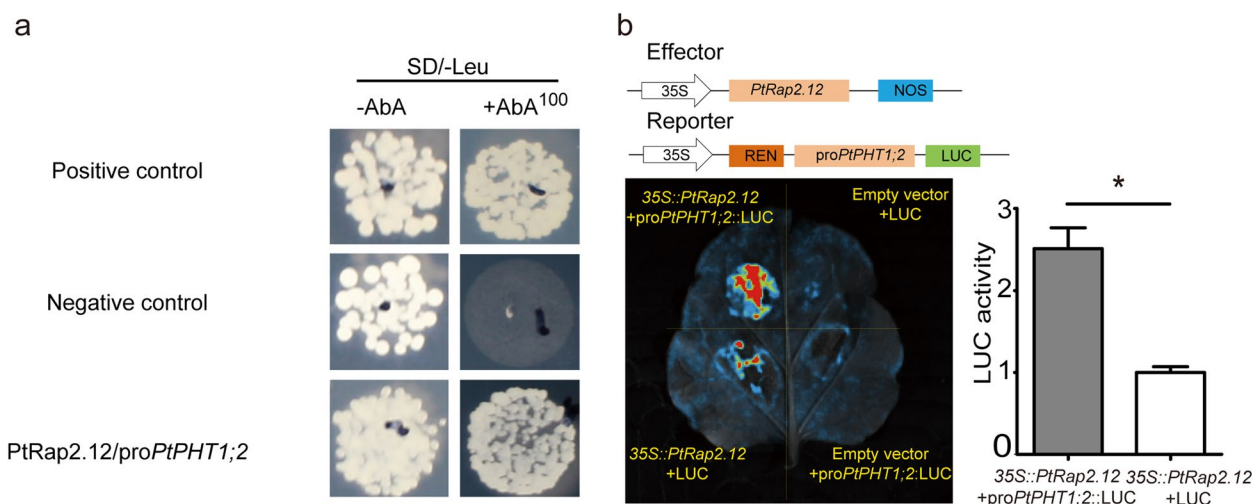


Fig. 7 Identification of *Poncirus trifoliata* Related to APETALA2.12 (PtRap2.12) as a transcriptional regulator of phosphate transporter 1;2 (PtPHT1;2). **a** Yeast one-hybrid (Y1H) point-to-point verification of Prey-PtRap2.12 and Bait-proPtPHT1;2. AbA, Aureobasidin A. **b** Analysis of dual-luciferase (LUC) activity and fluorescence detection of leaves in vivo. Positive control, P53-BD and P53-AD; Negative control, proPtPHT1;2-BD and pGADT7. Asterisks indicate statistically significant differences between two groups (*P < 0.05)

Compared with WT tobacco, the OE#1 transgenic line exhibited enhanced growth compared with WT plants, with increased plant height and canopy width, suggesting that *PtRap2.12* promotes vegetative growth in response to drought (Fig. 9c). Consistent with the observed phenotype, transgenic lines showed significantly lower electrolyte leakage and MDA content, along with higher activities of SOD and POD (Fig. 9d–i), indicating reduced cellular damage under stress conditions. Overall, these findings suggest that overexpression of *PtRap2.12* in tobacco substantially enhances drought tolerance.

Furthermore, to determine whether *PtRap2.12* influences phosphate transport, the expression of two tobacco phosphate transporter genes, *NtPHT1;1* and *NtPHT1;4*, was examined. Under PEG treatment, *NtPHT1;1* expression in shoots and *NtPHT1;4* expression in roots were significantly upregulated in transgenic lines, with increases of 2.68-fold and 2.07-fold, respectively (Fig. 9j, k). Correspondingly, total phosphorus content in both shoots and roots was significantly higher in transgenic plants than in WT controls (Fig. 9l).

Collectively, these results indicate that *PtRap2.12* functions as a nuclear transcriptional activator that enhances drought tolerance and promotes phosphate uptake, potentially through regulation of *PHT* gene expression.

Discussion

In citrus production, *P. trifoliata* is widely used as rootstock because of its resistance to abiotic stress, particularly drought stress. These adverse environmental conditions significantly reduce Pi uptake, adversely

affecting the yield and quality of citrus fruit (Quaggio et al. 2006; Cao et al. 2020; Zhang et al. 2020). P is essential for citrus plant growth and fruit development and is primarily absorbed by roots in the form of orthophosphate (H_2PO_4^-) (Rausch and Bucher 2002). This process is mediated by PHTs, which facilitate Pi movement across membranes via proton or sodium gradients (Bun-Ya et al. 1991; Rae et al. 2003).

PHT subfamilies are evolutionarily conserved and functionally diverse and play pivotal roles in Pi uptake, translocation, intracellular transport, and stress adaptation. The classification, structural characteristics, functional differentiation and evolutionary expansion of PHT members have been extensively investigated in diverse plants (Jia et al. 2011; Qin et al. 2012; Ren et al. 2014). Previous studies have demonstrated that the plant PHT family can be divided into multiple subfamilies with distinct functional roles, and functional divergence has been well characterized in *Arabidopsis*. PHT1 proteins are well known as key mediators of Pi absorption, accumulation and translocation (Shin et al. 2004; Remy et al. 2012; Chien et al. 2022); moreover, other PHT subfamilies (PHT2–PHT5) are involved mainly in intracellular Pi transport and homeostasis in different organelles (Versaw and Harrison 2002; Jia et al. 2015; Cubero et al. 2009; Wang et al. 2011, 2015a; Karlsson et al. 2015; Luan et al. 2018). In this study, 20 PtPHT members were identified and classified into five subfamilies (PtPHT1–PtPHT5). Among the PtPHT subgroups, PtPHT1 was the largest group, and the number of PtPHT1 members in *P. trifoliata* was relatively lower compared with that in other

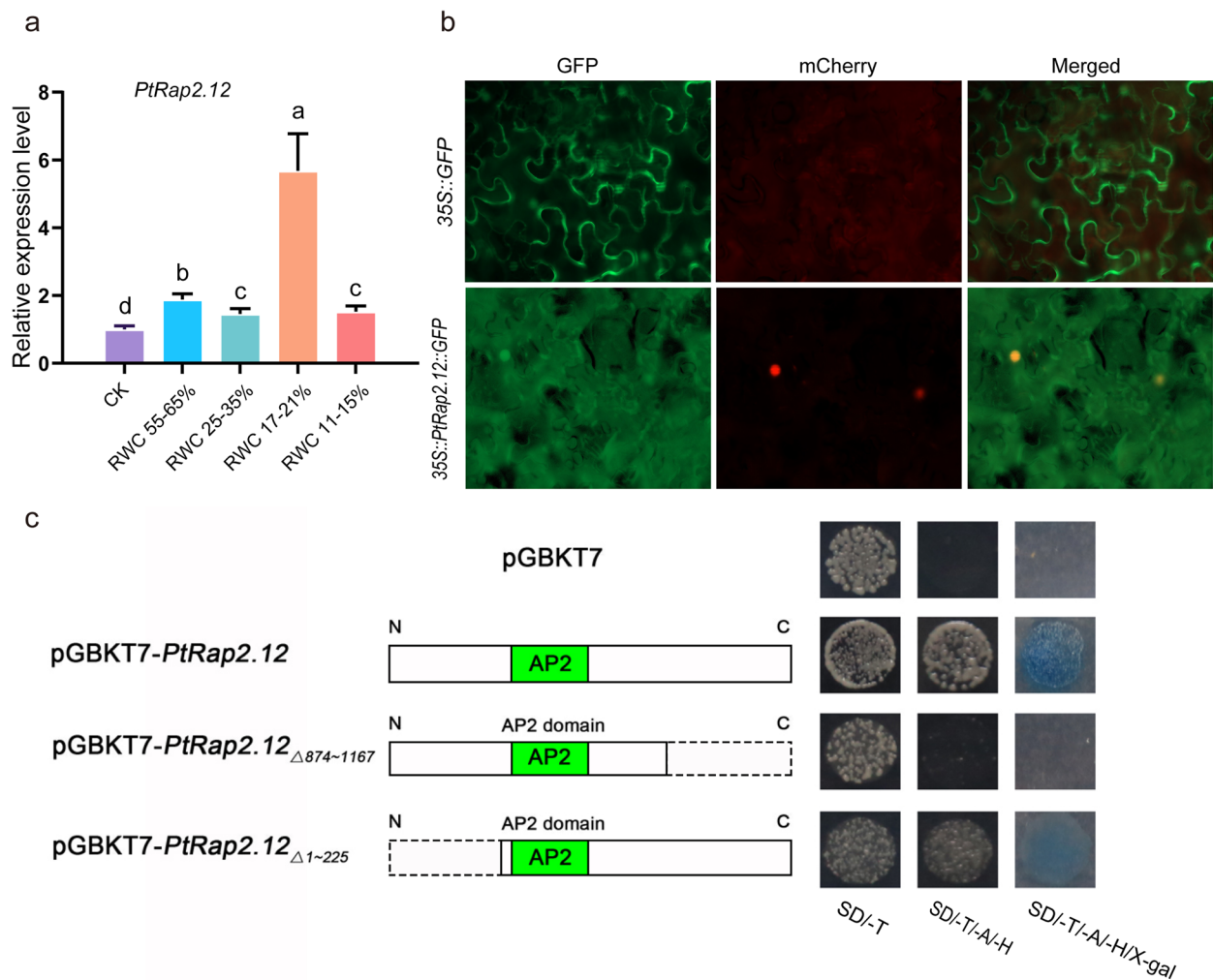


Fig. 8 Characterization of *Poncirus trifoliata* Related to APETALA2.12 (PtRap2.12): expression, subcellular localization and transcriptional activity. **a** Expression pattern of *PtRap2.12* in *P. trifoliata* roots under drought stress at different related water content (RWC) levels. **b** Subcellular localization of *PtRap2.12*. GFP and mCherry fluorescence were observed under green and red channels, respectively. "Merged" indicates the overlay of GFP and mCherry signals, and mCherry (marker) represents the red fluorescence signal of the control marker protein. **c** Identification of the *PtRap2.12* transcriptional activation domain. AP2, APETALA2; SD/-T/-A/-H, SD/-Trp/-Ade/-His/X- α -gal

species, suggesting possible gene loss events during evolution (Liu et al. 2011; Cai et al. 2020; Wan et al. 2020). All seven *PtPHT* proteins localize to the cell membrane (Table 1), contain the conserved 2A0109 domain and present the highest motif count, underscoring their central role in Pi uptake. Additionally, five segmental duplication events were detected in the *PtPHT* subfamily, and phylogenetic analysis revealed that *PtPHT1;1* and *PtPHT1;2* are closely related to *AtPHT1;1*, *AtPHT1;2*, and *AtPHT1;3*, which are tandem duplicates with partially redundant functions in Pi uptake (Shin et al. 2004). Together, these findings suggest that the core functions of Pi uptake and intracellular transport are conserved among the *PtPHT* subfamilies in *P. trifoliata* and

that segmental duplication events may result in robust Pi acquisition in *P. trifoliata* under P deficiency and drought conditions.

PHT proteins are well known for their roles in Pi uptake and homeostasis under phosphate deficiency and have also been implicated in responses to abiotic stresses such as salinity and drought (Versaw and Harrison 2002; Jia et al. 2011; Li et al. 2019). For instance, in apple (*Malus domestica*), *MdPHT1.5*, *MdPHT1;7*, and *MdPHT4;7* are markedly upregulated in stressed roots, whereas *MdPHT3;7* and *MdPHT4;3* are upregulated in leaves (Sun et al. 2017). Similarly, *PtPHT1;2* and *PtPHO9* are significantly upregulated in poplar under drought conditions (Zhang et al. 2016; Liu et al. 2023). In this study, transcriptome and

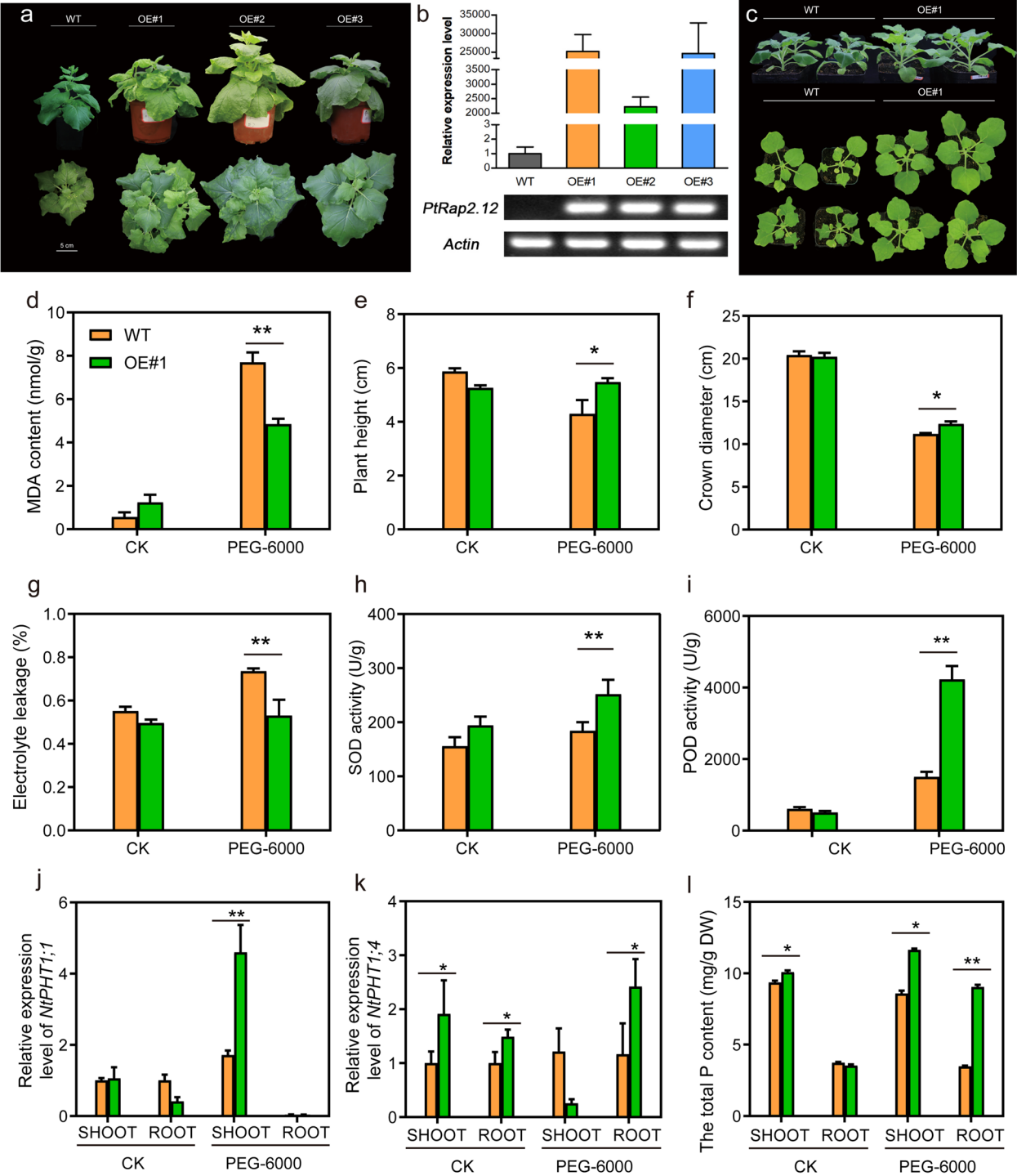


Fig. 9 Analysis of transgenic tobacco lines overexpressing *Poncirus trifoliata* Related to *APETALA2.12* (*PtRap2.12*) under PEG-6000 treatment. **a** Phenotypes of wild-type (WT) and three independent *PtRap2.12*-overexpressing transgenic tobacco lines (OE#1, #2, #3). **b** Identification of transgenic tobacco overexpressing *PtRap2.12* at the transcriptional level. **c** Phenotypes of WT and *PtRap2.12*-OE#1 transgenic tobacco after 10 d of PEG-6000 treatment. **d–i** Malondialdehyde (MDA) content (**d**), plant height (**e**), crown width (**f**), electrolyte leakage (**g**), superoxide dismutase (SOD) activity (**h**), and peroxidase (POD) activity (**i**) of WT and *PtRap2.12*-OE#1 tobacco under control (CK; well-watered) and PEG-6000 treatments. **j, k** Expression of *NtPHT1;1* (**j**) and *NtPHT1;4* (**k**) in the shoots and roots of transgenic tobacco after 10 d of PEG-6000 treatment. **l** Total phosphorus (P) content in shoots and roots. Asterisks indicate significant differences between transgenic and WT plants (* $P < 0.05$; ** $P < 0.01$)

RT-qPCR analyses revealed that several *PtPHT* genes, including *PtPHT1;1*, *PtPHT1;2*, *PtPHT1;3*, *PtPHT3;3*, *PtPHT4;3*, *PtPHT5;1*, and *PtPHT5;2*, were induced during the early stages of drought stress. However, the expression of *PtPHT1;1*, *PtPHT1;2*, *PtPHT1;3*, and *PtPHT3;3* declines as stress intensifies, indicating a biphasic response pattern typical of drought-responsive genes. This pattern may reflect an early adaptive response to increase Pi uptake, followed by metabolic adjustment under prolonged stress. Among these genes, *PtPHT1;2* exhibited the most pronounced response, with a 16.2-fold increase under drought conditions. Functional analysis further demonstrated that silencing *PtPHT1;2* significantly reduced phosphorus accumulation in citrus calli, confirming its key role in Pi transport. Similar findings have been reported in other species; for instance, *MdPHT1;7* is induced by both drought and Pi starvation, and its overexpression enhances Pi uptake and drought tolerance in apple and tomato (Sun et al. 2021), whereas *PtPHT1.2* in poplar is induced approximately 90-fold under drought stress (Zhang et al. 2016). These results suggest that drought stress triggers dynamic changes in Pi transport systems and that the transient induction of *PtPHT* genes—particularly *PtPHT1;2*—represents a key adaptive mechanism to maintain phosphate homeostasis under drought conditions.

Plants have evolved complex adaptive mechanisms to activate stress-related transcription factors (TFs) and remodel related metabolic processes to cope with drought stress. As members of a multifunctional TF family, AP2/ERF TFs play central roles in the response to drought, high salinity and low-temperature stress (Xie et al. 2019). Several AP2/ERF TFs have been shown to modulate multiple physiological processes, including stress resistance and nutrient adaptation, by regulating downstream target genes (Karlova et al. 2011; Mizoi et al. 2012; Wang et al. 2015b; Li et al. 2023; Kandhol et al. 2024). For example, AtRap2.11 regulates the potassium transporter *AtHAK5* under low-potassium conditions in *Arabidopsis* (Kim et al. 2012), whereas ZmEREB97 modulates nitrate uptake in maize (*Zea mays*) by repressing *ZmNRT* genes (Wu et al. 2024). The AP2/ERF TF TINY positively regulates drought responses by activating the expression of drought-responsive genes and promoting abscisic acid-mediated stomatal closure in *Arabidopsis* (Xie et al. 2019b). Similarly, overexpression of the *HDR* gene in rice enhanced drought tolerance and reduced water consumption by improving water use efficiency and photosynthetic assimilation (Karaba et al. 2007). In this study, a total of 22 upstream regulatory factors were identified through Y1H screening using the *PtPHT1;2* promoter as bait. Among them, the expression of PtRap2.12, an AP2/ERF family member, was rapidly induced in roots under drought stress, suggesting that it plays a role in stress

signaling. Our results suggest that PtRap2.12 may serve as a key regulator linking Pi homeostasis and drought adaptation and that the heterologous overexpression of *PtRap2.12* in tobacco resulted in improved growth, increased Pi accumulation and greater drought resilience, suggesting a regulatory model in which PtRap2.12 regulates the expression of *PtPHT1;2* to adapt to drought and the resulting Pi deficiency. Consistent with previous reports on the stress resistance function of plant AP2/ERF TFs (Jin et al. 2010; Romero et al. 2016; Li et al. 2022), the results of this study confirm that PtRap2.12 plays a pivotal role in enhancing drought tolerance and Pi uptake. Taken together, these findings suggest that Rap2.12 is a vital TF that links drought signaling with phosphate uptake, highlighting its role in coordinating stress adaptation and nutrient homeostasis in *P. trifoliata*.

Conclusion

In summary, we identified 20 members of the *PtPHT* family in *P. trifoliata* and classified them into five sub-families based on phylogenetic relationships and gene structures. RNA-seq profiling revealed the differential involvement of these genes in drought stress responses. Notably, *PtPHT1;2* emerged as a key drought-responsive regulator of phosphorus homeostasis in citrus. We further demonstrated that the transcription factor PtRap2.12 directly activates *PtPHT1;2*, thereby enhancing phosphate uptake and drought tolerance (Fig. 10).

Collectively, these findings provide new insight into the roles of *PtPHT* genes under drought stress and uncover a previously uncharacterized PtRap2.12–*PtPHT1;2* regulatory module. This module offers a potential genetic strategy for improving combined resistance to phosphate deficiency and drought in citrus rootstocks.

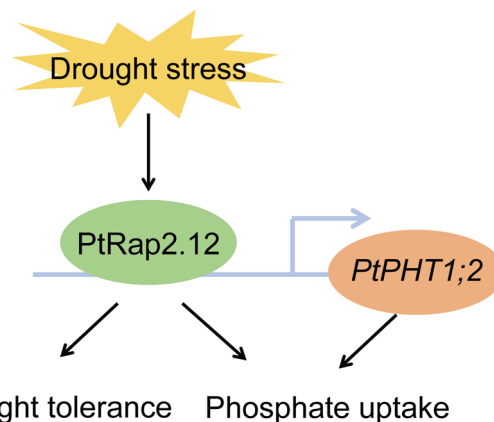


Fig. 10 Proposed model for the regulation of drought tolerance and phosphate uptake by PtRap2.12 and *PtPHT1;2*

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-026-00109-7>.

Supplementary Material 1: Figure S1. Identification of transgenic tobacco overexpressing *Poncirus trifoliata* Related to *APETALA2.12* (*PtRap2.12*) at the DNA level.

Supplementary Material 2: Table S1. Primers used in this study. Table S2. List of conserved motifs in phosphate transporter 1;2 (*PtPHT*) members. Table S3. List of candidate proteins that bind to the *PtPHT1;2* promoter.

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Authors' contributions

YWC performed the experiment and wrote the manuscript. QZ, JXL, RHL, MP and YYC analyzed the data. YJ, LS and JFY directed the experimental method. XPL and FFL revised the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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