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Proceeding Paper

Identification and Characterization of PHT1 Transporters Family and Differential Expression Patterns in Control and Blindness Broccoli Plants †

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Abstract: Phosphorous is predominantly taken up by the plant from the soil in its inorganic form (Pi). This energy-consuming process is carried out by a family of high-affinity Pi transporters (PHT). The objective of the present study was the identification and characterization of the PHT1 Pi transporter family in *Brassica oleracea var. italica*, broccoli plants. A total of 31 PHT1 gene sequences were identified in broccoli plants and were fully characterized. In addition, RNA sequencing expression of control and blinded broccoli plants was carried out with different tissues in order to understand the implication of these transporters, PHT1, in broccoli blindness.

Keywords: phosphorus transport; broccoli genome; broccoli rna-seq; plant nutrition

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1. Introduction

Brassica oleracea var. italica also known as broccoli is an important crop worldwide mainly due to its beneficial nutritional characteristics, displaying high concentrations of vitamins A, C, E, and K and other metabolites that present a wide range of beneficial bioactivities, known as glucosinolates and their derivate molecules, isothiocianates, and phenolic compounds [1]. However, when low amounts of available nutrients occur, this affects the biological properties of the plant. In this way, although phosphorus is one of the most important nutrients, its influence in broccoli plant physiology has been poorly studied [2].

Phosphorus is normally taken up from soil by plants in its inorganic form (Pi). The uptake of Pi by plants is an energy consuming process. In this way, we can find high and low-affinity transporters [3]. In the past, five high-affinity transporters families have been described in *Arabidopsis thaliana* (PHT1, PHT2, PHT3, PHT4, PHT5) [4]. In particular, the proteins belonging to the PHT1 family have been shown to be essential under Pi deficiency. In this sense, the highly conserved PHT1 group is crucial for Pi uptake from the soil [5,6]. In Arabidopsis, a total of nine PHT1 transporters have been identified (PHT1;1-PHT1;9). AtPHT1;1 and AtPHT1;4 are important in Pi uptake in low and high availability Pi environments [7], while AtPHT1;8 and AtPHT1;9 play important roles only during phosphorous starvation [8]. Recently, a total of 49 PHT1 family members have been identified and characterized in *Brassica napus*, describing multiple transcriptional regulation events that could refer to new roles of PHT1 genes in *B. napus* [9]. These findings should be researched as a possible interpolation in broccoli plants.

Therefore, the aim of this work was to determine the phosphorus transporters in broccoli and study their expression pattern in relation with a blindness physiopathy (malfunction of the apical meristem) that is very common in brassica plants.

2. Material and Methods

2.1. Identification of Putative Broccoli PHT1 Transporters (PHT1)

The complete set of PHT1 transporters of broccoli (*Brassica oleracea var. italica*) were identified using the blast protein algorithm against the broccoli (HDEM) proteome available in the Genoscope database (http://www.genoscope.cns.fr/plants, accessed on 12 September 2021) and using as template sequences those PHT1 proteins of *B. napus* identified by Li, Y et al. (2019).

2.2. Protein Characterization, Sequence Analysis and Phylogenetic Studies

Protein features such as amino acid length (no. aa) and molecular weight (Mw) were calculated with Expasy's ProtParam tool (https://web.expas/, accessed on 12 September 2021). The transmembrane helices were predicted using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/, accessed on 16 September 2021). The subcellular location was predicted with two different prediction software: Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on 16 September 2021) and a Eukaryotic protein subcellular localization predictor, the DeepLoc tool (http://www.cbs.dtu.dk/services/DeepLoc/, accessed on 16 September 2021).

Phylogenetic studies were performed with a tree construction using the sequences of the PHT1 transporters from *Arabidopsis thaliana*, *Brassica napus,* and *Brassica oleracea var. italica*. All the protein sequences were aligned with the MUSCLE algorithm and, to build the phylogenetic tree, a neighbor joining (NJ) algorithm with 1000 bootstrap replicates, a Poisson model and pairwise deletion was utilized with the help of Mega X software [10].

2.3. RNA-Seq Analysis

The quality of the raw data (reads) was analyzed and mapped onto the broccoli genome using HISAT2 software. For differential expression analyses, the DESeq2 algorithm was used and normalized with rLog. All analyses were carried out in the Galaxy web platform (https://usegalaxy.org/, accessed on 24 September 2021).

2.4. Data Analysis

Statistical analyses were performed using the SPSS 25.0.0.1 software package. Statistical differences were calculated via a Student's *t*-test and all parameters were determined at $p \leq 0.05$. The values presented are the means ± standard errors (SE).

3. Results

3.1. Genome-Wide Identification of PHT1 Genes in Broccoli and Phylogenetic Analysis

A search of the whole genome of broccoli for PHT1 transporters revealed a total of 31 matches. Three of the sequences found were partial and four other sequences were not completed, almost the full sequence, or lacking the final part. Due to this, the partial sequences were excluded from the phylogenetic analysis and tree construction, in order not to create interference in sequence alignments. The sequences were named according to the chromosome location from lower to higher numbers.

For the phylogenetic analyses, the protein sequences of Pi transporters found in the broccoli proteome were aligned against those from *B. napus* and *A. thaliana*. Arabidopsis PHT1 transporters are divided into nine subfamilies, forming nine groups in the phylogenetic tree (Figure 1).

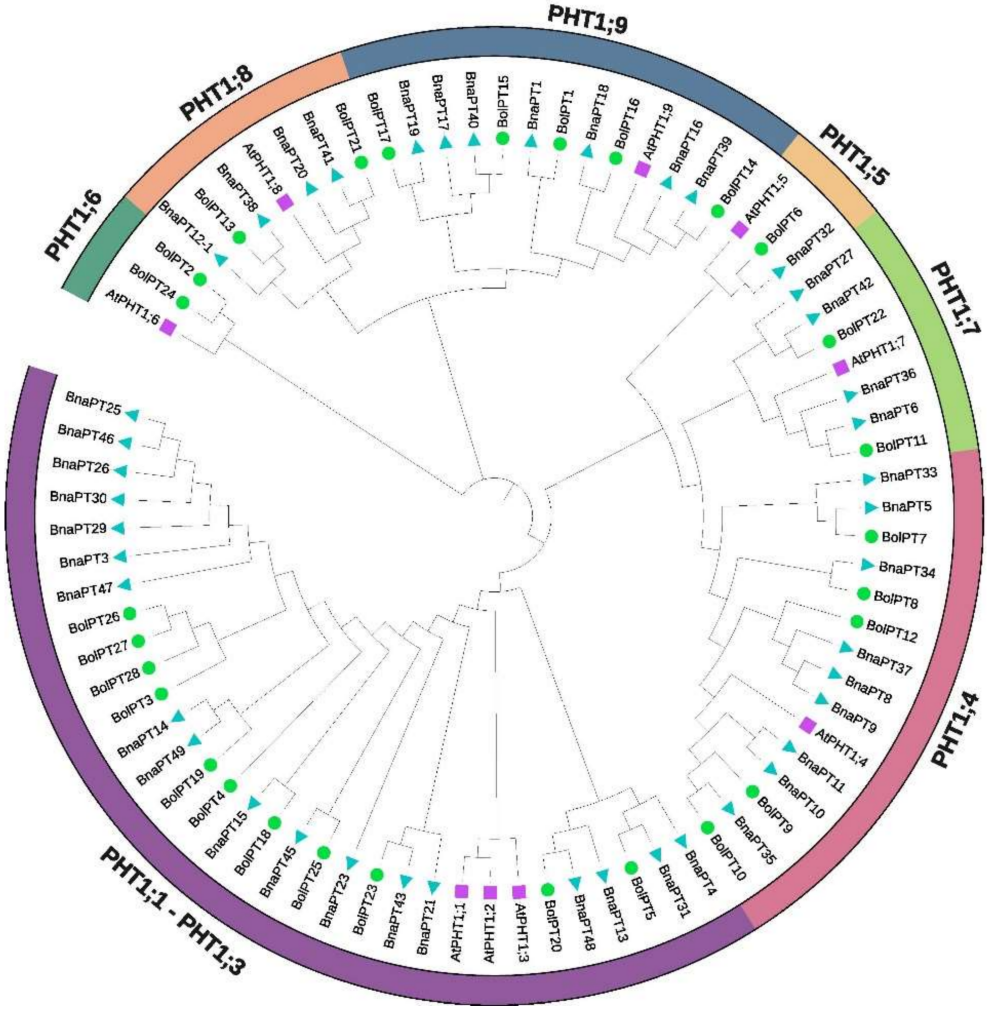

Figure 1. Phylogenetic analysis of PHT1 proteins of *B. oleracea var. italica* (circles), *A. thaliana* (squares), and *B. napus* (triangles). Muscle were used to align protein sequences and the NJ method (with 1000 bootstrap replication) was used to build the tree, all with MEGA X. The different PHT1 subfamilies are also represented.

3.2. Chromosomal Location and Protein Features and Subcellular Location Predictions

When focusing on gene location, all the genes were widely distributed in all chromosomes, except for chromosome 1, where no PHT1-like genes were found (Table 1). On the other hand, the chromosome with the highest number of PHT1 genes was chromosome 9, followed by chromosomes 2 and 4. When focusing on protein features, the complete sequences found were in the range of 509 to 557 amino acids in length, with a molecular weight between 56 and 59 kDa. Transmembrane transporters proteins normally have transmembrane domains formed by α-helixes forming the pore; in this case, a total of 12 α-helixes were predicted to form the PHT1 proteins. The 3D structures were also analyzed and almost all presented 12 transmembrane motifs (data not shown). Finally, subcellular location predictions determined that the main location of almost all the proteins was the plasma membrane when analyzed with Plant-m-Ploc. On the other hand, *deep loc* analysis also indicated that some proteins could also be located in the endoplasmic reticulum and vacuoles (tonoplast).

Table 1. List of 31 PHT1 transporter genes found in broccoli. Column identifiers (Gene name, ID), chromosome location (Chr loc), protein amino acid length (no. aa), molecular weight (Mw), number of transmembrane domains (TMHMM), and cellular location. Numbers 1 and 2 are related with the algorithm used to make the prediction, [1]: deep loc; [2]: Plant m-Ploc. † symbol indicates partial sequences and * symbols show non-completed sequences.

Name	Gene ID	Chr loc	No aa	Mw (g/mol)	TMHMM	Cellular Location
BolPT1	BolC2t09393H	2	538	59,106, 69	12	E.R. [1], P.M. [2]
BolPT2	BolC6t40089H	6	537	59,136, 52	12	E.R. [1], P.M. [2]
BolPT3	BolC6t38329H	6	551	60,907, 72	12	E.R. [1], P.M. [2]
BolPT4	BolC6t40088H	6	538	59,064, 55	12	E.R. [1], P.M. [2]
BolPT5	BolC8t49671H	8	557	61,462, 94	12	E.R. [1], P.M. [2]
BolPT6	BolC2t10437H	2	517	56,464, 16	11	P.M. [1,2]
BolPT7 *	BolC9t55444H	9	444	48,159, 46	10	P.M. [1,2]
BolPT8	BolC9t55475H	9	509	55,452, 65	11	P.M. [2], Vacuole [1]
BolPT9	BolC8t50596H	8	531	58,264, 55	11	P.M. [2], Vacuole [1]
BolPT10	BolC4t22465H	4	534	58,586, 23	11	P.M. [2], Vacuole [1]
BolPT11	BolC4t28201H	4	529	57,910, 35	11	P.M. [2], Vacuole [1]
BolPT12 *	BolC4t22466H	4	417	46,203, 77	8	P.M. [2], Vacuole [1]
BolPT13	BolC2t10439H	2	521	57,251, 2	11	P.M. [2], Vacuole [1]
BolPT14	BolC7t43120H	7	521	57,250, 22	11	P.M. [2], Vacuole [1]
BolPT15	BolC9t55490H	9	521	57,222, 17	11	P.M. [2], Vacuole [1]
BolPT16	BolC9t55477H	9	521	57,220, 24	11	P.M. [2], Vacuole [1]
BolPT17	BolC9t55480H	9	521	57,204, 24	11	P.M. [2], Vacuole [1]
BolPT18	BolC2t10445H	2	535	58,692, 65	11	P.M. [1,2]
BolPT19	BolC7t43121H	7	535	58,392, 07	11	P.M. [1,2]
BolPT20	BolC3t14963H	3	535	58,656, 36	12	P.M. [1,2]
BolPT21	BolC4t25357H	4	540	59,244, 65	11	P.M. [1,2]
BolPT22	BolC4t22464H	4	533	58,692, 05	12	P.M. [1,2]
BolPT29 †	BolC4t27979H	4	112	11,940, 99	1	P.M. [1,2]
BolPT23	BolC5t30648H	5	542	59,950, 3	12	P.M. [1,2]
BolPT24 *	BolC7t43115H	7	464	50,582, 37	11	P.M. [2], Vacuole [1]
BolPT25 *	BolC2t10440H	2	450	48,991, 7	11	P.M. [2], Vacuole [1]
BolPT26	BolC6t40092H	6	506	56,402, 57	12	P.M. [1,2]
BolPT27	BolC9t55476H	9	521	57,381, 33	11	P.M. [1,2]
BolPT30 †	BolC9t55487H	9	176	19,122, 03	4	P.M. [2], Vacuole [1]
BolPT31 †	BolC2t10438H	2	148	15,945, 88	3	P.M. [2], Vacuole [1]
BolPT28	BolC3t14547H	3	546	59,316, 62	10	P.M. [2], Vacuole [1]

3.3. RNA-Seq Analysis and Expression Studies

Different analyses of expression were carried out (Figure 2). The expression of PHT1 family genes was compared in different tissues, root, and leaves (Figure 2a) showing that the most expressed gene in leaves was BolPT1 with an almost ten-fold expression when compared with the root. Meanwhile, the BolPT6, BolPT7, BolPT9, BolPT10, BolPT11, BolPT16, BolPT17, and BolPT27 genes showed no statistical differences in expression between the two types of tissues. Alternatively, BolPT14, BolPT18, BolPT21, BolPT22, BolPT26, and BolPT29 showed higher expression levels in root when compared with leaves. Some of the PHT1 genes showed no expression either in roots and leaves and thus were not included in the graphical representation (Figure 2).

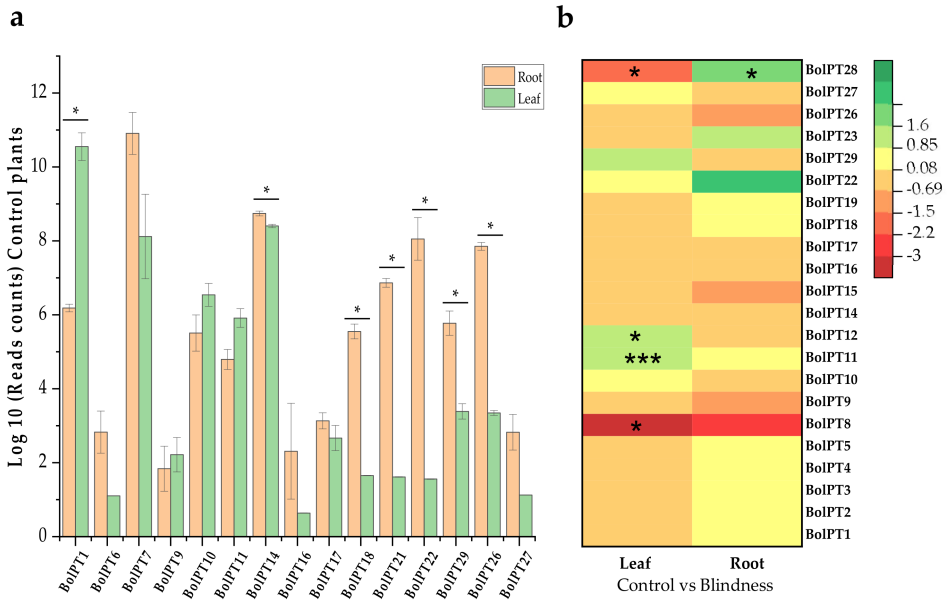

Figure 2. Analysis of RNA sequencing expression of PHT1 genes from broccoli plants in leaves and roots. (**a**) Expression of several PHT1 genes of control broccoli plants (SE errors bars); (**b**) Heat map representing fold change of PHT1 genes comparing broccoli control plants versus blindness broccoli plants in leaves and roots. Statistical differences were calculated with a Student's *t*-test and are shown with asterisks (* $p < 0.05$; *** $p < 0.0005$).

The differences in expression between control plants and plants with blindness were also measured (Figure 2b). On one hand, when analyzing gene expression, statistical differences in expression were found predominantly in the leaves when compared to the control with blindness, highlighting PT28 and PT8 that showed a repressed expression in the case of blindness broccoli plants leaves. However, BolPT11 and BolPT12 presented higher expression levels in blindness plants leaves when compared with control plants.

On the other hand, only PT28 showed a statistical difference, being over expressed in blindness plants roots, by almost two fold, when compared with control plants.

4. Discussion

Nowadays, the information available from the complete genome sequencing of all kinds of living organisms is increasing due to the amelioration of the techniques and the continuous price drop of the sequence cost per base [11]. Therefore, the increase of complete genome sequences requires an analysis and characterization of functional sequences such as genes.

In the case of broccoli plants, a total of 31 PHT genes were identified, in contrast with the 41 genes identified in *B. napus* [9] and 9 genes present in *A. thaliana* [8]. These findings reveal that the PHT1 family is heterogeneous and its presence, number, and functionalities depends on the plant species and family. Furthermore, there is a notably high copy number variation in PHT1 family genes between Brassica species.

Additionally, protein features such as molecular weight, transmembrane helix domains, and cellular location prediction revealed similarities between *B. napus* and *A. thaliana* [9,12]. Moreover, all PHT1 transporter proteins share the same three-dimensional structure. The analysis predicts that the members of this transporter family are characterized by the possession of 12 membrane-spanning domains (data not shown) [13].

The PHT1 transporters are one of the most studied plant phosphorous transporters [6]. In particular, these proteins are responsible of Pi uptake from soil [14]. In this sense, our expression analysis revealed an overall higher expression of PHT1 transporters in roots (Figure 2a). Despite this, BolPT1 showed higher expression levels in leaves when compared

root expression. These findings reveal a different role of BolPT1 in the case of broccoli plants as compared with its attributed role in *A. thaliana*. The BolPT1 protein is included in PHT1;9 (Figure 1) and this subfamily has been shown to be involved in Pi uptake by roots in Pi-starved plants in Arabidopsis [8]. Further analyses should be carried out in order to determine the role of BolPT1 in broccoli leaves.

Moreover, when comparing the expression of PHT1 transporters of control and blindness plants (Figure 2b), we found that changes mostly occur in leaf gene expression. This could be the result of blindness being a meristematic tissue disease resulting in major changes in the gene expression of leaves with apparently no effect on roots.

5. Conclusions

In this work, the sequences of PHT1 genes have been assessed in broccoli. The analysis of related plant species was useful to classify the genes by family. This analysis together with RNA-seq carried out in roots and leaves of control and blindness broccoli plants show an overview of the PHT1 transporters family. Additionally, the results showed different expression in some of the transporters (BolPT8, BolPT28, BolPT11, and BolPT12). Therefore, the involvement of this transporter in broccoli blindness opens a new line of research that points to the importance of phosphorus nutrition in the appearance of physiopathies such as blindness.

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Conflicts of Interest: The authors declare no conflict of interest.

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