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Characterization of *PHT* Genes in 'duli' (*Pyrus betulifolia Bunge*) and Expression Analysis of *PbPHTs* in Response to Plant Growth Regulators, P, and Salt Stress

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Abstract: The phosphate transporter (PHT) family plays an important role in the uptake and transport of P elements in plants. A total of 158 *PbPHTs* were identified from the genome of 'duli' (*Pyrus betulifolia Bunge*) in this study, including 70 *PbPHT1s*, 2 *PbPHT2s*, 70 *PbPHT3s*, 12 *PbPHT4s*, and 4 *PbPHT5s*. Among the 158 *PHT* genes, 150 were localized to 17 'duli' chromosomes. Gene duplication analysis identified 18 tandemly duplicated gene pairs. The promoter analysis showed that there were a large number of cis-acting elements related to phytohormones, growth, development, stress, and light response in *PbPHTs*. qRT-PCR analysis revealed that most *PHT* genes in 'duli' were highly expressed in the fruits, flowers, leaves, stems, and roots, and 15 *PbPHT* genes were responsive to 5 μM, 0.5 mM, 5 mM H_2PO_4, NaCl, GR24 (synthetic SL analog), GA3 (gibberellin 3), ABA (abscisic acid), and IAA (indole-3-acetic acid). GR24, GA3, IAA, and 5 mM KH_2PO_4 treatments could increase the concentration, absorption, transport, and distribution of P elements in the rhizomes and leaves of 'duli', but 5 μM KH_2PO_4, NaCl, and ABA had the opposite effect. This study therefore provides a list of *PbPHT* genes with substantial roles in abiotic stress response, as well as important information to understand the functional characteristics of *PbPHT* during 'duli' abiotic stress tolerance, and explores the function of PbPHTs in exogenous hormones, phosphorus, and salt stress in the future.

Keywords: 'duli'; PHT; gene expression analysis; hormonal; abiotic stress; physiological change

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1. Introduction

Phosphorus (P) is one of the most important macroelements necessary for plants and is an important component of nucleic acids, phospholipids, nucleoproteins, and ATP [1]. It is also involved in multiple biological processes in plants, such as photosynthesis, respiration, and sugar and starch transport. Although P is abundant in soil, it often forms insoluble phosphate with Ca^{2+}, Fe^{3+}, and Al^{3+}, which cannot be easily absorbed and utilized by plants [2]. However, P deficiency can reduce anthocyanin synthesis, reduce crop tillering, reduce the rate of bud and flower formation in fruit trees, and accelerate leaf shedding, thereby limiting plant growth [3]. Plants absorb P elements from the roots through the formation of inorganic phosphates through PHT proteins (phosphate transporters), including HPO_4^{2-} and $H_2PO_4^{-}$.

In plants, the *PHT* family is divided into five subfamilies (*PHT1*, *PHT2*, *PHT3*, *PHT4*, and *PHT5*) according to their subcellular localization and sequence [4,5]. The *PHT1* (phosphate transporter 1) family is a plasma membrane PHT protein. These are mainly responsible for P absorption in plant roots and P transport. *PHT1* is currently the most well-studied

family of phosphorus transporters [6]. The *PHT1* family contains nine members, namely, *AtPHT1.1-AtPHT1.9* in *Arabidopsis thaliana*, and *AtPHT1.1* and *AtPHT1.4* are mainly involved in the uptake of P elements from soil [7,8]. *AtPHT1.5* is involved in the transport and distribution of P elements from the source organ to the sink organ [9]. The *PHT2* family contains only one member in *Arabidopsis thaliana*. *AtPHT2* is a chloroplast phosphate transporter, which is involved in the transport and distribution of P elements in plants [10]. The *PHT3* family is a group of mitochondrial localized transporters, and three genes have been identified in *Arabidopsis thaliana*, which are responsible for Pi exchange between the mitochondrial matrix and the cytoplasm to support ATP [11]. There are six *PHT4* family members in *Arabidopsis thaliana*. *AtPHT4.1* is involved in the balance of Pi and pH in chloroplasts [12], *AtPHT4.2* is mainly involved in carbon metabolism in *Arabidopsis thaliana* [13], and *AtPHT4.6* is involved in the response of *Arabidopsis thaliana* to salt stress [14]. *PHT5* family proteins (SPX-MFS) are located in the vacuole membrane. It has been found that the *PHT5* family can regulate the homeostasis of P elements in plants. They play an important role in reproduction and plant growth. Under Pi-sufficient conditions, *PHT5* family members regulate excess Pi storage in vacuoles to prevent cytoplasmic toxemia, whereas in the case of Pi deficiency, the *PHT5* family regulates the export of Pi from vacuoles into the cytosol [15–17]. Although each subfamily plays an important role in the utilization of P in plants, the absorption, transport, and distribution of P depend on the interactions of each *PHT* family. With the development of genomics and bioinformatics, *PHT* families have been identified in the apple (*Malus domestica*) [18], tomato (*Solanum lycopersicum*) [19], sugarcane (*Saccharum officinarum*) [20], capsicum (*Capsicum annuum*) [21], poplar (*Populus simonii*) [22], arabidopsis (*Arabidopsis thaliana*) [23], and other species [24–26]. Such genomic analyses of phosphorus transporters have not been performed in 'duli' (*Pyrus betulifolia Bunge*).

Many soils in northern China are deficient in P. So, in pear production, abundant phosphorus fertilizers are applied to the fields every year. However, the heavy use of Pi fertilizers can pollute water resources, which has raised environmental concerns [27,28]. 'Duli' has high drought resistance and cold resistance, and it is a commonly used pear rootstock in northern China. Increasing the capacity of 'duli' roots to absorb P is important for improving the yield and quality of pear trees and reducing Pi pollution [29]. Phosphate transporters play an important role in the uptake and utilization of P elements in 'duli'. So, we identified the *PbPHT* gene; detected its expression patterns in the roots, stems, leaves, flowers, fruits, plant growth regulators, P, and salt stress; and analyzed the concentration, absorption, transfer, and distribution of P elements in the roots, stems, and leaves of 'duli' under hormone and Pi treatment. The results of our study will aid future investigations of the function of *PbPHT* family members. Genetic engineering approaches can be used to improve the efficiency of Pi uptake and transport by pear.

2. Materials and Methods

2.1. Identification of PbPHTs

In this study, the protein sequences and GFF/gff3 file of 'duli' were downloaded from GDR (https://www.rosaceae.org/organism/26137) (accessed on 21 April 2024). The protein sequences of the *AtPHTs* were downloaded from Tair (https://www.arabidopsis.org/) (accessed on 21 April 2024). To identify members of the 'duli' PHT family, 22 AtPHT protein sequences were used as queries and searched against the 'duli' genome database with an E-value $\leq 10^{-5}$. Profile information for the *PHT1* (MFS, PF00083), *PHT2* (PHO4, PF01384), *PHT3* (Mito_carr, PF00153), *PHT4* (MFS_1, PF07690), and *PHT5* (MFS_1, PF07690 and SPX, PF03105) domain was obtained from the Pfam database (http://pfam-legacy.xfam.org/) (accessed on 21 April 2024). After redundancies were removed, domains contained

in all protein sequences were verified by Pfam (http://pfam.janelia.org) (accessed on 21 April 2024) and SMART (https://smart.embl.de/) (accessed on 21 April 2024).

To analyze the physicochemical properties of 'duli' PHT proteins. The molecular weights, isoelectric points, hydrophilicity, and instability coefficients of each member of the horse 'duli' *PHT* gene family were analyzed using the Expasy website (https://web.expasy.org/protparam/) (accessed on 21 April 2024). Cell-PLoc 2.0 online software (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) (accessed on 21 April 2024) was used to analyze the subcellular localization of each *PbPHT*. The online software Some Secondary Structure Prediction (https://npsa-prabi.ibcp.fr) (accessed on 21 April 2024) was used to analyze the protein secondary structure of PbPHTs.

2.2. Phylogenetic Analysis and Chromosome Localization

Phylogenetic trees were made using the program MEGA11 with the neighbor-joining method with the Poisson correction model and 1000 bootstrap replicates; they were then annotated and visualized using Itol (https://itol.embl.de/) (accessed on 21 April 2024). The chromosomal distribution and collinearity of *PbPHTs* were analyzed using TBtools (version 2.119, College of Horticulture, South China Agricultural University, Guangzhou, China).

2.3. Conserved Motifs, Gene Structure, and Cis-Acting Elements

The shared conserved motifs and gene structure of the 'duli' *PHT* family were analyzed online using the MEME website (https://meme-suite.org/meme/tools/meme) (accessed on 24 April 2024) and visualized using Tbtools. After extracting 2000 bp sequences upstream of each member of the *PHT* family 'duli' using Tbtools, these sequences were then uploaded to the PlantCARE website (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (accessed on 24 April 2024) to predict cis-acting elements in the promoter region and visualized using TBtools.

2.4. Plant Materials and Treatments

Tissue samples (roots, stem, leaves, flowers, and fruits) and semi-annual 'duli' seedlings with consistent growth and no pests and diseases were obtained from the Innovation Experimental Park of Hebei Agricultural University. Semi-annual 'duli' seedlings were used in experiments. Seedlings of similar size were transferred to gray plastic basins (30 × 15 × 10 cm), and the seedlings with were fixed onto sponges, each containing 2.5 L of a 1/2-strength Hoagland nutrient solution, and 2 air pumps were placed in each basin. After 10 d of pre-cultivation, stress treatments were initiated. Each stress treatment had three biological replicates, and each replicate contained 15 plants. The treatments were as follows: (1) control KH_2PO_4 (500 μM); (2) low-P treatment KH_2PO_4 (5 μM); (3) high-P treatment KH_2PO_4 (5 mM) [18]; (4) NaCl treatment (200 mM); (5) GA_3 treatment (0.1 mM); (6) IAA treatment (100 μM); (7) ABA treatment (300 μM); (8) and GR24 treatment [30,31].

2.5. Expression Analysis

Samples were collected at 0, 24, 48, and 168 h after the start of the experiment. All tissue samples were flash-frozen in liquid nitrogen, triturated, and stored at −80 °C. RNA was extracted using an RNA kit (Omega, Beijing, China), and RNA was reverse-transcribed using a reverse-transcribed kit (Yeasen, Shanghai, China). Primers for the 15 *PbPHT* genes were designed using the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (accessed on 28 April 2024) (Table 1). The primers were synthesized by Beijing Biomed Company (Beijing, China). The qRT-PCR reaction system and procedures were carried out according to the method of Yuan Shuai et al. [32]. *PbeActin* was used as the internal reference standard [30]. The expression levels of *PbPHTs* were calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1. 'Duli' *PHT* family members and qRT−PCR primer design.

Gene Name	Forward Primer 5′-3′	Reverse Primer 5′-3′
PbPHT1.9	CGTTTGTTGTGCCAGTGGAG	GCCTTAGCCGGGTCTTGATT
PbPHT1.11	CGTAGCCGTTGGAGTTGTCA	GTAAGACCCACACCGAGCAA
PbPHT1.17	ATGTTCGGCCGATTTGTTGC	CGAGCCGTCCACCAACG
PbPHT1.64	TGGTGCATCGGTGATGGATA	AGATGCCTTGTGTGCCAAGT
PbPHT2.2	CATTTGCTCATGGCGGGAAT	TCGGAATAACAATCTCCGGACC
PbPHT3.32	CACAGCACATGATCTGGAGAGA	AGGAGTGTCTTCGCCGTTTT
PbPHT3.34	ATGATCAGAGAGAGCGCGG	ATCGCGGATAAAAATGCGGC
PbPHT3.49	CTGTGAAGAACATTGGCCTTG	TCCACTAGATGGCAGTCCACA
PbPHT3.66	CCACGCCGTTGTTTTTGTCT	TCATAGCACGCGATCCCTTC
PbPHT4.5	TGCTGTTTCGTTCGCGTTTC	GACTCCGCTTCCAAATGCTT
PbPHT4.7	ACCACGTTGACCTCAGACAA	AGTAAGCCAAGCAGATGCGA
PbPHT4.11	AGGAAATCGTAAAAGGACCTATGC	GTACAATCACCCAGCGCCTA
PbPHT5.2	TGCCTTTGGGAAAAAGCTGAA	TTCCAACAGGAAAAGGACGATAA
PbPHT5.3	TTCGGGAAGAAGTTGCGAGAA	GAGGCTTTCCCGTGGAACAT
PbPHT5.4	TTCGGGAAGAAGTTGCGAGAA	GCTTGCTAGCACTCCTTGTT

2.6. Determination of P Elements

The leaves, stems, and roots were harvested and washed with deionized water after 168 h of treatment. Samples were fixed at 105 °C for 15 min and then dried in an oven at 70 °C until a constant weight was reached; these samples were then used for analyses of nutrient concentrations. The samples (0.1 g) were placed in glasses, and 1 mL of ultrapure water was added to each glass, followed by 8 mL of concentrated sulfuric acid and perchloric acid mixture (10:1). The samples were then heated to 300 °C in a heavy-metal digestion apparatus (Multiwave PRO; Anton–Paar GmbH, Graz, Austria). Once the solution was clarified, the solution in the tube was reduced to 100 mL with ultrapure water. P was measured using a segmented flow analyzer (Auto Analyzer 3, SEAL Analytical, Norderstedt, Germany) [33]. Each treatment had three independent biological replicates. The distribution, transfer, and absorption of P were determined following the methods of Liang Bowen [34,35].

3. Results

3.1. Identification and Physicochemical Properties of PHT Genes in 'duli'

A total of 158 *PbPHTs* were identified in the 'duli' genome using bioinformatics methods. Based on conserved domain analysis, we divided the *PbPHT* genes into five subfamilies, *PHT1* (MFS), *PHT2* (PHO4), *PHT3* (Mito_Carr), *PHT4* (MFS_1), and *PHT5* (MFS_1 and SPX), and these genes were named *PbPHT1.1–PbPHT1.70*, *PbPHT2.1–PbPHT2.2*, *PbPHT3.1–PbPHT3.70*, *PbPHT4.1–PbPHT4.12*, and *PbPHT5.1–PbPHT5.4* according to their position on the chromosomes, respectively. The physical and chemical properties of the proteins encoded by *PbPHT* genes are summarized in Table S1 in the Supplementary Materials. The lengths of *PHT1* family members ranged from 294 aa to 1094 aa, the molecular weights ranged from 33.07 kDa to 120.77 kDa, and the pI ranged from 5.09 to 9.54. The lengths of PHT3 family members ranged from 229 aa to 1797 aa, and the pI ranged from 6.33 to 9.92. The lengths of *PHT4* family members ranged from 184 aa to 610 aa, the molecular weights ranged from 23.34 kDa to 203.17 kDa, and the pI ranged from 6.12 to 10.24. With the exception of PbPHT5.2, the other members of the PHT5 family were 699 aa in length, the molecular weights ranged from 77.82 kDa to 82.19 kDa, and the pI ranged from 5.97 to 8.30. *PHT2* and PHT5 family members are unstable proteins and hydrophobic proteins. With the exception of PbPHT3.26, PbPHT3.27, and PbPHT3.28, the other members of the *PHT3* family are hydrophilic proteins. Except for PbPHT4.1, PbPHT4.3, and PbPHT4.12, the other members of the *PHT4* family are stable proteins. The secondary structure of *PHT* family members was dominated by alpha helices, and the beta turn angle of all members was 0.

Subcellular localization plays an important role in studying gene function and studying protein interactions. In this study, subcellular localization prediction showed that *PHT1* and *PHT4* family members are located in cell membranes, *PHT2* family members are located in

chloroplasts, *PHT3* family members are located in mitochondria, PbPHT5.1 and PbPHT5.4 are located in vacuoles, and PbPHT5.2 and PbPHT5.3 are located in cell membranes.

3.2. Phylogenetic Analysis

To analyze the evolutionary relationships among 'duli' SMXL proteins, the phylogenetic relationships between 'duli' and Arabidopsis *PHT* genes were constructed. We divided the *PHT* gene (158 from 'duli' and 22 from Arabidopsis) and divided the *PHT* gene family into five subfamilies: *PHT1*, *PHT2*, *PHT3*, *PHT4*, and *PHT5* (Figure 1). The *PHT1* group contained the most members, with 79 PHT proteins. The *PHT2* group contained the least number of members, with only three *PHT* proteins. Both 'duli' and *Arabidopsis PHT* members were clustered under smaller branches, and the homology of members clustered in smaller branches was as high as 100%, suggesting that they had similar biological functions.

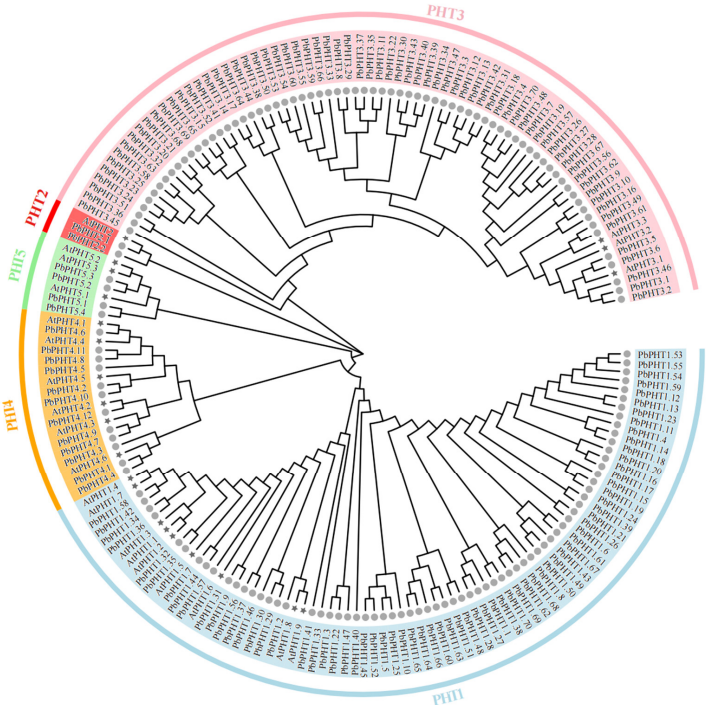

Figure 1. Phylogenetic analysis of PHT proteins. Light blue indicates the PHT1 family. Light red indicates the PHT2 family. Pink indicates the PHT3 family. Light yellow indicates the PHT4 family. Light green indicates the PHT5 family. Grey circles indicate *Pyrus betulifolia Bunge* (*Pb*), and black pentagrams indicate *Arabidopsis thaliana* (*At*).

3.3. Chromosome Localization and Gene Replication Analysis

To investigate the genomic distribution of *PHT* genes on the chromosomes, TBtools software was also used to analyze the chromosomal locations of *PHT* family genes. These 158 genes are located on 17 chromosomes (Figure 2A). Chr5 and Chr10 had the largest number of *PbPHT* genes, both containing 15 *PbPHT* members; this was followed by Chr 04 and Chr 11, with 13 *PbPHT* members each. Chr 14 had the lowest number of *PbPHT* genes, including three *PbPHT* members. There were also six unnamed chromosomes: Scaffold 21, Scaffold 5, Scaffold 2, Contig 15, Scaffold 28, and Contig 11. Gene clusters within a 100 kb range of the same chromosomal regions were defined as tandem duplicates [36]. We identified 18 pairs of *PbPHT* genes with tandem duplicates (Figure 2B,C). These pairs included *PbPHT1.29* and *PbPHT1.30*, *PbPHT1.37* and *PbPHT3.44*, *PbPHT1.53* and *PbPHT1.54*, *PbPHT1.54* and *PbPHT1.55*, *PbPHT1.18* and *PbPHT1.19*, *PbPHT1.57* and *PbPHT1.18*, *PbPHT1.68* and *PbPHT1.69*, *PbPHT4.3* and *PbPHT4.4*, *PbPHT1.40* and *PbPHT3.49*, *PbPHT1.41* and *PbPHT1.42*, *PbPHT1.35* and *PbPHT1.36*, *PbPHT1.32* and *PbPHT1.33*, *PbPHT1.33* and *PbPHT1.34*, *PbPHT1.63*

and *PbPHT1.64*, *PbPHT1.65* and *PbPHT1.66*, *PbPHT1.11* and *PbPHT1.12*, *PbPHT1.13* and *PbPHT1.14*, and *PbPHT1.15* and *PbPHT1.16*. These tandem replication events were most prevalent on Chr 01, Chr 04, Chr 05, Chr 06, Chr 07, Chr 10, Chr 11, and Chr 15 and were mainly observed in *PbPHT1* family members.

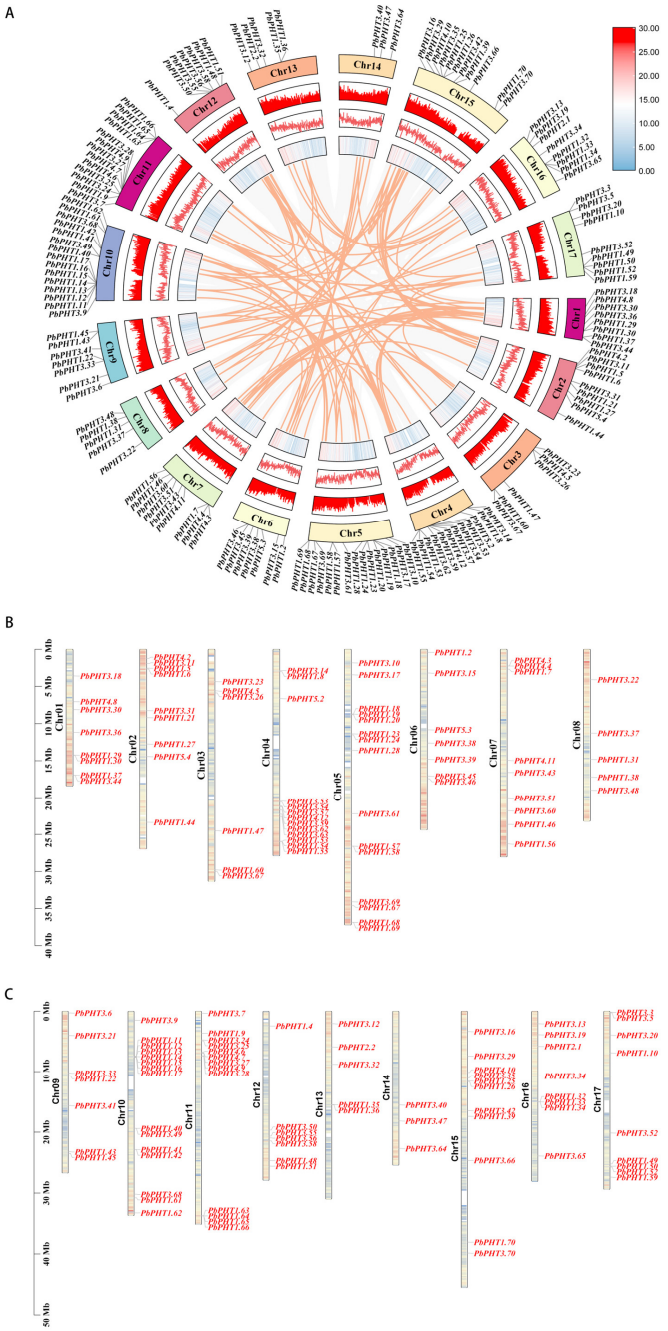

Figure 2. Gene replication and chromosome localization analysis of *PbPHTs*. (**A**) Collinearity analysis of *PbPHTs*. The outermost rectangular block represents the chromosomal skeleton, and the other three rectangular blocks represent the gene density of each chromosome. (**B**) The location of *PbPHTs* on chromosomes (Chr 01–Chr 08). (**C**) The location of *PbPHTs* on chromosomes (Chr 09–Chr 17).

3.4. Conserved Motif and Gene Structure Analysis

To further clarify the functions of *PbPHTs*, the structure and conserved motifs of all PHT proteins were analyzed using the 'duli' whole-genome sequence and genome annotation file. *PbPHTs* had the most diverse structures (Figure 3A), with the number of exons ranging from 0 to 17, which indicates a high degree of variation between genes. In the *PHT5* subfamily,

except for *PbPHT5.1*, which contains 9 introns, the other members contained 10 introns. The distribution of introns/exons is relatively uniform within each subfamily, and structurally similar genes may have similar functions. Furthermore, a total of 20 motifs (Motif 1–Motif 20) were identified in 158 *PbPHTs* (Figure 3B). *PHT1* subfamily members contained Motif 1. The *PHT2* subfamily members only contained Motif 15, Motif 19, and Motif 20. All members of the *PHT3* subfamily contained Motif 4 and Motif 5. The *PHT5* family contained only three motifs, and all *PHT5* family members contained Motif 7 and Motif 14.

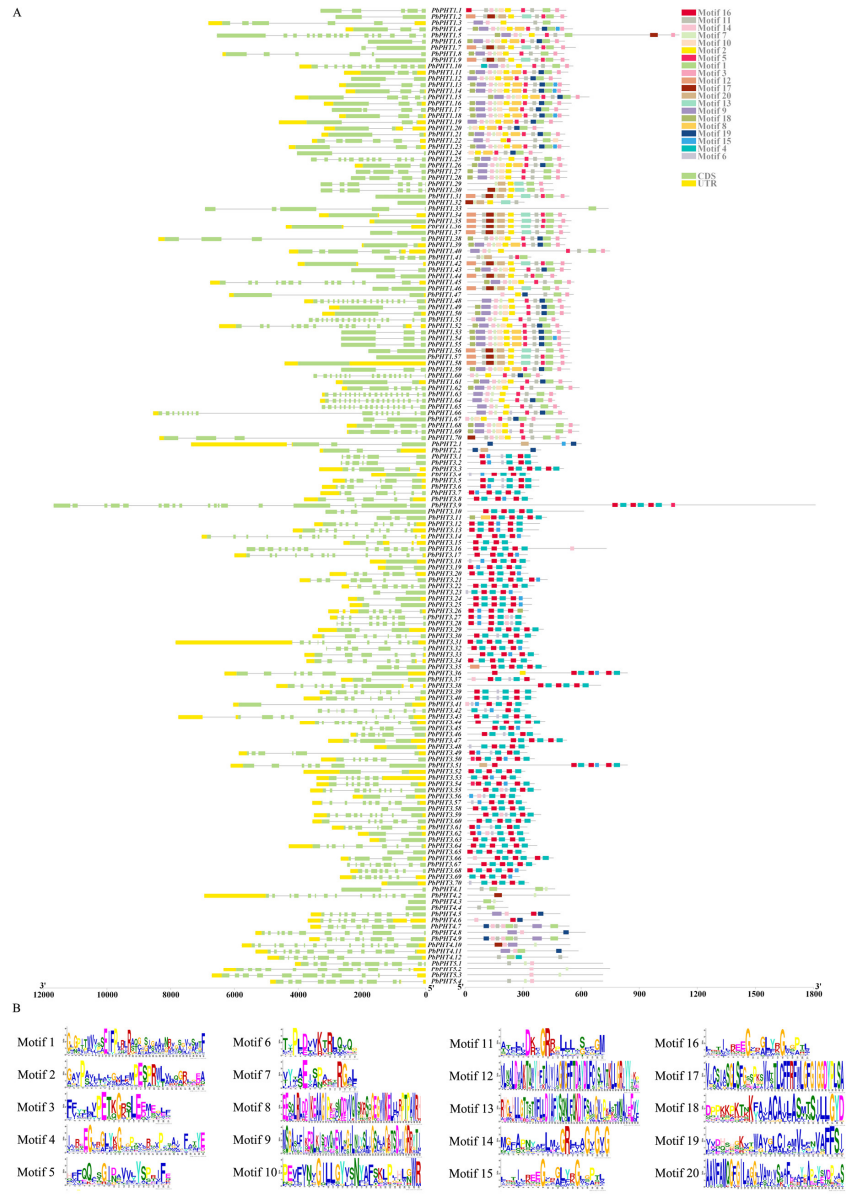

Figure 3. Conserved motifs and gene structure of *PbPHTs*. (**A**) Conserved motif and gene structure of *PbPHTs*. Yellow rectangles indicate UTR, and green rectangles indicate CDS. (**B**) Protein motifs (Motif 1–20).

3.5. Cis-Acting Element Analysis

The distribution and number of cis-acting elements of the 158 *PbPHTs* are shown in Figure 4. Light-responsive elements, short-effector elements, stress-responsive elements, and hormone-responsive elements were found in the promoter region upstream. Hormone-related elements included abscisic acid-related ABREs (427), jasmonic acid-related TGACG-motifs (274), and CGTCA-motifs (272), gibberellin-related P-box elements (86), GARE-motifs (49), auxin-related TGA-elements (96), and salicylic acid-related TCA-elements (101). *PHT2*, *PHT4*, and *PHT5* contained ABRE elements; both PHT2 and PHT5 contained

elements related to methy jasmonate. *PHT1.17*, *PHT3.22*, *PHT3.28*, *PHT3.56*, *PHT3.70*, and *PHT4.12* contained the highest number of ABRE components (each containing eight ABRE components). There were cis-elements associated with abiotic stress in *PbPHTs*, such as MBS (111), LTR (141), and AREs (365). In addition, *cis*-acting elements related to light responses and growth were identified, including 3-AF1 binding sites (17), Sp1 binding sites (39), and the GT1-motif (207) related to light reactions, the GCN4_motif (41) related to endosperm expression, and the CAT-box (81) related to the meristem. *PbPHT4.7* contained the largest number of *cis*-acting elements (28), followed by *PbPHT3.22* (24 *cis*-acting elements). Most *PbPHT* family members contained hormonal and abiotic stress-related elements, but the numbers of these genes varied among species.

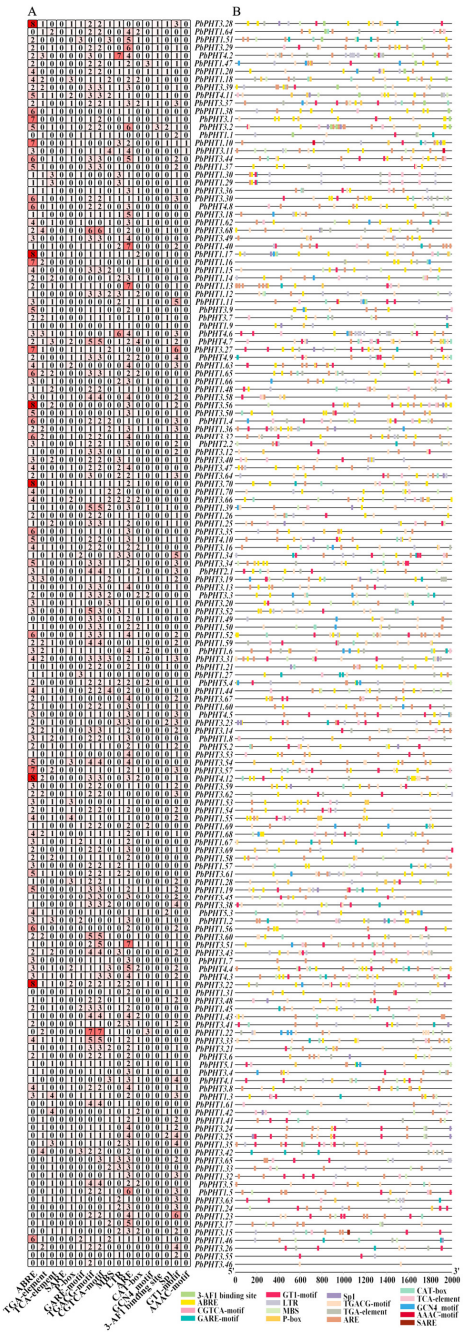

Figure 4. Cis-acting element analysis of *PbPHTs*. (**A**) The number of cis-acting elements in *PbPHTs*. The numbers inside the table cells indicate the numbers of cis-acting elements of the *PbPHTs*. The shade of red indicates the number of cis-acting elements in the *PbPHT* genes. (**B**) The distribution of cis-acting elements of *PbPHTs*. Rectangles of different colors represent different cis-acting elements.

3.6. Tissue-Specific Expression Patterns of PbPHTs

The qRT-PCR analysis revealed that 15 *PbPHTs* were ubiquitously expressed in 'duli' fruits, flowers, leaves, stems, and roots (Figure 5). For *PHT1* members, *PbPHT1.9* and *PbPHT1.17* were upregulated in the leaves. The expression levels of *PbPHT1.11* were higher in the leaves than in other tissues. The expression levels of *PbPHT1.11* were highest in the leaves, and their expression was 10.71 times higher in the leaves than in the roots. *PbPHT* 2.2 was highly expressed in almost all tissues, but its expression was highest in the stems, and its expression was 1.91 times higher in the stems than in the roots. *PbPHT4.5* was downregulated in the fruits but upregulated in other tissues. The expression levels of *PbPHT3.49*, *PbPHT3.66*, and *PbPHT4.7* were high in all tissues. The expression of *PbPHT1.17* was significantly higher in the roots than in the stems, flowers, and fruits. The expressions of *PbPHT1.17*, *PbPHT5.2*, and *PbPHT5.3* were significantly higher in the roots than in the stems and fruits. The expressions of *PbPHT4.5* and *PbPHT4.11* were high in the stems, leaves, and flowers, and the expressions of these genes were highest in the leaves. *PbPHT5.3* was upregulated in the leaves but downregulated in other tissues. Thus, the expression levels of *PbPHTs* in the roots, stems, leaves, flowers, and fruits of 'duli' were different.

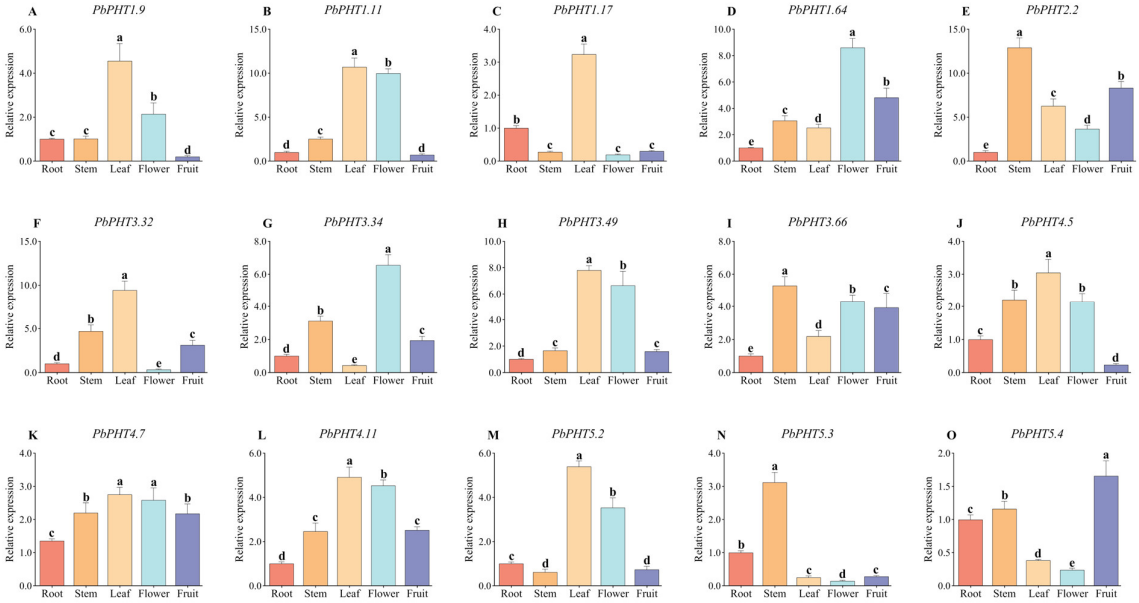

Figure 5. Expression patterns of *PbPHTs* in multiple tissues. The different lowercase letters indicate distinctiveness between the roots and other tissues. Different letters indicate significant differences, and the same letters indicate no significant differences at $p < 0.05$.

3.7. Expression Analysis of PbPHTs in Response to Hormone, Salt, and P Stress

We examined the expression patterns of 15 *PbPHTs* for 'duli' under 5 μM KH_2PO_4, 5 mM KH_2PO_4, 0.5 mM KH_2PO_4, NaCl, GR24, GA_3, IAA, and ABA treatments (Figure 6). After treatment with 5 mM KH_2PO_4, the expression levels of *PbPHT1.64*, *PbPHT2.2*, *PbPHT3.34*, and *PbPHT3.66* were highest at 48 h. After treatment with 5 μM KH_2PO_4, the expression level of *PbPHT4.11* was the highest at 168 h, and the expression level at 168 h was 8.77 times higher than that at 0 h. After treatment with NaCl, the expressions of eight *PbPHTs* peaked at 24 h, and the expressions of *PbPHT1.11*, *PbPHT1.17*, *PbPHT1.64*, *PbPHT1.9*, *PbPHT3.32*, *PbPHT3.34*, *PbPHT3.49*, and *PbPHT5.4* then decreased dramatically after 24 h. Thus, the majority of *PbPHTs* were induced to express by H_2PO_4 and NaCl but displayed with variant expression patterns.

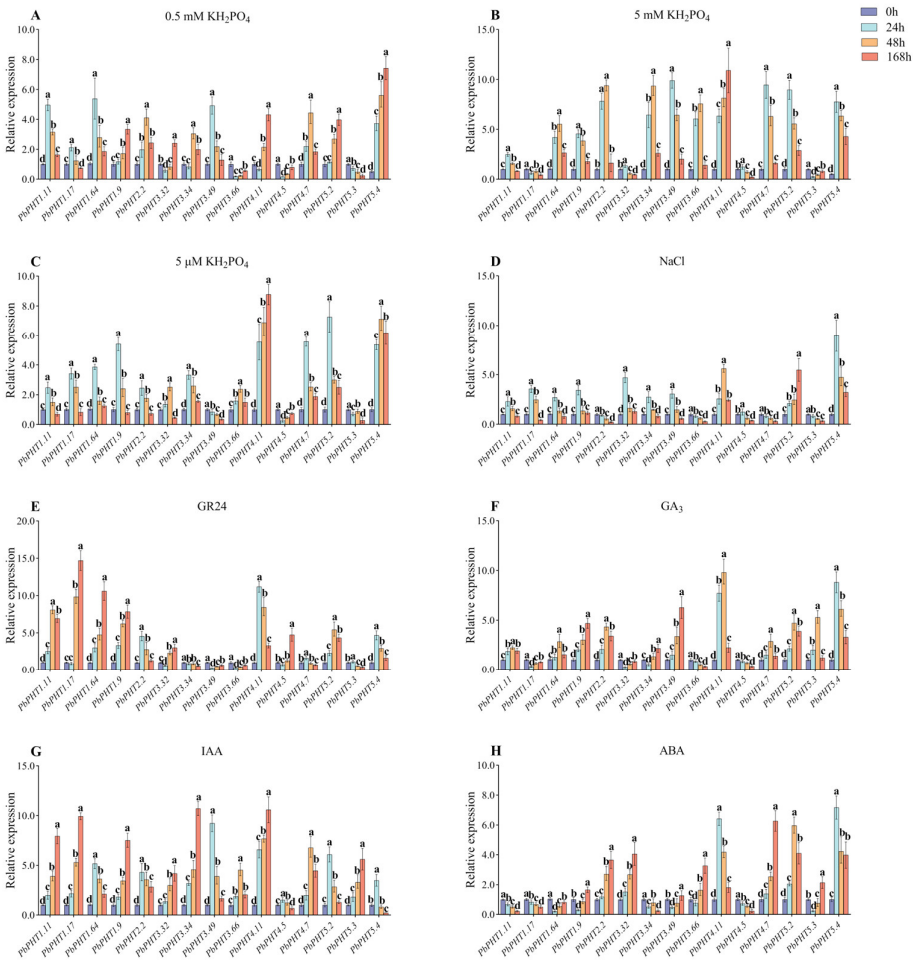

Figure 6. Expression analysis of *PbPHT* genes in response to hormone, salt, and phosphorus stress: (**A**) 0.5 mM KH_2PO_4 treatment, (**B**) 5 mM KH_2PO_4 treatment, (**C**) 5 μM KH_2PO_4 treatment, (**D**) NaCl treatment, (**E**) GR24 treatment, (**F**) GA_3 treatment, (**G**) IAA treatment, (**H**) ABA treatment. Different lowercase letters indicate distinctiveness between CK (0 h) and others (24 h, 48 h, and 168 h). Different letters indicate significant differences, and the same letters indicate no significant differences at $p < 0.05$.

After GR24 treatment, the expressions of *PbPHT1.9*, *PbPHT1.17*, and *PbPHT1.64* continued to increase. The expression level of *PbPHT1.17* was the highest at 168 h, and the expression level at 168 h was 14.69 times higher than that at 0 h. After GA_3 treatment, the expressions of *PbPHT4.7*, *PbPHT4.11*, *PbPHT5.2*, *PbPHT5.3*, and *PbPHT5.4* first increased and then decreased. After IAA treatment, the expressions of *PbPHT1.11*, *PbPHT1.17*, *PbPHT1.9*, *PbPHT3.32*, *PbPHT3.34*, *PbPHT4.7*, *PbPHT4.11*, and *PbPHT5.3* continued to increase. The expression level of *PbPHT3.34* was the highest at 168 h, and the expression level at 168 h was 10.71 times higher than that at 0 h. After 24 h of treatment at ABA, the expressions of *PbPHT4.11* and *PbPHT5.4* peaked. These analyses indicated that the expression of *PbPHTs* was induced by KH2PO4, NaCl, GR24, IAA, GA_3, and ABA treatment, but with differences in response speeds and expression levels.

3.8. Content, Absorption, Transfer, and Distribution of P Elements in 'duli'

The concentrations of P in the roots, stems, and leaves of 'duli' changed significantly under each treatment (Figure 7). The concentration of P elements in the leaves, stems, and roots was significantly increased in the 5 mM KH_2PO_4, GR24, GA_3, and IAA treatments. The concentrations of P were significantly increased by 9.42%, 39.65%, 15.06%, and 9.11% compared with 0.5 mM KH_2PO_4 in the roots; the concentrations of P were significantly increased by 8.92%, 61.35%, 32.75%, and 13.61% compared with 0.5 mM KH_2PO_4 in the

stems; the concentrations of P were significantly increased by 11.63%, 39.93%, 13.26%, and 15.11% compared with 0.5 mM KH_2PO_4 in the leaves. The P element absorption of 'duli' was significantly reduced by the 5 μM KH_2PO_4, NaCl, and ABA treatments, and it was 28.73%, 64.92%, and 33.44% lower in these treatments than in the 0.5 mM KH_2PO_4 treatment, respectively. The P absorption of 'duli' was significantly increased by the 5 mM KH_2PO_4, GR24, GA3, and IAA treatments, and the P absorption was highest under the GR24 treatment; specifically, it was 180.13% higher in the GR24 treatment than in the 0.5 mM KH_2PO_4 treatment. It is known through the transport (leaves and stems) and accumulation (roots) of P elements in 'duli' that the transport rate of P ranged from 0.22 to 3.54 μg g^{-1} day^{-1} in the stems, the transport rate of P ranged from 0.21 to 1.88 μg g^{-1} day^{-1} in the leaves, the accumulation of P ranged from 0.15 to 1.12 μg g^{-1} day^{-1} in the roots. It is known through the distribution of P elements in 'duli' (roots, stems, and leaves) that under the 5 mM KH_2PO_4, GR24, GA_3, and IAA treatment, compared with 0.5 mM KH_2PO_4 treatment, the content of P increased by 18.76%, 73.42%, 31.99%, and 26.88% in the roots; the content of P increased by 12.85%, 82.77%, 51.44%, and 28.30% in the stems; and the content of P increased by 26.23%, 57.89%, 30.11%, and 28.47% in the leaves. Under the of 5 μm KH_2PO_4, NaCl, and ABA treatments, the contents of P decreased by 23.87%, 22.93%, and 13.48% in the roots; the contents of P decreased by 25.07%, 33.32%, and 18.33% in the stems; and the contents of P decreased by 20.31%, 34.94%, and 20.16% in the leaves.

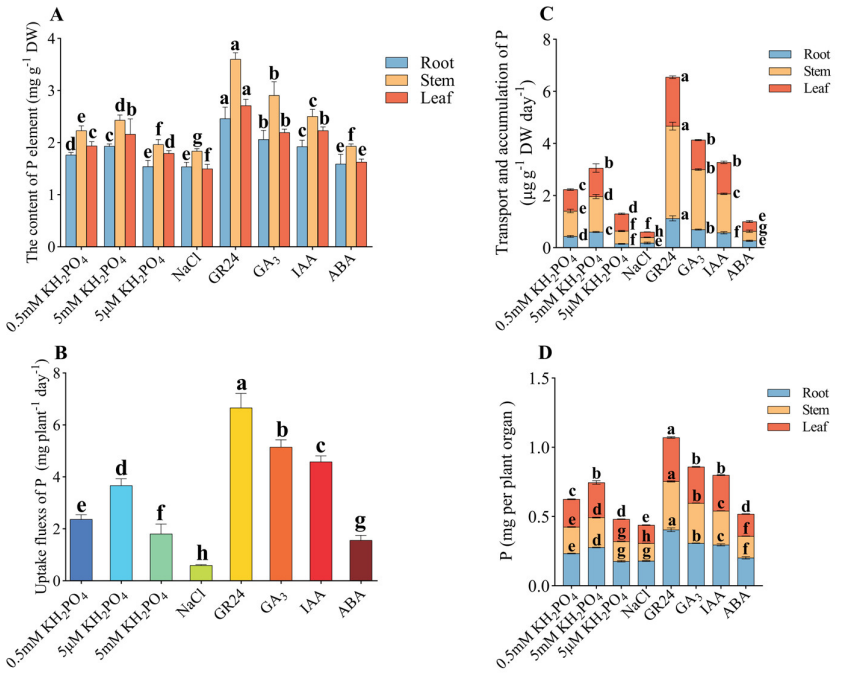

Figure 7. The content, absorption, transfer, and distribution of P elements in 'duli'. (**A**) The concentration of P elements (roots, leaves, and stems) in 'duli' plants. Different lowercase letters indicate distinctiveness between the roots and other tissues. (**B**) The absorption of P elements in 'duli' plants. Different colored columns indicate different stress treatments. (**C**) The transport (leaves and stems) and accumulation (roots) of P elements in 'duli' plants. (**D**) The distribution of P elements in 'duli' plants. Different letters indicate significant differences, and the same letters indicate no significant differences at $p < 0.05$.

3.9. Correlation Analysis

Correlation analysis was performed on 10 physiological indicators and the expression patterns of 15 genes (Figure 8). The expressions of *PbPHT1.9*, *PbPHT1.11*, *PbPHT1.17*, *PbPHT1.64*, and *PbPHT4.7* were significantly positively correlated with the uptake of P,

and the concentration, transport, and distribution of P in the roots, stems, and leaves. The expression of *PbPHT5.4* was significantly negatively correlated with the uptake of P and the concentration, transport, and distribution of P in the roots, stems, and leaves. The expression of *PbPHT1.9* was significantly positively correlated with the expressions of *PbPHT1.11*, *PbPHT1.17*, *PbPHT1.64*, *PbPHT3.32*, *PbPHT3.34*, *PbPHT4.7*, and *PbPHT5.3*. The expression of *PbPHT5.4* was significantly negatively correlated with the expressions of *PbPHT1.9*, *PbPHT1.1*, *PbPHT1.64*, *PbPHT3.32*, *PbPHT3.34*, and *PbPHT5.3*. The expression of *PbPHT5.2* was significantly negatively correlated with the expressions of *PbPHT3.34*, *PbPHT5.3*, and *PbPHT4.5*.

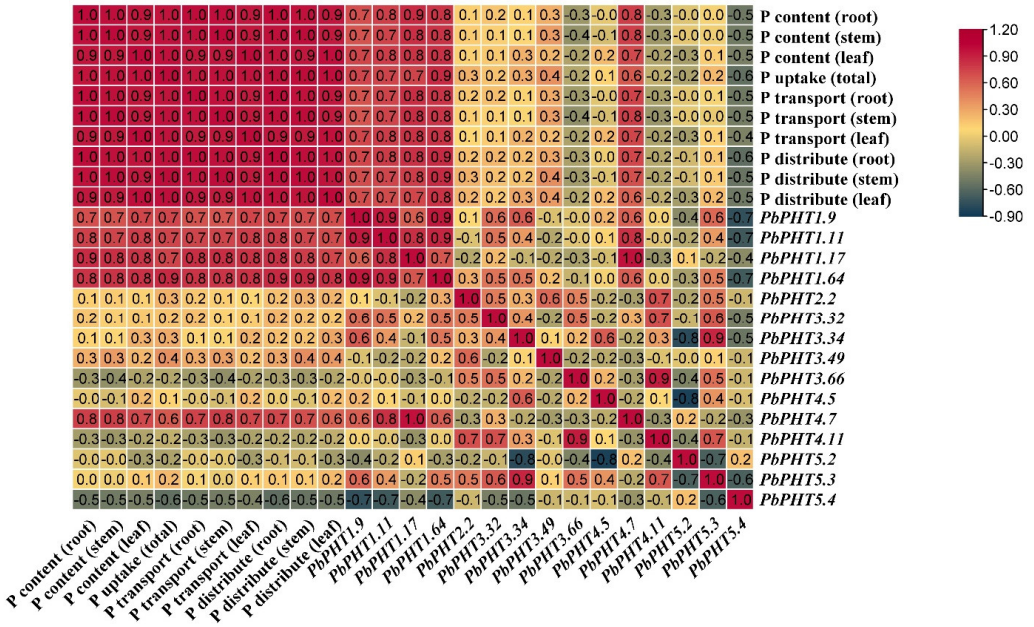

Figure 8. Correlation analysis between the expression levels of 15 *PbPHTs* and 10 physiological indexes. The numbers inside the table cells represent the correlation coefficients.

4. Discussion

4.1. Identification, Gene Replication, and Gene Structure Analysis of PbPHT Genes in 'duli'

P is a key component of nucleic acids, phospholipids, and ATP, which are involved in biological processes such as plant energy transfer and cell signaling. In plants, *PHT* genes mediate Pi uptake and translocation. *PHT* gene family members have been reported in many plants, including barley [37], wheat [38], finger millet [39], tomato [19], green algae [40], sugarcane [20], *Lupinus albus* [41], apple [18], sorghum [24], poplar [22], and *Camelina sativa* [1]. In this study, 158 *PbPHTs* were identified, including 70 *PbPHT1s*, 2 *PbPHT2s*, 70 *PbPHT3s*, 12 *PbPHT4s*, and 4 *PbPHT5s*. The number of *PHTs* was higher in the 'duli' genome than in rice, poplar, apple, and sorghum genomes, which may be related to differences in the size of the genome and the tandem duplication of genes.

Gene replication is the main driver of gene family amplification and includes tandem gene duplication and fragment gene duplication [20,39]. Gene duplication analysis identified 18 tandemly duplicated gene pairs. In this study, 18 pairs of tandemly duplicated genes were found from the genome of 'duli'; this exceeds the number of *PHTs* of sorghum [24], sugarcane [20], and *Brassica* [25], and the increase in the number of tandem repeats may increase the number of *PbPHT* family members.

In the early stages of gene amplification, some genes gradually lose their introns [42]. In the case of intron selection pressure, intron-free genes may evolve rapidly [43]. Gene structure and motif composition analysis showed that most of the *PbPHT* genes of the same subpopulation have similar structure and motif distribution. However, there are also some

PbPHT family genes that do not have introns. Therefore, it is speculated that some *PbPHTs* may gradually lose their introns and achieve functional evolution over time.

4.2. Expression Patterns of PbPHTs in Multiple Tissues

The expression patterns of *PbPHT1s*, *PbPHT2s*, *PbPHT3s*, *PbPHT4s*, and *PbPHT5s* varied among tissues. We found that *PbPHT1.9*, *PbPHT1.11*, and *PbPHT1.64* were highly expressed in the roots, leaves, and flowers. The same was observed in Arabidopsis, and most *AtPHT1* family members were highly expressed in the roots, but also in the leaves and flowers [44,45]. In apples, *MdPHT1.12* is highly expressed in the roots, leaves, and flowers [18]. These results indicate that *PHT1* family genes may play a role in Pi uptake in different species. *PbPHT 2.2*, *PbPHT3.49*, *PbPHT3.66*, and *PbPHT4.7* were highly expressed in almost all tissues, *PbPHT 5.2* was highly expressed in the leaves and flowers, *PbPHT 5.3* was highly expressed in the stems, and *PbPHT 5.4* was highly expressed in the fruits. The expressions of the *PbPHT* genes varied among tissues, suggesting that these genes may play different roles in the uptake, distribution, transport, and storage of Pi. A similar conclusion was reached in the correlation analysis. The expressions of *PbPHT1.9*, *PbPHT1.11*, *PbPHT1.17*, *PbPHT1.64*, and *PbPHT4.7* were positively correlated with the uptake of P and the concentration, transport, and distribution of P in the roots, stems, and leaves. The expression of *PbPHT5.4* was significantly negatively correlated with P uptake and the concentration, transport, and distribution of P in the roots, stems, and leaves. These results indicate that the expressions of *PbPHT1.9*, *PbPHT1.11*, *PbPHT1.17*, *PbPHT1.64*, *PbPHT4.7*, and *PbPHT5.4* genes play an important role in Pi uptake, transport, and storage. However, the specific functions of *PbPHT1.9*, *PbPHT1.11*, *PbPHT1.17*, *PbPHT1.64*, *PbPHT4.7*, and *PbPHT5.4* require further investigation.

4.3. Expression Analysis of PbPHTs in Response to P Stress

PHT1, which encodes a high-affinity Pi transporter, was mostly expressed in the roots, and its expression was induced by low P. In arabidopsis [46] and apples [18], the *PHT1* gene is a high-affinity transporter. The expressions of most *ATPHT1* genes were significantly upregulated under hypophosphate conditions. *MdPHT1.1* and *MdPHT1.7* were expressed under low Pi conditions. The qRT-PCR analysis of this study showed that the expressions of *PbPHT1.9*, *PbPHT1.11*, *PbPHT1.17*, and *PbPHT1.64* were upregulated after low P treatment. *PbPHT1* family members exhibited similar expression patterns in arabidopsis and apples. These results suggest that the protein encoded by the PbPHT1 gene may also be a high-affinity transporter. When plants are exposed to excess Pi, the expressions of *PbPHT3.66*, *PbPHT4.11*, *PbPHT4.7*, and *PbPHT5.4* are upregulated, suggesting that they encode low-affinity transporters. However, the detailed features of *PbPHTs* require further research.

4.4. Expression Analysis of PbPHTs in Response to Plant Growth Regulators

Gene expression is regulated by cis-acting elements [39,46,47]. The *PbPHT* promoters contain multiple *cis*-acting elements related to responses to stress, hormones, and light. qRT-PCR analysis of *PbPHT* showed that different genes exhibited different expression patterns under the same treatment, which may be related to the species and the distribution of cis-acting elements in the promoter region of *PbPHTs*. We detected abundant ABA-responsive elements, auxin-responsive elements, and GA-responsive elements within the promoter regions of different *PbPHT* members. The qRT-PCR assays revealed that ABA, IAA, GA, and GR24 could induce the expressions of most *PbPHT* genes. The expression patterns of *PbPHT1.9*, *PbPHT2.2*, *PbPHT4.7*, *PbPHT4.11*, *PbPHT5.2*, and *PbPHT5.4* were all responsive to exogenous GR24, GA, ABA, and IAA treatments, indicating that they might play a role in integrating the SL, GA, ABA, and IAA pathways in 'duli'. However, the mechanism by which *PbPHTs* regulate the interaction between hormones requires further investigation.

4.5. Expression Analysis of PbPHTs in Response to Salt Stress

Salt stress could reduce the uptake and utilization efficiency of P in plants. The expressions of *PbPHT3.32*, *PbPHT3.34*, and *PbPHT3.49* were significantly upregulated under salt treatment, and *PbPHT3* was localized to the mitochondrion. The respiration rate of 'duli' was accelerated, and more reactive oxygen species were produced under salt stress. Increases in the expressions of *PbPHT3.32*, *PbPHT3.34*, and *PbPHT3.49* promoted the transport of Pi and the conversion of ADP into ATP in 'duli', which provided energy to promote its resistance to salt stress. Similar conclusions were made by Takabatake et al. [11]. The *PHT3* family is a mitochondrial Pi transporter (MPT). It maintains the exchange of Pi between the cytoplasm and mitochondrial matrix through Pi/H^+ symport or Pi/OH^- antiport, catalyzing the phosphorylation reaction of ADP and Pi to form ATP [48]. *PHT3* family members thus might play an important role in the resistance of 'duli' to salt stress. Previous studies of the *PHT3* gene in *arabidopsis* have found that it is rapidly upregulated under salt stress and regulates levels of ATP and gibberellin in response to salt stress. Therefore, *PbPHT3.32*, *PbPHT3.34*, and *PbPHT3.49* may have similar functions to the *AtPHT3* gene. However, the specific functions of *PbPHT3.32*, *PbPHT3.34*, and *PbPHT3.49* require further study.

4.6. Arbuscular Mycorrhizal (AM) Fungi with P Elements

Some studies have found that through the extensive hyphal network of arbuscular mycorrhizal fungi (AMF), host plants can obtain water and nutrients from the soil, especially soil phosphorus elements [49]. In this study, it was found that, under GR24 treatment, the expression levels of the PbPHT1 family genes were significantly upregulated, and the expressions of *PbPHT1.9*, *PbPHT1.11*, *PbPHT1.17*, and *PbPHT1.64* were significantly positively correlated with the absorption of phosphorus elements. It is possible that strigolactones promote the symbiotic relationship between Pyrus betulaefolia and arbuscular mycorrhizal fungi and promote the branching of hyphae and that arbuscular mycorrhizal fungi induce the expression of the *PbPHT1* gene, thereby facilitating the absorption of phosphorus elements by Pyrus betulaefolia. The research by Hélène Javot et al. [50] also proved this point. The AM symbiotic relationship can affect the transcriptional level of *PHT1* transporters and influence the absorption of phosphorus elements by plants.

5. Conclusions

We identified *158 PbPHT* genes, including 70 *PbPHT1s*, 2 *PbPHT2s*, 70 *PbPHT3s*, 12 *PbPHT4s*, and 4 *PbPHT5s* in 'duli', and characterized their physiological and biochemical properties, gene replication, chromosomal position, evolutionary relationships, structures, and conserved motifs. Gene expression analysis revealed differential expression patterns of *PbPHTs* in different tissues, and in response to 5 μM KH_2PO_4, 5 mM KH_2PO_4, 0.5 mM KH_2PO_4, NaCl, GA_3, IAA, GR24, and ABA. Furthermore, we found that 5 mM KH_2PO_4, GR24, GA_3, and IAA treatments significantly increased the concentration, absorption, transport, accumulation, and distribution of P elements; the opposite effects were observed in the 5 μM KH_2PO_4, ABA, and NaCl treatments. Correlation analysis showed that *PbPHT1.9*, *PbPHT1.11*, *PbPHT1.17*, *PbPHT1.64*, *PbPHT4.7*, and *PbPHT5.4* were involved in the concentration, transport, and distribution of P. Our findings lay the foundation for future research on the function of the 'duli' PHT genes and in-depth identification of 'duli' abiotic-stress-resistant breeding candidate genes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture15020199/s1, Table S1: Physicochemical properties, protein secondary structure, and subcellular localization prediction analysis of 'duli' PHT proteins.

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